Sensitivity of *Calonectria pseudonaviculata*, the Pathogen of Boxwood Blight, to Strobilurin and Demethylation Inhibitor Fungicides in Connecticut[1]

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Abstract

Calonectria pseudonaviculata (Lombard, Crous, Wingfield & Wingfield) causes a severe blight disease on boxwood known as "boxwood blight". Three isolates: 11-9-4a and CTWH1 - wild types from Connecticut landscapes, and FC1 - an isolate of 11-9-4a that was selected for ability to grow on up to 250 µg a.i./ml pyraclostrobin fungicide, were evaluated for sensitivity to nine fungicides belonging to the demethylation inhibitor ([DMI] propiconazole, tebuconazole, triflumizole, myclobutanil, tetraconazole) and strobilurin (pyraclostrobin, azoxystrobin, trifloxystrobin, kresoxim-methyl) groups. The effects of fungicides on mycelial growth and conidial germination were analyzed using *in vitro* assays. All DMI fungicides strongly inhibited radial growth, but did not prevent conidial germination. Of the strobilurins, only pyraclostrobin inhibited mycelial growth and conidial germination of the 11-9-4a and CTWH1 isolates within label use rates. Pyraclostrobin, kresoxim-methyl, and trifloxystrobin inhibited mycelial growth for 11-9-4a and pyraclostrobin and kresoxim-methyl for CTWH1. All strobilurin fungicides inhibited the conidial germination of 11-9-4a and FC1; only pyraclostrobin affected CTWH1. FC1 and CTWH1 exhibited reduced sensitivity to strobilurin fungicides for mycelial growth. For effective control of mycelial growth and conidial germination, and to reduce the risk of resistance development, fungicides from both FRAC groups should be used and integrated with other best management practices.

Index words: fungicide resistance; *Buxus*; chemical disease management.

Chemicals used in this study: azoxystrobin (Heritage 50 WG); kresoxim-methyl (Cygnus 50 WG); myclobutanil (Rally 40WSP); propiconazole (Procon-Z 14.3 L); pyraclostrobin (Insignia 20 WG); tebuconazole (Torque 38.7 SC); tetraconazole (Minerva 11.6 SC); trifloxystrobin (Flint 50 WG); triflumizole (Procure 480 SC).

Species used in this study: boxwood (*Buxus* L.); boxwood blight (*Calonectria pseudonaviculata*).

Significance to the Horticulture Industry

Boxwood (*Buxus* L.) is a very important ornamental plant in landscapes in the United States and has a significant share of wholesale ornamental plant sales. The horticultural and landscape industries depend on the economical production and maintenance of healthy boxwood plants. The pathogen causing boxwood blight, *Calonectria pseudonaviculata*, first occurred in 2011 in Connecticut and North Carolina and has since spread to at least 21 states and three Canadian provinces. It has been found in landscapes, commercial production nurseries, garden centers, and in wholesale distribution. Total losses in Connecticut alone amount to an estimated $5.5 million (LaMondia 2015). Boxwood blight is difficult and costly to control with fungicides. Because of the requirement for repeated application of fungicides, the development of resistance is a real threat. Therefore, the assessment of the potential for development of resistance (or reduced fungicide sensitivity) is important to develop an effective spray program and to be able to avoid fungicide resistance. The use of multiple fungicide active ingredients in mixtures or rotational application as well as best management practices should be targeted.

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Introduction

Calonectria pseudonaviculata (Crous, J.Z. Groenew. & C.F. Hill) L. Lombard, M. J. Wingf. & Crous causes boxwood blight (Crous et al. 2002, 2004). The pathogen was introduced into the United States, and the disease was first observed in Connecticut and North Carolina in 2011 (Ivors et al. 2012). It has now been reported from 21 states (AL, CA, CT, DE, FL, IN, KS, KY, MA, MD, NC, NH, NJ, NY, OH, OR, PA, RI, SC, TN, VA) and three Canadian provinces (BC, ON, QC) (LaMondia 2015). Boxwood blight was first described in the United Kingdom in 1994 (Henricot et al. 2000) and New Zealand in 1998 (Crous et al. 2002). Since that time, boxwood blight has spread to at least 21 countries throughout the temperate regions of the world where boxwood is grown (Gehesquière et al. 2015).

C. pseudonaviculata causes aboveground symptoms, including brown leaf spots and black streaks on stems, resulting in defoliation and dieback. Control measures are based on integrated best management practices which include monitoring, cultural controls, cultivation measures, planting less susceptible or tolerant varieties, sanitation of equipment, and fungicide application. For successful disease management, an efficient fungicide spray program is necessary, but more needs to be known regarding fungicide efficacy and potential fungicide resistance development. *In vitro* studies showed inhibition of mycelium growth and conidial germination by several fungicides (Brand 2006, Henricot et al. 2008, LaMondia

Table 1. Fungicides evaluated for control of mycelial growth and conidial germination of *Calonectria pseudonaviculata* using *in vitro* assays

Fungicide a.i.	Formulation	FRAC group[z]	Efficacy[y]	Use rate (µg a.i./ml)[x]
Myclobutanil	Rally 40WSP	3	S	60-120
Propiconazole	Procon-Z 14.3 L	3	S	70-140
Tebuconazole	Torque 38.7 SC	3	S	135-335
Tetraconazole	Minerva 11.6 SC	3	S	235-390
Triflumizole	Procure 480 SC	3	S	150-600
Azoxystrobin	Heritage 50 WG	11	S	40-300
Kresoxim-methyl	Cygnus 50 WG	11	P	40-240
Pyraclostrobin	Insignia 20 WG	11	P	30-240
Trifloxystrobin	Flint 50 WG	11	P	40-150

[z]FRAC = Fungicide Resistant Action Committee.

[y]S = systemic effect; P = protectant effect.

[x]Use rate represents the range of active ingredient concentration in label recommendations on ornamentals or other crops.

2014). LaMondia (2014) reported that the demethylation inhibitor (DMI) fungicides propiconazole, triflumizole, tebuconazole, and the combined fungicides fludioxonil + cyprodinil had the most inhibition efficacy against mycelial growth, but the DMIs had no effects on conidial germination. The strobilurins kresoxim-methyl, pyraclostrobin, trifloxystrobin, and the combined fungicides boscalid + pyraclostrobin and fludioxonil + cyprodinil inhibited conidial germination. Studies from Europe demonstrated that the fungicide kresoxim-methyl and the combined fungicides epoxiconazole + kresoxim-methyl + pyraclostrobin (Opponent), epoxiconazole + pyraclostrobin (Opera), and boscalid + pyraclostrobin (Signum) were the most effective products at inhibiting mycelial growth and conidial germination (Henricot et al. 2008). Tolylfluanid, mancozeb, chlorothalonil, and fludioxonil + cyprodinil inhibited conidial germination and prochloraz, propiconazole, thiophanate-methyl, and carbendazim + flusilazole inhibited mycelial growth (Brand 2006).

The first boxwood blight management studies under field conditions were described by Henricot and Wedgwood (2013) and LaMondia (2015). Propiconazole, myclobutanil, thiophanate-methyl, fludioxonil, pyraclostrobin, kresoxim-methyl, and chlorothalonil showed efficacy against *C. pseudonaviculata* (LaMondia 2015). The products epoxiconazole + kresoxim-methyl + pyraclostrobin (Opponent) and the combination treatments of prochloraz, epoxiconazole, chlorothalonil, boscalid, pyraclostrobin, and kresoxim-methyl had significant efficacy (Henricot and Wedgwood 2013). *Calonectria henricotiae*, a closely related species present in Europe but not in the United States, differs in fungicide sensitivity from *C. pseudonaviculata* (Gehesquière et al. 2015). Not all of the tested fungicides are registered for use on boxwood or on ornamentals in the United States. The combination of systemic and protectant fungicides in a single application seemed to be an effective disease management strategy.

This study focused on two fungicide classes important for managing boxwood blight: demethylation inhibitor (DMI) fungicides (FRAC Group 3, sterol biosynthesis inhibitors [SBI]) and strobilurin fungicides (FRAC Group 11, Quinone outside Inhibitors [QoI]) (Table 1); both fungicide groups are prone to development of fungicide resistance. To our knowledge, wide-spread resistance to fungicides has not been described yet for *C. pseudonaviculata*, but reduced sensitivity to azoxystrobin and kresoxim-methyl has been observed (LaMondia 2014). However, the risk of fungicide resistance development is high and optimal use of these products will require stewardship. Strobilurin fungicides belong to an important class of fungicide and they have worldwide applications because of their broad spectrum of efficacy (Bartlett et al. 2002). QoI fungicides, to which strobilurins belong, block the mitochondrial respiration in fungi at the Qo site of the cytochrome bc1 enzyme complex (Gisi et al. 2000). DMI fungicides inhibit demethylation required for biosynthesis of fungal sterols like ergosterol, which is essential for proper function of fungal cell membranes. DMI fungicides have a broad spectrum of fungicidal and systemic effects as well, and a wide range of crops can be treated (Ziogas and Malandrakis 2015). The risk for resistance development has been classified as high for strobilurin fungicides and the risk of resistance to DMI fungicides is considered medium according to the fungicide resistance action committee (FRAC). QoI fungicides are active against a broad range of plant pathogens and their usage is very common. QoI fungicide resistance due to target site reduced sensitivity readily leads to cross-resistance within this FRAC group, and has been reported for 56 plant pathogens on many crops (Fungicide Resistance Action Committee 2013). Therefore, it is important to consider the FRAC guidelines and to follow resistance management, including good agricultural practice, resistant varieties, sanitation, disease free seed, and disease forecasting. The use recommendations for both fungicide groups include the use of mixtures, alternation of chemicals with different modes of action, and/or restricting the number of applications used during a season.

Our previous work suggested that reduced sensitivity to fungicides in *C. pseudonaviculata* may have developed in response to selection pressure as isolates previously affected by pyraclostrobin were selected for ability to grow on increasing concentrations of the fungicide (LaMondia 2014). Therefore, we focused on three isolates and two classes of fungicides and compared the data with the literature. The objectives of the present study were to determine the potential sensitivity of selected isolates of *C. pseudonaviculata* to four strobilurin (pyraclostrobin, azoxystrobin, trifloxystrobin, kresoxim-methyl) and five DMI (propiconazole, tebuconazole, triflumizole, myclobutanil, tetraconazole) fungicides. Some of these fungicides are not currently registered for use on boxwood in the United States.

In a previous study, 20 different active ingredients from 13 different Fungicide Resistance Action Committee groups were evaluated for their effects on the mycelial growth and conidial germination of the *C. pseudonaviculata* isolates L1 (Cps-CT-L1) and S1 (Cps-CT-S1) using *in vitro* assays (LaMondia 2014). In this present work, different isolates were evaluated. The isolate FC1 was chosen for its demonstrated ability to grow on up to 250 µg a.i./ml pyraclostrobin-amended media (data not shown). It

was compared with isolate 11-9-4a, from which FC1 was derived through selection. The CTWH1 isolate was included because it was recently isolated from diseased boxwood and was an aggressively virulent pathogen. These isolates represent a sample of *C. pseudonaviculata* strains found in CT.

Materials and Methods

Prior to initiating these experiments, seven *C. pseudonaviculata* isolates were initially screened for the ability to grow on pyraclostrobin-amended media over several serial stepwise transfers on media with increasing concentrations of pyraclostrobin, starting at 1 and ending at up to 250 µg a.i.·ml^{-1}. Isolates screened included: L1 and L2 that were isolated from leaves, and isolates S1 and S2 were recovered from stems of *Buxus sempervirens* 'Suffruticosa' in Connecticut December 5, 2011; isolates 11-9-4a, 12-1-2a, and 12-5-3 were isolated from boxwood plants (variety unknown) from Connecticut landscapes at the Connecticut Agricultural Experiment Station in New Haven, CT on September 4, 2011, January 2, 2012 and May 3, 2012, respectively, and were provided to the Valley Laboratory in Windsor, CT. All isolates were grown on half-strength PDA (19g Difco™ Potato Dextrose Agar with 15 g Difco™ Agar Technical, Fisher Scientific, Pittsburgh, PA, added to 1 liter (0.26 gal) deionized water; henceforth ½ PDA) and isolates were grown from single spores by dilution of a spore suspension in sterile water, streaking on ½ PDA, and subsequently growing a colony from an excised hyphal tip arising from growth from a single conidium. One isolate, designated as FC1, was selected from isolate 11-9-4a for its ability to grow repeatedly on 1 µg a.i.·ml^{-1} up to 250 µg a.i.·ml^{-1} pyraclostrobin.

After selection, three *C. pseudonaviculata* isolates were used in this study. Isolate: 11-9-4a - the wild type from a Connecticut landscape, FC1 - the isolate of 11-9-4a selected for its ability to grow on up to 250 µg a.i.·ml^{-1} pyraclostrobin, and isolate CTWH1, recovered from boxwood in a landscape from West Hartford, CT on November 11, 2013.

The sensitivity of the isolates CTWH1, 11-9-4a and FC1 to four strobilurins: Azoxystrobin (Heritage 50% WG, Syngenta Crop Protection LLC, Basel, Switzerland), kresoxim-methyl (Cygnus 50% WG, BASF Corp., Ludwigshafen, Germany), pyraclostrobin (Insignia 20% WG, BASF Corp., Ludwigshafen, Germany), trifloxystrobin (Flint 50% WG, Bayer CropScience LP, Leverkusen, Germany) and five demethylation inhibitor (DMI) fungicides: Myclobutanil (Rally 40WSP, Dow AgroScience LLC, Zionsville, IN), propiconazole (Procon-Z 14.3% L, Loveland Products, Inc., Loveland, CO), tebuconazole (Torque 38.7% SC, Cleary Chemicals LLC, Dayton, NJ), tetraconazole (Minerva 11.6% SC, SipcamAdvan, Durham, NC), and triflumizole (Procure 408 SC 42.14% WG, Chemtura Corp., Philadelphia, PA) was assessed (Table 1). Fungicides were evaluated for efficacy against both mycelial growth and conidial germination.

Inhibition of mycelial growth. Fungicide concentrations were prepared by dilution of the stock solution prepared from formulated commercial fungicide products of 5,000 and 1,000 µg a.i.·ml^{-1}. Mycelial plugs with a diameter of about 1.5 mm^2 were transferred to ½ PDA amended with fungicide. Three plates of each concentration of 0, 3.16, 10, 31.6, 100, 316, and 1,000 µg a.i.·ml^{-1} for each of the strobilurin fungicides and three plates of each concentration of 0, 0.001, 0.00316, 0.01, 0.0316, 0.1, 0.316, and 1 µg a.i.·ml^{-1} for each DMI fungicide were prepared. The plug with fungal mycelia was inverted and placed on the edge of the plate. The plates were incubated in the dark at 21 C (70 F) for four weeks. The colony diameter in cm was measured weekly and values of the fourth week were used for the statistical analyses. Each experiment was done twice.

The programs Statistix 9.0 and 10 (Analytical Software, Tallahassee, FL) were used for the statistical analyses. Growth data were normalized with the following formula: Formula 1:

$$normalized\ value = \frac{untreated\ value - treated\ value}{untreated\ value}$$

Data were linearized using log-concentration versus logit-transformed values for the proportion reduction of growth relative to the untreated control (i.e., the normalized value, defined above). Statistically significant differences among linearized data were determined by linear regression and use of the homogeneity of slopes test.

The EC_{85} (effective dose at 85%) was calculated because the authors believe that the EC_{50} value does not describe the efficacy required for control of such a devastating disease. To determine the EC_{85} value, the concentration that suppressed fungal growth to 15% of that on unamended media for each fungicide – isolate combination, the regression estimates for the y-intercept and the slope for the dose response were used with the following formula:
Formula 2:

$$\log(EC85) = \left(\log\left(\frac{0.85}{0.15}\right) - b\right)/m$$

where b is the intercept, and m is the slope.

Some values of very low concentrations did not show a consistent trend and could be misleading, resulting in false interpretations. Therefore, a minimum EC_{85} value of 0.1 µg a.i.·ml^{-1} was used as a threshold to exclude data from the statistical analysis. Because of variations between some repetitions, the 95% confidence interval was not calculated and observations were interpreted from individual trials.

Inhibition of conidial germination. Boxwood plants 'Green Velvet' (*B. sinica* var. *insularis* × *B. sempervirens*) were inoculated with FC1 conidia and incubated at 21 C (70 F) in darkness. The leaves were kept moistened for the next four hours. After approximately 7 days, when the plants showed symptoms, small pieces of the lesions were cut and transferred onto ½ PDA. Detached boxwood leaves were surface sterilized with bleach (0.6% sodium hypochlorite) for 30 s, washed in sterile water for 30 s and air dried. Sterile 'Green Velvet' boxwood leaves were inoculated with conidia of 11-9-4a and CTWH1 and

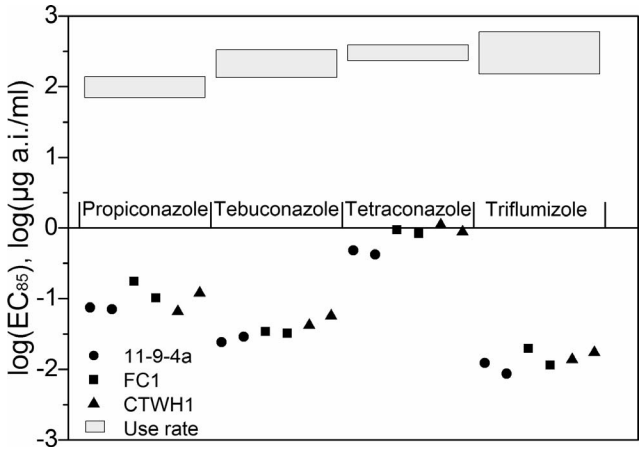

Fig. 1. The effective concentration of demethylation inhibitor fungicides that suppressed fungal growth by 85% (expressed as log(EC_{85})) of *Calonectria pseudonaviculata* isolates 11-9-4a (●), FC1 (■), and CTWH1 (▲). The grey boxes represent the use rate for spray application.

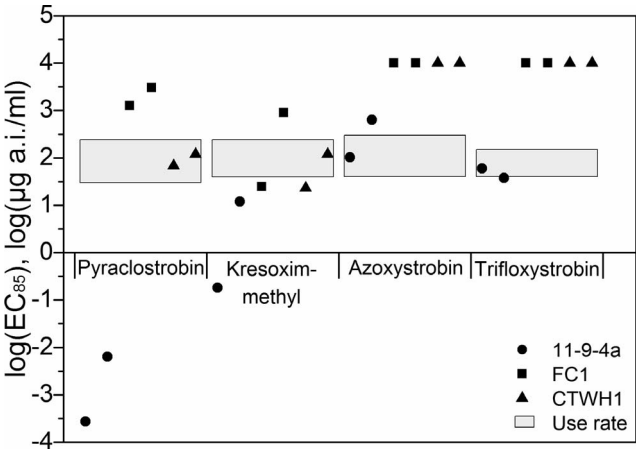

Fig. 2. The effective concentration of strobilurin fungicides that suppressed fungal growth by 85% (expressed as log(EC_{85})) for *Calonectria pseudonaviculata* isolates 11-9-4a (●), FC1 (■), and CTWH1 (▲). The grey boxes represent the use rate for spray application. Log(EC_{85}) values above 4 (e.g. 10 000 µg a.i./ml) had to be extrapolated and were displayed as 4 to permit visualization (actual estimated log(EC_{85}) values were FC1: azoxystrobin 6.5 and 7.3, trifloxystrobin 7.6 and 11.2; CTWH1: azoxystrobin: 4.8 and 5.7, trifloxystrobin: 18.9 and 7.0).

incubated at 21 C (70 F) in darkness. Small pieces of infected leaves with conidia were transferred to ½ PDA plates and incubated at 21 C (70 F) in the dark for several days. Plugs with conidia were transferred to new ½ PDA plates. Conidia were harvested by adding sterile distilled water with one drop of Tween 80 per 200 ml of water, (Tween 80 polyoxyethylene sorbitan monooleate, Sigma-Aldrich, St. Luis, MO) and rubbing with a sterile glass rod. One drop (approximately 1,200 to 2,000 conidia/drop, $3-5 \times 10^4$ conidia/ml) was placed on each of two strobilurin amended ½ PDA plates (0, 3.16, 10, 31.6, 100, and 316 µg a.i.·ml^{-1}). The plates were incubated at 21 C (70 F) in the dark overnight. On the next day, the first 100 conidia observed under a microscope were assessed for germination. Conidia were counted as having germinated if the germ tube extended to the length of the conidia or beyond. Experiments were conducted twice. To analyze the inhibition of germination by each fungicide and for the three isolates, the data were normalized using Formula 1. Existence of a dose-response for the tested concentrations of fungicides was determined if there was a linear relationship in the data when plotted on a log-concentration versus probability (germination) graph, as visualized with Sigma Plot (Systat Software, Inc, San Jose, CA). Comparisons of dose-responses were made via the homogeneity of slopes test for linear regression, using the program Statistix. The proportion of germinated spores was normalized relative to the untreated check, and then data were subjected to linear regression with log-transformed concentration and logit-transformed proportion of germinating spores.

Results and Discussion

C. pseudonaviculata demonstrated reduced sensitivity to strobilurin fungicides, especially on comparison to DMI fungicides (LaMondia 2014). In general, the EC_{85} values showed a wide range within the different isolates (Fig. 1 and 2). The EC_{85} values for DMI fungicides against mycelial growth were 0.28% or less of the maximum label use rates (Fig. 1). The most effective fungicide against mycelial growth was triflumizole (EC_{85} = 0.009 to 0.020 µg a.i. ml^{-1}) followed by tebuconazole (EC_{85} = 0.024 to 0.057 µg a.i.·ml^{-1}), propiconazole (EC_{85} = 0.065 to 0.176 µg a.i.·ml^{-1}) and tetraconazole (EC_{85} = 0.442 to 1.098 µg a.i.·ml^{-1}) (Fig. 1). Myclobutanil did not consistently inhibit the mycelial growth and as a result the EC_{85} value could not be determined. For the strobilurins, only pyraclostrobin inhibited mycelial growth and conidial germination of the 11-9-4a and CTWH1 isolates within the label use rate. Pyraclostrobin, kresoxim-methyl, and trifloxystrobin inhibited mycelial growth for the 11-9-4a isolate and pyraclostrobin and kresoxim-methyl for CTWH1, as well. Pyraclostrobin and kresoxim-methyl had the highest control of the tested strobilurins against mycelial growth of the pathogen. Fig. 2 shows the EC_{85} values for the three isolates and the use rate for the fungicides. Log-transformed EC_{85} values above 4 (e.g., 10,000 µg a.i.·ml^{-1}, or a 1% solution) could not be measured and were displayed as 4 to communicate their lack of efficacy (Fig. 2). Extrapolated log(EC_{85}) values from the linear regression for FC1 were 6.5 and 7.3 for azoxystrobin and 7.6 and 11.2 for trifloxystrobin and values for CTWH1 were 4.8 and 5.7 for azoxystrobin and 18.9 and 7.0 for trifloxystrobin. However, these high EC_{85} values are either not achievable in practice (due to label constraints) or physically impossible. Note that a log(EC_{85}) value of 6 would be pure active ingredient.

Growth in the presence of strobilurin fungicides differed among the isolates ($P < 0.05$ for slope and/or elevation), the only exception being similar effects for kresoxim-methyl ($P = 0.435$ and 0.362; slope and elevation, respectively) and trifloxystrobin ($P = 0.719$ and 0.801) when tested against the FC1 versus CTWH1 isolates (Table 2). The isolates 11-9-4a and FCI differed in their response

Table 2. Effective fungicide concentration against *Calonectria pseudonaviculata*, at which *in vitro* mycelial growth was inhibited by 85% (EC_{85}) and by 50% (EC_{50}). Values are for duplicate trials.

		EC_{85} (µg a.i./ml)		EC_{50} (µg a.i./ml)	
Fungicide	**Isolates**	**Trial 1**	**Trial 2**	**Trial 1**	**Trial 2**
Propiconazole	11-9-4a	0.07	0.07	0.02	0.03
	FC1	0.18	0.10	0.02	0.02
	CTWH1	0.07	0.12	0.02	0.04
Tebuconazole	11-9-4a	0.02	0.03	0.01	0.01
	FC1	0.03	0.03	0.01	0.01
	CTWH1	0.04	0.06	0.01	0.01
Tetraconazole	11-9-4a	0.48	0.42	0.14	0.21
	FC1	0.94	0.83	0.11	0.20
	CTWH1	1.10	0.88	0.21	0.27
Triflumizole	11-9-4a	0.01	0.01	< 0.01[v]	< 0.01[v]
	FC1	0.02	0.01	0.01	< 0.01[v]
	CTWH1	0.01	0.02	< 0.01[v]	0.01
Azoxystrobin	11-9-4a	102.56	635.91	2.14	2.74
	FC1	> 1,000[u]	> 1,000[u]	52.46	29.64
	CTWH1	> 1,000[u]	> 1,000[u]	14.00	2.11
Kresoxim-methyl	11-9-4a	0.18	12.04	< 0.01[v]	0.01
	FC1	24.63	892.91	0.02	0.06
	CTWH1	22.93	119.99	0.09	0.07
Pyraclostrobin	11-9-4a	< 0.01[v]	0.01	< 0.01[v]	< 0.01[v]
	FC1	> 1,000[u]	> 1,000[u]	0.19	0.59
	CTWH1	68.11	119.88	1.37	0.83
Trifloxystrobin	11-9-4a	59.80	37.49	< 0.01[v]	< 0.01[v]
	FC1	> 1,000[u]	> 1,000[u]	5.75	2.46
	CTWH1	> 1,000[u]	> 1,000[u]	8.70	1.22

[v] < 0.01: The EC value is lower than 0.01 µg a.i./ml

[u] > 1,000: The EC value is higher than 1000 µg a.i./ml

to strobilurin fungicides ($P < 0.05$ for slope and/or elevation) as well as the isolates 11-9-4a and CTWH1 ($P < 0.05$ for slope and/or elevation). The isolates FC1 and CTWH1 demonstrated reduced sensitivity to strobilurin fungicides compared to the 11-9-4a isolate ($P < 0.05$ for slope and/or elevation), which was sensitive to all tested strobilurins, whereas FC1 was operationally resistant, as suppressive rates are greater than those labeled and used for spray application (Fig. 2). Only pyraclostrobin and kresoxim-methyl had good efficacy against CTWH1, and this isolate was not as insensitive to fungicides as FC1. Some of the EC_{85} values for the strobilurin fungicides have a wide range and differed significantly between the two repetitions, thus the experiments were examined separately.

The DMI fungicides did not show significant differences in effects on growth between the isolates ($P > 0.05$ for slope and elevation), with the exception of the CTWH1 isolate and propiconazole ($P = 0.576$ and 0.005; slope and elevation, respectively). Differences in sensitivity were only observed for the isolates 11-9-4a and FC1 for propiconazole ($P = 0.004$ and 0.925) and tetraconazole ($P = 0.010$ and 0.991) as well as for the isolates 11-9-4a and CTWH1 for triflumizole ($P = 0.664$ and 0.023), but these differences have little practical significance because the effective concentrations were well below the field application rate. Note, however, that the FC1 isolate was not selected for resistance to this fungicide group.

Several studies have described reduced sensitivity to fungicides with a concentration of 30 to 50 µg a.i.·ml^{-1} (Gehesquière et al. 2015, LaMondia 2014), because the *in vitro* inhibition might be much higher than what is reached under environmental field conditions. Assuming that 50 µg a.i.·ml^{-1} is a threshold for defining a practical level of reduced sensitivity, only pyraclostrobin and kresoxim-methyl effectively inhibited the strain 11-9-4a by 85%. This threshold has no impact on the effect on the DMI fungicides, because the EC_{85} values ranged from 1.098 to 0.009 µg a.i.·ml^{-1}. Henricot et al. (2008) noted that mycelial growth of *C. pseudonaviculata* was more sensitive to the systemic fungicides than to the protectant fungicides. However, their data showed that the protectants kresoxim-methyl (strobilurin fungicide) and prochloraz (DMI fungicide) were very effective ($EC_{50} = 0.03$ to 0.09 and 0.01 to 0.03 µg a.i.·ml^{-1}). Moreover, the individual fungicides they tested varied widely in EC_{50} and in label use rates.

Our current and previous work showed that the EC_{85} value of azoxystrobin, a systemic strobilurin fungicide, was higher than the use rate as the concentration of the active ingredient in the spray tank (40 to 300 µg a.i.·ml^{-1}) and that azoxystrobin had no effect on mycelial growth (LaMondia 2014). In direct comparison, the EC_{50} values are up to 131 times higher (2.1 to 52.46 µg a.i.·ml^{-1}) in the present study than Henricot et al. reported (0.4 to 1.65 µg a.i.·ml^{-1}) (Henricot et al. 2008), except kresoxim-methyl, which had a similar impact on the pathogen. In general, in the Henricot et al. (2008) study, all strobilurin fungicides were effective against *C. pseudonaviculata* with regard to EC_{50} values. Compared to the results of LaMondia (2014), who used different isolates of *C. pseudonaviculata,* the EC_{85} of the present data values are higher for the strobilurin (except 11-9-4a isolate for pyraclostrobin) and lower for the DMI fungicides (0.01 to 0.18 versus 0.34 to 0.87 µg a.i.·ml^{-1})

Table 3. *In vitro* inhibition of conidial germination of *Calonectria pseudonaviculata* isolates as a percentage of the untreated control

Isolate	Concentration [µg a.i./ml]	Inhibition of germination (%)			
		Pyraclostrobin	Kresoxim-methyl	Azoxystrobin	Trifloxystrobin
11-9-4a	1	99	99	25	98
	3.16	99	100	70	99
	10	100	100	91	98
	31.6	100	100	96	99
	100	100	100	98	100
	316	100	100	100	100
FC1	1	99	100	14	97
	3.16	99	99	48	99
	10	99	99	87	99
	31.6	99	100	94	99
	100	100	100	99	100
	316	100	100	100	100
CTWH1	1	55	42	7	24
	3.16	91	45	12	33
	10	98	69	18	32
	31.6	100	58	23	28
	100	99	54	30	35
	316	99	67	55	40

(Table 2). Myclobutanil did not show a consistent inhibition trend in the present study, which is not the case for the other studies (LaMondia 2014, Henricot et al. 2008). Furthermore, the isolates FC1 and CTWH1 demonstrated reduced sensitivity to strobilurin fungicides compared to the 11-9-4a isolate. Strobilurin fungicides resulted in significant differences in growth rates between the isolates 11-9-4a and FC1. Isolate 11-9-4a was sensitive to all tested strobilurins except azoxystrobin, whereas FC1 was resistant compared to the use rate for spray application. Furthermore, Gehesquière et al. (2015) described two different clades of *C. pseudonaviculata*: *C. pseudonaviculata sensu stricto* (G1 clade) and *C. henricotiae* (G2 clade) as having reduced sensitivity to specific triazole as well as strobilurin fungicides in Europe. Gehesquière et al. (2015) concluded that *C. henricotiae* isolates (G2) showed reduced sensitivity to both kresoxim-methyl and tetraconazole but not to other strobilurin or DMI fungicides compared to G1 isolates. This differed from our results for the three isolates analyzed in this study. On the contrary, the Connecticut isolates had a higher sensitivity to kresoxim-methyl than to the other strobilurin fungicides. Tetraconazole had higher EC_{85} and EC_{50} values than the other DMI fungicides, but the concentrations were still below the label use rate. The current EC_{50} values for tetraconazole are lower than the data shown by Gehesquière et al. (2015), 0.14 to 0.26 versus 0.31 to 0.39 for G1 and 3.15 to 3.89 µg a.i.·ml^{-1} for G2. However, G2 (*C. henricotiae*) isolates have not been identified in the U.S. The range of differences of the EC values compared to other studies as well as the differences between the isolates in the present study regarding the sensitivity to fungicides convince us that *C. pseudonaviculata* isolates are more variable than has been assumed. Furthermore, this study demonstrates that reduced fungicide sensitivity in *C. pseudonaviculata* can be selected for *in vitro* as was done with the strobilurin fungicide pyraclostrobin (FC1) and that reduced strobilurin sensitivity can also occur naturally in pathogenic isolates in the landscape, as demonstrated with CTWH1.

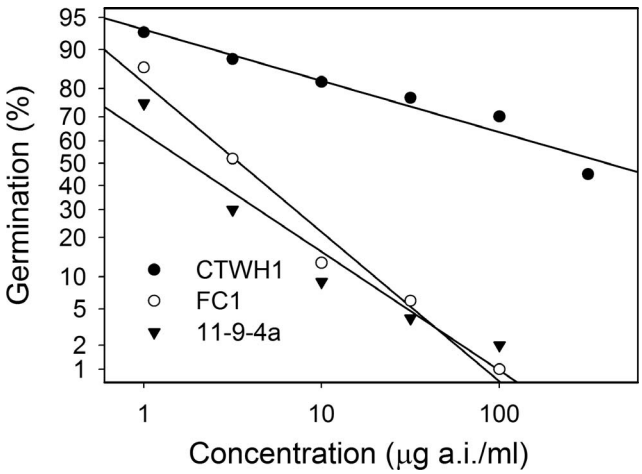

Fig. 3. Germination response for the *Calonectria pseudonaviculata* isolates 11-9-4a, FC1, and CTWH1 relative to the concentration of the strobilurin fungicide azoxystrobin. The y-axis is plotted using a probability scale to linearize the dose-responses.

SBI fungicides, which include the DMI fungicides, have no effect on conidial germination (Henricot et al. 2008, LaMondia 2014); apparently, sterol synthesis may only be relevant for fungal growth, and plays no role in the process of spore germination. Hence, only the strobilurin fungicides were considered for targeting conidial germination in our studies. The strobilurin fungicides fell into two categories for the conidial germination experiment: (1) A dosage-dependent inhibition of the conidial germination was observed for the fungicides or (2) there was such uniformly high control at the concentrations tested that a dose-response was not observed (Table 3). Fig. 3 (variation in germination among isolates 11-9-4a, FC1, and CTWH1 for the strobilurin fungicide azoxystrobin) and Fig. 4 (variation in germination response of CTWH1 to different strobilurin fungicides) visualize the relations of category 2, which were analyzed with the programs Sigma Plot and

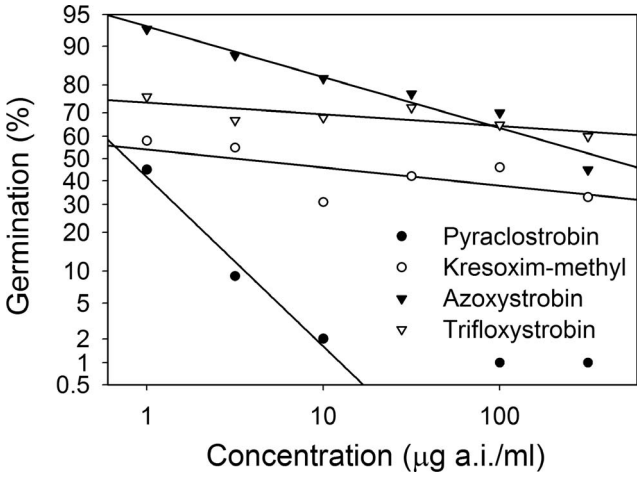

Fig. 4. Germination response of *Calonectria pseudonaviculata* isolate CTWH1 relative to strobilurin fungicide concentration. Pyraclostrobin data at 100 and 316 µg a.i./ml were excluded from the statistical analysis. The y-axis is plotted using a probability scale to linearize the dose-responses.

Statistix. Significant differences were found between isolates 11-9-4a and CTWH1 ($P = 0.001$ and 0.000, slope and elevation, respectively) as well as FC1 and CTWH1 ($P = 0.000$ and 0.001), but not between strains 11-9-4a and FC1 ($P = 0.114$ and 0.269) (Fig. 3). The analysis of the response of the isolate CTWH1 to various fungicides detected significant differences ($P < 0.05$) between either slope or elevation for all pairwise comparisons of fungicides (Fig. 4), with the exception of the non-significant differences for slope for the kresoxim-methyl versus trifloxystrobin comparison, and non-significant differences for elevation between the azoxystrobin versus trifloxystrobin comparison. The strobilurins pyraclostrobin, trifloxystrobin, and kresoxim-methyl strongly inhibited the conidial germination of 11-9-4a and FC1 at all tested concentrations (Table 3). Azoxystrobin had reduced efficacy against those isolates at concentrations of at least 3.16 µg a.i.·ml^{-1} (Table 3), which was also reported previously (LaMondia 2014). However, azoxystrobin, trifloxystrobin, and kresoxim-methyl had no effect on CTWH1 conidial germination within the tested concentrations; only pyraclostrobin inhibited the conidial germination of this isolate at concentrations of less than 1 µg a.i.·ml^{-1} (Table 3). The fungicides differed significantly (Fig. 4), the comparison of conidial germination for each isolate and fungicide confirmed the conclusion that reduced sensitivity for certain fungicides may occur naturally in pathogenic isolates.

In general, strobilurins are described to have curative effects because of their biochemical mode of action, as well as the effects on spore germination (Bartlett et al. 2002). According to best practices for managing resistance risk, strobilurin fungicides are recommended for preventative application (FRAC), those fungicides are best applied prior to infection or in the early stages of disease development because they inhibit spore germination (Bartlett et al. 2002). However, in this study, strobilurin fungicides had insufficient impact on mycelial growth and conversely, DMI fungicides have no effects on conidial germination, whereas they were very effective against mycelial growth. The combination of both fungicide groups might then be an effective chemical control strategy. For management of boxwood blight, integrated best management practices include inspection, sanitation, and cultural controls as well as an effective spray program (CAES, Boxwood Blight – Information and News).

Multiple fungicide active ingredients from different mode-of-action groups in mixture and rotational usage are necessary to avoid potential development of fungicide resistance. The combination of systemic fungicides with a high effect on mycelial growth as well as protectant fungicides with effect on both conidial germination and mycelial growth should result in high efficacy and also protect against the development of fungicide resistance.

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