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Geographical Distribution of Strobilurin Resistance of *Alternaria alternata,* Causal Agent of Alternaria Brown Spot in Florida Citrus Groves

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Alternaria brown spot is the most important fungal foliar disease affecting tangerines and tangerine hybrids. Control is based primarily on fungicide applications such as copper and strobilurins; however, control failure with strobilurins was detected recently. A statewide survey was initiated to evaluate the sensitivity of *A. alternata* isolates to azoxystrobin and pyraclostrobin. In total, 143 monoconidial isolates of *A. alternata* from 15 groves in eight counties were evaluated using a resazurin (RZ)-based microtiter assay. Strobilurin resistance occurred in all surveyed counties. On average, 68% of tested isolates were highly resistant to strobilurins. The effective concentration needed to inhibit 50% growth (EC_{50}) values were greater than 5 µg/mL for azoxystrobin and 1 µg/mL for pyraclostrobin, while mean EC_{50} values for sensitive isolates were 0.2135 and 0.0266 µg/mL, respectively. Resistance to the two fungicides was highly correlated (*P* < 0.001), indicating cross resistance. The majority of isolates were from cultivars Minneola (45%) and Murcott (27%); the remainders were from 'Sunburst' (11%), 'Dancy' (10%), 'Orlando' (5%), and 'Lee' (2%). The highest proportion of resistant isolates was from 'Minneola' (92%), followed by 'Orlando' (77%), 'Dancy' (60%), 'Sunburst' (50%), and Murcott' (36%). Based on our observations, strobilurin resistance occurred more frequently on susceptible cultivars with intense fungicide use.

Alternaria brown spot (ABS), caused by the fungus *Alternaria alternata* (Fr.) Keissl. tangerine pathotype, is the most important foliar disease affecting tangerines (*Citrus reticulata* Blanco) and tangerine hybrids in commercial citrus orchards. The most susceptible cultivars commonly grown in Florida include 'Minneola' tangelo, 'Dancy' tangerine, 'Sunburst' tangerine, and 'Murcott' tangor (Canihos et al., 1999). In the last decade, ABS incidence has increased in Florida and other citrus-producing regions around the world (Canihos et al., 1997; Peres et al., 2003). ABS management includes the use of cultural control methods as well as chemical fungicide applications; but the use of fungicides is the most effective tool for disease control (Timmer et al., 2000). Calendar applications of fungicides have been used extensively in citrus production for ABS control. In Florida, applications can be made every 10 to 14 d from March to July in groves with heavy disease pressure (Dewdney and Timmer, 2012).

Strobilurin fungicides (formally named Qinone outside inhibitors or QoI), such as azoxystrobin, pyraclostrobin, and trifloxystrobin, are often used against ABS in Florida. Strobilurins block the electron transfer between cytochrome *b* and cytochrome c_1 by binding at the ubiquinol oxidation center (Qo-center) in the mitochondria (Bartlett et al., 2002). Because the mode of action is very specific, the risk of resistance development in the population exposed to the fungicide is high. A single point mutation within the cytochrome *b* gene confers strobilurin resistance. The most important mutation that confers a high level of resistance is from the amino acid substitution glycine (G) to alanine (A) at position 143 (Bartlett et al., 2002; Ypema and Gold, 1999).

Since the first report of *A. alternata* strobilurin resistant isolates in Florida tangerine groves (Mondal et al., 2009), the level of resistance as well as the resistance distribution in commercial citrus groves in Florida remains unknown. The objective of this study was to identify the geographical distribution of strobilurin resistant *A. alternata* in Florida citrus groves.

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Materials and Methods

Sample collection and monoconidial isolates. During 2010–11, a statewide survey was conducted in commercial tangerine and tangerine hybrid groves. Overall, 15 groves encompassing eight counties were sampled. Samples consisted of 60 to 80 Alternaria-infected leaves or fruit randomly selected per grove. Isolations were done as previously described (Peever et al., 1999). To differentiate between saprophytes and other *A. alternata* pathotypes, pathogenicity was confirmed by inoculating young leaves of 'Minneola', 'Dancy', or 'Murcott' in a detached leaf assay as previously described (Canihos et al., 1999). Once a pathogenic isolate was confirmed, a monoconidial culture was produced when a single germinating conidia was transferred from 2% water agar to potato dextrose agar (PDA). Isolates were stored at –20 °C on colonized filter paper for future work.

Screening for strobilurin resistance. Isolates were screened for resistance to azoxystrobin and pyraclostrobin using the resazurin-based microtiter assay described by Vega et al. (2012). Technical-grade azoxystrobin and pyraclostrobin were dissolved in acetone at 10 mg/mL of active ingredient. The final fungicide concentrations evaluated were 0.001, 0.01, 0.05, 0.1, 0.5, 1, and 10 µg/mL for azoxystrobin and 0.001, 0.005, 0.01, 0.05, 0.1, 1, and 10 µg/mL for pyraclostrobin. In total, 143 isolates were tested to estimate the effective concentration necessary to produce 50% inhibition (EC_{50}) using the exponential decay function as previously described (Vega et al., 2012).

Results and Discussion

In the 2010 survey, 385 isolates of *A. alternata* were recovered from commercial citrus groves in Florida; while in the 2011 survey, 216 isolates were recovered. Out of the 601 isolates recovered, 342 were tested for pathogenicity and 302 isolates (approximately 88%) were confirmed as pathogenic. The majority of isolates were collected from Polk County (57%).

Strobilurin sensitivity survey. The presence of high levels of strobilurin resistance was confirmed for 68% of *A. alternata* isolates tested (98 out 143). As expected, a strong correlation ($P < 0.001$; $R^2 = 0.9432$) between azoxystrobin and pyraclostrobin was observed. Both strobilurins [fungicide resistance action committee: (FRAC group 11)] have a common mode of action, blocking electron transport in the mitochondria by binding to the Qo site of the cytochrome bc_1 complex; therefore cross resistance between strobilurins has been shown (Gisi et al., 2002). The strobilurin sensitivity distribution split the sampled population into two well defined groups: "sensitive" and "resistant." The sensitive population displayed a normal distribution with an EC_{50} mean of 0.2135 µg/mL for azoxystrobin and 0.0266 µg/mL for pyraclostrobin, while the resistant population showed a typical left-skewed distribution with an EC_{50} mean > 10 µg/mL for azoxystrobin and 5.046 µg/mL for pyraclostrobin. The presence of resistant isolates was widespread among counties; however the distribution of resistant isolates varied by region and groves (Table 1). Polk County is the most important specialty fruit producing county in Florida and resistance was confirmed in almost 40% of the isolates tested, which means the resistant isolates have become a larger proportion of the population and the total loss of this important fungicide for ABS management is likely. Counties located on the East Coast (Indian River and St. Lucie) had a completely resistant population likely due to the selection pressure produced by multiple strobilurin applications per season (Genet et al., 2006). More than 90% and 75% of isolates tested from 'Minneola' and 'Orlando', respectively, were strobilurin resistant (Table 2). Typically, 'Minneola' is one of the most susceptible cultivars (Timmer et al., 2000); therefore multiple fungicide applications are needed to ensure a good fruit quality and yield, increasing the selection pressure against sensitive population. The proportion of resistant and sensitive isolates by host is linked to the susceptibility of the cultivar. The less susceptible cultivars, such as 'Murcott' and 'Sunburst', had the lowest proportion of resistance found (Table 2). Only in two out of 15 groves sampled was the entire tested population sensitive. In contrast, in 13 groves the population was resistant. These findings show that strobilurin resistance of *A. alternata* is widely present in Florida citrus production areas and could make management of this important disease difficult.

Thus, the presence of strobilurin-resistant populations of *A. alternata* in Florida-citrus groves increases the need to find alternate disease control measures for ABS management in Florida.

Table 1. Distribution of *Alternaria alternata* isolates resistant to strobilurins by Florida counties

County	N[z]	Resistant[y]	Sensitive[x]	Resistant isolates (%)
Hardee	10	10	0	100
Highlands	9	9	0	100
Indian River	15	15	0	100
Lake	12	12	0	100
Osceola	9	5	4	55.5
Polk	68	27	41	39.7
Sarasota	10	10	0	100
St. Lucie	10	10	0	100

[z]Number of isolates tested.
[y]Resistant isolates grew on complete medium in resazurin-based microtiter assay amended with azoxystrobin at 5 µg/mL or pyraclostrobin at 1 µg/mL.
[x]Sensitive isolates did not grow on complete medium in RZ-based microtiter assay amended with azoxystrobin at 5 µg/mL or pyraclostrobin at 1 µg/mL.

Table 2. Distribution of *Alternaria alternata* isolates resistant to strobilurins by host.

Host[z]	N[y]	Resistant[x]	Sensitive[w]	Resistant isolates (%)
'Dancy' mandarin	10	6	4	60
'Lee' citrus hybrid	9	0	9	0
'Minneola' tangelo	73	67	6	91.8
'Murcott' tangor	28	10	18	35.7
'Orlando' tangelo	13	10	3	76.9
'Sunburst' tangerine	10	5	5	50

[z]Mandarin or tangerine = *Citrus reticulata*, citrus hybrid = mandarin × tangelo, tangelo = *C. paradisi* × *C. reticulata*, tangor = *C. reticulata* × *C. sinensis*
[y]Number of isolates tested.
[x]Resistant isolates grew on complete medium in resazurin (RZ)-based microtiter assay amended with azoxystrobin at 5 µg/mL or pyraclostrobin at 1 µg/mL.
[w]Sensitive isolates did not grow on complete medium in RZ-based microtiter assay amended with azoxystrobin at 5 µg/mL or pyraclostrobin at 1 µg/mL.

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