Differential Responses of *Colletotrichum gloeosporioides* and *C. truncatum* Isolates from Different Hosts to Multiple Fungicides Based on Two Assays

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Abstract

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Anthrachnose is one of the most important postharvest diseases of many economically important crops worldwide. This study was conducted with the objective of investigating the sensitivity of *Colletotrichum gloeosporioides* and *C. truncatum* isolates to multiple fungicides with different modes of action. The study analyzed quantitative sensitivity data derived from conventional amended agar (AA) assays and qualitative spore responses obtained from a novel microtiter bioassay that is based on reduction of a viability dye, Alamar blue (AB). Generally, for AA assays, the percent growth inhibition (%RGI) increased with increasing concentration for all isolates and all fungicides, except for copper hydroxide. *C. truncatum* isolates reacted differently to increasing concentrations of the various fungicides depending on whether the isolates originated from pepper or papaya. *C. truncatum* from pepper had generally less %RGI than *C. truncatum* isolates from papaya. *C. gloeosporioides* isolates from papaya had generally higher %RGI than *C. truncatum* isolates for all concentrations tested for pyraclostrobin, chlorothalonil, and fosetyl-aluminum. *C. gloeosporioides* isolates from pepper had generally higher %RGI than *C. truncatum* isolates for all

concentrations tested for most fungicides. In all cases, *Colletotrichum* sp. and fungicide had significant ($P \leq 0.001$) effects on the log concentration of fungicide for which relative growth was inhibited by 50 and 90% (log EC50 and log EC90, respectively) calculated for all isolates, regardless of whether values were compared for only *C. gloeosporioides* isolates or only *C. truncatum* isolates. Correlation analyses of log EC50 and log EC90 values of all the isolates revealed a nonsignificant association for pyraclostrobin. In AB assays, all fungicides had an equivalent effect at inhibiting spore germination at the lower concentrations. According to binary logistic regression analyses, species, isolate, and fungicide concentration had significant predictive value in determining whether an AB test would be positive. Sequence alignments between *C. gloeosporioides* isolates and *C. gloeosporioides* f. sp. *aeschyromene* revealed no base substitutions at codons 198, 199, 200, and 240; however, sequence comparisons between *C. truncatum* isolates and *C. gloeosporioides* f. sp. *aeschyromene* revealed two codon changes located outside of the identified codon 198 or 200 associated with the benzimidazole-resistant phenotype of *C. gloeosporioides* isolates.

Filamentous fungi that are members of the genus *Colletotrichum* are one of the most destructive plant pathogens worldwide (10). These fungi are responsible for anthracnose disease, which is one of the most economically important postharvest diseases of a wide range of tropical and subtropical fruit (33,47,53). Infection results in severe losses due to significant reductions in fruit quantity, quality, and product value. Effective control of *Colletotrichum* infection usually involves the use of a combination of cultural, biological, and chemical control and intrinsic resistance. The pathogen's ability to cause quiescent or latent infections at both the pre- and post-harvest stages; spore dispersal by surface water or rain splash; continuous cropping practices supported by tropical climates, which ensure rapid build-up of inoculum; a lack of commercially available resistant cultivars, especially for fruit crops; and development of resistance to certain fungicides challenge successful management of *Colletotrichum* infection (23,29,34).

Fungicides are used extensively for post-harvest disease control in fruit and vegetables caused by *Colletotrichum* spp.; however, their use may be restricted by the relative efficacy of the fungicide, the *Colletotrichum* sp. associated with infection, the time of infection, the availability of the product for a specific host species, inoculum density and disease pressure, negative effects on fruit ripen-

ing, phytotoxic effects, prevailing climatic conditions, and pesticide regulations that apply to producing countries as well as destination countries with specific importation restrictions (47).

Avocado (*Persea americana* Mill.), mango (*Mangifera indica* L.), and papaya (*Carica papaya* L.) are among the top 10 tropical fruit crops that are produced worldwide (27). In Trinidad, avocado, mango, and papaya production increased between 30 and 40% over the last 10 years to accommodate export and various processing markets (27). Anthracnose disease of these fruit is arguably one of the most economically important diseases throughout production areas and is usually caused by *Colletotrichum gloeosporioides* although *C. acutatum* has also been implicated in some locations (21,24,61,68). In addition to *C. gloeosporioides*, *C. truncatum* (synonym *C. capsici*; 19) has also been reported to infect papaya in Trinidad (57), Indonesia (60), Florida (64), Mexico (63), and Japan (70).

Generally, chemical control of anthracnose in these fruit crops includes the use of dithiocarbamates such as zineb, maneb, and captan but mancozeb use is restricted in fruit carded for export to certain countries due to residue accumulation on fruit surfaces (2,8). Copper fungicides and benomyl, when applied as pretreatments, delayed onset of symptoms in mango, avocado, and papaya (4,5,59). In Trinidad, alternating applications of mancozeb, benomyl, copper fungicides, and, more recently in some fields in South Trinidad, pyraclostrobin are used to control a number of fungal diseases in papaya, including anthracnose and *Phytophthora* fruit rot. Little to no chemical control is used for anthracnose of mango and avocado in Trinidad.

C. gloeosporioides and *C. acutatum* are commonly associated with anthracnose of pepper; however, in Trinidad, the disease on red-ripe fruit is caused by both *C. gloeosporioides* and *C. truncatum* (Ramdial and S. N. Rampersad, unpublished data). To date, there are no reports of the disease on immature green fruit (40). Chemical control of bell pepper anthracnose include the use of

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*The e-Xtra logo stands for "electronic extra" and indicates that Figures 1 and 2 appear in color online.

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copper fungicides, chlorothalonil (alone or in mixes), maneb, and, more recently, strobilurins such as azoxystrobin, trifloxystrobin, and pyraclostrobin (alone or in mixes; 40,62). In Trinidad, chlorothalonil, benomyl, and copper fungicides are used to control fungal pathogens in bell pepper. Thiophanate-methyl and tolclofos-methyl are generally used in soil preparation prior to planting.

Conventional methods to assess fungicide sensitivity are based on measuring radial diameter of colonies on amended agar (AA) compared with growth on nonamended agar (6,7); however, these methods only monitor mycelial growth and cannot determine the effects of chemicals on spore viability. The Alamar blue (AB) assay monitors the reducing environment that is indicative of metabolically active cells or spores. The reducing environment of the cells or spores is measured through the conversion of resazurin (oxidized form of the dye is blue) to resorufin (reduced form of the dye is pink) either colorimetrically or fluorimetrically. The assay, therefore, can be performed with the objective of attaining qualitative (visual inspection for color change of the dye) or quantitative (absorbance or fluorescence) measurements of cell or spore viability. Compared with other viability dyes, AB dye is (i) water soluble; (ii) nontoxic to cells, resulting in no metabolic interference; (iii) nontoxic to user because it is noncarcinogenic; (iv) stable in culture, which allows continuous monitoring of cells over a period of time; and (v) nontoxic to the environment, so that there are no organic waste disposal issues (1,54,65). One disadvantage of this assay, however, is that it is not a direct cell-counting technique like hemocytometry, and the absorbance or fluorescence signal can be affected by a number of factors, including cell or spore density, incubation time and temperature, pH and nutrient content of the medium, and addition of chemicals that reduce the dye (1,54,57). Initially, AB has been used over the past 50 years in studies pertaining to cell viability and cytotoxicity in a range of biological and environmental systems, including the assessment of antimicrobial compounds (3,17,28,39,43,44,51,52). It offers several advantages over other metabolic indicators and other methods of measuring cell viability and cell proliferation. However, as with any bioassay, suitability must be determined for each application and cell model. Recently, the assay has been applied to studying fungicide sensitivity of plant pathogens *Monilinia fruticola* (18), *Botrytis cinerea* (49), *Verticillium dahliae* (56), and *Alternaria alternata* (66). By investigating fungicide sensitivity using both conventional AA and spore-viability assays, a more complete assessment of the impact of various fungicides with different modes of action on survival of the fungus can be obtained because two fitness parameters, mycelial growth and spore viability, can be evaluated.

To effectively manage anthracnose disease in a number of economically important plant species, it is critical to determine the relative fungicide sensitivity of the *Colletotrichum* spp. responsible for these infections. In Trinidad, it is uncertain whether *Colletotrichum* isolates have developed resistance to commonly used fungicides to control anthracnose disease of avocado, mango, papaya, and bell pepper or whether a lack of effective control is due to use of fungicides that have little effect on a given *Colletotrichum* sp. responsible for disease. The objectives of this study were to (i) evaluate the relative fungicide sensitivities of *C. gloeosporioides* and *C. truncatum* isolates from various host species (avocado, mango, papaya, and bell pepper) to fungicides with different modes of action based on two methods of chemical toxicity testing, (ii) determine the percent relative growth inhibition and log EC values for these isolates based on AA tests, (iii) assess the effects of increasing concentration of fungicides on spore respiration based on qualitative AB tests, (iii) evaluate the effects of *Colletotrichum* spp. on fungicide sensitivity, and (iv) characterize benomyl-resistant genotypes based on amino acid sequence comparisons of the β -tubulin gene.

Materials and Methods

Collection of isolates. Isolates were collected during the period August 2009 to June 2010 from avocado, mango, papaya, and bell

pepper cultivations throughout Trinidad. Isolations were made by first surface sterilizing the infected material (rinsing in 0.6% sodium hypochlorite for 1 min, rinsing in 70% ethanol for 2 min, then a final washing in sterile distilled water for 2 min). A 4-mm³ section of tissue taken from the edge of the lesion was plated on 2% water agar (BactoAgar; Difco Laboratories) supplemented with streptomycin, tetracycline, and chloramphenicol (Sigma-Aldrich) at 50 mg/liter each. Cultures were incubated at 25 $\pm$ 1°C for 5 days. Emerging colonies were then transferred to potato dextrose agar (PDA; Oxoid Ltd.). Monoconidial cultures were then prepared and used in subsequent assays. Avocado and mango isolates were considered to give baseline responses for all of the fungicides tested in this study because none of these fungicides were used in the orchards from which these isolates were collected. Conversely, papaya and pepper isolates were collected from fields with a 5-year spray history of Acrobat, Banrot, benomyl, Mankocide, Rizolex, Vacomil, and copper-based fungicides. Bellis, Cabrio, and Polyram were relatively new fungicides on the market and there was only one report of Bellis use that started in 2010 in papaya cultivations in South Trinidad. Importantly, it should be noted that growers generally apply a cocktail of pesticides, and the mix ratio and application rates may or may not follow the recommended guidelines of the manufacturer.

Molecular characterization of isolates. Total genomic DNA was isolated using the E.Z.N.A. SP fungal DNA extraction kit (Omega Bio-tek Inc.). Polymerase chain reaction (PCR) amplification was carried out using internal transcribed spacer (ITS)4 and -5 primers that target the internally transcribed spacer region of the nuclear ITS1-5.8S-ITS2 rDNA (67) and Bt2a/b primers that partially amplify the β -tubulin gene (31). PCR products were sequenced in both directions (Amplicon Express). Comparisons were made with sequences available on the GenBank database using the gapped BLAST algorithm in order to confirm the identity of the isolates. To determine whether sensitivity to benomyl was due to base substitutions in the β -tubulin region, TUB2-L/R primers were utilized (51). PCR products were sequenced and the translated nucleotide sequences were compared with cognate sequences of the β -tubulin gene of reference type *C. gloeosporioides* f. sp. *aeschynomene* (GenBank accession number U14138) (14). Amino acid sequence alignments and identification of codon changes were carried out using the MegAlign module of Lasergene software (version 7.0; DNASTAR, Inc.).

Selection of chemicals. Seven chemicals were selected based on four criteria: (i) fungicides currently used in Trinidad to control anthracnose in papaya, bell pepper, avocado, and mango; (ii) fungicides currently used or registered in other countries but not necessarily for the plant species of this study; and (iii) fungicides not registered for anthracnose specifically. Commercial formulations were purchased from local retailers (with the exception of Cabrio, which was supplied directly by BASF Corp.) and stored in a desiccator at 25°C to preserve their activity (Table 1). Commercial formulations, and not the active ingredients, were evaluated in an attempt to improve the predictive value of the in vitro sensitivity assays by ensuring that pathogen responses were based on the formulation that they would be exposed to in the field. Each chemical was prepared in dimethyl sulfoxide (DMSO, ACS reagent $\geq$ 99.9%; Sigma-Aldrich) as stocks of 1, 0.1, and 0.01 g/ml and then filter sterilized (0.22- μ m filter). The final concentration of DMSO was maintained below 0.1% (56). The copper-based fungicide was prepared in sterile deionized water.

AA assays. Sensitivity of the isolates to different fungicides was determined by AA assays. Molten PDA was amended with stock fungicide solution to give final concentrations of 0.0, 0.01, 0.1, 1.0, 10.0, 100.0, and 1,000.0 μ g/ml for each fungicide. A 4-mm³ agar block was cut from the advancing edge of an actively growing culture on PDA and placed centrally, mycelia-side down, on the surface of the AA. Plates were incubated in the dark at 25°C for 6 days. The radial diameter (perpendicular measurements in millimeters) was recorded for each colony and the corrected diameter (mean radial diameter minus the length of the agar block) was used

to calculate percent relative growth (%RG = [mean diameter of colony on AA/mean diameter of colony on control or nonamended agar] × 100) and percent relative growth inhibition (%RGI = 100 – %RG) compared with the nonamended controls. Control plates consisted of a 4-mm³ agar block placed on nonamended PDA. Each isolate was plated in quadruplicate. The assays were conducted twice and the mean corrected colony diameters were used in all calculations.

AB assays. Sensitivity of the isolates to different fungicides was qualitatively determined by an AB assay according to the optimization procedure by Rampersad (56). Two isolates from each host species were selected (eight isolates in total). The AB test was carried out in a laminar flow hood using aseptic techniques. Particulates in canned V8 juice were first removed by filtering the juice through four layers of mira cloth (Calbiochem). The clarified supernatant was then used to prepare a 10% broth concentration using deionized water. The diluted juice was then buffered with morpholinepropanesulphonic acid (Sigma-Aldrich), the pH was adjusted to 7.4 at 25°C, and the juice was then autoclaved at 121°C for 20 min.

Nine-day old cultures that were actively growing on 10% V8 juice agar (10% clarified, buffered V8 juice containing 2% BactoAgar) were used to prepare stock conidia suspensions for each isolate. The plates were flooded by adding 5 ml of clarified, buffered 10% V8 juice broth; then, the surface of the agar was gently scraped with a sterile rubber spatula and the resulting suspension that contained dislodged conidia was filtered through two layers of sterile mira cloth. The stock conidia concentration was determined by dark-field contrast microscopy at ×100 using a hemocytometer and a 10⁶ conidia/ml working dilution was prepared from this stock suspension. Then, 100 µl of a suspension of 10⁶ conidia/ml was added to test wells in duplicate, and stock fungicide solutions were added to give a final concentrations of each fungicide (0.0, 0.01, 0.1, 1.0, 10.0, and 100.0 µg/ml). AB dye (AbD Serotec) was added as 10% of the final volume in the test wells of Nunc Maxisorp flat-bottomed plates (Thermo Fisher Scientific). Plates were covered with sterile plastic plate covers, gently rotated horizontally to mix the well contents, placed in clean sealable containers lined with moistened tissue paper, and incubated in the dark at 25°C for 48 h. Duplicate negative control wells contained 200 µl of 10% V8 juice broth and 10% AB dye only. Duplicate positive control wells contained 100 µl of 10% V8 juice broth, 100 µl of 10⁶ conidia/ml, and 10% AB dye only. A chemical control plate was also prepared to ensure that the fungicides themselves did not reduce the AB dye and consisted of 100 µl of stock fungicide to achieve the final concentration of each fungicide, 100 µl of 10% V8 juice broth, and 10% AB in each test well. A positive test result was recorded as a color change from blue to pink, which indicated that the dye had been reduced due to the presence of viable conidia. A negative test result was recorded as no color change or the dye remained blue, which indicated that the dye was not reduced due to the absence of viable conidia. One mean inhibitory concentration (MIC) endpoint was visually determined and defined as the lowest concentration of fungicide that prevented a color change from blue to pink (MIC-blue) after 48 h of incubation (43). Optimal incubation time was determined by monitoring the color of the negative control wells

that contained spores and AB only. A fluorescent pink color in these control wells was optimal and a bleached-out or faded pink appearance meant that a second redox step had occurred, which would result in lower-than-expected AB reduction. A purple color was considered to be a “trailing” result which would eventually turn pink after a longer incubation period (44).

Statistical analyses. All experiments were conducted twice and the mean values for both trials are presented. Statistical analyses were performed using MINITAB (v. 16 for Windows; Minitab Inc.). Replications did not serve as sources of variation and the mean values and standard error of the mean for all experiments were reported. All percentages were arcsine-transformed and data were compared by analysis of variance. Means were separated by Fisher’s least significant difference test with a significance threshold of $P \leq 0.05$. Log concentrations of fungicide for which RG was inhibited by 50 and 90% (log EC50 and log EC90 values, respectively) were calculated by probit analysis from log-concentration-response curves (26,37). The binary response data from the AB tests (positive = 1 or negative = 0 test result for each concentration of fungicide tested) was analyzed by binary logistic regression (BLR).

Results

Isolates. In total, 118 *Colletotrichum* isolates were collected from the various crop hosts. *C. gloeosporioides* and *C. truncatum* isolates were identified from papaya and bell pepper collections and only *C. gloeosporioides* isolates were obtained from the avocado and mango collections. In papaya, *C. gloeosporioides* was more common than *C. truncatum*; however for bell pepper, *C. truncatum* was the predominant species associated with anthracnose disease of red-ripe fruit. In all, 68 representative isolates were selected for the study: 46 *C. gloeosporioides* isolates (12 from avocado, 12 from mango, 12 from papaya, and 10 from bell pepper,) and 22 *C. truncatum* isolates (10 from papaya and 12 from bell pepper).

Comparison of percent growth inhibition for *C. gloeosporioides* isolates from all host species. For most of the fungicides and concentrations tested, there were no significant differences in %RGI for *C. gloeosporioides* isolates from avocado and mango when compared separately with those from papaya and bell pepper. However, for benomyl at 1.0 µg/ml, *C. gloeosporioides* isolates from avocado and mango had significantly greater %RGI than those from papaya and bell pepper (Table 2). For benomyl at 0.1 µg/ml, papaya isolates had the least %RGI compared with the other isolates which shared similar %RGI. Conversely, for chlorothalonil at 1.0 µg/ml and metiram at 10.0 µg/ml, *C. gloeosporioides* isolates from bell pepper had significantly higher %RGI than the other isolates. Growth inhibition was not significantly different ($P \geq 0.05$) between *C. gloeosporioides* isolates previously exposed to fungicides and those with no known history of fungicide exposure. There were also no significant interaction effects.

Comparison of percent growth inhibition for all *C. truncatum* and *C. gloeosporioides* isolates. Generally, the percent growth inhibition (%RGI) increased with increasing concentration for all isolates and all fungicides, except for copper hydroxide. This fungicide did not inhibit mycelial growth in AA assays regardless of concentration. Hence, %RGI values could not be calculated for this fungicide. Generally, *C. truncatum* isolates had significantly

Table 1. Fungicides used for in vitro amended agar and Alamar blue assays

Trade name	Active ingredient	Manufacturer	Formulation ^x	FRAC chemical class ^y
Benomyl	50% Benomyl	VAPCO Mfg. Co. Ltd., Jordon, Israel	WP	Methyl benzimidazole carbamates
Cabrio	20% Pyraclostrobin	BASF Corp., Germany	EG	Quinone Outside inhibitors
Chlorothalonil	29.6% Chlorothalonil	ISK Biotech Corporation, OH, United States	WP	Chloronitriles
Kocide	30.7% Copper hydroxide	DuPont, United States	WP	Inorganic
Polyram ^z	80% Metiram	BASF Corp., Germany	DF	Dithio-carbamates
Rizolex	10% Tolclofos-methyl	Sumitomo Chemical Australia Pty. Ltd.	WP	Aromatic hydrocarbons
Valete	80% Fosetyl aluminum	VAPCO Mfg. Co. Ltd., Jordon, Israel	WP	Phosphonates

^x EC = emulsifiable granule, WP = wettable powder, and DF = dust base fungicide.

^y Chemical Class according to Fungicide Resistance Action Committee (FRAC) code list 2010 (30).

^z Polyram was not used in Alamar blue (AB) assays because it reduced the dye in the absence of spores.

less %RGI than *C. gloeosporioides* isolates. At 10.0 µg/ml, *C. truncatum* isolates had significantly less %RGI compared with *C. gloeosporioides* isolates for benomyl, tolcllofos-methyl, and fosetyl-aluminum. At 100.0 and 1,000.0 µg/ml, there was no significant difference in %RGI for *C. truncatum* and *C. gloeosporioides* isolates for all fungicides except for benomyl and fosetyl-aluminum (Table 3).

Comparison of percent growth inhibition for only *C. truncatum* isolates. *C. truncatum* isolates reacted differently to increasing concentrations of the various fungicides depending on whether the isolates originated from pepper or papaya (Table 4). *C. truncatum* from pepper had generally less %RGI than *C. truncatum* isolates from papaya. Significantly less %RGI occurred for benomyl at concentrations ≥10.0 µg/ml. *C. truncatum* from papaya had signifi-

Table 2. Comparison of percent relative growth inhibition (RGI) for all *Colletotrichum gloeosporioides* isolates tested according to host species

		RGI (%) for each fungicide concentration (µg/ml) ^z				
<i>C. gloeosporioides</i> isolates	<i>N</i>	0.1	1.0	10.0	100.0	1,000.0
Benomyl						
Avocado and mango	24	28.9 b	79.8 b	82.2	89.1	99.1
Papaya	12	16.1 a	80.0 b	80.0	100.0	100.0
Pepper	10	35.7 b	46.4 a	99.9	99.9	99.9
<i>P</i> value	...	*0.001	*0.021	0.169	0.423	...
Pyraclostrobin						
Avocado and mango	24	17.9	30.1	34.1	90.2	99.1
Papaya	12	31.4	35.6	42.0	100.0	100.0
Pepper	10	27.0	38.5	36.1	100.0	100.0
<i>P</i> value	...	0.174	0.944	0.817	0.839	...
Chlorothalonil						
Avocado and mango	24	10.9 b	27.7	67.5	90.2	99.1
Papaya	12	0.0 a	60.6	85.0	100.0	100.0
Pepper	10	7.7 b	27.6	81.1	100.0	100.0
<i>P</i> value	...	*0.033	0.057	0.785	0.839	...
Metiram						
Avocado and mango	24	10.5	21.3	33.8	90.8	100.0
Papaya	12	4.4	30.9	69.0	100.0	100.0
Pepper	10	9.9	28.8	37.0	100.0	100.0
<i>P</i> value	...	0.489	0.814	0.064	0.838	...
Tolclofos-methyl						
Avocado and mango	24	9.1	25.1	59.3	91.9	99.0
Papaya	12	3.2	30.5	72.0	100.0	100.0
Pepper	10	5.0	19.1	81.1	99.9	99.9
<i>P</i> value	...	0.624	0.882	0.799	0.398	...
Fosetyl-aluminum						
Avocado and mango	24	2.8	8.6	24.1	89.0	99.0
Papaya	12	0.0	0.5	11.5	90.3	100.0
Pepper	10	4.2	3.8	27.5	99.9	99.9
<i>P</i> value	...	0.139	0.202	0.064	0.552	...

^z Asterisk (*) indicates $P \leq 0.05$.

Table 3. Comparison of percent relative growth inhibition (RGI) for all *Colletotrichum truncatum* and *C. gloeosporioides* isolates tested

		RGI (%) for each fungicide concentration (µg/ml) ^z				
Isolates	N	0.1	1.0	10.0	100.0	1,000.0
Benomyl						
All <i>C. gloeosporioides</i>	46	22.0	78.9	91.2	99.9	99.9
All <i>C. truncatum</i>	22	13.7	17.7	16.2	63.0	95.1
P value	...	0.300	≤0.001	≤0.001	≤0.001	≤0.001
Pyraclostrobin						
All <i>C. gloeosporioides</i>	46	21.1	32.9	37.1	100.0	100.0
All <i>C. truncatum</i>	22	12.6	19.0	27.6	100.0	100.0
P value	...	0.100	≤0.001	≤0.001	...	...
Chlorothalonil						
All <i>C. gloeosporioides</i>	46	5.3	35.0	79.6	100.0	100.0
All <i>C. truncatum</i>	22	5.0	28.1	82.6	100.0	100.0
P value	...	0.700	0.200	0.500	...	...
Metiram						
All <i>C. gloeosporioides</i>	46	7.3	24.2	44.0	100.0	100.0
All <i>C. truncatum</i>	22	7.8	34.0	40.2	100.0	100.0
P value	...	0.800	0.500	0.500	...	...
Tolclofos-methyl						
All <i>C. gloeosporioides</i>	46	4.0	20.9	78.2	99.9	99.9
All <i>C. truncatum</i>	22	15.0	30.3	87.9	99.9	99.9
P value	...	≤0.001	0.100	≤0.001	0.300	0.300
Fosetyl-aluminum						
All <i>C. gloeosporioides</i>	46	1.8	3.5	19.3	97.4	99.9
All <i>C. truncatum</i>	22	4.0	3.5	6.9	23.1	99.9
P value	...	0.400	0.900	≤0.001	0.001	0.300

cantly less %RGI than *C. truncatum* from pepper in the presence of pyraclostrobin and fosetyl-aluminum at ≥ 10.0 $\mu\text{g/ml}$.

Comparison of percent growth inhibition for *C. truncatum* and *C. gloeosporioides* isolates from papaya. *C. gloeosporioides* isolates from papaya had generally higher %RGI than *C. truncatum* isolates for all concentrations tested for pyraclostrobin, chlorothalonil, and fosetyl-aluminum. Significant differences were recorded at concentrations ≥ 10.0 $\mu\text{g/ml}$. Significantly less %RGI were obtained for benomyl for *C. gloeosporioides* isolates (Table 5).

Comparison of percent growth inhibition for *C. truncatum* and *C. gloeosporioides* isolates from pepper. *C. gloeosporioides* isolates from pepper had generally higher %RGI than *C. truncatum* isolates for all concentrations tested for most fungicides (Table 6). Significantly higher %RGI was recorded for benomyl and fosetyl-aluminum at 10.0 $\mu\text{g/ml}$. Both *Colletotrichum* spp. reacted equally

to all tested concentrations of pyraclostrobin, chlorothalonil, and metiram.

Comparison of log EC50 and log EC90 values. Log EC50 and EC90 values were calculated for all isolates according to fungicide (Table 7). *Colletotrichum* spp. and type of fungicide had significant effects on the calculated log EC values ($P \leq 0.0001$ for both variables). When log EC values were compared for only *C. gloeosporioides* isolates, there were no significant differences ($P = 0.136$ and 0.231 for log EC50 and log EC90, respectively). Similarly, for only *C. truncatum* isolates, there were no significant differences in log EC values ($P = 0.311$ and 0.504 for log EC50 and log EC90, respectively). There was no gradient response to increasing concentrations of benomyl (%RGI was absolute for all *C. gloeosporioides* isolates at ≥ 10.0 $\mu\text{g/ml}$), and copper hydroxide (%RGI was effectively zero for all isolates at all concentrations tested); hence,

Table 4. Comparison of percent growth inhibition (RGI) for only *Colletotrichum truncatum* isolates from pepper and papaya

<i>C. truncatum</i> isolates	N	RGI (%) for each fungicide concentration ($\mu\text{g/ml}$) ^z				
		0.1	1.0	10.0	100.0	1,000.0
Benomyl						
Pepper	12	11.1	11.1	8.8	32.1	91.0
Papaya	10	16.7	25.5	25.2	100.0	100.0
P value	...	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001	≤ 0.001
Pyraclostrobin						
Pepper	12	15.9	28.7	31.8	100.0	100.0
Papaya	10	8.7	7.4	22.5	100.0	100.0
P value	...	0.700	≤ 0.001	0.100	>0.05	>0.05
Chlorothalonil						
Pepper	12	7.9	13.8	84.1	100.0	100.0
Papaya	10	1.4	45.3	80.8	100.0	100.0
P value	...	0.300	≤ 0.001	0.500	...	...
Metiram						
Pepper	12	8.3	41.7	44.7	100.0	100.0
Papaya	10	7.2	24.7	34.8	100.0	100.0
P value	...	0.900	0.200	0.300	...	...
Tolclofos-methyl						
Pepper	12	19.0	30.2	83.2	99.9	99.9
Papaya	10	10.2	30.4	93.7	100.0	100.0
P value	...	0.200	1.000	0.100	...	...
Fosetyl-aluminum						
Pepper	12	0.0	1.2	10.3	28.6	99.9
Papaya	10	8.8	6.2	2.8	16.5	100.0
P value	...	≤ 0.001	0.100	≤ 0.001	≤ 0.001	...

Table 5. Comparison of percent growth inhibition (RGI) for only *Colletotrichum truncatum* and *C. gloeosporioides* isolates from papaya

Isolates, papaya	N	RGI (%) for each fungicide concentration ($\mu\text{g/ml}$) ^z				
		0.1	1.0	10.0	100.0	1,000.0
Benomyl						
<i>C. truncatum</i>	10	0.0	25.1	24.8	100.0	100.0
<i>C. gloeosporioides</i>	12	16.1	80.0	80.0	100.0	100.0
P value	...	≤ 0.001	≤ 0.001	≤ 0.001	...	...
Pyraclostrobin						
<i>C. truncatum</i>	10	8.7	7.4	22.5	100.0	100.0
<i>C. gloeosporioides</i>	12	31.4	35.6	42.0	100.0	100.0
P value	...	≤ 0.001	≤ 0.001	≤ 0.001	...	...
Chlorothalonil						
<i>C. truncatum</i>	10	1.4	45.3	80.8	100.0	100.0
<i>C. gloeosporioides</i>	12	0.0	60.6	85.0	100.0	100.0
P value	...	0.300	0.300	0.400	...	...
Metiram						
<i>C. truncatum</i>	10	7.2	24.7	34.8	100.0	100.0
<i>C. gloeosporioides</i>	12	4.4	30.9	69.0	100.0	100.0
P value	...	0.500	0.700	0.100	...	...
Tolclofos-methyl						
<i>C. truncatum</i>	10	10.2	30.4	93.7	100.0	100.0
<i>C. gloeosporioides</i>	12	3.2	30.5	72.0	100.0	100.0
P value	...	0.100	0.800	0.100	...	...
Fosetyl-aluminum						
<i>C. truncatum</i>	10	8.8	6.2	2.8	16.5	100.0
<i>C. gloeosporioides</i>	12	0.0	0.5	11.5	90.3	100.0
P value	...	≤ 0.001	0.100	≤ 0.001	≤ 0.001	...

regression analyses could not be conducted for these fungicides and, subsequently, log EC values could not be calculated for the isolates.

Correlation of EC values for strobilurin fungicide. Correlation analyses of log EC50 and log EC90 values of all the isolates revealed a nonsignificant association for pyraclostrobin (log EC50 $r^2 = 0.305$, $P = 0.079$; log EC90 $r^2 = 0.329$, $P = 0.057$). Neither were the correlations significant for log EC values for only *C. gloeosporioides* isolates (log EC50 $r^2 = 0.328$, $P = 0.126$; log EC90 $r^2 = 0.297$, $P = 0.169$) nor for log EC values for only *C. truncatum* isolates (log EC50 $r^2 = 0.187$, $P = 0.581$; log EC90 $r^2 = 0.348$, $P = 0.295$).

AB assays. AB assays were conducted to determine the effects of different classes of fungicides on spore viability of *Colletotrichum* isolates. Generally, positive AB test results occurred at the lower concentrations of fungicides used in the assay. The visually

determined MIC-blue values for each isolate according to fungicide were recorded (Fig. 1; Table 8). Values could not be obtained for metiram because this fungicide caused a reduction of the dye in the absence of any spores. A significantly higher percentage of positive AB test results was obtained for papaya and pepper isolates when compared with all other isolates for all fungicides tested, except for chlorothalonil (Table 9). BLR analysis was significant ($P = 0.001$; Table 10) and adequately described the relationship between the test response (positive or negative AB test result) and the predictor variables in the data set because the Pearson, Deviance, and Hosmer-Lemeshow tests gave very large P values ($P > 0.990$). Species and fungicide concentration were significant ($P \leq 0.001$ for both) predictors of whether a positive AB test result would be obtained. Among the *C. gloeosporioides* isolates, the probability of obtaining a positive AB test result was significantly higher for isolates PAP-41-papaya ($P \leq 0.001$), SPE-

Table 6. Comparison of percent growth inhibition (RGI) for only *Colletotrichum truncatum* and *C. gloeosporioides* isolates from pepper

Isolates, pepper	N	RGI (%) for each fungicide concentration ($\mu\text{g/ml}$) ^a				
		0.1	1.0	10.0	100.0	1,000.0
Benomyl						
<i>C. truncatum</i>	12	11.1	11.1	8.8	32.1	91.0
<i>C. gloeosporioides</i>	10	35.7	46.4	99.9	99.9	99.9
P value	...	≤ 0.001	≤ 0.001	≤ 0.0001	≤ 0.001	≤ 0.001
Pyraclostrobin						
<i>C. truncatum</i>	12	15.9	28.7	31.8	100.0	100.0
<i>C. gloeosporioides</i>	10	27.0	38.5	36.1	100.0	100.0
P value	...	0.200	0.100	0.600	...	...
Chlorothalonil						
<i>C. truncatum</i>	12	7.9	13.8	84.1	100.0	100.0
<i>C. gloeosporioides</i>	10	7.7	27.6	81.1	100.0	100.0
P value	...	0.900	0.100	0.400	...	...
Metiram						
<i>C. truncatum</i>	12	8.3	41.7	44.7	100.0	100.0
<i>C. gloeosporioides</i>	10	9.9	28.8	37.0	100.0	100.0
P value	...	0.600	0.100	0.977	...	...
Tolclofos-methyl						
<i>C. truncatum</i>	12	19.0	30.2	83.2	99.9	99.9
<i>C. gloeosporioides</i>	10	5.0	19.1	81.1	99.9	99.9
P value	...	≤ 0.001	≤ 0.001	0.989	...	...
Fosetyl-aluminum						
<i>C. truncatum</i>	12	0.0	1.2	10.3	28.6	99.9
<i>C. gloeosporioides</i>	10	4.2	3.8	27.5	99.9	99.9
P value	...	≤ 0.001	0.400	≤ 0.001	≤ 0.001	...

Table 7. Means and ranges of 50 and 90% effective concentration (log EC50 and EC90) values for all isolates^a

Isolates	log EC50		log EC90	
	Mean	Range	Mean	Range
Pyraclostrobin				
All isolates	0.65	0.01–1.00	2.14	1.00–1.69
Only <i>C. gloeosporioides</i>	0.39	0.01–0.42	1.38	1.00–1.68
Only <i>C. truncatum</i>	0.53	0.09–1.00	1.51	1.27–1.69
Chlorothalonil				
All isolates	0.81	0.01–1.14	2.04	0.8–2.31
Only <i>C. gloeosporioides</i>	0.46	0.01–0.95	1.35	0.8–2.31
Only <i>C. truncatum</i>	0.69	0.23–1.14	1.39	1.23–1.73
Metiram				
All isolates	0.86	0.01–1.29	2.23	0.64–1.94
Only <i>C. gloeosporioides</i>	0.55	0.01–1.29	1.48	0.64–1.88
Only <i>C. truncatum</i>	0.63	0.01–1.13	1.51	1.10–1.94
Tolclofos-methyl				
All isolates	0.51	0.09–1.05	1.99	0.68–3.05
Only <i>C. gloeosporioides</i>	0.48	0.4–1.05	1.46	0.73–3.05
Only <i>C. truncatum</i>	0.07	0.09–0.71	1.06	0.68–1.93
Fosetyl-aluminum				
All isolates	2.03	0.30–2.32	3.22	1.47–3.29
Only <i>C. gloeosporioides</i>	1.16	0.42–1.56	1.85	1.47–3.00
Only <i>C. truncatum</i>	1.75	0.30–2.32	2.73	2.41–3.29

^a Log EC values could not be determined for benomyl and copper hydroxide because a linear range was not obtained with respect to inhibition of growth on amended agar tests.

3A-pepper ($P \leq 0.001$), and Man-Sc-mango ($P = 0.008$). Of the *C. truncatum* isolates tested, a significantly higher probability ($P \leq 0.001$) of obtaining a positive AB test result was identified for isolate SPE-13A from pepper. The calculated odds ratio for these isolates also indicated a very high probability of obtaining a positive AB test result. All fungicides had a similar inhibitory effect on isolates and species at increasing concentrations. Interaction effects were nonsignificant.

Molecular characterization of benzimidazole-resistant isolates. Sequences of the β -tubulin amplicons (475 bp in length) of two *C. gloeosporioides* isolates each from the four host species and two *C. truncatum* isolates each from papaya and bell pepper were compared with cognate sequences of the reference isolate, *C. gloeosporioides* f. sp. *aeschnomene* (Fig. 2). Sequence alignments between *C. gloeosporioides* isolates and *C. gloeosporioides* f. sp. *aeschnomene* revealed no base substitutions at any of the codons, including 198, 199, 200, and 240. Sequences of the *C. gloeosporioides* isolates corresponded with nucleotide positions 1,183 to 1,551 of the cognate β -tubulin sequence of *C. gloeosporioides* f. sp. *aeschnomene*.

Fig. 1. Assessment of spore response to different concentrations of fungicides based on qualitative analysis of reduction of the Alamar blue dye. The spore response of a representative isolate (*Colletotrichum gloeosporioides*, PAP-41) is shown. Concentration of fungicide was in microliters per milliliter. Negative controls were 1, 100 μ l of media and 10 μ l of Alamar blue redox dye only (no spores) and 2, 100 μ l of fungicide (final concentration of 1.0 μ g/ml) and 10 μ l of Alamar blue redox dye only (no spores). The positive control was 100 μ l of stock spore suspension at 10^6 conidia/ml and 10 μ l of Alamar blue dye only (no fungicide).

PCR amplification of *C. truncatum* isolates from papaya and bell pepper produced amplicons of expected band size for only *C. truncatum* isolates from papaya. No amplicons were obtained for bell pepper isolates, which may suggest mutation changes in primer annealing sites. Sequences of the *C. truncatum* isolates corresponded to nucleotide positions 1,144 to 1,561 of the cognate β -tubulin sequence of *C. gloeosporioides* f. sp. *aeschnomene*. Sequence alignments between *C. truncatum* isolates and *C. gloeosporioides* f. sp. *aeschnomene* revealed base substitutions that led to two codon changes; codons 154 (K-R: lysine-arginine), and 170 (V-G: valine-glycine). The relationship between the benzimidazole (BEN)-resistant phenotype of these *C. truncatum* isolates and the identified codon changes in the β -tubulin gene sequence needs further verification because no other similar studies were previously carried out for this *Colletotrichum* sp.

Discussion

This comparative study evaluated the sensitivities of *Colletotrichum* isolates from different host species to various fungicides with different modes of action utilizing two methods that assess two fitness parameters: the conventional AA test, which monitors mycelial growth, and a novel bioassay that is based on the ability of AB viability dye to react to certain respiratory activities of fungal spores, which is indicative of spore viability. According to AA tests, log EC values and RGI were a function of *Colletotrichum* sp. and type of fungicide. Results also indicated that *Colletotrichum* spp., isolate, type, and concentration of fungicide were significant variables that affected whether a positive AB test result would be obtained. When the results of both techniques were compared, both were useful at discerning chemicals that effectively reduced mycelial growth or spore viability according to *Colletotrichum* spp.

Developing an appropriate assay that is tailored to meet specific requirements can be challenging. Understanding what the assay has to measure, the cell type to be used in the study, the need for end-point determinations, the correlation between measurement and cell viability (number of live cells), and the potential limitations of assay chemistries are all important for either manual or automated high-throughput platforms. Some assays may be more suited to certain cell lines or only primary cell lines or some may be more applicable to adherent cells than cell suspensions. Another consideration when using metabolic assays is that metabolic activity can vary depending on the life cycle of a given cell and the cell type (41,55). In addition, assay responsiveness is influenced by a number of factors, including the culture medium, cell density, evaporation, edge effects (the microtiter plate format), temperature, and pH; interactions between the culture media and test compound components, assay chemistry, dose, and exposure; sensitivity and linearity of the assay; reagent toxicity; stability for time-course monitoring; instrumentation availability (spectrophotometer, fluorometer, and luminometer); total cost of the assay; safety to the user and the environment; reproducibility of the data and level of intra- and inter-laboratory agreement; and, finally, whether the in vitro data can reliably predict meaningful in vivo effects. It is also worth noting that the data derived from these assays represent an

Table 8. Mean inhibitory concentration-blue (MIC-blue) values according to an Alamar blue assay²

Isolate	Benomyl	Pyraclostrobin	Chlorothalonil	Copper hydroxide	Tolclofos-methyl	Fosetyl-aluminum
PAP-14 (<i>C. truncatum</i>)	10.0	10.0	1.0	10.0	10.0	100.0
PAP-41 (<i>C. gloeosporioides</i>)	10.0	10.0	10.0	100.0	10.0	100.0
SPE-13B (<i>C. truncatum</i>)	10.0	10.0	10.0	10.0	10.0	100.0
SPE-3A (<i>C. gloeosporioides</i>)	10.0	10.0	10.0	10.0	10.0	100.0
Avo1 (<i>C. gloeosporioides</i>)	1.0	10.0	1.0	1.0	1.0	10.0
Avo2 (<i>C. gloeosporioides</i>)	1.0	10.0	1.0	1.0	10.0	100.0
Man-Sc (<i>C. gloeosporioides</i>)	10.0	10.0	1.0	10.0	0.1	10.0
Man-Lc (<i>C. gloeosporioides</i>)	10.0	10.0	1.0	10.0	10.0	10.0

² MIC-blue was defined as the lowest concentration of fungicide that prevented a color change from blue (negative test result) to pink (positive test result) after 48-h incubation at 25°C. Polyram caused a reduction of the dye and a color change from blue to pink in the absence of any spores in chemical control wells; hence, MIC-blue values could not be obtained for any of the isolates.

estimated signal of the entire population in vitro and may not always reflect an in vivo response.

The optimum pH of the AB assay is between 7.0 and 7.4, which means that the liquid culture medium must be clarified and buffered with phosphate. The optimum temperature is 37°C and plates must be sealed to prevent evaporation. It is critical to run the assays at a uniform temperature to ensure reproducibility across a single plate. Stacking large numbers of assay plates together in close proximity should be avoided to ensure temperature equilibration. The redox indicator is also photosensitive and the incubations must be done in the dark. The culture medium and the test compound must be nonreducing. It is also preferred that the culture medium be synthetic and defined; however, it also must allow sufficient growth so that stimulatory or inhibitory effects of test compounds are not exaggerated. Proper negative and positive controls are required to test whether compounds being measured have an effect on the assay chemistry or result in artifacts. The duration of incubation and cell density must be empirically determined because too-low cell density means slower growth and lower-than-expected levels of dye reduction. The optimal end point of the assay depends on the cell density used; generally, it is recommended that the cells be in the exponential stage of growth. Quenching of fluorescence or bleaching out of the pink color of the reduced state of the dye occurs after prolonged incubation times due to the formation of a colorless product called dihydroresorufin. Exhaustion of the buffering capacity of the dye's formulation may explain the formation of this product (AbD Serotec). Apart from prolonged incubation times, 10% fetal bovine serum and bovine serum albumin may also cause quenching of the assay (32,48). Phenol red does not affect assay chemistry except to shift all values 0.03 units higher than without phenol red. Microbial debris and contamination will reduce the redox indicator and lead to false results; hence, the assay must be set up under aseptic conditions and the medium may require supplementation with antibiotics (1). Originally, AB as a redox indicator was thought to be reduced by mitochondrial enzymes. However, other enzymes may be able to reduce AB such as the diaphorases (dihydroipoamine

dehydrogenase; EC 1.8.1.4.) (42), NAD(P)H:quinone oxidoreductase (EC 1.6.99.2) (12), and flavin reductase (EC 1.6.99.1) (16), which can be found in the cytoplasm (EC 1.6.99.1) and mitochondria (EC 1.6.99.2). Hence, a decline in AB reduction indicates an impairment of cellular metabolism rather than specific mitochondrial dysfunction through interruption of electron transport.

Rampersad (56) demonstrated a quantitative application of the AB assay by determining the percent reduction of the AB dye deduced from the spectrophotometric relationship between the corrected recorded absorbance at two wavelengths for the oxidized and reduced forms of the dye in test wells and the molar extinction coefficients of the dye at these wavelengths. This study demonstrated a qualitative application of the assay where presence or absence of a color change in the dye indicated sensitivity to different concentrations of various fungicides. The AB assay detected that *C. gloeosporioides* isolates from papaya and pepper and one from mango were more likely to give a positive AB test result than the other isolates according to BLR analyses. When the overall combined percentage of positive AB test results obtained for each fungicide over the range of concentrations tested were compared, there was a significantly higher percentage of positive AB test results for papaya and pepper isolates, with the exception of chlorothalonil. Intensive year-round production of papaya and pepper with concomitant chemical use would have exposed these isolates to a number of chemicals, more so than isolates from mango and avocado hosts, which are seasonally produced with limited to no chemical use. The AB results also suggested a higher sensitivity of fungal spores to copper hydroxide and pyraclostrobin when compared with their effects on hyphal extension. This can be explained by the modes of action of these fungicides and greater sensitivity of the AB assay in detecting spore viability (e.g., spore germination is an energetically demanding stage of fungal development and is particularly sensitive to strobilurin fungicides and copper chemistries; 69).

BEN fungicides inhibit microtubule formation and assembly. Single-site activity facilitates development of resistance to BENs

Table 9. Percentage of *Colletotrichum gloeosporioides* isolates with a positive Alamar blue test result according to fungicide^y

Isolates from	Percentage + test results				
	Pyraclostrobin	Chlorothalonil	Copper hydroxide	Tolclofos-methyl	Fosetyl-aluminum
Papaya and pepper	60	40	70	50	60
Avocado and mango	40	40	30	30	40
<i>P</i> value ^z	0.004	...	0.001	0.003	0.004

^y Percentages to the nearest whole number from tests conducted twice.

^z $P \leq 0.05$.

Table 10. Binary logistic regression of responses obtained from an Alamar blue assay^z

Predictor	Coeff	SE Coef	Z	P	Odds ratio	95% CI	
						Lower	Upper
Constant	6.3504	1.1013	5.77	0.000	...	...	...
Log concentration	-3.4634	0.3842	-9.02	0.000	0.03	0.01	0.07
Species							
<i>Colletotrichum gloeosporioides</i> isolate	-5.3581	0.9889	-5.42	0.000	0.00	0.00	0.03
Avo2	1.5775	0.8133	1.94	0.052	4.84	0.98	23.83
Man-Lc	1.5772	0.8133	1.91	0.025	4.84	0.98	23.83
Man-Sc	2.2085	0.8309	2.66	0.008	9.10	1.79	46.39
PAP-14	5.3581	0.9889	5.43	0.000	212.32	30.56	1,475.15
PAP-41	4.0988	0.9129	4.49	0.000	60.26	10.07	360.66
SPE-13B	1.2618	0.8065	1.56	0.118	3.53	0.76	17.16
SPE-3A	3.7845	0.8965	4.22	0.000	44.01	7.59	255.09
Fungicide							
Tolclofos-methyl	-0.8855	0.9450	-0.94	0.349	0.41	0.06	2.63
Copper hydroxide	-1.7572	0.9537	-1.84	0.050	0.17	0.03	1.12
Benomyl	-1.7572	0.9538	-1.84	0.065	0.17	0.03	1.12
Pyraclostrobin	-0.0000	0.9364	-0.00	1.000	1.00	0.16	6.27
Chlorothalonil	-1.7572	0.9537	-1.84	0.170	0.17	0.03	1.12

^z Log likelihood = -87.247. Test that all slopes are zero: $G = 435.81$, $DF = 18$, P value = 0.000. CI = confidence interval.

and selects for pathogen genotypes bearing certain mutations in the β -tubulin gene which can persist in the pathogen population due to disruptive selection long after the chemical has been withdrawn (15,36). Such mutations have been related to specific amino acid substitutions at several regions of the β -tubulin gene. There have been reported cases of resistance to BENs in a number of different fungal species (15,20,21,24,35,45,46,50). In this study, none of the *C. gloeosporioides* isolates (exposed and underexposed isolates) showed resistance to benomyl, and growth inhibition was comparable with sensitive *C. gloeosporioides* isolates reported elsewhere (29,50). *C. truncatum* isolates from both pepper and papaya were only sensitive to benomyl at concentrations ≥ 100.0 $\mu\text{g/ml}$. In AB assays, increasing concentrations of benomyl resulted in reduced spore survival of *C. gloeosporioides* and *C. truncatum* isolates; however, the differential responses between *C. gloeosporioides* and *C. truncatum* isolates as recorded for AA assays was not apparent. This can be explained by the mode of action of these fungicides, where microtubule assembly impacts mitosis and cell division during hyphal extension and fungal growth (29); however, there is no effect on oxidative phosphorylation processes in spore mitochondria.

Sequence alignments between *C. truncatum* isolates from papaya and *C. gloeosporioides* f. sp. *aeschynomene* revealed two codon changes outside of the recognized codons usually associated with the BEN-resistant phenotype. The relationship between the BEN-resistant phenotype of these *C. truncatum* isolates and the identified codon changes in the β -tubulin gene sequence needs further verification because similar studies were not previously conducted for this *Colletotrichum* sp. It is unclear why no amplicons were obtained for *C. truncatum* isolates from pepper but one explanation may be that sufficient variation existed in the primer binding sites for these isolates, which reduced primer binding efficiency. Mutations in specific amino acids that confer BEN resistance occur at codon 198 or 200 in a number of fungal species (36,38,71). Peres et al. (50) examined cognate β -tubulin gene sequences of *C. acutatum* isolates from citrus (generated by the TUB2-L/R primers) and determined that mutations that conferred resistance to BENs in these isolates were located outside of the 198 or 200 codons. Mutations reported to confer BEN resistance in fungal pathogens can have intra-specific or even intra-strain differ-

ences with codon changes outside of the recognized 198 and 200 positions (e.g., at codon 165 in *Aspergillus nidulans*; 36), and may be more complex than previously thought, because stabilization dynamics and functioning of microtubule heterodimers in BEN-resistant isolates are also affected by temperature (58).

Copper-based fungicides have been used to control anthracnose in a number of fruit and vegetable crops; however, their use may be restricted in some cropping systems because of phytotoxic effects (2). The copper-based fungicide screened in this study had no effect on radial mycelial growth of *Colletotrichum* isolates regardless of species. However, the AB assay results indicated that increasing concentration inhibited spore survival for most isolates. This distinction was a result of the mode of action of copper-based chemistries. During germination, copper ions passively migrate into spores and accumulate to levels which become toxic (61). This emphasizes the importance of the AB assay in assessing sensitivity of fungal spores to different fungicides. Accuracy of fungicide sensitivity screening is improved when both AA and AB assays are conducted. The AB assay can identify chemicals that affect spore physiology as opposed to those that only affect hyphal extension (AA assay) (56).

Strobilurins or quinone outside inhibitor fungicides are an important class of fungicides with a site-specific mode of action. They interfere with mitochondrial respiration by binding to the outer quinone oxidizing pocket (Q_o complex) located on cytochrome b mitochondrial membrane protein. Transfer of electrons from cytochrome b to cytochrome c_1 is disrupted and production of ATP is halted. Inhibition of ATP production directly prevents mycelial growth and spore germination, which are both energy- and ATP-dependent processes (9,11,25). The pyraclostrobin fungicide tested in this study caused significant reduction in mycelial growth regardless of species and host origin, according to AA and AB assays.

There have been numerous cases of resistance developing in the major classes of single-site inhibitors such as the BENs, pyrimidines, carboxanilides, phenylamides, sterol biosynthesis inhibitors, morpholines, and Q_o inhibitors for various fungal pathogens (11). Prevention of fungicide resistance is a priority and it is recommended that (i) mixes of single-site and multi-site fungicides

Fig. 2. Partial tubulin protein sequence alignment to illustrate amino acid changes at codons 154 and 170 in the *Colletotrichum truncatum* sequence (papaya isolates only) based on comparisons with the corresponding reference sequence of *C. gloeosporioides* f. sp. *aeschynomene* (GenBank accession number U14138).

be used in rotation rather than at-risk fungicides alone, (ii) application of fungicides ideally should be on a needs basis instead of by calendar or prescription basis, (iii) only the recommended doses and application rates should be used, and (iv) integrated chemical and non-chemical strategies should be implemented for disease management (13,58). Continuous monitoring of fungicide sensitivity based on in vitro bioassays that assess mycelial growth and spore germination, such as the ones utilized in this study, is essential to (i) detect shifts in EC values toward greater resistance, which would indicate directional selection; (ii) reduce the rate at which these shifts develop; and (iii) understand pathogen population dynamics under various chemical control regimes over time (13,22). As with many in vitro bioassays, results may not always correlate with in vivo efficacy, and the predictive limitation of any bioassay must be considered in designing respective monitoring strategies.

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