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Baseline Sensitivities of Fungal Pathogens of Fruit and Foliage of Citrus to Azoxystrobin, Pyraclostrobin, and Fenbuconazole

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ABSTRACT

Mondal, S. N., Bhatia, A., Shilts, T., and Timmer, L. W. 2005. Baseline sensitivities of fungal pathogens of fruit and foliage of citrus to azoxystrobin, pyraclostrobin, and fenbuconazole. *Plant Dis.* 89:1186-1194.

The baseline sensitivities for mycelial growth of foliar fungal pathogens of citrus, *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri*, the causal agents of postbloom fruit drop, brown spot of tangerine, citrus scab, melanose, and greasy spot, respectively, were determined in vitro for azoxystrobin, pyraclostrobin, and fenbuconazole. The effective dose to reduce growth by 50% (ED₅₀ values) was determined for each pathogen–fungicide combination using five isolates from different citrus areas of Florida and eight fungicide concentrations. A discriminatory dose for each combination was selected near the ED₅₀, and the range of sensitivity of 50 to 62 isolates of each fungal species was determined. The effect of salicylhydroxamic acid (SHAM) on the sensitivity of the five fungal species to azoxystrobin and pyraclostrobin was determined. Since mycelial growth of *A. alternata* was insensitive to azoxystrobin, the effect of that fungicide with and without SHAM on spore germination was assessed. The ED₅₀ values for most fungal pathogens of citrus were relatively high compared with foliar pathogens of other tree crops. Values for azoxystrobin ranged from a low of 0.06 µg/ml with *E. fawcettii* to a high of >100 µg/ml with *A. alternata*. With pyraclostrobin, the values ranged from a low of 0.019 µg/ml with *D. citri* to a high of 0.87 µg/ml with *A. alternata*. With fenbuconazole, the lowest ED₅₀ value was 0.21 µg/ml with *M. citri* and the highest was 1.01 µg/ml with *C. acutatum*, but *A. alternata* and *D. citri* were not tested. SHAM was inhibitory to all species and reduced growth of *D. citri* greatly. Inclusion of SHAM in the medium did not greatly affect the sensitivity of mycelial growth of these fungi to azoxystrobin or pyraclostrobin, nor did it affect the ED₅₀ values for conidial germination of *A. alternata*. The coefficients of variation for the sensitivity of 50 to 62 isolates of each species to these fungi ranged from 7.3% with the pyraclostrobin–*C. acutatum* combination to a high of 55.0% with the fenbuconazole–*M. citri* combination. Discriminatory doses have been established for these pathogen–fungicide combinations that should be useful for detecting major shifts in fungicide sensitivity.

Fungal pathogens of fruit and foliage cause serious losses to the Florida citrus industry and reduce yield and fruit quality (37,39,40). Postbloom fruit drop (PFD), caused by *Colletotrichum acutatum*, affects flowers and results in abscission of young fruitlets and the formation of persistent calyces. Losses are greatest on Navel and Valencia sweet oranges (*Citrus sinensis*) in years where significant rainfall occurs during the bloom period. *Alternaria* brown spot, caused by *Alternaria alternata*, affects tangerines and their hybrids and produces lesions on fruit, leaves, and twigs. The disease has become more widespread in recent years and affects yield and

fruit quality of high-value cultivars such as Murcotts (*C. sinensis* × *C. reticulata*) and Minneola tangelos (*C. paradisi* × *C. reticulata*). Citrus scab, caused by *Elsinoe fawcettii*, exhibits warty pustules on the leaves, twigs, and fruit of susceptible citrus species. The disease causes external blemishes and reduces the acceptability of grapefruit and many tangerines and their hybrids produced for the fresh market. Melanose, caused by *Diaporthe citri*, exhibits brick red lesions on the fruit, leaves, and twigs of most citrus. This disease reduces the value of fruit for the fresh market and is most important on grapefruit (*C. paradisi*), navel oranges, and tangerines (*C. reticulata*) and their hybrids. Greasy spot, caused by *Mycosphaerella citri*, exhibits lesions on mature leaves and rind blotch on fruit of most citrus. The leaf lesions induce premature leaf drop and can affect yield and fruit size of all citrus. Rind blotch is most serious on grapefruit grown for the fresh market.

Modifications of cultural practices are helpful in reducing the impact of these diseases, but the primary means of control is by application of fungicides (37). For

postbloom fruit drop, one to two well-timed sprays during bloom are usually sufficient for disease control, even in years with rain during the bloom. Benzimidazole fungicides, such as benomyl or thiophanate methyl, have been the most effective for disease control, but strobilurins also control the disease (31,32,41). Benomyl has been withdrawn from the market, and thiophanate methyl has only an emergency registration for use on Florida citrus (29). *Alternaria* brown spot is difficult to control, and sprays may be needed every 7 to 10 days from the beginning of growth in the spring to mid-July, when the fruit becomes resistant. Copper and strobilurin fungicides are the primary products used for control of brown spot. For control of citrus scab, up to three applications may be needed to protect the new growth and young fruit in the spring. Ferbam and the strobilurin fungicides are currently recommended, but fenbuconazole is also very effective (29,34,38,49). Melanose is controlled by several fungicide applications from late April until early July when the fruit become resistant. Copper and strobilurin fungicides are the most effective products for melanose control (1). For greasy spot control on foliage, one or two fungicide applications are needed in early to midsummer, and an additional spray may be required for rind blotch control on fruit. A wide range of products is effective for greasy spot control, including petroleum oils, copper fungicides, fenbuconazole, and strobilurins.

The strobilurin fungicides, azoxystrobin, pyraclostrobin, and trifloxystrobin, have recently been registered for use on citrus in Florida (37). Fenbuconazole currently has an emergency registration for control of greasy spot on grapefruit. This fungicide is also quite effective for control of scab (13,49) and potentially useful for postbloom fruit drop. Fenbuconazole is much less effective for control of *Alternaria* brown spot and melanose (13,37,49). Fenbuconazole is the only sterol-biosynthesis-inhibiting fungicide ever registered on citrus in Florida.

Little research has been conducted on the fungicide sensitivity of any of the fungal pathogens of fruit and foliage, and little is known about effective resistance management of fungicides for citrus. Benomyl was first registered in the early 1970s, and *M. citri*, the greasy spot pathogen, quickly developed resistance (43). *E. fawcettii*, the

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causal agent of citrus scab, developed resistance to benomyl as well (34), but resistance is not nearly as widespread. Benomyl was recommended for scab, but not greasy spot, control until recently when it was withdrawn from the market.

Baseline sensitivity of some important fungal pathogens of other crops to the strobilurin fungicides has been established (25,47,48). Resistance of these fungi has developed on several different plants (4,5,18–20,42). Baseline sensitivities of several pathogens to fenbuconazole and other triazole fungicides have been determined as well (20,33,34). Although large shifts in the sensitivity of isolates has not occurred, resistance is a problem nevertheless (20,21,34).

Three strobilurin fungicides are now fully registered on citrus, and fenbuconazole could be fully registered in the near future. The objective of this research was to establish the baseline sensitivity of the major citrus fungal pathogens to these products prior to their widespread use. This information should facilitate detection and verification of the development of resistance to these products.

MATERIALS AND METHODS

Isolation and storage of fungal pathogens. Since the intent was to establish baseline sensitivity values for the major fungal pathogens of citrus, all isolates were collected from groves not previously treated with the fungicides studied. All of the isolates collected early in the 1990s would never have been exposed to any of these products since these fungicides were not yet available. Subsequently, isolates were collected from abandoned groves or where the fungicide usage in the blocks had been verified with the grower. All isolates of *C. acutatum* were obtained from flower petals of sweet oranges affected by postbloom fruit drop. Symptomatic petals were dipped in 70% ethanol for 30 s, then in 1% sodium hypochlorite for 1 min, then rinsed with sterile distilled water and blotted dry on sterilized filter paper (28,30). Tissue pieces were plated on potato dextrose agar (PDA) and incubated at 24 to 27°C. All isolates were identified as *C. acutatum* by colony morphology, conidial shape, and their ability to grow on PDA + 10 µg/ml benomyl (30).

All of the isolates of *A. alternata* were of the tangerine pathotype (39,40). Leaf tissue of affected tangerines or tangerine hybrids with typical symptoms of *Alternaria* brown spot was collected. Tissue pieces about 4 mm² were dipped in 95% ethanol for 10 s, in 1% sodium hypochlorite for 60 s, rinsed with sterile, distilled water, blotted dry on sterile filter paper, and plated on PDA + 10 µg/ml benomyl (31). Since the tangerine pathotype is morphologically indistinguishable from saprophytes and other pathotypes (29), pathogenicity was verified by inoculating

young leaves of *Minneola* tangelo in vitro (15).

Isolates of *E. fawcettii* were collected from affected leaves or fruit. Fruit or leaves were washed thoroughly with soap and water and allowed to dry for 2 to 3 days. Symptomatic tissues were dipped in 95% ethanol and allowed to air dry. Using a scalpel, small flakes of affected tissues were scraped from the surface and allowed to fall on the selective medium consisting of PDA + 400 mg of dodine, and 100 mg each of streptomycin and tetracycline per liter (46). Cultures were incubated at 24 to 27°C for about 10 days, and typical colonies of *E. fawcettii* were selected (39) and transferred to fresh PDA.

For isolation of *D. citri*, twigs affected by melanose were collected and dried for 10 days in the laboratory (22). They were then soaked in water for 3 to 4 h every other day and maintained at 28°C for 60 to 90 days until pycnidia formed. Spore tendrils exuding from pycnidia were aseptically transferred to plates of PDA + 0.1% tartaric acid, and colonies were allowed to grow at 24 to 27°C. This fungus was identified by the presence of characteristic alpha and beta conidia in the spore tendrils and by colony morphology (37).

For isolation of *M. citri*, mature leaves affected by greasy spot were surface-sterilized by dipping in 70% ethanol for 30 s, rinsing with sterile distilled water, and placing them in 1% sodium hypochlorite for 5 to 10 min and rinsing thoroughly with sterile distilled water. The surface of the lesion was removed with a sterile scalpel, and small bits of mesophyll were transferred to PDA plates (45). Cultures were incubated for 10 to 14 days at 24 to 27°C, and characteristic slow-growing, gray-green colonies were selected and transferred to fresh plates (36). Alternatively, decomposing leaves with mature pseudothecia of *M. citri* were collected from the grove floor, soaked for 10 to 15 min, blotted dry, and affixed to the lid of a petri plate containing 5% water agar using petroleum jelly to allow pseudothecia to eject ascospores onto the agar (23). Cultures were incubated overnight at 23 to 25°C. Germinated ascospores were transferred to PDA + 0.1% tartaric acid. After formation of characteristic colonies, mycelial bits were transferred to fresh PDA.

Cultures of all pathogens obtained before 1998 were stored on PDA slants and transferred every 30 to 90 days to maintain them. Subsequently, all cultures were stored on filter paper (28). Mycelial bits were transferred to PDA plates placed next to a sterile 3 × 3 cm piece of filter paper. Plates were incubated at 24 to 27°C until the filter paper was colonized. Then, the filters were lifted from the agar surface with sterile forceps, placed in a sterile coin envelope, and dried overnight in a laminar flow hood. Envelopes were placed in a closed plastic container with a desiccant

and stored at –20°C until needed. All cultures and their source and year of isolation are listed in Table 1.

Determination of ED₅₀ values. For all studies, commercial formulations of the products were used: azoxystrobin, Abound 2.08 EC (Syngenta Crop Protection, Greensboro, NC); pyraclostrobin, Headline 2.09 EC (BASF Corporation, Research Triangle Park, NC); and fenbuconazole, Enable 2F (Dow AgroSciences, Indianapolis, IN). These fungicides were diluted in sterile water and added to molten PDA at about 50°C to obtain final concentrations of 0, 0.001, 0.01, 0.05, 0.1, 1.0, 10, and 100 µg of active ingredient per ml. PDA was delivered into petri dishes with a Brickman Bottletop Dispenser (Brickman Instruments, Westbury, NY) using 20 ml per 95 × 15 mm diameter plate or 10 ml per 65 × 15 mm diameter dish.

Five isolates of each fungal species were evaluated for sensitivity to all three fungicides except that fenbuconazole was not tested with *A. alternata* or *D. citri* since it is not effective against those species (13,37,49). Seed plates for each species were grown on PDA. Five-millimeter-diameter mycelial plugs were cut from the margin of colonies on the seed plates. These plugs were transferred to three or four replicate plates of each concentration of fungicide. Plates were maintained at ambient laboratory temperatures (23 to 25°C) with about 12 h per day of fluorescent lighting. Colonies were grown until the no-fungicide plates were 50 to 75% covered with mycelium. Time required varied with the species and was 5 to 10 days with *C. acutatum*, *A. alternata*, and *D. citri* and 20 to 30 days with *M. citri* and *E. fawcettii*. Colony diameter was measured across two axes, averaged, and the diameter of the mycelial plug subtracted from the average. The percent inhibition was calculated in relation to the no-fungicide controls. Each experiment was conducted twice.

The effective dose to reduce growth by 50% (ED₅₀ value) for the three fungicides was calculated for each experiment with each isolate by fitting the dose response to a sigmoidal function. Each fungal species was evaluated in two experiments and all isolates tested each time. Thus, separate analyses for variances (ANOVAs) were conducted for each species. The effect of fungicide, isolate, and experiment plus all of the two-way interactions were investigated. Since in all cases, the variances were equal within fungal species, data were pooled and a response curve developed for each fungal species to each fungicide. The mean and range of ED₅₀ values among isolates were calculated and presented.

Effect of SHAM on mycelial growth. Two isolates of each species were evaluated to determine the effect of salicylhydroxamic acid (SHAM) on growth of the fungi and their reaction to azoxystrobin

and pyraclostrobin. SHAM was dissolved in methanol and added to molten PDA at 100 µg/ml. Growth of each fungal species was evaluated as above using each fungicide at 0.0, 0.01, 0.1, 1.0, and 10 µg/ml in the presence or absence of SHAM. The isolates of each species used are given in Table 1. The experiment was conducted three times using four replicate dishes in each test. Since fungicides at low doses reversed the inhibition of mycelial growth by SHAM, percent inhibition and ED₅₀ values could not be calculated. The effects

of SHAM, isolate, fungicide concentration, and experiment were determined using ANOVAs of colony diameters.

Effect of SHAM and azoxystrobin on germination of conidia of *A. alternata*. The effects of azoxystrobin and SHAM on the conidial germination of *A. alternata* were also determined. Five isolates were used for these tests: Ran, LOR, SH, IMK, and EV (Table 1). Conidia were produced as described by Peever et al. (28). PDA plates with 0.0, 0.01, 0.1, 1.0, and 10 µg/ml azoxystrobin with and without 100

µg/ml SHAM were prepared as described above. Suspensions of conidia at 2,000 spores per milliliter were prepared and 0.1 ml spread on each petri dish. Conidia on dishes were incubated overnight at 25°C. Four replicate dishes were used for each azoxystrobin–SHAM combination, and the experiment was repeated three times. One hundred conidia per petri dish were observed under a stereomicroscope to determine the percent germination.

ED₅₀ values were calculated for each isolate–SHAM–experiment combination

Table 1. Fungal species and host, location, and dates of collection of isolates evaluated for sensitivity to fungicides

Pathogen and isolates	No. of isolates used	Host ^a	Location	Year
<i>Colletotrichum acutatum</i>				
CLR-IMK (7)* ^{†b}	5	Valencia sweet orange	Immokalee	1999
OCO-ARC (1S)*	5	Navel sweet orange	Arcadia	1999
GND	5	Navel sweet orange	?	1998
ALB IND	5	Valencia sweet orange	Indiantown	2000
STL	4	Navel sweet orange	Frostproof	1999
VTP-IND*	5	Navel sweet orange	Indiantown	2000
SGR-FTP* [†]	5	Navel sweet orange	Frostproof	1999
MRN-IND	6	Valencia sweet orange	Indiantown	2000
RCO-IMK	5	Valencia sweet orange	Immokalee	2000
SRL	5	Valencia sweet orange	Frostproof	2000
LB	1	Navel sweet orange	La Belle	1990
LB-4	1	Navel sweet orange	La Belle	1991
CS-1	1	Valencia sweet orange	Ft. Pierce	1993
PS-1	1	Navel sweet orange	Lake Placid	1993
PS-2	1	Valencia sweet orange	Lake Placid	1993
Coca Cola	1	Navel sweet orange	Indiantown	1993
Maran-91	1	Valencia sweet orange	Indiantown	1991
SM-3*	1	Valencia sweet orange	Lake Placid	1993
LP-2	1	Valencia sweet orange	Lake Placid	1991
MAYO	1	Navel sweet orange	Vero Beach	1993
<i>Alternaria alternata</i>				
WP-MIL	5	Minneola tangelo	West Palm Bch.	1996
Ran* [†]	5	Sunburst tangerine	Immokalee	1997
LOR*	4	Sunburst tangerine	Lorida	1997
SH*	5	Minneola tangelo	Polk City	1996
FM	3	Minneola tangelo	Fort Meade	1996
CPI	4	Murcott tangor	Immokalee	1996
EV*	4	Minneola tangelo	Lake Alfred	1996
SWAMP-1	1	Minneola tangelo	Polk City	?
IMK* [†]	5	Minneola tangelo	Immokalee	1997
VB	5	Grapefruit	Vero Beach	1997
AR	5	Sunburst tangerine	Arcadia	1997
Re-ISO	1	Orlando tangelo	Winter Haven	?
IRA	1	Minneola tangelo	?	?
10 AR	2	Minneola tangelo	?	?
<i>Elsinoe fawcettii</i>				
Ft. Pierce	4	Temple tangor	Ft. Pierce	2001
IMK Temple	7	Temple tangor	Immokalee	2001
Con-Lal	2	Calamondin	Orange Co.	2001
Duda-IMK-TEMPLE	5	Temple tangor	Immokalee	2001
STL-TEM	1	Temple tangor	Frostproof	2001
SS-IMK TEM	3	Temple tangor	Immokalee	2001
LKPCD-TEM	2	Temple tangor	Lake Placid	2001
TBL-LA TEM	5	Temple tangor	Lake Alfred	2001
EUS MUR	2	Murcott tangor	Eustis	2001
EUR MUR LV-1	1	Murcott tangor	Eustis	2001
SWFRC-IMK-TEM	1	Temple tangor	Immokalee	2001
SWFRC-MUR-LV-1	2	Murcott tangor	Immokalee	2001
Ft. Meade	2	Grapefruit	Ft. Pierce	2001

(continued on next page)

^a Sweet orange = *Citrus sinensis*, tangelo = *C. paradisi* × *C. reticulata*, tangor = *C. sinensis* × *C. reticulata*, tangerine or mandarin = *C. reticulata*, grapefruit = *C. paradisi*, calamondin = *C. limonia*? × *Fortunella* sp.?, Tahiti lime = *C. latifolia*, Volkamer lemon = *C. volkameriana*, rough lemon = *C. jambhiri*, citrange = *Poncirus trifoliata* × *C. sinensis*.

^b * Indicates isolates used for determination of the effective dose to reduce growth by 50% (ED₅₀) for each species; where more than one isolate is listed, only one from that location was used for the ED₅₀ determination; [†] indicates isolates used for determination of the effect of salicylhydroxamic acid (SHAM) on the ED₅₀ values for mycelial growth.

^c Isolate used for all pyraclostrobin assays to replace “Felsmere” after it was lost.

using sigmoidal functions. ED₅₀ values for azoxystrobin with SHAM were based on comparison with the SHAM plates with no fungicide. Then ED₅₀ values were subjected to ANOVAs to determine the effects of isolate, addition of SHAM, and experiment on the sensitivity of conidial germination.

Range of sensitivity. A discriminatory concentration near the ED₅₀ value was selected for each fungicide–species combination. The 50 to 62 isolates collected for each fungal species were evaluated at that concentration in a similar manner to that described above. All experiments were

conducted twice and results averaged to determine the percent inhibition of each isolate for the fungicide tested. The range and the coefficients of variation were calculated for each fungicide–fungal species combination.

RESULTS

Determination of ED₅₀ values. In the analysis of variance of the response of different pathogens to fungicides, the effect of fungicide was highly significant (Table 2). The effect of isolate as well as the fungicide × isolate interaction was

significant for *E. fawcettii*, *D. citri*, and *M. citri*, but not for the other two species. There was no significant effect of experiment and no significant interaction of experiment with other factors (Table 2). Since variances were equal, data from different isolates and experiments were pooled to calculate a mean ED₅₀ for each pathogen–fungicide combination using sigmoidal functions (Figs. 1 to 3), and the means and ranges for different isolates were calculated (Table 3). The regression coefficients ranged from 0.91 to 0.99, and all were highly significant.

Table 1. (continued)

Pathogen and isolates	No. of isolates used	Host ^b	Location	Year
<i>Elsinoe fawcettii</i> (continued)				
Manatee Co.	3	Temple tangor	Manatee Co.	2001
Lef GF	1	Grapefruit	Bowling Green	?
Lef-FF-1	1	Grapefruit	Bowling Green	?
Brigg	2	Grapefruit	Babson Park	2003
IRREC-MUR	2	Murcott tangor	Ft. Pierce	2001
SS IMK Lime	2	Tahiti lime	Immokalee	2001
Lefond*	1	Grapefruit	Bowling Green	2000
Navarro	4	Grapefruit	Frostproof	2003
CC-3	1	Volkamer lemon	Indiantown	1990
R-31	1	Temple tangor	Lake Placid	1991
Roesseto Bent. R	1	Grapefruit	Ona	2003
May	2	Grapefruit	Avon Park	2003
Volk*	1	Volkamer lemon	Indiantown	1990
RL	1	Rough lemon	Lake Alfred	?
Cal-WH-1	1	Calamondin	Winter Haven	2002
Hunt Bent R	1	Temple tangor	Frostproof	2003
Carrizo*	1	Carrizo citrange	Lake Alfred	1996
SCSK [†]	1	SunChaShaKat mandarin	Sebring	1992
Hastings*	1	Grapefruit	Hastings	1991
Scott* [†]	1	Temple tangor	Davenport	1990
<i>Diaporthe citri</i>				
East LW	5	Grapefruit	Lake Wales	2003
Lake Wales	5	Grapefruit	Lake Wales	2003
SS-29	7	Grapefruit	Immokalee	2003
Ft-Pierce-SW	4	Sweet orange	Ft. Pierce	2003
Budwood Flame	4	Grapefruit	Immokalee	2002
Immokalee-Temple	1	Temple tangor	Immokalee	2002
Ft Pierce-Temple	5	Temple tangor	Fort Pierce	2003
PC Ft Pierce	5	Sweet orange	Fort Pierce	2003
Lefond	5	Grapefruit	Bowling Green	2001
Berry	5	Grapefruit	LaBelle	2002
Berkovitz	2	Grapefruit	Frostproof	2001
Ever Glades	5	Grapefruit	Immokalee	2002
Lake Placid	4	Sweet orange	Lake Placid	2003
STL	6	Temple tangor	Frostproof	2003
Ser-2-2* [†]	1	Grapefruit	Lake Alfred	1995
D-1* [†]	1	Sweet orange	Dundee	2000
JMP-2*	1	Grapefruit	Bowling Green	2000
P-94*	1	Grapefruit	Lake Alfred	1991
Nova-2*	1	Grapefruit	Lake Alfred	1997
<i>Mycosphaerella citri</i>				
SLT	6	Temple tangor	Frostproof	?
FTP	6	Grapefruit	Frostproof	2000
MC-IMK-SS* [†]	5	Grapefruit	Immokalee	2000
Conserve* [†]	5	Grapefruit	Orange Co.	2001
SS-IMK	4	Grapefruit	Immokalee	2001
Lef-1*	1	Sweet orange	Bowling Green	2001
MC LA	8	Grapefruit	Lake Alfred	2000
MC Lef G	4	Grapefruit	Bowling Green	2001
Felsmere*	1	Navel sweet orange	Felsmere	1996
PREC	4	Grapefruit	Ft. Pierce	2001
APR-EC	4	Grapefruit	Ft. Pierce	2001
Duda	5	Grapefruit	Immokalee	2001
Raley	6	Valencia sweet orange	Lake Placid	2001
EV*	2	Minneola tangelo	Lake Alfred	?
ARC* ^c	1	Sweet orange	Arcadia	1997

E. fawcettii and *D. citri* were the most sensitive to azoxystrobin, and *C. acutatum* and *M. citri* had slightly higher ED₅₀ values (Table 3). Mycelial growth of *A. alternata* was insensitive to azoxystrobin. The ED₅₀ values for all five species were lower for pyraclostrobin than for azoxystrobin. The sensitivity of isolates of *E. fawcettii* and *M. citri* to fenbuconazole was similar to their sensitivity to pyraclostrobin (Table 3, Fig. 3). Fenbuconazole was more active against *C. acutatum* than was azoxystrobin, but less active than pyraclostrobin.

Effect of SHAM on mycelial growth. ANOVAs indicated that fungicide concentration and SHAM had significant effects on the colony diameter of all five species of fungi. In all combinations of fungicide–fungal species, except for the *E. fawcettii*–pyraclostrobin pair, the SHAM × fungicide interaction was significant. Thus, the response to fungicide concentration is altered by SHAM, but it does not obviously increase the sensitivity of the fungi to azoxystrobin or pyraclostrobin (Fig. 4).

In fact, addition of low rates of fungicide appeared to reverse the inhibition of mycelial growth by SHAM (Fig. 4). With all species of fungi, colony diameter was slightly greater in the presence of pyraclostrobin at 0.01 µg/ml plus SHAM than with SHAM alone. In the case of *M. citri*, colony diameter in the presence of 10 µg/ml pyraclostrobin was still greater than with SHAM alone. The effects were less dramatic with azoxystrobin and were noted only with *E. fawcettii*, *D. citri*, and *M. citri*. With *D. citri*, addition of 0.01 µg/ml azoxystrobin to dishes with SHAM had three times greater colony diameter than with SHAM alone.

Sensitivity of conidia of *A. alternata* to azoxystrobin and SHAM. The ANOVA indicated that SHAM had no effect on the sensitivity of conidial germination to azoxystrobin. Neither isolate nor experiment had significant effects in the analysis. The mean ED₅₀ value for all isolates without SHAM was 0.12 µg/ml and with SHAM was 0.06 µg/ml. These values are much lower than the values for mycelial growth (Table 3).

Range of fungicide sensitivity. A discriminatory concentration was selected based on the ED₅₀ values of the five iso-

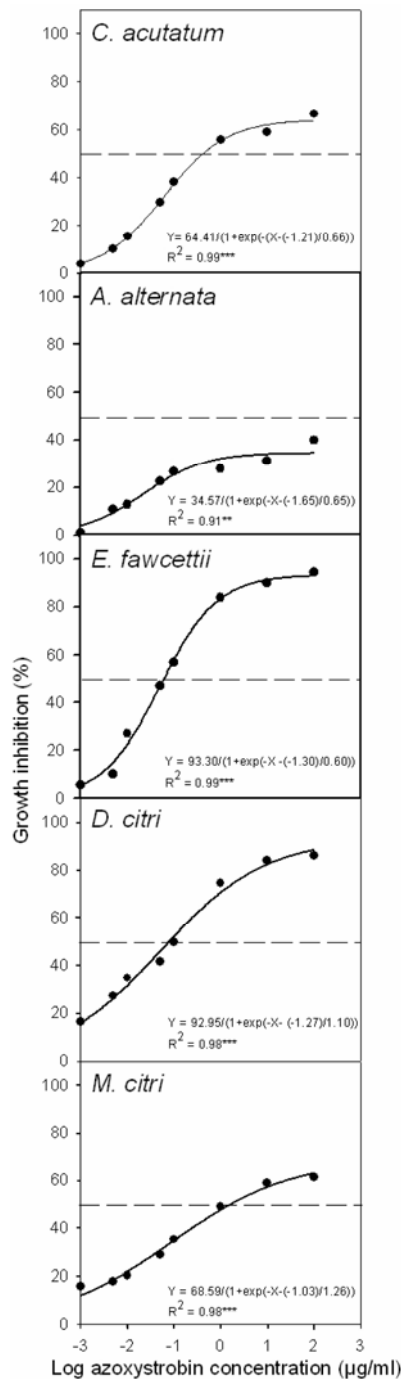


Fig. 1. Inhibition of mycelial growth of *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* by different concentrations of azoxystrobin.

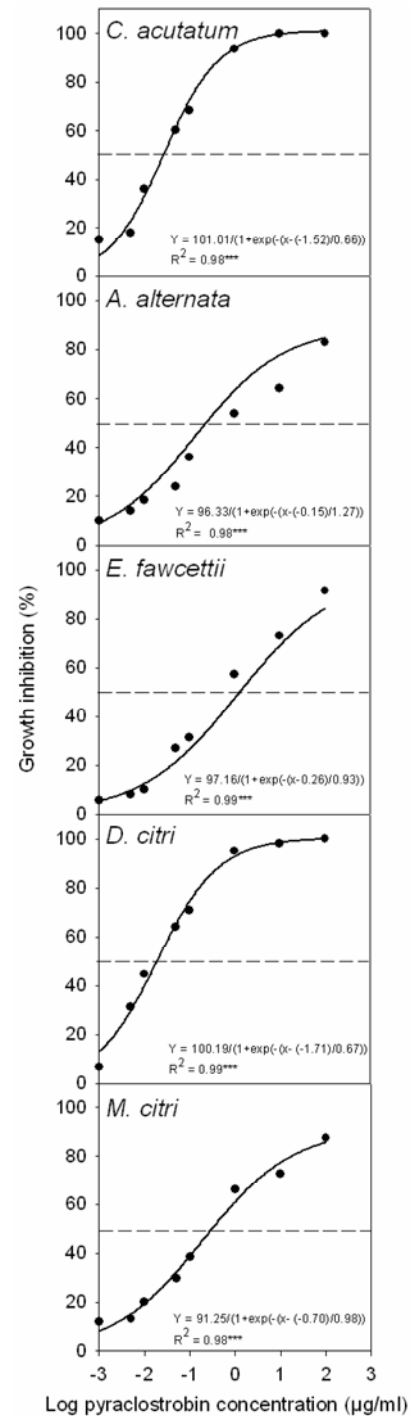


Fig. 2. Inhibition of mycelial growth of *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* by different concentrations of pyraclostrobin.

Table 2. Analysis of variance of the effect of the concentration of azoxystrobin, pyraclostrobin, and fenbuconazole on the mycelial growth of five fungal pathogens of citrus

Sources of variation	df	P values				
		<i>Colletotrichum acutatum</i>	<i>Alternaria alternata</i>	<i>Elsinoe fawcettii</i>	<i>Diaporthe citri</i>	<i>Mycosphaerella citri</i>
Fungicide (F)	2 (or 1) ^a	<0.001 ^b	<0.001	<0.001	<0.001	<0.001
Isolate (I)	4	0.27	0.59	0.01	0.001	0.006
Experiment (E)	1	0.68	0.61	0.23	0.30	0.21
F × I	8 (or 4)	0.61	0.61	<0.001	0.002	0.03
F × E	2 (or 1)	0.47	0.62	0.20	0.71	0.57
I × E	4	0.84	0.50	0.44	0.58	0.70

^a Number in parentheses for *A. alternata* and *D. citri*.

^b Significant effects are underlined.

lates (Table 3) and used to test the field isolates. The range of sensitivity of *C. acutatum* and the coefficient of variation (CV) of *C. acutatum* was low for both strobilurin fungicides (Table 3), and most isolates fell into one or two sensitivity categories (Figs. 5 and 6). With *A. alternata* and *D. citri*, there were a few isolates that were quite tolerant to azoxystrobin and some that were relatively sensitive

(Fig. 5). With the other pathogens, the number of isolates falling in any given category, e.g., 40 to 60% inhibition, was similar to the number in other categories. The response of the same isolates to pyraclostrobin was similar, but the ranges and CV were generally lower than for azoxystrobin (Table 3). There were a few isolates of *A. alternata* that were especially sensitive or tolerant to pyraclostrobin (Fig. 5). Single isolates of *E. fawcettii* and a few *M. citri* were especially insensitive to this fungicide. Other than those isolates, most fell into a continuum in their response to pyraclostrobin.

With fenbuconazole, the ranges of sensitivity and CVs were generally higher than with the strobilurin fungicides (Table 3). There were several isolates of *M. citri* that were especially sensitive to fenbuconazole (Fig. 7).

DISCUSSION

In this study, the ED₅₀ values and the ranges of sensitivity of the five principal foliar fungal pathogens of citrus to azoxystrobin, pyraclostrobin, and fenbuconazole were established. These fungicides were registered for use on Florida citrus in recent years. Both the strobilurin and SBI fungicides are prone to development of resistance. We now have established discriminatory concentrations for each pathogen–fungicide combination based on mycelial growth of isolates recovered prior to the use of these products in groves. When control failures occur in groves, isolates obtained from those groves can be compared with baselines now established.

In general, the citrus pathogens were much less sensitive than other fungi tested for sensitivity to strobilurin fungicides. In our study, the ED₅₀ values for mycelial growth of citrus pathogens to azoxystrobin ranged from 0.06 µg/ml for *E. fawcettii* to greater than 100 µg/ml for *A. alternata*. In contrast, the ED₅₀ values for conidial germination for azoxystrobin for *Plasmopara*

viticola on grapes ranged from 0.05 to 0.94 µg/ml (47) and for *Uncinula necator* ranged from 0.0037 to 0.028 µg/ml (48). However, both of these organisms are obligate biotrophs, and the sensitivity of these pathogens to azoxystrobin was based primarily on spore germination rather than mycelial growth. In studies based on mycelial growth in vitro, results were more comparable to those obtained with citrus pathogens (5,25). The ED₅₀ values of *Venturia inaequalis* isolates to kresoxim methyl as determined by mycelial growth in vitro ranged from 0.11 to 0.75 µg/ml (25). With *Colletotrichum graminicola*, the ED₅₀ values in relation to azoxystrobin were 0.01 to 0.1 for sensitive isolates (16). The sensitivity of pathogens of other crops to pyraclostrobin has not been reported.

The citrus pathogens were much less sensitive to fenbuconazole than was *Monilinia oxycocci*, a cranberry pathogen (21). The ED₅₀ values of sensitive isolates of *M. oxycocci* were about 0.0001 to 0.001 µg/ml compared to 0.24 to 1.49 for citrus pathogens. *Cladosporium caryigenum*, the cause of pecan scab, had ED₅₀ values of 0.19 to 0.30 µg/ml (33), which were more similar to those found for citrus pathogens.

Fungicide sensitivity to strobilurin fungicides determined in vitro may not be an accurate reflection of their sensitivity to these products in vivo (4–6,25,26). Many fungi respond to Q_oI fungicides by expression of alternative respiration, a pathway that avoids electron transport through the target site of the Q_oI fungicides, cytochrome *b*. Azoxystrobin did not inhibit growth of *A. alternata* by 50% even at 100 µg/ml. Thus, fungi attacking citrus could be using alternative respiration to avoid effects of the Q_oI fungicides on the cytochrome *b* system in vitro, or mycelium may not take up these fungicides well. Alternative respiration is usually not active in fungi in the plant (4,5,15), and thus, many fungi are more sensitive in vivo than in vitro. Salicylhydroxamic acid (SHAM),

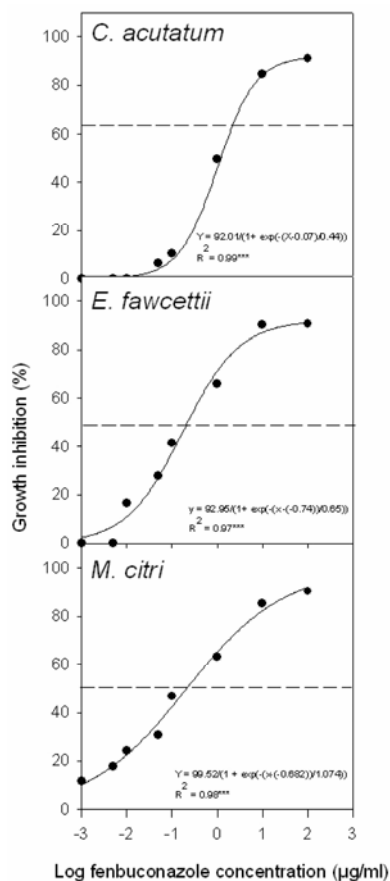


Fig. 3. Inhibition of mycelial growth of *Colletotrichum acutatum*, *Elsinoe fawcettii*, and *Mycosphaerella citri* by different concentrations of fenbuconazole.

Table 3. Mean effective dose to reduce growth by 50% (ED₅₀ value) for each fungicide–species combination, selected discriminatory concentration, and range and coefficient of variation (CV) for the 50 to 62 isolates evaluated at that concentration

Fungicide Fungal pathogen	ED ₅₀ (µg/ml) ^a	Selected discriminatory concentration	Field isolates inhibition (%)	
			Range	CV (%)
Azoxystrobin				
<i>Colletotrichum acutatum</i>	0.40 (0.09-0.79)	1.0	23.18	8.36
<i>Alternaria alternata</i>	>100 (all >100)	100.0	64.18	39.71
<i>Elsinoe fawcettii</i>	0.06 (0.03-0.45)	0.1	69.84	25.04
<i>Diaporthe citri</i>	0.08 (0.03-0.45)	0.1	60.40	28.35
<i>Mycosphaerella citri</i>	1.62 (0.37-4.2)	1.0	45.82	30.11
Pyraclostrobin				
<i>C. acutatum</i>	0.029 (0.01-0.034)	0.01	18.36	7.30
<i>A. alternata</i>	0.87 (0.37-1.58)	10.0	46.04	22.11
<i>E. fawcettii</i>	0.62 (0.21-1.31)	0.1	53.92	32.17
<i>D. citri</i>	0.019 (0.003-0.059)	0.01	31.05	19.16
<i>M. citri</i>	0.03 (0.02-0.89)	0.1	52.49	31.70
Fenbuconazole				
<i>C. acutatum</i>	1.01 (0.85-1.26)	0.1	36.81	31.64
<i>E. fawcettii</i>	0.22 (0.11-0.46)	1.0	49.00	20.84
<i>M. citri</i>	0.21 (0.13-0.75)	0.1	59.96	55.02

^a Average and range in parentheses of five isolates of each species and two experiments with each.

which blocks alternative respiration, can be incorporated into the medium to determine the sensitivity of fungi to Q₀I fungicides (5,25). However, with the citrus pathogens, SHAM itself was quite inhibitory. SHAM did not increase the sensitivity of mycelial growth of any of the citrus pathogens to the strobilurins. Thus, there is no indication that mycelium of these fungi is utilizing alternative respiration to avoid the toxic effects of the strobilurin fungicides.

Spore germination may be more sensitive than mycelial growth to the inhibitory

effects of strobilurin fungicides (26,27). In the current study, mycelial growth of *A. alternata* was rather insensitive to this fungicide, but the ED₅₀ for spore germination was much lower. As with mycelial growth, the addition of SHAM did not increase the activity of azoxystrobin against *A. alternata*. Pasche et al. (27) found that SHAM did not inhibit spore germination of *A. solani* from potatoes; but they used SHAM in all of the evaluations and thus did not evaluate the effect of this chemical on the sensitivity of the fungus to azoxystrobin.

Despite the comparatively low activity of strobilurins and fenbuconazole against mycelial growth of most citrus fungal pathogens, these fungicides are moderately to highly effective for control of most diseases (37). Azoxystrobin and trifloxystrobin were effective for control of PFD but generally were less active than the benzimidazoles for this disease (26,27,38). They are recommended in Florida in combination with contact fungicides for improved control and for resistance management (37). Fenbuconazole has not been evaluated for PFD control, but other SBI

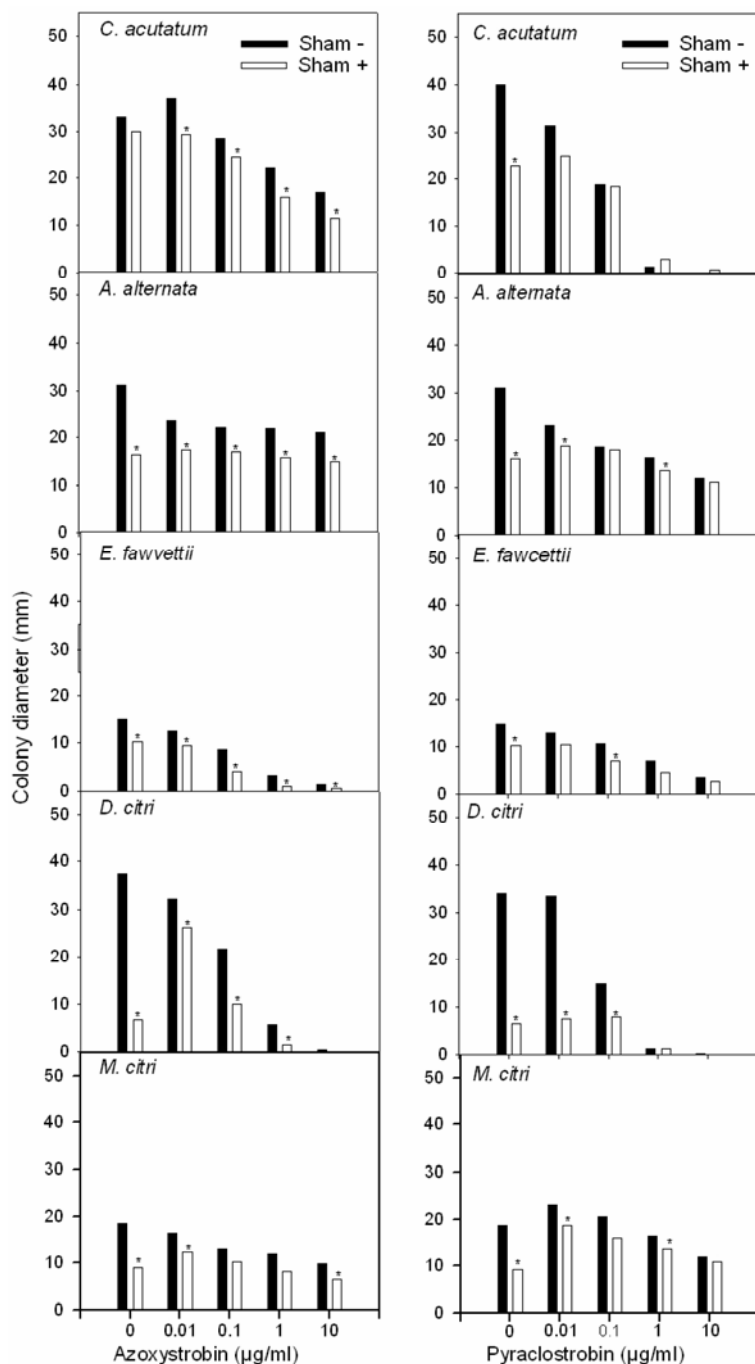


Fig. 4. Effect of salicylhydroxamic acid (SHAM) on the activity of azoxystrobin and pyraclostrobin on mycelial growth of *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* at different concentrations of fungicide. * Indicates that colony diameter is significantly lower with 100 ppm of SHAM than in its absence according to LSD, $P = 0.05$.

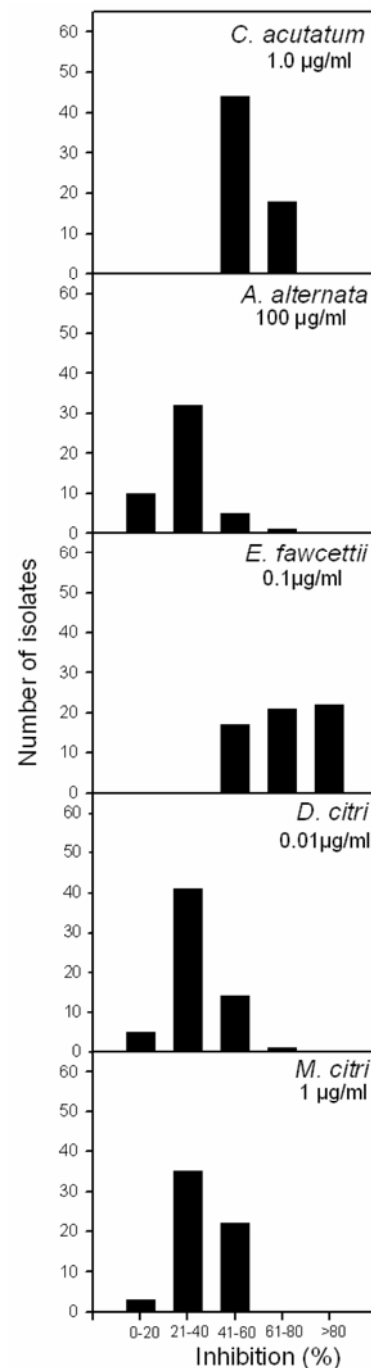


Fig. 5. Range of isolate sensitivity of *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* to azoxystrobin.

fungicides are moderately active against PFD (30). In the field, azoxystrobin and pyraclostrobin are highly effective for control of *Alternaria* brown spot (8,9,11,36). This contrasts sharply with the high ED₅₀ values observed for these fungicides against mycelial growth of *A. alternata* in vitro. The activity of strobilurins in the field is due to their ability to inhibit spore germination. Pyraclostrobin, azox-

ystrobin, and fenbuconazole are all highly effective against citrus scab under field conditions (2,10,38,49). Azoxystrobin and fenbuconazole have good preventive activity against scab infection and also proved effective as eradicants in greenhouse studies (14). Azoxystrobin and pyraclostrobin are moderately to highly effective for melanose control used alone or in programs with copper fungicides (1,49). Azoxystrobin had good preventive activity in greenhouse tests but was not effective as a postinfectious application (14). Fenbuconazole is relatively ineffective for melanose control in the field (13,49). In vitro, pyraclostrobin was more active against *D. citri* than was azoxystrobin. Azoxystrobin, pyraclostrobin, and fenbuconazole are effective for control of greasy spot in the field, especially when used in combination with petroleum oil (12,35). Fenbuconazole was also very effective in reducing the epiphytic growth of *M. citri* and in reducing disease severity (7,24).

The ED₅₀ and discriminatory values determined in this study should be useful for detection of resistance to these fungicides in most cases. When isolates resistant to strobilurin fungicides occur, they usually tolerate high levels of fungicide, often

greater than 10 µg/ml (5). Thus, the situation should be comparable to resistance to benzimidazole fungicides in *E. fawcettii* and *M. citri*, where isolates with resistance were readily detected (43,44). Ma et al. (18) also found that resistant isolates of *Alternaria* spp. tolerated high levels of azoxystrobin even though the mutation was different from the one commonly found. However, Pasche et al. (27) found that *A. solani* isolates on potatoes can develop an insensitivity to azoxystrobin and that the average ED₅₀ can increase steadily with continued use. If decreases in sensitivity of citrus pathogens are incremental, the development of resistance may be more difficult to detect.

In the case of the *A. alternata*-azoxystrobin combination, the discriminatory concentration of 100 µg/ml is probably not useful for detection of resistance in future studies. Since spore germination is much more sensitive than mycelial growth, resistance to azoxystrobin will be much more easily detected using conidia. The range of sensitivity of a large number of isolates remains to be determined. However, use of spores for assessment of fungicide sensitivity with some of the other species may be difficult. Conidia of *D. citri* require weeks to produce (22), *M. citri* produces few conidia in culture or in nature, and production of conidia with *E. fawcettii* is laborious. Only *C. acutatum* produces abundant conidia rapidly in culture.

Resistance to fenbuconazole could also be difficult to detect. With the SBI fungicides, resistance usually occurs as incremental shifts in tolerance in the population (21,33). Thus, to detect resistance shifts requires determination of the ED₅₀ values from relatively large numbers of isolates. So the situation may be comparable to the benzimidazole-*C. acutatum* relationship, where resistance would be difficult to detect (30). Large numbers of isolates will be needed to assess resistance levels of populations of these pathogens.

In most cases, evaluations of large numbers of isolates of citrus fungal pathogens can be difficult and time-consuming. *A. alternata* isolates can be collected at any time of year and grow rapidly, but isolates must be evaluated for pathogenicity since saprophytes of the same species are common on citrus (32). *C. acutatum* is readily isolated from infected petals but is much more difficult to recover from vegetative tissues (3), and thus, larger numbers of isolates can only be collected during epidemic years at bloom time. *M. citri* and *E. fawcettii* can be isolated relatively easily at most times during the year. However, both are very slow growing and require weeks to evaluate resistance. *D. citri* grows more rapidly but can be consistently isolated only from twigs (22), which requires weeks. Despite the difficulties, methods are available to collect sufficient isolates to

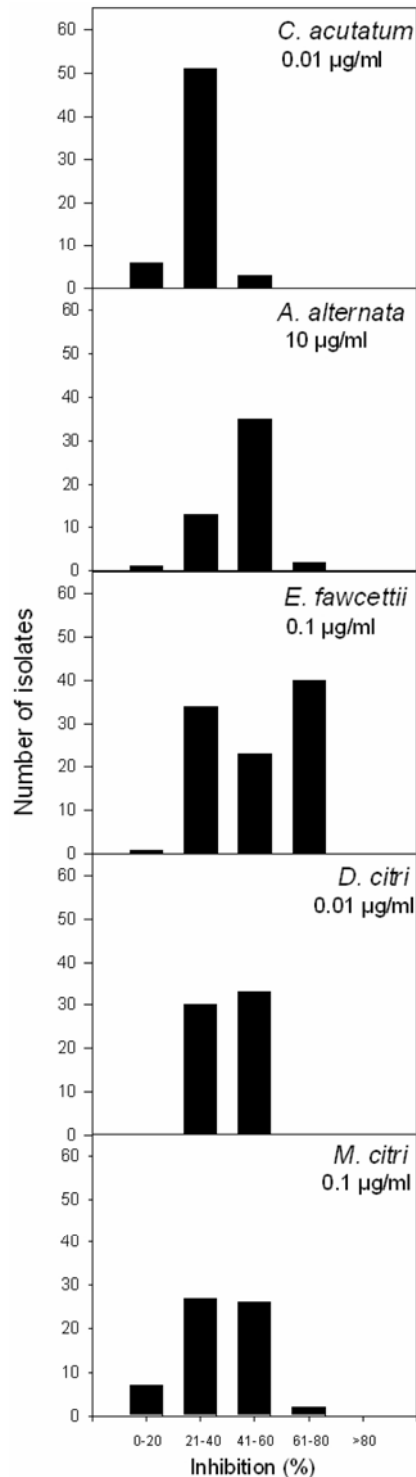


Fig. 6. Range of isolate sensitivity of *Colletotrichum acutatum*, *Alternaria alternata*, *Elsinoe fawcettii*, *Diaporthe citri*, and *Mycosphaerella citri* to pyraclostrobin.

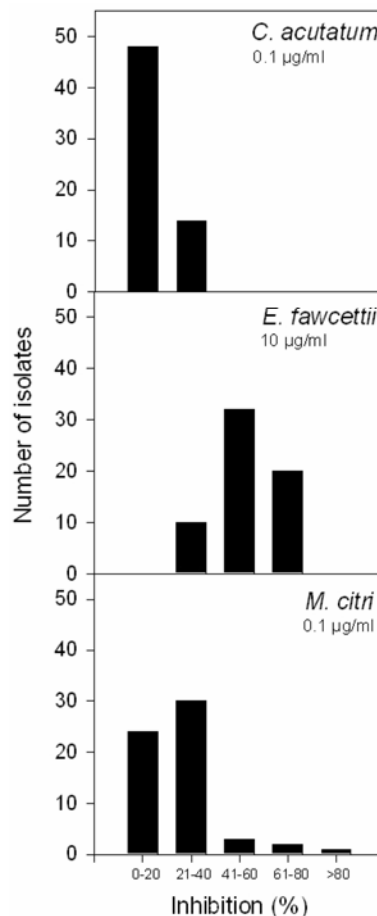


Fig. 7. Range of isolate sensitivity of *Colletotrichum acutatum*, *Elsinoe fawcettii*, and *Mycosphaerella citri* to fenbuconazole.

evaluate control failures to determine whether they are due to resistance development or to other problems. The recent development of the spiral gradient dilution for use with fungi (17) should be helpful in evaluating the sensitivity of large numbers of isolates, but collection of isolates will still be time-consuming.

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