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Sensitivity of *Zasmidium citri-griseum* to Fenbuconazole and Control of Citrus Greasy Spot in Panama

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Abstract: Citrus greasy spot, caused by *Zasmidium citri-griseum* (= *Mycosphaerella citri*) is the most prevalent fungal disease of citrus in Panama, causing premature defoliation and yield reduction. Fungicide applications are generally needed for the control of greasy spot. In this study, the sensitivity to fenbuconazole of 34 isolates of *Z. citri-griseum* from Panama was determined by calculating the effective concentration needed to reduce mycelial growth by 50% (EC_{50}). Two field trials were conducted from 2011 to 2013, to evaluate the efficacy of fenbuconazole to reduce disease severity and yield loss. The EC_{50} values for fenbuconazole ranged from 0.09 to 7.62 µg mL^{-1}, with a mean EC_{50} value of 2.66 $\pm$ 0.36 SE µg mL^{-1} for mycelial growth. These data can be used for monitoring sensitivity shifts in *Z. citri-griseum* to fenbuconazole and reduce risk of fungicide resistance in Panama. In the field trials, sprays with fenbuconazole significantly reduced ($p < 0.0001$) the severity of greasy spot on leaves compared with the non-treated control. Greater disease control was obtained when three sprays of fenbuconazole were applied instead of one. Nevertheless, no significant differences ($p > 0.05$) were detected in yield.

Keywords: Central America; disease management; fungicide; *Mycosphaerella citri*

1. Introduction

Citrus greasy spot, caused by *Zasmidium citri-griseum* (F.E. Fisher) U. Braun and Crous (=*Mycosphaerella citri* Whiteside), is one of the most important fungal diseases of citrus in the Caribbean Basin, including Florida (US), the Caribbean islands, Mexico, and Central America [1–3]. Greasy spot is widely distributed in Panama affecting mainly sweet orange [*Citrus sinensis* (L.) Osbeck] and Tahiti lime [*C. latifolia* (Yu.Tanaka) Tanaka] [4]. Trees affected by greasy spot typically show yellow mottle on the adaxial surface of leaves and yellow-brown spot, slightly raised pustules on the abaxial surface, resulting in premature defoliation and yield reduction. In Florida, fruit symptoms have been reported mainly in grapefruit (*C. paradisi* Macf.), consisting of minute black flecks on the peel which coalesce to form rind blotch [3]. In Florida, losses up to 45% have been reported in grapefruit and 25% in sweet orange [2]. In Cuba, yield losses up to 5 tons ha^{-1} were reported in sweet orange associated with greasy spot [5].

The main inoculum source of *Z. citri-griseum* are the ascospores formed in pseudothecia on decaying leaves on the orchard floor. Ascospores are discharged in response to wetting and dispersed by wind currents [6]. Once deposited on the leaf surface, ascospores germinate and the resulting mycelia grow epiphytically [7]. Conidia of *Z. citri-griseum* have also been reported, but they are considered of minor epidemiological relevance [2].The disease is characterized by a relatively long

incubation period, and foliar symptoms of greasy spot on lemon [*C. limon* (L.) Burm f.] and grapefruit are visible 3–4 months after infection, while in sweet orange the appearance of symptoms takes much longer [2].

Leaf litter removal can be effective in reducing the inoculum of the pathogen in orchards [8]. Application of urea to the leaf litter reduced the production of pseudothecia and ascospores of *Z. citri-griseum*, but did not affect the rate of leaf decomposition [9]. In contrast, the application of lime and additional irrigations accelerate litter decay, and leaf tissues are decomposed so rapidly that pseudothecia do not form [3]. Foliar sprays with fertilizers such as zinc, manganese, and iron, are effective for greasy spot control if applied at sufficiently high rates [10]. In Texas (US), foliar sprays with aqueous organic mixtures and aqueous suspension of vegetable oil reduced the incidence of greasy spot on grapefruit leaves. [11].

Nevertheless, the primary means of greasy spot control is the application of fungicides to reduce disease severity on the leaves and subsequent defoliation. *Z. citri-griseum* is sensitive to a wide range of fungicides from different groups and mode of actions such as benzimidazoles, demethylation inhibitors (DMIs), quinone outside inhibitors (QoIs), copper compounds and petroleum oils [2,3]. The pathogen is affected by fungicides mainly during its epiphytic growth on the leaf surface [3]. In Florida, fungicide sprays applied any time in May or June provided the best control of disease on the spring flush [3,12]. In Costa Rica, fungicide applications in June and August provided effective disease control [1].

The group of DMI fungicides was introduced in the mid-1970s and they are effective against numerous fungal diseases, especially powdery mildews, rusts and leaf spots in general [13]. The chemical group of triazoles is the most represented within the DMI fungicides [14]. These fungicides inhibit the removal of the C14-methyl group from 24-methylenedihydrolanosterol or eburicol, and disrupt sterol production by the fungus [14]. According to the Fungicide Resistance Action Committee (FRAC) classification [15], there is medium risk for the development of resistance to DMI fungicides.

The fungicide fenbuconazole is a triazole first introduced in 1988 [16]. Fenbuconazole is a systemic fungicide with protectant and curative activity, used for the control of rusts, powdery mildews, bunts and smuts on different crops [17]. This triazole fungicide has also been reported to provide effective control of greasy spot and rind blotch [18–23]. The establishment of the sensitivity distribution is the first step to develop a strategy for monitoring and managing fungicide resistance [24]. Baseline studies with fenbuconanzole have been performed in Florida with *Z. citri-griseum*, *Colletotrichum acutatum* J. H. Simmonds and *Elsinoë fawcettii* Bitanc. and Jenkins on citrus [25], *Venturia effusa* (G. Winter) Rossman and W.C. Allen in pecan [26] and *Monilinia oxycocci* (Woronin) Honey in cranberry in Wisconsin (US) [27]. There is no information on the sensitivity of *Z. citri-griseum* isolates from Panama to fenbuconazole. Neither is there any information on the efficacy of fenbuconazole for the control of greasy spot under the particular agroclimatic conditions of Panama. Therefore, the objectives of this study were to determine the sensitivity of *Z. citri-griseum* isolates from Panama to fenbuconazole and evaluate its efficacy for the control of greasy spot under field conditions.

2. Materials and Methods

2.1. Mycelial Growth Inhibition Assay

A total of 34 isolates of *Z. citri-griseum* were collected during the rainy season from May to December in 2010, 2011 and 2012 on symptomatic citrus trees in three provinces of Panama, namely Chiriquí, Veraguas and Coclé (Table 1). Isolates were obtained from symptomatic leaves of different citrus species. Leaves were surface disinfested with 1% sodium hypochlorite solution for 5 min and rinsed twice in sterile distilled water [25]. On the abaxial side with lesions, small pieces of mesophyll were removed with a sterile scalpel [28]. Small pieces were plated on potato dextrose agar (PDA) (Liofilchem, Italy) amended with 0.5 g L^{-1} of streptomycin sulphate (Sigma-Aldrich, USA)

(PDAS), and incubated at 25 °C in the dark for 10 to 14 days. The fungal colonies obtained were transferred to PDA, and monohyphal isolates were obtained by serial dilution [29].

Isolations from leaf litter were carried out by attaching wetted small leaf pieces (∼5 mm^2) with pseudothecia to the top of Petri dishes using double-sided adhesive tape (Scotch, 3M, USA) and allowing the ascospores to be ejected onto PDAS. Plates were incubated at 25 °C in the dark for one week and examined daily. Individual germinating ascospores were selected under the stereomicroscope and transferred with a sterile needle to malt extract agar (MEA) (Oxoid Ltd., England) plates, which were incubated at 25 °C in the dark for two weeks [30].

All isolates were identified by morphological analysis, sequencing of the internal transcribed spacer region (ITS) and translation elongation factor 1-alpha (EF-1α) based on previously described procedures by Aguilera et al. [31]. For long-term storage, all isolates were kept in 15% glycerol solution at −80 °C in 1.5 mL cryovials until further use. For mycelium production, *Z. citri-griseum* isolates were transferred to PDA and grown at 25 °C in the dark for 30 days.

Table 1. Isolates of *Zasmidium citri-griseum* included in the sensitivity study for fenbuconazole with their corresponding values of the effective concentration needed to reduce mycelial growth by 50% (EC$_{50}$).

Isolate	Year of Isolation	Province	Locality	Plant Species	Affected Tissue	EC$_{50}$ µg mL^{-1}
2NTV1	2010	Veraguas	La Dormilona	*Citrus sinensis*	Canopy leaf	1.28
4NTV1	2010	Veraguas	Santiago	*C. sinensis*	Canopy leaf	0.55
6NCV4	2010	Veraguas	Bajo de la Honda	*C. sinensis*	Canopy leaf	1.82
9NCV1	2010	Veraguas	El Espino	*C. sinensis*	Canopy leaf	3.40
9NCV4	2010	Veraguas	El Espino	*C. sinensis*	Canopy leaf	3.27
10NCV6	2010	Veraguas	El Espino	*C. sinensis*	Canopy leaf	1.12
15NCV1	2010	Veraguas	Alto Piedra	*C. sinensis*	Canopy leaf	0.41
12NCC9	2010	Coclé	Churuquita	*C. sinensis*	Canopy leaf	7.40
17NCC3	2010	Coclé	Mira Flores	*C. sinensis*	Canopy leaf	6.02
17NCC5	2010	Coclé	Mira Flores	*C. sinensis*	Canopy leaf	4.68
19NCC3	2010	Coclé	El Guabal	*C. sinensis*	Canopy leaf	4.44
26LCC5	2011	Coclé	Santa Clara	*C. latifolia*	Canopy leaf	0.96
27LCC2	2011	Coclé	Santa Clara	*C. latifolia*	Canopy leaf	1.96
31TCC4	2011	Coclé	Platanal	*C. paradisi*	Canopy leaf	2.33
33TCC2	2011	Coclé	Platanal	*C. paradisi*	Canopy leaf	0.09
34NCC4	2011	Coclé	Toabré	*C. sinensis*	Canopy leaf	4.87
37LCC2	2011	Coclé	Tambo	*C. latifolia*	Canopy leaf	1.05
37LCC3	2011	Coclé	Tambo	*C. latifolia*	Canopy leaf	1.78
38NCC2	2011	Coclé	Tambo	*C. sinensis*	Canopy leaf	4.26
43NCCh2	2011	Chiriquí	Rovira	*C. sinensis*	Canopy leaf	2.08
45NCCh2	2011	Chiriquí	Banco de Rovira	*C. sinensis*	Canopy leaf	2.98
48NCCh1	2011	Chiriquí	Potrerillo	*C. sinensis*	Canopy leaf	7.62
Myc-14	2011	Coclé	Churuquita	*C. sinensis*	Leaf litter	2.97
Myc-21	2011	Coclé	Tambo	*C. sinensis*	Leaf litter	1.10
Myc-23	2011	Coclé	Churuquita	*C. sinensis*	Leaf litter	6.20
Myc-26	2011	Coclé	Churuquita	*C. sinensis*	Leaf litter	1.51
Myc-36	2011	Coclé	Toabré	*C. paradisi*	Leaf litter	0.84
Myc-37	2011	Coclé	Toabré	*C. paradisi*	Leaf litter	1.16
61LCC4	2012	Coclé	Tambo	*C. latifolia*	Canopy leaf	0.33
62LCC4	2012	Coclé	Tambo	*C. latifolia*	Canopy leaf	5.18
65NCC3	2012	Coclé	Caimito	*C. sinensis*	Canopy leaf	3.03
68NCC2	2012	Coclé	Toabré	*C. sinensis*	Canopy leaf	2.01
69NCC2	2012	Coclé	Toabré	*C. sinensis*	Canopy leaf	0.70
69NCC3	2012	Coclé	Toabré	*C. sinensis*	Canopy leaf	1.12

A commercial formulation of fenbuconazole (Indar 25% w/v OF; Dow Agrosciences, US) was used in the mycelial growth inhibition assay of *Z. citri-griseum*. Appropriate volumes of fungicide were added to PDA at about 50 °C to obtain final concentrations of active ingredient (a.i.) of 0.01, 0.1, 1, 10 and 100 µg mL^{-1}. Non-amended PDA medium was used as the control. Amended and non-amended PDA (20 mL) was poured into 90-mm-diameter Petri dishes. Mycelial plugs, 5 mm in diameter, from the actively growing area of the fungal colonies were placed in the center of each plate. A sample size of

four replicate plates was used for each combination of isolate and fungicide concentration. Plates were incubated at 25 °C in the dark for 30 days until the control plates were 50% covered with mycelium. Colony diameter was determined as the average of two perpendicular measurements, substracting the 5 mm of the mycelial plug. Growth inhibition (%) was calculated relative to the non-amended control for each isolate. Dose-response curves were obtained and the effective concentration needed to reduce mycelial growth by 50% (EC_{50}) was calculated with the `nplr` package for `R` [32]. This package is commonly used for n-parameter logistic regression models, in our case with four-parameter logistic regressions. The box-whisker plot and histogram of EC_{50} values were obtained. The shape of frequency distribution was analyzed by examining curve shape, range, and mean values of EC_{50}. The `R` version 3.2.5 was used in all statistical and graphical analyses [33].

2.2. Field Trials

To evaluate the efficacy of fenbuconazole for the control of greasy spot, two field experiments were conducted in commercial citrus orchards severely affected by the disease. Experiment 1 was located in a 'Star Ruby' grapefruit orchard at Tambo with 6-year-old trees grafted on 'Swingle' citrumelo [*C. paradisi* x *Poncirus trifoliata* (L.) Raf.] rootstock and with 5 x 10 m tree spacing. Experiment 2 was located in a 'Valencia' sweet orange orchard at Miraflores with 5-year-old trees grafted on 'Swingle' citrumelo and with 3.5 x 7 m tree spacing. Both orchards were rainfed. The same commercial formulation of fenbuconazole indicated for the mycelial growth inhibition assay was used. According to the label rate, a concentration of fenbuconazole of 0.12 g a.i. L^{-1} was compared with a non-treated control. Treatments were arranged in a completely randomized design with six replications of 10 trees each. A guard tree was located between plots within rows. Trees were sprayed with a volume of $\sim$ 6.5 L $tree^{-1}$ in experiment 1 and $\sim$ 3 L $tree^{-1}$ in experiment 2 using a hydraulic sprayer (Arbus 2000 Jacto, Brasil) at 300 psi. In 2011, spray applications were performed on 20 May, 7 June and 30 June in experiment 1 and 9 June, 1 July and 21 July in experiment 2. In order to evaluate a more cost-effective program, in 2012 only one spray application was carried out on 18 May in experiment 1 and 15 May in experiment 2. In 2013, one spray application was made on 21 May in experiment 1 and no fungicides were applied in experiment 2. In all cases, the first spray coincided with the appearance of new shoots at phenological stage BBCH 32–39 (between 20% and 90% of final shoot length) [34].

Disease severity was assessed in December 2011–2013 in experiment 1 and December 2011–2012 in experiment 2. Ten shoots were arbitrarily selected in each tree ($\sim$ 70 leaves $tree^{-1}$) and tagged before the first spray. All leaves in the tagged shoots were assessed according to the following rating scale: 0 = no lesions observed; 1 = 1–10 leaf spots; 2 = 11–20 leaf spots; 3 = more than 20 leaf spots per leaf [31]. Descriptive statistics were obtained for disease severity categories in each experiment. Disease severity categories were treated as an order factor, which was modelled with a proportional odds logistic regression model using the `polr` function of the `MASS` package for `R` [35].

$$logit[P(Y_i \leq j)] = \alpha_{j0} - \beta_{tr} I_{tr}(i) \qquad i = 1, ..., n \qquad (1)$$

where $P(Y_i \leq j)$ is the cumulative probability between 0 and 1 for category j of Yi, $j = 0, \ldots, 2$ are categories of greasy spot severity, α_{j0} are the thresholds for greasy spot severity categories, β_{tr}, is the coefficient for the fungicide treatment evaluated (I_{tr}), and n is the total number of ten-tree plots. The proportional odds model assumes that the *logit* of the cumulative probabilities changes linearly as the explanatory variables change, and also that the slope of this relationship is the same regardless of the disease severity category [36]. In our case, the non-treated control was used as the reference level and the odds ratio for the fungicide treatment was calculated as $e^{-\beta}$ based on the cumulative probabilities. Owing to the proportional odds assumption, the odds ratio for the fungicide treatment stays the same no matter how disease severity is dichotomized into two levels. This proportional odds assumption was evaluated by the Likelihood Ratio Test (LRT) between the proportional odds model and the non proportional odds model [37] using the `VGAM` package for `R` [38]. The goodness of fit was assessed by a χ^2–test of the residual deviance against the null model (i.e., including only the intercept).

A significant effect for the fungicide treatment was considered when the 95% confidence interval of the odds ratio did not overlap the null value of 1 [36].

All fruit in each tree were harvested and weighed. In each experiment, descriptive statistics and analysis of variance (ANOVA) were performed for yield data (kg tree[1]) using the `stats` package for `R` [39]. Prior to ANOVA the normality assumptions were verified by the test by Shapiro–Wilk and the homogeneity of variance using the Bartlett test. A diagnosis of the studied residues was made applying the test of Breusch–Pagan to determine the homocedasticity of the variance.

3. Results

3.1. Mycelium Growth Inhibition Assay

The EC_{50} values of 34 isolates of *Z. citri-griseum* collected from different provinces of Panama exposed to fenbuconazole ranged from 0.09 to 7.62 µg mL^{-1} (Table 1), with a mean $\pm$ standard error (SE) of 2.66 ± 0.36 µg mL^{-1}. The box-whisker plot (Figure 1) showed a median value of 1.98 µg mL^{-1} with a interquartile range from 1.11 to 4.04 µg mL^{-1} and no outliers were observed. The frequency distribution of EC_{50} to fenbuconazole of the 34 isolates of *Z. citri-griseum* included in the assay showed a unimodal curve with a positive skew (Figure 1). The frequency of isolates with EC_{50} values <1 µg mL^{-1} represented 20.6% (n = 7). For EC_{50} from 1 to 5 µg mL^{-1} the frequency was 64.7% (n = 22) and for EC_{50} values >5 µg mL^{-1} represented 14.7% (n = 5).

3.2. Field Trials

In experiment 1 in 2011, 57.22% of the leaves were in the severity category 0 (no lesions) in the trees treated with three fenbuconazole sprays while only 3.95% in the non-treated control (Figure 2). The non-treated control showed 81.39% of the leaves in the severity category 3 (>20 leaf spots) in contrast to the 10.82% in the trees treated with fenbuconazole. In 2012 and 2013, the percentages of leaves in the severity category 0 in the trees with one spray of fenbuconazole decreased to 5.83% and 3.14%, respectively. The percentage of leaves in category 0 in the non-treated control was 0.54% in 2012 and 0.27% in 2013. Values for category 3 in the non-treated control were 74.96% in 2012 and 74.26% in 2013. In the trees with one spray of fenbuconazole, the percentage of leaves in category 3 was 33.38% and 33.15% in 2012 and 2013, respectively.

In experiment 2 in 2011, the percentage of leaves in the severity category 0 (no lesions) in the trees with three sprays of fenbuconazole was 51.55% whereas only 2.62% in the non-treated control (Figure 3). The percentage of leaves in the severity category 3 (>20 leaf spots) was 14.66% in the trees treated with fenbuconazole and 64.31% in the non-treated control. In 2012, the percentage of leaves in category 0 decreased to 8.34% in the trees treated with one spray of fenbuconazole and 0.82% in the non-treated control. On the contrary, the percentage of leaves in category 3 increased to 42% in trees treated with fenbuconazole and 66.12% in the non-treated control.

In experiment 1, the proportional odds logistic regression model applied to the disease severity categories showed a significant effect ($p < 0.0001$) of the factor treatment in the three years of study (2011, 2012 and 2013) (Table 2). The negative values of the estimate for the treatment factor in the years 2011 (-3.55), 2012 (-1.79) and 2013 (-1.80) indicated a higher probability of obtaining lower values of disease severity in trees treated with fenbuconazole. In experiment 2, the results of the proportional odds logistic regression model also showed a significant effect ($p < 0.0001$) of the factor treatment in the two years of study (Table 3). Likewise, the negative values of the estimate in the years 2011 (-2.70) and 2012 (-1.05) indicated a higher probability of obtaining lower values of disease severity in the trees treated with fenbuconazole.
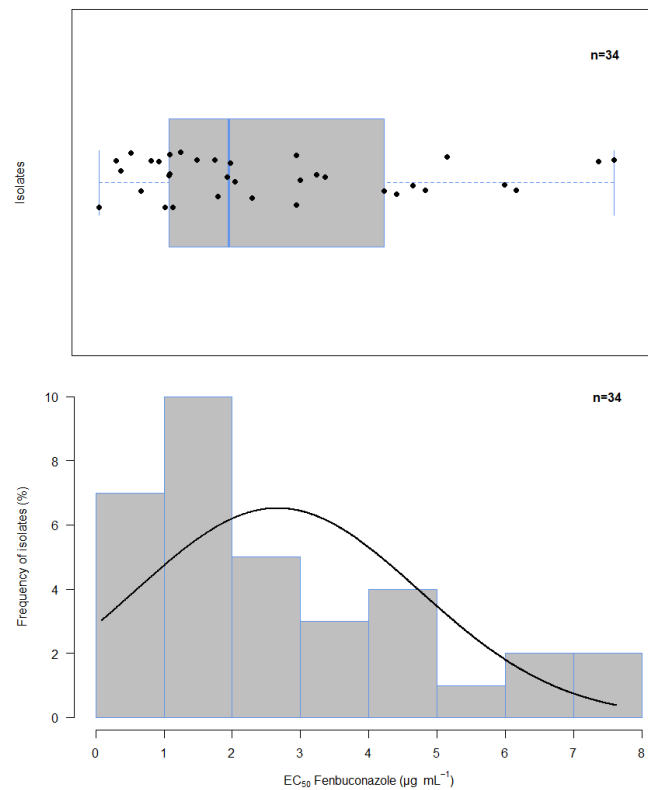
Figure 1. Box-whisker plot and frequency distribution of fenbuconazole sensitivity of *Zasmidium citri-griseum* isolates from Panama (n = 34) expressed as the effective concentration needed to reduce mycelial growth by 50% (EC$_{50}$).

Odds ratio and 95% confidence interval values for the trees treated with fenbuconazole in experiment 1 are shown in Table 4. As it is observed, for non-treated trees, the odds of presenting higher disease severity were 34.81, 5.99 and 6.05 times than for treated trees in 2011, 2012 and 2013, respectively. Fenbuconazole applied 1 or 3 times reduced disease severity compared with the non-treated control in all years as the 95% confidence interval of the odds ratio did not overlap the null value of 1. The predicted probabilities for disease severity categories in the trees treated with fenbuconazole and the non-treated control in experiment 1 are shown in Table 4. In 2011, a high probability of occurrence of severity category 0 (no lesions) was observed in the trees treated with three sprays of fenbuconazole (0.57), in contrast with the low probability (0.04) of occurrence of this severity category in the non-treated control. On the other hand, a high probability (0.81) of occurrence of severity category 3 (>20 leaf spots) was obtained for the non-treated control compared with a low probability (0.11) for the trees treated with fenbuconazole. In 2012 and 2013, the predicted probability for the severity category 3 in the non-treated control was higher (0.74) than in the trees treated with one spray of fenbuconazole (0.33).

Odds ratio and 95% confidence interval values for the trees treated with fenbuconazole in experiment 2 are shown in Table 4. As it is observed, for non-treated trees, the odds of presenting higher disease severity were 14.88 and 2.86 times than for treated trees in 2011 and 2012, respectively. Fenbuconazole applied 1 or 3 times reduced disease severity compared with the non-treated control in all years as the 95% confidence interval of the odds ratio did not overlap the null value of 1. The predicted probabilities for disease severity categories in the trees treated with fenbuconazole and the non-treated control in experiment 2 are shown in Table 4. In 2011, the trees with three sprays of fenbuconazole showed a high probability (0.50) of occurrence for the severity category 0 (no lesions) in contrast with the non-treated control that showed a very low probability (0.06). On the other hand, the non-treated control showed a high probability (0.66) of occurrence of severity category 3 (>20 leaf

spots) compared with the trees treated with fenbuconazole that showed a low probability (0.11) for this same severity category. In 2012, the predicted probability for the severity category 3 in the non-treated control was still high (0.67) compared with the trees with one spray of fenbuconazole (0.41) (Table 4).
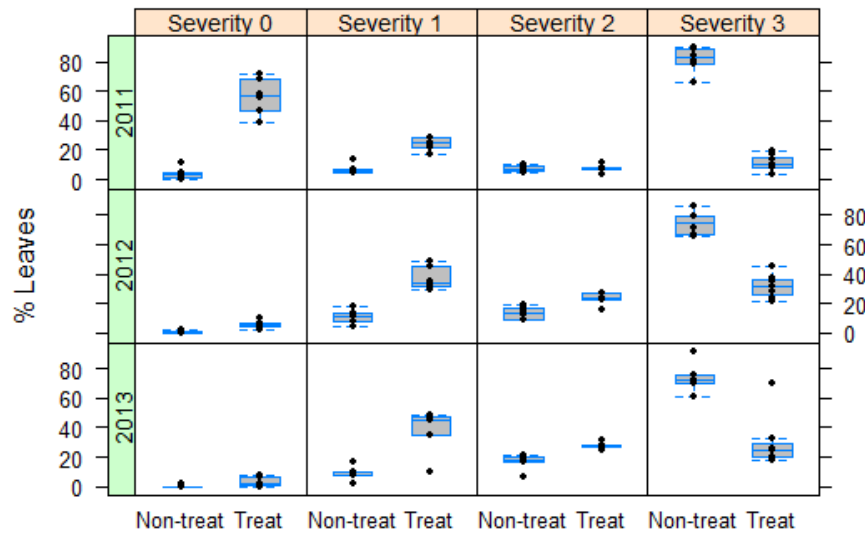

Figure 2. Box-whisker plots of percentages of leaves in the categories of greasy spot severity observed in trees treated with fenbuconazole and the non-treated control in experiment 1 on 'Star Ruby' grapefruit at Tambo, Panama, from 2011 to 2013 (0 = no lesions; 1 = 1–10 leaf spots; 2 = 11–20 leaf spots; 3 = more than 20 leaf spots). Three sprays of fenbuconazole were applied in 2011 and one spray in 2012 and 2013.
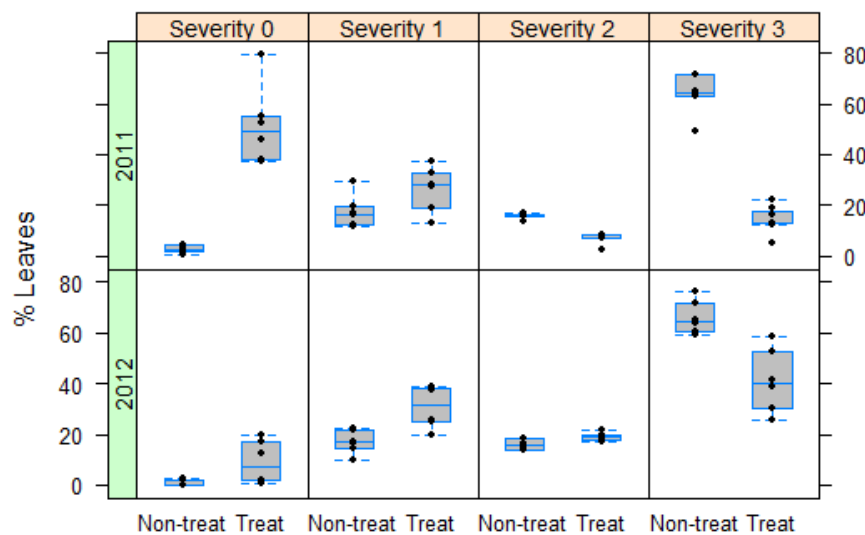

Figure 3. Box-whisker plots of the percentages of leaves in the categories of greasy spot severity observed in trees treated with fenbuconazole and the non-treated control in experiment 2 on 'Valencia' sweet orange at Miraflores, Panama, in 2011 and 2012 (0 = no lesions; 1 = 1–10 leaf spots; 2 = 11–20 leaf spots; 3 = more than 20 leaf spots). Three sprays of fenbuconazole were applied in 2011 and one spray in 2012.

Table 2. Results obtained with the proportional odds logistic regression of greasy spot severity categories in experiment 1 on 'Start Ruby' grapefruit at Tambo, Panama from 2011 to 2013.

Source of Variation	Estimate	Standard Error	t Value	p-Value
2011				
Treatment factor [a]	−3.55	0.06	−55.79	<0.0001
Cut points [b]				
0|1	−3.26	0.06	−55.03	<0.0001
1|2	−2.10	0.05	−41.75	<0.0001
2|3	−1.47	0.05	−33.07	<0.0001
2012				
Treatment factor	−1.79	0.05	−40.26	<0.0001
Cut points				
0|1	−4.65	0.07	−67.73	<0.0001
1|2	−2.07	0.04	−53.16	<0.0001
2|3	−1.10	0.03	−32.06	<0.0001
2013				
Treatment factor	−1.80	0.05	−34.62	<0.0001
Cut points				
0|1	−5.30	0.10	−51.20	<0.0001
1|2	−2.20	0.05	−47.90	<0.0001
2|3	−1.07	0.04	−27.42	<0.0001

[a] The non-treated control was used as the reference level. [b] Thresholds defining the disease severity intervals.

Table 3. Results obtained with the proportional odds logistic regression of greasy spot severity categories in experiment 2 on 'Valencia' sweet orange at Miraflores, Panama in 2011 and 2012.

Source of Variation	Estimate	Standard Error	t Value	p-Value
2011				
Treatment factor [a]	−2.70	0.06	−43.55	<0.0001
Cut points [b]				
0|1	−2.72	0.06	−48.17	<0.0001
1|2	−1.33	0.04	−30.18	<0.0001
2|3	−0.65	0.04	−16.11	<0.0001
2012				
Treatment factor	−1.05	0.05	−20.28	<0.0001
Cut points				
0|1	−3.67	0.07	−51.44	<0.0001
1|2	−1.50	0.04	−35.42	<0.0001
2|3	−0.69	0.04	17.94	<0.0001

[a] The non-treated control was used as the reference level. [b] Thresholds defining the disease severity intervals.

In experiment 1 conducted at Tambo, average yield in the grapefruit trees treated with fenbuconazole was 42.98 kg tree^{-1} in 2011, 68.12 kg tree^{-1} in 2012 and 64.45 kg tree^{-1} in 2013 (Figure 4). Average yield in the non-treated control was 35.01 kg tree^{-1} in 2011, 50.87 kg tree^{-1} in 2012, 62.46 kg tree^{-1} in 2013. In experiment 2 conducted at Miraflores, average yield in the sweet orange trees treated with fenbuconazole was 23.19 kg tree^{-1} in 2011 and 24.71 kg tree^{-1} in 2012 (Figure 5). Average yield in the non-treated control trees was 20.97 kg tree^{-1} in 2011 and 24.10 kg tree^{-1} in 2012.

Table 4. Probabilities and odds ratios obtained with the proportional odds logistic regression of greasy spot severity categories in experiment 1 on 'Start Ruby' grapefruit at Tambo and experiment 2 on 'Valencia' sweet orange at Miraflores, Panama from 2011 to 2013.

| Experiments | Evaluation Year | Treatments | Probabilities [a] | | | | Odds Ratio |
			Sev. 0	Sev. 1	Sev. 2	Sev. 3	
Experiment 1	2011	Fenbuconazole	0.57	0.24	0.08	0.11	34.81 (30.75–39.47) [b]
		Non-treated	0.04	0.07	0.08	0.81	
	2012	Fenbuconazole	0.05	0.38	0.24	0.33	5.99 (5.50–6.55)
		Non-treated	0.01	0.10	0.14	0.75	
	2013	Fenbuconazole	0.03	0.37	0.27	0.33	6.05 (5.47–6.70)
		Non-treated	0.00	0.09	0.16	0.74	
Experiment 2	2011	Fenbuconazole	0.50	0.30	0.09	0.11	14.88 (13.21–16.85)
		Non-treated	0.06	0.15	0.13	0.66	
	2012	Fenbuconazole	0.07	0.32	0.20	0.41	2.86 (2.85–3.16)
		Non-treated	0.02	0.16	0.15	0.67	

[a] 0 = no lesions observed; 1 = 1–10 leaf spots; 2 = 11–20 leaf spots; 3 = more than 20 leaf spots. [b] In brackets 95% confidence interval.

Yield data (kg tree^{-1}) from experiments 1 and 2 were normally distributed ($p > 0.05$) in all years, with homogeneous variances ($p > 0.05$). In experiment 1, no significant differences in yield were detected between the grapefruit trees treated with fenbuconazole and the non-treated control in 2011 ($F = 1.96$, d.f. = 1, 10, $p = 0.19$), 2012 ($F = 1.57$, d.f. = 1, 10, $p = 0.24$) and 2013 ($F = 0.05$, d.f. = 1, 10, $p = 0.83$). Likewise, in experiment 2 no significant differences in yield were detected between the sweet orange trees treated with fenbuconazole and the non-treated control in 2011 ($F = 0.53$, d.f. = 1, 10, $p = 0.48$) and 2012 ($F = 0.04$, d.f. = 1, 10, $p = 0.84$). The Breusch-Pagan test performed on the models residuals did not detect heterocedasticity of the variances ($p > 0.05$).
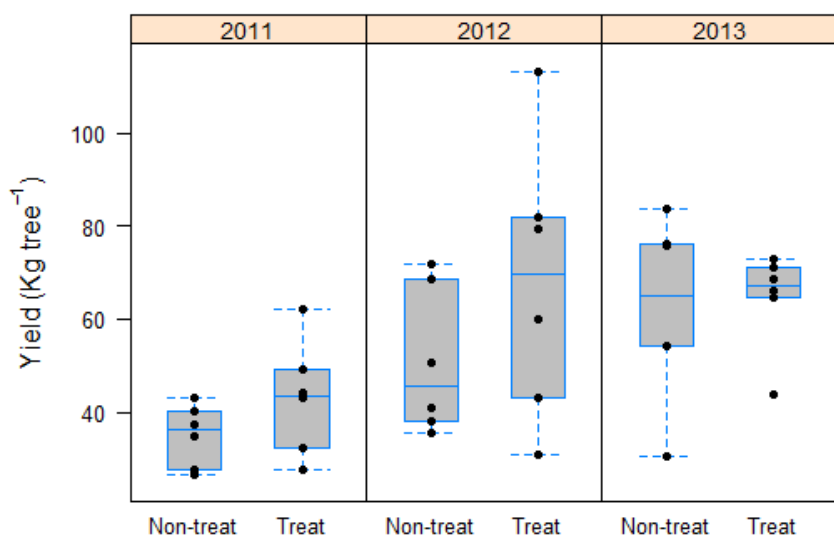

Figure 4. Box-whisker plots of yield of trees treated with fenbuconazole and the non-treated control in experiment 1 on 'Star Ruby' grapefruit at Tambo, Panama, from 2011 to 2013.
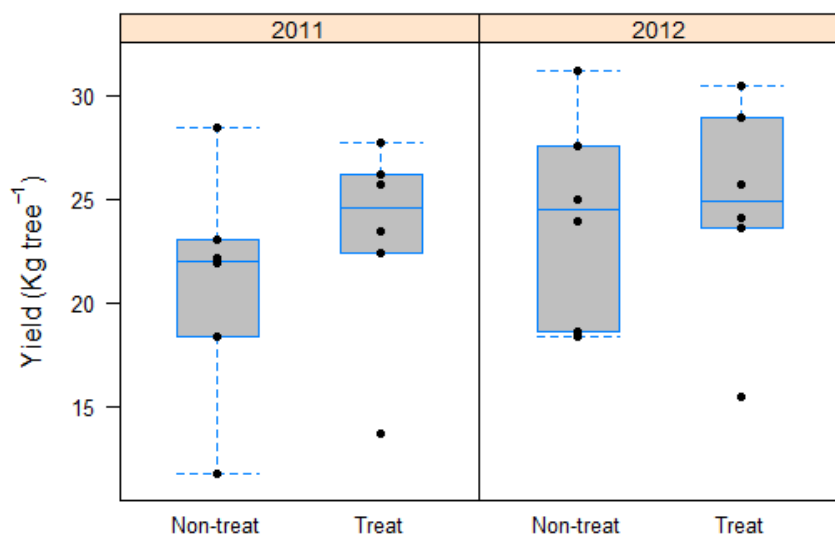
Figure 5. Box-whisker plots of yield of trees treated with fenbuconazole and the non-treated control in experiment 2 on 'Valencia' sweet orange at Miraflores, Panama, in 2011 and 2012.

4. Discussion

In this study, the EC_{50} values for mycelial growth of *Z. citri-griseum* isolates from Panama (n = 34) to fenbuconazole ranged from 0.09 to 7.62 µg mL^{-1} (Table 1, Figure 1). In Florida, Mondal et al. [25] reported EC_{50} values for *Z. citri-griseum* to fenbuconazole ranging from 0.13 to 0.75 µg mL^{-1}. Our results showed a considerable variability of EC_{50}, with some *Z. citri-griseum* isolates showing a phenotype of reduced sensitivity to fenbuconazole. In our study, 20.6% of *Z. citri-griseum* isolates tested had EC_{50} <1 µg mL^{-1}, similar to the EC_{50} values reported by Mondal et al. [25] indicating sensitivity to fenbuconazole. Isolates with lower sensitivity to fenbuconazole had EC_{50} values from 1 to 5 µg mL^{-1} (64.7%) and >5 µg mL^{-1} (14.7%). Rosenzweig et al. [40] categorized phenotypically the sensitivity of *Cercospora beticola* Sacc. to fenbuconazole and other DMI fungicides based on mean EC_{50} values as being sensitive (<1 µg mL^{-1}); with reduced sensitivity (1–10 µg mL^{-1}); moderately insensitive (10–50 µg mL^{-1}); insensitive (50–100 µg mL^{-1}), and resistant (>100 µg mL^{-1}). Based on this classification, our results indicated that 20.6% of the isolates of *Z. citri-griseum* analyzed could be considered sensitive to fenbuconazole and 79.4% with reduced sensitivity.

The reduced sensitivity of *Z. citri-griseum* isolates to fenbuconazole detected in our study suggest that the isolates tested were previously exposed to this fungicide and/or other DMIs. Corio-Costet [41] indicated that the resistance to one DMI fungicide can confer positive cross-resistance to other molecules in the same group. Nevertheless, to elucidate the presence of resistance to DMIs in *Z. citri-griseum* populations in Panama, it would be necessary to detect point mutations or overexpression of the C14α-demethylase (CYP51) by molecular analysis [16]. Another important aspect to consider when interpreting our results is the sample size used to determine the EC_{50}. Franke et al. [42] indicated that is important to define the appropriate sample size to detect differences in fungicide sensitivity. In our study, a total of 34 isolates of *Z. citri-griseum* from different geographic areas of Panama were collected to calculate the EC_{50}. However, in the study carried out by Mondal et al. [25] only five isolates were evaluated. The greater variability in EC_{50} values of *Z. citri-griseum* to fenbuconazole detected in our study might be associated with the greater sample size used, capturing differences associated with the origin of the isolates and potential previous exposure to DMIs. Actually, Franke et al. [42] indicated that the minimum sample size depends on the variation of the EC_{50} values, as a number of individuals less sensitive to the fungicides could significantly change the variance. Our results provide

important data for monitoring sensitivity shifts in *Z. citri-griseum* to fenbuconazole and reduce the risk of fungicide resistance in Panama.

The efficacy of fenbuconazole to reduce the severity of foliar symptoms of greasy spot was evaluated on grapefruit (experiment 1) and sweet orange (experiment 2). In 2011, three sprays with fenbuconazole were applied from May to July resulting in lower disease incidence and severity than the non-treated controls. The reduction of greasy spot severity was illustrated by the 95% confidence intervals of the odds ratios in experiment 1 (30.75–39.47) and experiment 2 (13.21–16.85), not overlapping the null value of 1 in either case. Our results are in line with those obtained in Costa Rica, where two sprays with copper compounds applied in June and August coinciding with the first leaf flush of 'Valencia' sweet orange significantly reduced the incidence and severity of greasy spot and subsequent defoliation [1]. Similar results were reported by Timmer et al. [2] in experiments carried out in Florida on 'Marsh White' and 'Ruby Red' grapefruits with two copper applications in June and August during the spring flush. Mondal and Timmer [21] indicated that one or two fenbuconazole applications before the development of epiphytic mycelium in July completely controlled greasy spot in the spring flush. In our study, the first fenbuconazole spray was applied at the beginning of shoot emergence in May, coinciding with the onset of the infection period of *Z. citri-griseum* in Panama [43].

When a single spray of fenbuconazole was applied in 2012 and 2013 in experiment 1 and 2012 in experiment 2, disease incidence doubled that of 2011 when three sprays were applied. The 95% confidence intervals of the odds ratios for the experiment 1 in 2012 (5.50–6.55) and 2013 (5.47–6.70) were lower than those obtained in 2011, but did not overlap the null value of 1 in either case. Similar results were obtained in experiment 2, with a 95% confidence interval for the odds ratio in 2012 (2.85–3.16) lower than in 2011. The more effective disease control achieved in 2011 was likely due to the two additional sprays applied during the infection period, which probably inhibited the epiphytic growth of the pathogen for a longer period of time.

Although with lower efficacy, disease severity was significantly reduced with a single spray of fenbuconazole compared with the non-treated control. Our results are in line with those by Timmer et al. [2], who reported effective control of greasy spot with a single application of copper applied in June in three experiments carried out in Florida on grapefruit. Similarly, other experiments on grapefruit in Florida also reported reductions in greasy spot severity with a single spray of fenbuconazole applied in June or July [18–20]. Actually, a single spray with petroleum oil applied in mixture with copper from mid-May to June is recommended for greasy spot control in processing sweet oranges in Florida [44]. For processing grapefruits, two sprays are recommended instead [44]. QoI and DMI fungicides, including fenbuconazole, are also recommended for greasy spot control with or without petroleum oil [45]. In the case of fenbuconazole, it provides effective control of the disease on both leaves and fruit [44]. In our case, no rind blotch symptoms on fruit were observed in the field trials, nor in our previous surveys [4,31].

Despite the significant reduction of greasy spot severity on leaves in the trees treated with fenbuconazole, particularly in 2011 when three sprays were applied, no significant differences in yield were found in any of the experiments. Timmer et al. [46] reported similar results in Texas, where several fungicide spray programs were evaluated for greasy spot control on grapefruit during 4 consecutive years. Despite greasy spot severity on leaves and rind blotch incidence on fruit were significantly reduced, yields (kg tree^{-1}) were not significantly improved. Likewise, Showler et al. [11] reported in Texas that sprays with aqueous organic mixtures applied for three consecutive years reduced disease incidence in grapefruit, but differences in yield (No. fruit tree^{-1}) were only detected the first year. In our case, experiments for greasy spot control should probably have covered a longer period of time, allowing the treated trees to completely recover their leaf area and thus have an impact on yields. Furthermore, the higher control efficacy observed when three fenbuconazole sprays were applied instead of one indicates that intensive spray program would be probably needed under the rainfed conditions of Panama. In fact, in Florida two applications are needed when severe defoliation due to greasy spot occurred in the previous year [44]. In those cases, the first spray should

be applied from mid-May to June and the second soon after the major summer flush has expanded [44]. In addition to the disease itself, it is important to consider the concurrence of other limiting agronomic factors. Rainfed conditions and typical crop management practices in Panama may result in nutritional deficiencies and seasonal water stress (January to March), which could seriously limit citrus yields and thus masking the effects of disease control.

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