

Fungicide Sensitivity among Isolates of *Colletotrichum truncatum* and *Fusarium incarnatum-equiseti* Species Complex Infecting Bell Pepper in Trinidad

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Bell pepper is an economically important crop worldwide; however, production is restricted by a number of fungal diseases that cause significant yield loss. Chemical control is the most common approach adopted by growers to manage a number of these diseases. Monitoring for the development to resistance to fungicides in pathogenic fungal populations is central to devising integrated pest management strategies. Two fungal species, *Fusarium incarnatum-equiseti* species complex (FIESC) and *Colletotrichum truncatum* are important pathogens of bell pepper in Trinidad. This study was carried out to determine the sensitivity of 71 isolates belonging to these two fungal species to fungicides with different modes of action based on *in vitro* bioassays. There was no significant difference in log effective concentration required to achieve 50% colony growth inhibition (LogEC_{50}) values when field location and fungicide were considered for each species separately based on ANOVA analyses. However, the LogEC_{50} value for the Aranguez-Antracol location-fungicide combination was almost twice the value for the Maloney/Macoya-Antracol location-fungicide combination regardless of fungal species. LogEC_{50} values for Benomyl fungicide was also higher for *C. truncatum* isolates than for FIESC isolates and for any other fungicide. Cropping practices in these locations may explain the fungicide sensitivity data obtained.

Keywords : anthracnose, *Fusarium*, integrated pest management, sweet pepper

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The top five ranked producers of bell peppers (*Capscium annuum* L.), between 2007 and 2011, were China, Mexico, Turkey, Indonesia, and Spain (FAOSTAT, 2011). For the grower, selection of bell pepper cultivars is dependent on disease resistance, growing conditions, performance and yield. Protected-culture technologies for pepper production offers the grower a greater level of control over growing conditions especially with respect to chemical use and fertilizer input (Correll and Thornsbury, 2013). Field-grown bell peppers are more susceptible to a range of bacterial, virus and fungal diseases and this continues to be a limiting factor to pepper production (Black et al., 1991).

Bell pepper is an important crop grown throughout Trinidad and field production is mainly concentrated in the Northeast and Southeast regions (Mohammed, 2013). The cost of greenhouse production systems remains prohibitive, USD 1.13 per 0.5 kg for greenhouse production vs USD 0.48 per 0.5 kg for open field production (Seepersad et al., 2013). Barbados is the only current export market and exports have fluctuated between 2006 and 2010 perhaps as a result of changing market value. There has been a decline in the importation of bell peppers from 2008 to 2012 with the lowest recorded trade occurring in 2012 (Mohammed, 2013). Over the last five years, yield has been affected by fungal diseases that cause fruit rot. Several *Colletotrichum* species are implicated as causal agents of anthracnose in bell pepper, including *C. gloeosporioides*, *C. coccodes*, *C. acutatum*, and *C.*

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truncatum (syn. *C. capsici*; Damm et al., 2009). Different *Colletotrichum* species infect pepper at different stages of maturity, e.g., *C. gloeosporioides* and *C. acutatum* have been reported to infect both young and mature green fruit, whereas *C. truncatum* has been shown to infect mature red-fruits (Ramdial and Rampersad, 2015; Than et al., 2008). *Colletotrichum* is capable of causing quiescent infection where infection of the plant in the early stages of development, the fungus exhibits biotrophy and inactivity for most of the plant's development, and then becomes necrotrophic during the ripening and senescence stages (Harp et al., 2008; Prusky and Lichter, 2007). Fruit rot can also extend to the seed cavity which may lead to infection of the seed. In Trinidad, *C. truncatum* infects mature red fruit and causes severe fruit rot. Symptoms are seen as a single, dry, large lesion or as multiple lesions (Fig. 1A). Field occurrence and yield loss are estimated between 90% and 100% and 40% and 60%, respectively.

Members of the *Fusarium* genus are economically important plant pathogens. They are ubiquitous fungi, found in almost every terrestrial ecosystem (Ploetz, 2006) and are known for causing disease in a wide range of weed, forest, ornamental and crop host species (Abu Bakar et al., 2013). Several *Fusarium* species are associated with fruit rot in susceptible *Capsicum* sp.; however, in Trinidad, *Fusarium incarnatum-equiseti* species complex (FIESC) has been identified as the causal agent of fruit rot in bell pepper (Ramdial et al., 2016). Symptoms are seen as black, water-soaked lesions that begin around the calyx but can develop anywhere on the mature fruit (Fig. 1B). There is evidence of internal decay in some cases. To date, the disease has been detected in fields located in Northwest and Northeast Trinidad and yield loss is estimated between 30% and 50% of mature red fruit.

Although anthracnose disease resistance is available in

some varieties of chili peppers, there are no bell pepper cultivars that are resistant to anthracnose or *Fusarium* (AVRDC, 1999, 2000; Yoon et al., 2004). Development of cultivars with a shorter ripening period or with a thicker waxy cuticle may allow the fruit to escape infection by the fungus (Harp et al., 2008; Lewis-Ivey et al., 2004). Chemical control is among the most commonly used strategies for management of fungal diseases (Gang et al., 2015). Chemical control of anthracnose includes use of benzimidazoles, strobilurins, dicarboximides, and demethylation inhibitors which are single-site mode-of-action fungicides (Young et al., 2010). However, for late maturing red peppers, maneb (Group M3), azoxystrobin (Quadris, Fungicide Resistance Action Committee [FRAC] Group 11), trifloxystrobin (Flint, FRAC Group 11), and pyraclostrobin (Cabrio, FRAC Group 11) have been suggested for the control of anthracnose (Alexander and Waldenmaier, 2000, 2001, 2003; Zitter, 2011). Marvel (2003) stated that azoxystrobin in combination with maneb, offers a high level of protection against anthracnose in green pepper in field trials. There are no fungicides registered to control *Fusarium* fruit rot in bell pepper.

Fungicide resistance can occur as a result of genetic mutations of target proteins; exclusions and expulsion of fungicidal compounds via intrinsic efflux pumps such as ATP-binding cassette (ABC) transporters; overexpression of target proteins; and detoxification of the fungicides through development of catabolic pathways (FRAC, 2014, 2015). Resistance arising from mutations in target proteins is reported for single-site inhibitors and there is less risk of developing resistance with multi-site inhibitors (FRAC, 2014). Monitoring the development of fungicide resistance is necessary to (i) detect for selection of resistant phenotypes after prolonged chemical use, (ii) streamline chemical control strategies and cost associated with



Fig. 1. (A) Symptoms of *Colletotrichum truncatum* infection on mature bell pepper fruit. (B) Symptoms of *Fusarium* sp. infection on mature bell pepper fruit.

chemical use, and (iii) reduce environmental impact due to accumulation of fungicides (Gang et al., 2015). This study was, therefore, conducted to determine the sensitivity of *Fusarium* and *Colletotrichum* isolates infecting bell pepper to fungicides with different modes of action.

Materials and Methods

Isolate collection. Seventeen fields were sampled for anthracnose fruit rot infection in the major production areas in Trinidad (see Tables 1 and 2 for sampling locations). Symptomatic fruit with diagnostic symptoms were collected and surface disinfested by rinsing in 70% ethanol for 1 min followed by another rinse in 0.6% sodium hypochlorite solution for 1 min. Samples were then washed three times in sterilized distilled water and dried on sterilized tissue paper in a laminar flow hood. A 4-mm³ block

of tissue was cut from the edge of the lesion and placed in the center of STEA plates (2% water agar amended with 50 mg/l streptomycin and 50 mg/l tetracycline [Sigma-Aldrich Co., St. Louis, MO, USA] and 5% absolute ethanol). STEA plates were incubated for five days at 25°C in the dark. After incubation, a 4-mm³ block of agar taken from the advancing mycelial edge of a 5-day old STEA culture was removed and placed in the center of a potato dextrose agar plate (PDA; Oxoid Ltd., Basingstoke, UK) supplemented with 50 mg/l streptomycin and 50 mg/l tetracycline (Sigma-Aldrich Co.). Cultures were incubated for seven days at 25°C in the dark. Monoconidial cultures were subsequently obtained through serial dilution. Isolates were maintained on PDA slants at 4°C for temporary storage, and as conidial suspensions in 50% glycerol at -70°C for long-term storage.

Table 1. LogEC₅₀ values determined for isolates belonging to the *Fusarium-incarnatum*-*equiseti* species complex

n	Location	Region*	Fungicide	Regression equation	R ² -value	LogEC ₅₀
25	Arang	Northwest	Antracol	$y = 3.531x + 4.381$	0.996	12.919
13	Mal/Mac	North Central	Antracol	$y = 5.467x + 8.152$	0.992	7.654
25	Arang	Northwest	Criptan	$y = 3.423x + 5.104$	0.927	13.116
13	Mal/Mac	North Central	Criptan	$y = 1.722x + 0.574$	0.600	28.703
25	Arang	Northwest	Benomyl	$y = 4.182x + 3.552$	0.883	11.107
13	Mal/Mac	North Central	Benomyl	$y = 13.624x + 11.327$	0.835	2.839
25	Arang	Northwest	Valete	$y = 7.459x + 9.562$	0.961	5.421
13	Mal/Mac	North Central	Valete	$y = 7.985x + 6.907$	0.955	5.397

LogEC₅₀, log effective concentration required to achieve 50% colony growth inhibition.

*Region key: Arang, Aranguez (5 fields); Mal and Mac, Maloney (3 fields) and Macoya (3 fields).

Table 2. LogEC₅₀ values determined for *Colletotrichum truncatum* isolates infecting bell pepper in Trinidad

n	Location	Region*	Fungicide	Regression equation	R ² -value	LogEC ₅₀
12	Arang	Northwest	Antracol	$y = 3.569x + 5.902$	0.991	12.356
10	Mal/Mac	North Central	Antracol	$y = 4.573x + 13.127$	0.988	8.063
11	BA/P	South	Antracol	$y = 6.025x + 11.457$	0.998	6.397
12	Arang	Northwest	Criptan	$y = 4.628x + 3.775$	0.711	9.988
10	Mal/Mac	North Central	Criptan	$y = 5.394x + 8.779$	0.978	7.642
11	BA/P	South	Criptan	$y = 4.776x + 12.937$	0.925	7.76
12	Arang	Northwest	Benomyl	$y = 2.43x$	0.777	20.58
10	Mal/Mac	North Central	Benomyl	$y = 7.105x$	0.736	27.04
11	BA/P	South	Benomyl	$y = 2.339x + 0.086$	0.526	21.34
12	Arang	Northwest	Valete	$y = 5.973x + 7.732$	0.963	7.076
10	Mal/Mac	North Central	Valete	$y = 7.168x + 12.305$	0.989	5.259
11	BA/P	South	Valete	$y = 8.733x + 12.216$	0.969	4.327

LogEC₅₀, log effective concentration required to achieve 50% colony growth inhibition.

*Region key: Arang, Aranguez (5 fields); BA, Bonne Aventure (4 fields); Mal and Mac, Maloney (3 fields) and Macoya (3 fields); P, Penal (2 fields).

PCR amplification, sequencing and identification of isolates. Fresh mycelium was harvested from cultures grown in potato dextrose broth and used in genomic DNA extraction using the cetyl trimethylammonium bromide (CTAB) extraction method. PCR amplification was carried out using two primers pairs that target the ITS1-5.8S-ITS2 rDNA region (ITS4/5 primers; White et al., 1990) and the translation elongation factor 1 alpha (EF1/2 primers) under published thermal cycling conditions (Geiser et al., 2004; O'Donnell et al., 2015). For a single 25 µl reaction, PCR components (Invitrogen; Life Technologies Co., Gaithersburg, MD, USA) included 1× PCR buffer; 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U Taq DNA polymerase and 50 pmoles of each primer (Integrated DNA Technologies, Coralville, IA, USA). PCR amplification thermal conditions consisted of an initial denaturation of 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C with a final extension of 5 min at 72°C. PCR products were sequenced directly and in both directions (Amplicon Express, Pullman, WA, USA). Isolates were then identified based on Blast queries with cognate sequences deposited in GenBank and the FUSARIUM-ID sequence repositories.

In vitro bioassay. The sensitivity of isolates to four fungicides (Antracol, Criptan, Benomyl, and Valete) was determined using the amended agar screening method. The selection of fungicides was based on confirmed use in bell pepper fields by growers in Trinidad (survey conducted by Ramdial, unpublished). A summary of each fungicide's FRAC grouping, mode of action, registered for controlling anthracnose or *Fusarium* and evidence of resistance is given in Supplementary Table 1 (Davidse, 1986; Dirou and Stovold, 2005; Gordon, 2011; Jarvis et al., 1994; Lucas et al., 2015; Lukens, 1969; Owens and Novotny, 1959; Richmond and Somers, 1963; Rosenberger, 2013; Turechek, 2004). Stock solutions of the commercially available formulation of each fungicide were prepared in acetone (Rampersad, 2011; Zhang et al., 2010). PDA media were amended with 0, 0.1, 1.0, 10.0, and 100.0 µg/ml of fungicide. A total of 71 isolates were screened ($N = 38$ FIESC; $N = 33$ *C. truncatum*). Four replicates of each fungicide concentration were used for each isolate and the experiment was conducted twice. Cultures were prepared by removing a 4-mm³ block of mycelia from the leading edge of an actively growing 7-day-old culture and placing it mycelium-side-down in the center of fungicide-amended media. The plates were incubated at 25°C for five days and the radial diameter of each colony was measured (orthogonal measurements) for each isolate to determine percentage of relative growth inhibited compared to the growth on non-amended media. Mean values were used

in subsequent analyses as values did not differ significantly between experiments. The log effective concentration required to achieve 50% colony growth inhibition (LogEC₅₀) on fungicide-amended media was calculated for isolates based on *in vitro* bioassays.

Statistical tests. Linear regression analyses were conducted to determine the LogEC₅₀ values for the fungal isolates according to field location. Analysis of variance (ANOVA) of the LogEC₅₀ values (mycelia growth of the control vs the Log₁₀ of the fungicide concentration) was carried out (Minitab v.17; Minitab, State College, PA, USA). A comparison of means LogEC₅₀ values using Fisher's least significant difference and Tukey's multiple comparison of means tests was also done.

Results

The influence of species, fungicides used, as well as the location of isolate collection, on calculated LogEC₅₀ values for each of the two fungal species were determined based on ANOVA analyses (Table 1, 2). The *t*-tests were also conducted to compare the LogEC₅₀ values of FIESC isolates against those of *C. truncatum* isolates. With respect to FIESC, there was no significant influence of fungicide on LogEC₅₀ values ($P = 0.219$). This was further confirmed by Tukey's and Fisher's tests where there were no significant differences among LogEC₅₀ values of various fungicide groupings ($P > 0.05$), e.g., Criptan-Antracol ($P = 0.456$). There was also no significant influence of location on LogEC₅₀ values ($P = 0.938$). It should be noted that the Bon Aventure/Peñal location was not included in this analysis as no FIESC isolates were collected here.

ANOVAs were also performed on *C. truncatum* isolates, to assess the influence of fungicide use, and the location of sample collection, on the LogEC₅₀ values calculated (Supplementary Table 2). It must be noted that *C. truncatum* isolates were obtained from the Bon Aventure and Peñal locations, and, therefore, LogEC₅₀ values were included in this analysis. Results showed significant influence of fungicide on LogEC₅₀ values ($P = 0.000$). Both Tukey's and Fisher's tests indicated that mean LogEC₅₀ values were grouped separately for Benomyl as these values were significantly higher than for the other fungicides for *C. truncatum* isolates. For the combined data set, however, results showed no significant influence of fungicides on LogEC₅₀ values ($P = 0.082$). This was further confirmed by Tukey's and Fisher's tests where there were no significant differences among mean LogEC₅₀ values and all means fell within the same grouping.

The effect of species and fungicide on LogEC₅₀ values, the effect of species and location on LogEC₅₀ values and

the effect of location and fungicide on LogEC₅₀ values were also determined separately (Supplementary Table 2). None of the paired factors had a significant effect on LogEC₅₀ values ($P > 0.05$), e.g., species \times fungicide ($P = 0.293$), species \times location ($P = 0.711$), and location \times fungicide ($P = 0.314$).

The t -tests were also performed to compare LogEC₅₀ values of each species according to fungicide and field location. Equal variances were assumed for all tests, with 95% confidence interval. When the test was carried out to compare LogEC₅₀ values for all Aranguez isolates versus all Maloney and Macoya isolates of both species regardless of fungicide screened (combined data set), there was a significant difference in mean LogEC₅₀ values ($P = 0.005$). Aranguez isolates had a significantly higher LogEC₅₀. When LogEC₅₀ values of all *C. truncatum* and FIESC isolates for individual locations and fungicides were compared, the Aranguez-Antracol combination was significantly different from all other pairwise comparisons ($P = 0.004$). Similarly, the Maloney/Macoya-Antracol combination ($P = 0.017$) was significantly different from other pairwise combinations. All other location-fungicide pairwise comparisons of LogEC₅₀ values for all isolates were not significant ($P > 0.05$). For example, Aranguez-Criptan ($P = 0.086$), Maloney/Macoya-Criptan ($P = 0.334$), Aranguez-Benomyl ($P = 0.185$), Maloney/Macoya-Benomyl ($P = 0.256$), and Aranguez-Valete ($P = 0.084$).

Discussion

Two fungal species, FIESC and *C. truncatum* are important pathogens of bell pepper in Trinidad. There is no single fungicide that is effective against all fungal pepper diseases. Therefore, it is necessary to screen different chemistries to optimize chemical use for management of different fungal diseases in bell pepper. This study was carried out to determine the efficacy of various fungicides with different modes of action to control 71 isolates belonging to two fungal species that affect bell pepper in Trinidad.

Another important consideration is fungicide resistance management. *Colletotrichum* spp. are reported to become resistant to benzimidazole fungicides after prolonged use with ultimate selection for resistant isolates in the fungal population (Ramdial et al., 2016). The risk factors for developing fungicide resistance in a given fungal population depends on a number of interacting factors including, (i) the fitness advantage offered to resistant mutants, (ii) the population size, reproductive rate and history of resistance of the target pathogen, (iii) repetitive and sustained fungicide use especially single-site inhibitors, (iv) no integration with other complementary non-chemical

control methods, (v) exceeding the dose rate and timing of application, (vi) use of chemicals as an eradicator rather than preventative application, and (vii) cross-resistance among single-site inhibitors as is the case with benzimidazoles (Brent and Hollomon, 2007). Alternating products in different fungicide groups is, therefore, highly recommended.

The findings of this study indicated that there was no significant difference in LogEC₅₀ values when field location and fungicide were considered for each species separately based on ANOVA analyses. However, the LogEC₅₀ value for the Aranguez-Antracol location-fungicide combination was almost twice the value for the Maloney/Macoya-Antracol location-fungicide combination regardless of fungal species. Antracol is a multi-site inhibitor and the risk of developing resistance is low. The higher LogEC₅₀ values obtained for this fungicide for Aranguez may be a reflection of difference in dosage requirement for these two fungal species infecting bell pepper in Trinidad and not necessarily a case of resistance. Resistance to Antracol has not yet been reported (Bayer CropScience Egypt, 2016).

There was a significant difference in LogEC₅₀ values calculated for Benomyl fungicide compared to the values obtained for the other three chemicals. *C. truncatum* had the highest LogEC₅₀ values for this fungicide compared to the other three chemicals. Ramdial et al. (2016) determined that *C. truncatum* infecting bell pepper was resistant to Benomyl. This fungicide was included in this study for comparison with the LogEC₅₀ values for FIESC isolates infecting bell pepper. These values, therefore, reflect resistance to this fungicide by *C. truncatum* isolates. FIESC isolates had significantly lower LogEC₅₀ values compared to *C. truncatum*. Molecular work to characterize FIESC isolates for the detection of the E198A genotype among Benomyl resistant isolates would have to be carried out to confirm whether the LogEC₅₀ values obtained for this fungus species reflect resistance or dosage requirements for this fungicide.

By examining the history of agriculture at the Aranguez, Maloney and Macoya locations, the fungicide sensitivity data obtained in this study may be explained. In the late 1800s to early 1900s, Aranguez was originally run as a sugar cane estate (North Aranguez) and rice plantation (South Aranguez) (Gomes, 1981). By the late 1930s, as the petroleum industry expanded in Trinidad, sugar cane and rice production declined, and vegetable crop cultivation which was previously done on a small scale, progressively increased and completely replaced sugar cane cultivation. Year-round vegetable crop cultivation was boosted by the implementation of an irrigation scheme through the construction of a dam, access roads, rapidly expand-

ing urban districts, and food shortages during World War II (1941–1945). However, by 1950, growers reported a decline in soil fertility perhaps as a result of soil depletion from sugar cane cultivation, and a difficulty in cultivating in certain soil types, e.g., silty-clay loam, with some areas remaining unproductive due to salt water infiltration (Bell, 1950). As a result, fields were developed outside of Aranguez, in Maloney, Wallerfield, Macoya, and Valencia areas. To date, Aranguez is still known as a major vegetable production area in Trinidad with intensive crop cultivation supported by a heavy reliance on agro-chemical use, e.g., fertilizers, pesticides, fungicides and herbicides (Kenny, 2008). It is proposed that chemical use, particularly in Aranguez, is related to a history of (i) poor soil quality, (ii) monoculture practices, (iii) unavailability of high performing cultivars which may have contributed to plants with relatively higher sensitivity to infection, and (iv) indiscriminate use of chemicals all of which may have resulted in fungal populations with detectable levels of resistance to certain chemicals or with relatively higher LogEC₅₀ values to certain chemicals.

Apart from screening for sensitivity to differently acting fungicides, to further optimize strategies to control anthracnose and *Fusarium* fruit rot in bell pepper in Trinidad, it may be useful to quantify the environmental spore load in both productive and abandoned fields. Research should also be conducted to determine the relationship between fruit rot and spore pressure, fruit load, fruit position on the plant, growing conditions, and cultivar. Although anthracnose has been detected in all fields sampled in this study, FIESC has only been found in fields located in Northwest and Northeast Trinidad.

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