Monitoring Fungicide Sensitivity Levels and Mycelial Compatibility Groupings of *Sclerotium rolfsii* Isolates from Florida Peanut Fields

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ABSTRACT

Sclerotium rolfsii, the causal agent of peanut stem rot, is a diverse pathogen that has exhibited decreases in sensitivity to fungicides in areas where they are frequently applied. To better understand this pathogen's diversity and its response to various fungicides in Florida, a monitoring survey was done to examine isolates from several peanut producing areas using morphological characteristics, mycelial compatibility groupings and fungicide sensitivity profiles. A high level of morphological diversity was observed among a small number (N = 15) of isolates which was affirmed by both Shannon-Weiner (E = 0.812) and Simpson's (D = 0.280) indices. However, despite this high level of diversity, fungicide sensitivity of these isolates to flutolanil (EC_{50} = 0.031 ppm) and tebuconazole (EC_{50} = 0.008 ppm) appears to remain relatively unchanged when compared to a previous baseline study. Utilizing a small number of isolates, this monitoring survey indicated the EC_{50} values for the products azoxystrobin (EC_{50} = 0.050 ppm), prothioconazole (EC_{50} = 0.213 ppm), penthiopyrad (EC_{50} = 0.016 ppm) and solatenol (EC_{50} = 0.005 ppm). A trend for hormesis was also noted in this survey (e.g. flutolanil), but further research is necessary to better understand sub-lethal fungicide dose effects on increasing mycelial growth. It is apparent from these results that despite the high levels of phenotypic diversity in *S. rolfsii* populations, current fungicide management practices should remain effective for disease control.

Keywords: Diversity indices, fungicide sensitivity, peanut white mold, phenotypic population diversity, southern stem rot.

Introduction

One of the most important diseases in peanut is stem rot also known as southern blight, caused by *Sclerotium rolfsii* Sacc. (teleomorph *Athelia rolfsii* (Curzi) C.C. Tu and Kimbr.), which was first reported by Rolfs in 1892 on tomato (Aycock 1966). This pathogen causes reductions in pod quality and numbers resulting in yield losses ranging from 10 to 80% (Melouk and Backman 1995). Production value losses from this disease in 2011 were estimated to be $50 million including both yield reductions and additional management costs such as fungicides (Woodward 2011). While fungicides are an effective strategy for managing stem rot in peanut (Kemerait et al. 2015), they can cost producers approximately $60 to $150 per acre each year. However, many producers consider this cost to be acceptable compared to overall risk associated from not utilizing them. An improved understanding about *S. rolfsii* populations in peanut could provide the industry with more information about how to manage this devastating pathogen while reducing production expenses such as fungicides.

S. rolfsii has a high level of phenotypic and genetic diversity (Xie et al. 2014; Remesal et al. 2012; Bagwan 2010; Punja and Sun 2001; Okabe and Matsumoto 2000). Regional samples of this fungus have been observed to produce multiple mycelia compatibility groupings with isolates of a particular MCG typically clustering together genetically (Punja and Sun 2001; Okabe and Matsumoto 2000). MCGs can also provide insights about *S. rolfsii*'s relative aggressiveness and virulence (Xie et al. 2014; Remesal et al. 2012) However, considerable variation is present among the MCGs of this pathogen that does not allow these trends to remain consistent. Assessing the diversity of this pathogen in southeastern peanut production areas is a critical step in developing comprehensive resistance screening programs as well as understanding variation in the biological traits of this peanut pathogen.

Knowledge about pathogen diversity is an important component for developing useful integrated management strategies, but so is monitoring the pathogen phenotype fungicide sensitivity (Brent, 1998). There are multiple fungicidal products (e.g. tebuconazole, prothioconazole, flutolanil) available for *S. rolfsii* control, however, extensive sampling for pathogen sensitivity to these products has been limited in recent years (Franke et al. 1998a; Franke et al. 1998b; Le et al. 2012).

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Reduced sensitivity to the fungicide pentachloronitrobenzene (PCNB) was observed in Texas during the 1985 peanut growing season (Nalim et al. 1995). However, it was noted that this reduced sensitivity was limited to one isolate. This isolate belonged to a unique mycelial compatibility group which was not found in isolates collected between 1990 and 1994. Thus, it is possible that this trait is limited to *S. rolfsii* individuals that were no longer observed in the field. There is also an indication that in areas where fungicides are frequently used to manage *S. rolfsii* that the pathogen's sensitivity to these fungicides decreases (Franke et al., 1998a; Le et al. 2012). Thus, continued monitoring through intensive sampling (many single infections on several occasions) (Brent, 1998) of *S. rolfsii* population responses to different fungicide chemistries is needed to be sure that this pathogen remains relatively sensitive to these products.

Based on the *S. rolfsii* diversity reported by Xie et al. (2014), it was hypothesized that a high level of phenotypic diversity exists within *S. rolfsii* individuals from Florida's major peanut producing areas. We also hypothesized that reduced sensitivity to fungicides would be present in isolates collected from peanut fields. The objectives of our study were to phenotypically characterize *S. rolfsii* isolates collected from Florida peanut fields based on morphology and mycelial compatibility, and assess the difference in sensitivity of these *S. rolfsii* isolates for two commercial fungicides (flutolanil and tebuconazole) and survey the sensitivity of four novel commercial fungicides (azoxystrobin, penthiopyrad, prothioconazole, and solatenol) using intensive sampling techniques.

Materials and methods

Isolate collection

During the 2013 growing season, 15 *S. rolfsii* isolates were collected from diseased peanut plants in the Florida counties of Gadsden (6 isolates), Jackson (1 isolate), Hamilton (1 isolate), Levy (1 isolate), Marion (4 isolates) and Alachua (2 isolates). Twelve of the isolates were collected from production fields growing the runner type variety 'Georgia 06-G', and 3 were from the perennial peanut forage variety 'Floragraze'. Production fields were irrigated, had a history of peanut plantings with 2 to 3 years between crops, and consistently used 5 to 7 spray fungicide programs. Fields from Gadsden and Jackson counties typically used more flutolanil, estimated at 2 sprays per year, than production fields in the other counties sampled in this study. Tebuconazole and azoxystrobin were commonly used in all the production fields ranging between 2 to 4 sprays a year. Penthiopyrad was more commonly used in Levy, Marion, Alachua and Hamilton Counties with sprays ranging between 1 and 3 each year. Fungicide sprays had not been used in the perennial peanut site at the time the isolates had been collected with the previous crop being Bahiagrass. Plant samples were kept in cold storage at 6 ± 2°C for 3 to 5 days after which the fungus was isolated from 1 cm long diseased stem segment containing mycelium or a lesion. These segments were surface sterilized for 5 minutes using a 10 % bleach solution and rinsed with sterile distilled water (Domsch et al. 1980; Okabe and Matsumoto 2000). Quarter strength potato dextrose agar (QPDA) was prepared by mixing 5 g of potato dextrose agar (PDA, Difco™) in 1 L of distilled water. The sterilized stem segments were transferred to QPDA and incubated at 26 ± 2°C for 3 days. After incubation, a 6 mm diameter hyphal disk was harvested from the actively growing edge of the colony and transferred to another QPDA plate. This process was repeated 3 times to ensure that isolations were clean of contamination from other fungal species and bacteria. Single sclerotia from 10-day-old cultures were transferred to new QPDA plates for long- term storage at room temperature (22 ± 5°C) (Bagwan, 2011). To maintain the original characteristics (e.g. pathogenicity) of the isolates (Ryan et al. 2012), isolates were inoculated onto peanut plants and then re-isolated as previously described every six months (Shokes et al. 1996). To obtain mycelial plugs for each experiment isolates from long-term storage were revived by inoculating one sclerotium from each isolate onto QPDA plates and incubating for 4 days at 26 ± 2°C in complete darkness.

Morphological characterization. Morphological characteristics of each isolate were assessed using a media growth assay. Actively growing mycelial plugs of 6 mm diameter were harvested from the edge of the growing colony, transferred to the center of a PDA petri plate and incubated for 2 days at 26 ± 2°C in complete darkness. Digital images of the plates with a scale were captured for intrinsic growth analysis. The total area of the colony growth was measured using the digital image measurement software KLONK (KLONK Image Measurement, Denmark, Copenhagen). The area of the inoculum plug was subtracted from the total colony area to find colony growth after inoculation. Petri dishes were placed back in the growth chamber (26 ± 2°C; complete darkness) for an additional 14 days to assess sclerotial characteristics. Again, digital images of the plates with a

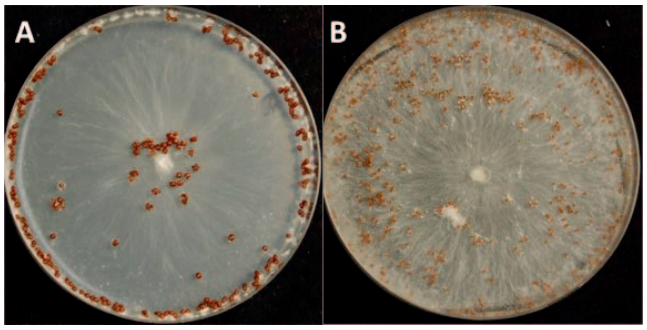

Fig. 1. *S. rolfsii* culture plates 14 days after inoculation use to assess sclerotial characteristics of size and number. The figure shows an example of an isolate A) producing larger and less numerous sclerotia; B) producing small and more numerous sclerotia.

scale were captured for analysis (Fig. 1). The number of sclerotia and average size of sclerotia were determined by using the ImageJ software's (National Institute of Health, United States) particle counting and analysis protocol based on the ImageJ tutorials (https://imagej.nih.gov/ij/docs/examples/index.html, accessed June of 2016).

Mycelial compatibility groups (MCGs). Isolates were tested for mycelial compatibility by placing 2 agar plugs of the same isolate and 1 agar plug of a different isolate onto PDA (20 g PDA, Difco™ in 1 L of distilled water) plates equidistant (5 - 6 cm) from each other (Kohn et al. 1990; Kohn et al. 1991). The plugs of the same isolate served as the control for determining compatible mycelial reactions. After inoculation, the plates incubated at 26 ± 2°C in complete darkness. Plates were rated 2 days after inoculation once the control colonies had grown together for a compatible reaction. Compatibility between plugs was visually assessed to determine if they form a merged confluent colony as seen for a compatible reaction or a dark inhibition line as seen for an incompatible reaction (Leslie, 1993; Xie et al. 2014). Each replication of the experiment consisted of 3 sub-samples and the whole experiment was repeated 3 times.

One representative isolate from each MCG identified within the peanut isolates was also compared to a selection of the established MCGs reported by Xie et al. (2014) which included *S. rolfsii* isolates from cotton, peanut, tomato and other vegetable crops. Only 15 MCGs were tested because 8 type isolates (MCG 4, 8, 14, 15, 16, 17, 18, and 20) were no longer viable.

Mycelial growth inhibition assay. A mycelial growth assay was used to test for isolates sensitivities to the commercial fungicides Abound® (azoxystrobin 22.9%, Syngenta® U.S., Wilmington, DE), Proline® (prothioconazole 41%, Bayer® U.S., Research Triangle Park, NC), TebuStar® 3.6 L (tebuconazole 38.7%, Agristar® US, Ankeny, IA), Convoy® (flutolanil 40%, Nichino America, Inc. U.S., Wilmington, DE), Fontelis™ (penthiopyrad 20.4%, DuPont® U.S., Wilmington, DE), and Solatenol® (benzovindiflupyr, Syngenta® U.S., Wilmington, DE). The commercial fungicide products were diluted in distilled water to amend QPDA with the concentrations of 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0.0001 µg of the fungicide's active ingredient per ml of medium. Fungicide solutions were added to sterile QPDA that had been cooled to 50°C using a water bath. Solutions were regularly (~ every 1 to 2 min) swirled for homogenous mixing. For the azoxystrobin sensitivity test, a stock solution of salicylhydroxamic acid (SHAM) (Sigma Chemical Co., St. Louis, MO) with methanol was prepared by adding 100 mg of SHAM to 1 ml of methanol in a microcentrifuge tube and warming in a water bath at 50°C for 1-2 minutes to completely dissolve SHAM in methanol. 70 µl of this SHAM stock solution (7 mg) was added to every 100 ml of autoclaved liquid QPDA media that was cooled in a water bath to 60°C (Pasche et al. 2004). Azoxystrobin sensitivity assay without SHAM was also conducted to evaluate the effect of SHAM on azoxystrobin sensitivity since it has been indicated that other fungal pathogens can survive media amended with the fungicide by using an alternative respiration pathway (Wood and Hollomon, 2003).

Mycelial inhibition was calculated by transferring an actively growing mycelial plug (6 mm diameter) of *S. rolfsii* to the center of a petri-dish (100 mm*15 mm, Fisherbrand™, USA) containing approximately 22 ml of fungicide amended PDA. Inoculated plates were incubated in complete darkness at 26 ± 2°C for 48 hours. After incubation, digital images of the plates with a scale were captured and stored for analysis. The total area of the colony growth was measured using the digital image measurement software KLONK (KLONK Image Measurement, Denmark, Copenhagen). The area of inoculated plug was subtracted from the total colony area to find the total colony growth after inoculation.

Isolates were exposed to these 10 concentrations for two experimental repeats and analyzed using nonlinear regression as described below. The resulting regression model for each isolate was used to estimate the concentrations causing percentage inhibition of 5%, 20%, 35%, 50%, 65%, 80%, and 95% which were used in the final three repeats to estimate EC_{50} values. The concentrations of fungicide used for the final three repeats are listed in Table 1.

Table 1. Concentration of the commercial fungicide's active ingredient (a.i.) used for the final three runs of fungicide sensitivity assay. These concentrations were the estimated values that caused 5, 20, 35, 50, 65, 80, 95 percent growth inhibition of the isolates based on 2 previous runs using standard concentrations.

Fungicide[a]	Concentration of commercial fungicide a.i. (μg/ml of media)
prothioconazole	0.003,0.03,0.09,0.21,0.47,1.1,3.4
tebuconazole	0.00003,0.0008,0.004,0.01,0.04,0.1,0.8
flutolanil	0.005,0.02,0.03,0.04,0.07,0.14,0.79
penthiopyrad	0.002,0.008,0.02,0.03,0.05,0.1,3.7
solatenol	0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5
azoxystrobin	0.001, 0.009, 0.02, 0.05, 0.1, 0.5, 1
azoxystrobin with SHAM[b]	0.0009, 0.009, 0.01, 0.09, 0.5, 1, 2

[a]Common names of the fungicide products.

[b]azoxystrobin assays were conducted with salicylhydroxamic acid (SHAM; 70 μg of SHAM dissolved in methanol was added in 100μl amended PDA)

The growth inhibition at each fungicide concentration was calculated using the following equation:

$$\text{PGI} = 1 - \left(\frac{\text{CAF}}{\text{CA}}\right)*100 \qquad (1)$$

In which, PGI is the percentage of colony growth inhibited by the fungicide compared to un-amended checks, CAF is the recorded colony area in plates amended with fungicide and CA is the colony area in the unamended control plates (Franke et al. 1998a). A total of 41 PGI values were calculated for each fungicide product using all five replications of the inhibition assay. The sensitivity of each isolate was determined by fitting a sigmoid nonlinear regression model with three parameters using SigmaPlot 12.5 (Systat Software Inc. San Jose, CA) (Franke et al. 1998a). The nonlinear equation was:

$$f = \frac{a}{1 + \exp^{\left(\frac{x - x_o}{b}\right)}} \qquad (2)$$

in which f is the percent growth inhibition at fungicide concentration x, a is the maximum percent growth inhibition with the fungicide, x_o is the inflection point or EC_{50} value, and b is the shape parameter determining steepness of the curve (Damgaard and Nielsen 1999). This equation was fit to each of the repetitions and analyzed for goodness of fit to calculate the mean EC_{50} value for each isolate.

Data analysis. All phenotypic data analyses were completed using SAS (version 9.4; SAS Institute Inc, Cary, NC, USA). Phenotypic data were analyzed as a completely randomized design using the PROC GLM command to conduct an analysis of variance (ANOVA) with *S. rolfsii* isolate as the fixed effect for each of the dependent variables of radial hyphal growth (mm), sclerotia size (mm) and sclerotia number. Pairwise comparisons of isolate means for each dependent variable were analyzed using Fisher's protected least significant difference test ($\alpha = 0.05$). Both the Simpson's and Shannon-Weiner's diversity indices were calculated using the formulas from Zar, 1999 and Magurran, 2004 to quantify the diversity of *S. rolfsii* related to MCGs observed in this study and for the isolate data from different hosts published in Xie et al. 2014. The distributions of the EC_{50} values for all the isolates in relation to the various fungicide products were compared to a normal distribution using the Shapiro-Wilks Test (shapiro.test()) in RStudio (package {stat}version 0.99.451, RStudio, Inc.). and Pearson correlation coefficients were calculated using cor() in RStudio to examine the linear relationship between the EC_{50} results of the six different fungicide products across the *S. rolfsii* isolates (Erickson and Wilcox, 1997). The Rf was calculated by dividing the maximum EC_{50} by the mean EC_{50} value for the respective fungicides.

Results

Phenotypic Traits. Isolates differed in their average colony growth on PDA two days after inoculation and sclerotial traits, including color, size and total number produced 14 days after inoculation (Table 2). In general, isolates were divided into two groups, those that produced large (> 1 mm^2), less numerous (< 200), dark brown sclerotia, and small (< 1 mm^2), more numerous (> 200), light brown sclerotia (Table 2, Fig. 1). These morphological characters varied within and across the Florida counties and fields from which the isolates were collected.

A total of seven MCGs were identified among the 15 isolates of *S. rolfsii* examined in this study (Table 2). These groups were arbitrarily assigned as MCG 1 to MCG 7. A total of seven isolates collected belonged to MCG 1, three isolates were

Table 2. Morphological characteristics of *Sclerotium rolfsii* isolates from peanut fields in Florida.

Isolate[a]	County[b]	Colony area (mm^2)[c]		Sclerotia Number[d]		Sclerotia Size (mm^2)[e]		Sclerotia Color	MCG[f]
AL31	Alachua	1337	abc	173	cd	1.46	a	Dark brown	1
GA39	Gadsden	1034	defg	132	d	1.35	a	Dark brown	1
HA32	Hamilton	1052	defg	167	d	1.35	a	Dark brown	1
LE948	Leon	1460	a	135	d	1.34	a	Dark brown	1
MA22	Marion	1082	cdefg	173	cd	1.35	a	Dark brown	1
MA23	Marion	1162	bcde	144	d	1.52	a	Dark brown	1
MA24	Marion	1049	defg	140	d	1.47	a	Dark brown	1
GA37	Gadsden	859	fg	236	bc	0.62	b	Light brown	2
GA38	Gadsden	840	g	283	b	0.62	b	Light brown	2
HA33	Hamilton	900	efg	377	a	0.54	b	Light brown	2
JA25	Jackson	1284	abcd	260	b	0.56	b	Light brown	3
MA26	Marion	1247	abcd	274	b	0.53	b	Light brown	4
GA17	Gadsden	1128	bcdef	362	a	0.65	b	Light brown	5
GA18	Gadsden	1377	ab	156	d	1.41	a	Dark brown	6
GA19	Gadsden	1207	abcd	128	d	1.25	a	Dark brown	7

[a]Isolate code used to originally identify isolates collected at the various field sites. The two letters in the isolate number denote the name of the counties where they were collected; the numbers following the letters were assigned arbitrarily.

[b]Florida county name from which the isolate was collected.

[c]Area (mm^2) of colony mycelial growth for the isolate 48 hours after inoculation onto potato dextrose agar at 26±2°C. Values followed by different letters are significantly different based on Fisher's protected least significant difference (LSD = 269.50, $\alpha = 0.05$).

[d]Total number of sclerotia produced per petri plate (78.50 cm^2) by *S. rolfsii* isolates 2 weeks after inoculation onto potato dextrose agar and kept at 26±2°C. Values followed by different letters are significantly different based on Fisher's protected least significant difference (LSD = 63.52, $\alpha = 0.05$).

[e]Mean sclerotia diameter (mm^2) recorded per petri plate (78.50 cm^2) by *S. rolfsii* isolates 2 weeks after inoculation onto potato dextrose agar and kept at 26±2°C. Values followed by different letters are significantly different based on Fisher's protected least significant difference (LSD = 0.30, $\alpha = 0.05$).

[f]Mycelial compatibility group (MCG) designations for the 15 *S. rolfsii* isolates examined in this study.

categorized in MCG 2, and MCGs 3, 4, 5, 6 and 7 each contained one isolate. The three isolates collected from one perennial peanut site in Gadsden County (GA17, GA18, and GA19) belonged to three separate MCGs and varied significantly in their sclerotia size, number and color (Table 2). The representative isolate from MCG 1 in this study was compatible with MCG 11, reported by Xie et al. 2014, but MCG 2 through 7 were not compatible with any of the other MCGs from Xie et al (2014) that were tested.

Simpson's diversity index indicated the probability of randomly selecting two isolates of the same MCG in this study is approximately 0.28 and 0.09 when studies were combined with published data by Xie et al. 2014 (Table 3). The Shannon-Weiner diversity index values were greater than one for all studies reported indicating a high level of diversity. Evenness variables were greater than 0.80 indicating that the abundance in each MCG is similar or homogenous.

Fungicide Sensitivity Monitoring and Survey. Ranges of EC_{50}, mean EC_{50} and overall inhibition response of the *S. rolfsii* isolates were different for all fungicide products tested (Table 4, Fig. 2). Prothioconazole had the highest mean and range EC_{50} values and solatenol had lowest mean and range EC_{50} values (Table 4). The ranges calculated for tebuconazole and flutolanil in this study were 4.8 and 4.7 times smaller than those reported previously by Franke et al. 1998a. The coefficient of variation (CV) based on the EC_{50} values was more than 30% for all the products except flutolanil and penthiopyrad. Tebuconazole had the highest CV, which was about 1.68 times higher than the next highest fungicide product, solatenol (Table 4). Tebuconazole had the highest resistance factor which was 1.81 times greater than the next highest products, prothioconazole and solatenol.

The distribution of EC_{50} values for the 15 isolates was not significantly different ($p > 0.05$) from a normal distribution for all the isolates tested except tebuconazole (Table 4). The Shapiro-Wilk's test indicated the EC_{50} distribution for tebuconazole isolates was skewed ($p < 0.01$) with a long tail towards the maximum (0.031 ppm) value observed. The distribution for prothioconazole was not significantly different from normal ($p = 0.06$), however, the largest EC_{50} range difference (0.360 ppm) was observed for this product. This range

Table 3. Quantitative diversity indices for phenotypic results based on mycelial compatibility groupings (MCGs) observed in the current and previous studies.

	Simpson's Index[b]		Shannon-Weiner Index[c]	
Study type[a]	D	1-D	H'	E
Current	0.280	0.720	1.580	0.812
Previous	0.081	0.919	2.769	0.883
Combined	0.086	0.914	2.875	0.854

[a]The MCG values used in the calculations came from the 7 delineated in this study (Current) and the 22 established by Xie et al. 2014 (Previous) as well as combined results.

[b]Simpson's diversity index calculated from MCGs. D is the probability that 2 randomly selected individuals belong to the same MCGs. 1-D is the probability that two randomly selected individuals belong to different MCGs.

[c]Shannon-Weiner diversity index measures the order observe within a particular system. H' is a quantitative expression used to measure diversity with values greater than 1 indicating high diversity. E is the evenness variable which measures how similar the abundance of different isolates are. A value of 1 would be a completely even distribution.

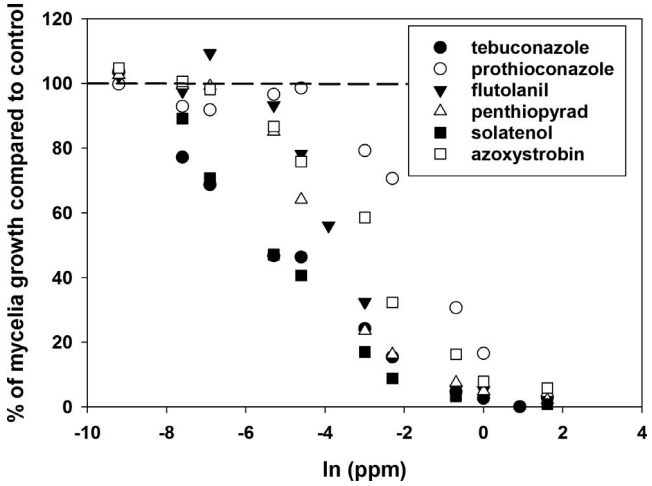

Fig. 2. The average response of the *S. rolfsii* isolates towards different concentrations of the various fungicide products tested. Mycelia growth of the isolates on the media amended with the various concentrations of fungicide were compared to a control, which consisted of media not amended with the fungicide compound. Non-amended plates were considered to have 100% of the possible growth for the isolates. A trend was apparent that the isolates produced more mycelia growth than the un-amended media (hormesis) for select fungicides at values of ~ ln (–6 ppm) or less.

difference was 12.46 times greater than other triazole, tebuconazole (0.029 ppm).

A significant correlation was observed between the EC_{50} values of flutolanil and penthiopyrad ($r = 0.69$, p-value $= 0.004$). No significant correlations (p-value > 0.100) were observed for the rest of the fungicide products tested; however, a positive correlation coefficient was noted between flutolanil and solatenol ($r = 0.41$, p-value $= 0.134$).

Discussion

Phenotypic traits, such as morphological characteristics and MCGs, have been used to assess the diversity of *S. rolfsii* on different hosts and in many regions throughout the world (Xie et al. 2014; Remesal et al. 2012; Sarma et al. 2002; Punja and Sun 2001; Cilliers et al. 2000; Harlton et al. 1995; Nalim et al. 1995). Previous studies have shown

Table 4. The effective concentrations that limit the growth of *Sclerotium rolfsii* by 50% (EC_{50}) for the 15 isolates from Florida. Results are shown for the 6 fungicide products tested based on nonlinear analysis of the percent inhibition for 7 fungicide product concentrations (ppm).

						Shapiro-Wilk's Test[f]	
Fungicides[a]	Range[b]	Mean	Diff.[c]	CV(%)[d]	Rf[e]	W	p-value
prothioconazole	0.0900-0.4501	0.2130	0.3601	42.70	2.1	0.89	0.059
tebuconazole	0.0023-0.0312	0.0082	0.0289	91.16	3.8	0.74	<0.001
flutolanil	0.0138-0.0425	0.0310	0.0287	21.68	1.4	0.93	0.260
penthiopyrad	0.0091-0.0222	0.0163	0.0131	20.93	1.4	0.97	0.917
solatenol	0.0020-0.0112	0.0053	0.0092	54.28	2.1	0.91	0.153
azoxystrobin	0.0238-0.0734	0.0407	0.0496	30.59	1.8	0.91	0.145
azoxystrobin+ SHAM[g]	0.0253-0.0805	0.0458	0.0552	36.55	1.8	0.93	0.298

[a]The various fungicidal active ingredients used to test the EC_{50} values (ppm) for the 15 *S. rolfsii* isolates

[b]Minimum and maximum EC_{50} values (ppm) recorded for the 15 *S. rolfsii* isolates tested with each fungicide.

[c]Difference between the minimum and maximum EC_{50} values (ppm).

[d]Calculated coefficient of variation (standard deviation/mean multiplied by 100).

[e]Resistance factor (Rf) calculated by dividing the maximum EC_{50} value by the mean EC_{50} value.

[f]Statistics for the Shapiro-Wilks test for normality. The null hypothesis was that the distribution of EC_{50} values for 15 isolates is normally distributed ($\alpha = 0.05$).

[g]Azoxystrobin fungicide sensitivity assays conducted with media containing salicylhydroxamic acid (SHAM; 70 μg of SHAM dissolved in methanol was added in 100 μl amended PDA)

that *S. rolfsii* isolates can vary both genetically and morphologically within the same MCG (Punja et al. 2001; Cilliers et al. 2000), and that the diversity of MCGs can be high within a field or across a region (Xie et al. 2014; Remesal et al. 2012). In this study, our results support these general conclusions about *S. rolfsii* MCGs. For example, seven separate MCGs were recognized among the six Florida counties in this study of which three distinct MCGs (5, 6, & 7) were all from a single field site location. This high level of diversity was also supported by both the Shannon-Weiner and Simpson's indices. The Shannon-Weiner index implies that the MCGs will have similar abundances across the Florida peanut regions and the southern region when combining this data with results from Xie et al (2014). Simpon's index indicates that the likelihood of randomly selecting two individuals from the same MCG is low for Florida peanut fields by county. It is important to note that all diversity indices are affected by sample size, which was low in this study (Gotelli and Ellison, 2013; Barrantes and Sandoval, 2009; Soetaert and Help, 1990). However, both the Shannon and Simpson indices are widely used for discerning population diversity and their agreement supports the conclusion that high level of diversity was present. This high level of diversity is likely a result of the relatively high MCG category number, which can mask the effects of large proportions being present within one category as observed in this study (Gotelli and Ellison, 2013). This means that the large number of MCG categories with low isolate numbers (< 4) is what the indices are indicating for the high diversity in the population. Thus, these results support the conclusion that *S. rolfsii* has a diverse population, but there does appear to be some clustering of isolates into one MCG.

Despite the high level of diversity indicated by MCGs, other morphological characteristics, such as number, size and color of sclerotia, remained more consistent with each phenotypic grouping. Sclerotia that were dark brown tended to have larger and less numerous sclerotia produced on media when compared to light brown sclerotia. Also, all the sclerotia in the largest MCG, group 1, were dark brown compared with light brown sclerotia for the second largest MCG, group 2. These results are different from Xie et al. (2014) and Le et al. (2012) in that they reported peanut isolates of *S. rolfsii* generally producing larger sclerotia compared to those from other crops such as tomato. These studies also did not notice a relationship between sclerotia size and number, which was apparent in this study. Xie et al. (2014) observed that most of their peanut isolates had tan to brown sclerotia with very few having dark brown color compared to a majority of the isolates in this study having dark brown color. Even though these studies had significantly higher sample sizes, they were still not able to capture all the variability associated with morphological traits of *S. rolfsii*, especially on peanut. In fact, it was discovered that possibly six new MCGs were identified in this study when compared to those previously established by Xie et al. (2014). Thus, high levels of diversity in *S. rolfsii* make it difficult to adequately describe a population using MCGs as well as other phenotypic traits even with an 80 isolate sample size. These results indicate that some population inferences can be observed from both large and small sample sizes, especially when monitoring is the goal of the study. However, it will be important in future studies to further classify *S. rolfsii* populations and possibly the genus using genetic data.

Fungicide sensitivity is another important phenotypic trait that can be used to better understand fungal populations exposed to selection pressures (Franke et al. 1998a; Franke et al. 1998b; Brent and Hollomon 2007; Milgroom 2015). However, *S. rolfsii* is considered a low risk pathogen for the development of fungicide insensitivity, and thus there should be minimal variation in the response of this pathogen to various fungicidal products. Our monitoring results support this hypothesis with low levels of variation observed between the isolates in regards to their fungicide sensitivity when exposed to commercial grades of the different active ingredients. Only tebuconazole had a coefficient of variation greater than 90% and produced an EC_{50} distribution that was not normally distributed. These results indicate that monitoring the effectiveness of tebuconazole products in the field will be important in the years to come, as a trend towards possible isolate insensitivity was noticed in this small sample size. As for the other products tested, no case of field resistance has currently been reported in Florida indicating if resistance is present it is in low proportions. Based on the sample size of 15, Russel (2008) indicated that we would have a 97% chance of identifying at least one resistant isolates in the population if the true frequency of resistance was 0.20 or greater. Thus, based on these results it appears that a majority, greater that 80%, of the *S. rolfsii* population is sensitive to fungicides surveyed in this study, but that it will be important to continue field monitoring for reductions in efficacy of any of these products when managing this pathogen.

The efficacy of various fungicidal compounds on the inhibition of *S. rolfsii* intrinsic growth *in-vitro* has been previously examined in multiple studies (Bhulyan et al. 2012; Akgul et al. 2011; Franke et al. 1998a; Shim et al. 1998; Waterfield and Sisler 1989; Csinos 1987). In these studies, only Franke et al. (1998a) and Csinos (1987) examined similar compounds which were tebuconazole and flutolanil. Csinos (1987) indicated that the EC_{50} value for flutolanil was < 0.01 ppm, which was more than 2 times lower than the mean EC_{50} value reported in the extensive study by Franke et al. (1998a) and in this intensive, monitoring study. One possible reason for this difference could be that Csinos (1987) only used 1 isolate to test for flutolanil sensitivity, and the range from Franke et al. (1998a) and this study both had isolates with EC_{50} values near or below 0.01 ppm. Thus, a better comparison for shifts in sensitivity would be by comparing these monitoring results EC_{50} values with those of Franke et al. (1998a). This comparison shows no indication of changes in sensitivity of S. rolfsii to flutolanil as the values reported in this small sample size study tended to match the lower values reported Franke et al. (1998a). While continued monitoring will be important for this fungicide, it is apparent from these results that current fungicide usage has not selected for large populations with reduced-sensitivity in Florida.

Similar conclusions can be made about tebuconazole, in that the mean EC_{50} values reported in this monitoring study were generally lower than those observed by Franke et al. (1998a). Our observation can be further supported by the comparison of triazole product results from this study with those previously reported for propiconazole (Waterfield and Sisler 1989; Bhulyan et al. 2012). In these studies, the EC_{50} identified for propiconazole was around 0.25 ppm. This EC_{50} value was similar to the one identified for prothioconazole, and provides relative support that reduced sensitivity to triazole fungicides is still not prevalent in *S. rolfsii* populations in Florida. Overall, even though our results indicated a non-normal EC_{50} distribution was observed for tebuconazole, there was no indication from these comparisons that major changes in fungicide efficacy have occurred in this class of fungicides.

Correlation analysis is commonly used to identify the possibility of cross-resistance between fungicide compounds. Positive correlation values were among between the SDHI compounds of flutolanil, penthiopyrad and solatenol, in which the greatest correlations were seen between flutolanil and the other two compounds. This is concerning since many new products being released for peanut disease control often contain a SDHI product. This means that there could be an increased selection pressure put on *S. rolfsii* populations in the coming years that could lead to the establishment of resistant populations in peanut fields. Thus, it will be important to continue monitoring product efficacy in the field and test isolates that exhibit insensitivity to SHDI products by using the estimated EC_{50} values indicated here and the baseline value established Franke et al. (1998a) for flutolanil.

A unique outcome observed while conducting the fungicide sensitivity assay was that sub-lethal concentrations of flutolanil, as well as four other fungicide products, produce apparent increases in mycelial growth compared to the non-amended checks. This process, called hormesis, has been documented with other fungicidal compounds, primarily pertaining to oomycete control (Flores and Garzon, 2013). While current field doses of these products should provide effective control of *S. rolfsii,* there are concerns about the effects this observation will have on management. The dilution of fungicide products by plant canopies as well as producers reducing fungicide rates to save costs, could create a situation in which products can promote the development of the pathogen producing what appears to be an insensitive response. Further research is needed to better understand the impact hormesis will have on *S. rolfsii* growth and infection, and how it might influence disease development in the field. This trait could lead to false identification of field resistance related to the various fungicidal compounds.

Summary and Conclusion

Despite the high levels of phenotypic variation noticed in MCGs of *S. rolfsii*, from this small sample size study there was no indication of changes in sensitivity of S. rolfsii to flutolanil or tebuconazole. Phenotypic traits provide some insights about the population structure of this pathogen in Florida's peanut producing regions; however, more detailed information could be gained through the use of genetic data analysis and extensive sampling. These results imply that current fungicide reommendations should continue to provide effective control of this disease in peanut. Further research is needed to determine the true variations in the pathogen's diversity as well as the population's responses to the novel fungicidal compounds tested here, but our results provide essential reference points for researchers

working on this pathogen in Florida and possibly throughout the southeast.

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