Isolate–Cultivar Interactions, In Vitro Growth, and Fungicide Sensitivity of *Fusarium oxysporum* Isolates Causing Seedling Disease on Soybean

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Abstract

Fusarium oxysporum is frequently associated with soybean root rot in the United States. Information about pathogenicity and other phenotypic characteristics of *F. oxysporum* populations is limited. The objective of the research described herein was to assess phenotypic characteristics of *F. oxysporum* isolates from soybean, including the interaction between isolates and soybean cultivars, fungal growth characteristics in culture, and sensitivity to fungicides commonly used as seed treatment products. The pathogenicity of 14 isolates was evaluated in rolled-towel and Petri-dish assays using 11 soybean cultivars. In the rolled-towel assay, seed were inoculated with a conidial suspension and disease severity was observed. In the Petri-dish assay, *F. oxysporum* isolates were grown on 2%

water agar and seed were placed on the *F. oxysporum* colony to observe the symptoms that developed. Cultivars differed in susceptibility to *F. oxysporum*, and significant ($P = 0.0140$) isolate–cultivar interactions were observed. *F. oxysporum* isolates differed in radial growth on potato dextrose agar at 25°C. Pyraclostrobin and trifloxystrobin reduced conidial germination with average 50% effective concentration (EC_{50}) of 0.15 and 0.20 µg active ingredient (a.i.)/ml, respectively. Ipconazole reduced fungal growth with average EC_{50} of 0.23 µg a.i./ml, whereas fludioxonil was ineffective. Our results illustrate soybean *F. oxysporum* isolate variability and the potential for their management through cultivar selection or seed treatment.

Fusarium root rot is an important soybean (*Glycine max* (L.) Merr.) disease in the United States and Canada. The common symptoms of Fusarium root rot include wilting, damping-off, slow emergence, root rot, and vascular discoloration (Nelson 1999). Diverse species of *Fusarium* have been associated with soybean root rot. Although fungal populations may vary due to geographic and environmental conditions, the most prevalent and frequent species of *Fusarium* recovered from roots, soil, or crop residues in a number of surveys performed across soybean fields in the United States included *Fusarium oxysporum* Schltdl., *F. solani* (Mart.) Sacc., and *F. graminearum* Schwabe (Díaz Arias et al. 2013b; Leslie et al. 1990; Marburger et al. 2015; Nyvall 1976; Zhang et al. 2010).

F. oxysporum is composed of diverse cryptic species known as the *F. oxysporum* species complex (FOSC) (O'Donnell et al. 2009). Members within this group are responsible for causing vascular wilts, damping-off, and root rots in a wide range of economically important crops (Michiels and Rep 2009; O'Donnell et al. 2009). In previous studies, new species of *Fusarium* have been identified within the FOSC, including *F. commune* K. Skovg., O'Donnell & Nierenberg (Skovgaard et al. 2003), which was previously reported to be a component of the FOSC from Iowa soybean root isolates (Ellis et al. 2013). Isolates of *F. oxysporum* associated with soybean seedling and root rot diseases in Iowa were previously placed into five clades within the FOSC, whereas *F. commune* isolates correspond to clade 6 within the same complex (Ellis et al. 2014).

Soybean isolates within the FOSC are genotypically and phenotypically diverse. Pathogenic capabilities of these isolates to soybean range from highly pathogenic to nonpathogenic and saprophytic (Ellis et al. 2014; Laurence et al. 2014). Even though *F. oxysporum* is the species most commonly associated with soybean root rot, there have been inconsistent findings about its role and importance in the Fusarium root rot complex. For example, in greenhouse studies using artificially infested soils, *F. oxysporum* produced the greatest seed

mortality and caused less severe root rot compared with *F. graminearum*, *F. avenaceum*, and *F. tricinctum* (Zhang et al. 2010). In artificially infested microplots, *F. oxysporum* did not significantly reduce yield, but regression analysis performed on individual *F. oxysporum* isolates indicated a negative correlation between root rot severity and yield (Díaz Arias et al. 2013a). At present, little information is available regarding the range of aggressiveness of *F. oxysporum* isolates toward different soybean cultivars. One of the first reports regarding *F. oxysporum* suggested a range of sensitivity between a single *F. oxysporum* isolate and multiple cultivars (Leath and Carroll 1982).

Currently, one of the most efficient disease management strategies to control seedling diseases in soybean is the use of fungicide seed treatments (Munkvold et al. 2014). These are typically used to prevent infections by soilborne pathogens that cause pre- and post-emergence damping-off, in circumstances where environmental conditions cause delayed germination. The use of seed treatments on soybean is becoming more common because many commercial seed treatments generally include a combination of multiple fungicides as well as a nematicide (Hartman et al. 1999; Mueller et al. 2013). Among the most widely used active ingredients, the quinone outside inhibitors (QoI), such as trifloxystrobin and pyraclostrobin are extensively used on soybean and maize (*Zea mays* L.) in combination with fludioxonil to improve efficacy against a wide range of soilborne pathogens, including *Fusarium* spp. (Broders et al. 2007a; Munkvold 2009). However, QoI have site-specific activity and are characterized by a high risk for resistance development (Mueller et al. 2013). Increased insensitivity to QoI have been reported for *Fusarium* spp. in tomato (Chapin et al. 2006), *Alternaria alternata* in citrus and apple (Mondal et al. 2005; Reuveni and Shenglov 2002), and *Pythium* spp. in soybean (Broders et al. 2007b).

The objectives of this research were to clarify the importance of *F. oxysporum* as a soybean seedling pathogen by (i) studying the reactions of soybean cultivars to different *F. oxysporum* isolates collected from commercial soybean fields in Iowa, (ii) examining the impact of commonly used fungicidal active ingredients on conidial survival and radial growth of *F. oxysporum* isolates, and (iii) comparing radial growth of *F. oxysporum* isolates under optimum temperature conditions.

Materials and Methods

Inoculum and plant source. Fourteen *F. oxysporum* isolates collected from symptomatic soybean roots across Iowa in 2007 (Díaz Arias et al. 2013a) were used in all experiments (Table 1). Isolates

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originated from single conidia and were maintained on silica gel beads at 4°C until use. These 14 isolates had been previously tested in greenhouse studies, and information regarding their aggressiveness in causing root rot and seedling mortality on the soybean cultivar Asgrow 2403 (soybean death syndrome [SDS] susceptible; Monsanto Company, St. Louis) was available (Díaz Arias et al. 2013a). In addition, the same isolates had been previously characterized phenotypically in a rolled-towel assay with the *F. oxysporum*-susceptible cultivar MN1805 (Ellis et al. 2014).

Each *F. oxysporum* isolate was grown on potato dextrose agar (PDA) for 7 to 14 days in an incubator at 25°C with a 12-h photoperiod in order to promote conidial formation. Macro or microconidia were collected by adding sterile distilled water (SDW) to the culture and gently scrubbing with a sterile stick. The suspension was passed through sterile cheesecloth to remove mycelial fragments. Inoculum concentration was calculated by counting conidia in a hemacytometer (Bright-line Hemacytometer; American Optical, Buffalo, NY) under the microscope, and conidial suspensions were adjusted to a concentration of 1×10^4 conidia/ml.

Eleven soybean cultivars were selected for these studies, including the *F. oxysporum*-susceptible cultivar MN1805. The cultivars were

selected from a range of soybean maturity groups (MG), representing MG I to V, and represented a wide range of susceptibility to SDS and soybean cyst nematode (Table 2).

Roller-towel pathogenicity assay. Fifteen soybean seeds were placed equidistantly, approximately 1 cm apart, in a row on a moist paper towel. Each seed was inoculated with 100 µl of a suspension of 1×10^4 conidia/ml. After inoculation, seed were covered with another moist towel to keep them in place and to provide moisture for germination, and the towels were rolled. There were three *F. oxysporum*-inoculated rolls and one noninoculated roll (used as a control) per cultivar. Inoculations were performed with one *F. oxysporum* isolate on 11 soybean cultivars at a time. The 44 rolls were placed in a 25-liter bucket containing 300 ml of distilled water and incubated for 7 days at room temperature (25°C). Mock-inoculated rolls were confined within the bucket with a plastic tray to avoid cross contamination. One roll was considered an experimental unit, with 15 seeds or subsamples on each roll. Each of the 14 *F. oxysporum* isolates were tested 1 at a time. The experimental design was a randomized complete block, in which each bucket was considered as a random factor for the statistical analysis. The experiment was conducted two times.

Table 1. Description of *Fusarium oxysporum* isolates collected from soybean roots across Iowa in 2007 (Díaz Arias et al. 2013a) and genotypic characterization, including mating type loci and clades within the *F. oxysporum* species complex (FOSC) (Ellis et al. 2014)

Isolate ID	County	Stage ^b	Mating type	FOSC clade	Type of conidia ^a	
					Microconidia	Macroconidia
FO36	Lyon	V3	MAT1-2	5	P	A
FO37	Lyon	V3	MAT1-2	5	A	P
FO38	Hamilton	R1	MAT1-2	5	P	P
FO39	Lee	V3	MAT1-2	5	P	A
FO40	Butler	V3	MAT1-1	3	P	P
FO41	Jefferson	V3	MAT1-1	6	P	A
FO42	Lee	V3	MAT1-2	2	P	P
FO43	Crawford	V2	MAT1-2	5	P	P
FO44	Winneshiek	V2	MAT1-2	5	P	A
FO45	Lyon	V3	MAT1-2	5	P	A
FO46	Crawford	V2	MAT1-2	5	A	P
FO47	Dickinson	V3	MAT1-2	3	P	A
FO48	Crawford	V2	MAT1-1	3	A	P
FO49	Allamakee	V3	MAT1-2	6	P	A

^a Type of conidia produced on potato dextrose agar after 7 days of incubation with a 12-h light period at 25°C. P = present and A = absent.

^b Soybean developmental stage at the time of root sampling.

Table 2. Description of 11 soybean cultivars used to study their interaction with *Fusarium oxysporum*

Cultivar	MG ^b	Pedigree	Released	Developer reference ^c	Disease reactions ^a		References
					SDS	SCN	
Ripley	IV	Hodgson × V68-1034	1985	Ohio ARDC and USDA-ARS	PR	S	Cianzio et al. 2016; Cooper et al. 1990; Luckew et al. 2012; Tatalović 2014
299N	II	...	...	Mycogen Seeds	S	MS	Cianzio et al. 2016; Luckew et al. 2012; Tatalović 2014
Forrest	V	Dyer × Bragg	1972	Mississippi, Tennessee AES, and USDA	R	R	Hartwig and Epps 1973; Tatalović 2014
MAC02	IV	...	...	University of Missouri	S	MR	Luckew et al. 2012; Tatalović 2014
MN1606	I	...	...	...	R	S	Luckew et al. 2012; Tatalović 2014
CM396	III	...	...	...	R	S	Schmidt et al. 2014; Tatalović 2014
MYC5171	I	...	...	Mycogen Seeds	MS	S/MS	Luckew et al. 2012; Tatalović 2014
Williams 82	III	Williams (7) × Kingwa	1981	Illinois AES and USDA	S	S	Bernard and Cremeens 1988; Schmidt et al. 2009; Tatalović 2014
H2494	II	...	...	...	S	S	Cianzio et al. 2016; Tatalović 2014
MN1805	I	...	...	...	MR	S	Luckew et al. 2012; Tatalović 2014
Jack	II	Fayette × Hardin	1989	Illinois AES	MR	R	Cianzio et al. 2016; Nickell et al. 1990; Schmidt et al. 2009; Tatalović 2014

^a SDS = sudden death syndrome and SCN = soybean cyst nematode. Abbreviations: S = susceptible, MS = moderately susceptible, MR = moderately resistant, PR = partially resistant, and R = resistant.

^b Maturity group.

^c ARDC = Agricultural Research and Development Center, USDA-ARS = United States Department of Agriculture–Agricultural Research Service, and AES = Agricultural Experiment Station.

Disease severity was evaluated by two methods, as described by Ellis et al. (2011). Disease severity index (DSI) was calculated as the ratio between the length of discolored hypocotyl lesion and the total plant length, multiplied by 100. When seed did not germinate and were completely colonized, they were assigned a DSI value of 100%. Seedlings were also evaluated using a 1-to-5 severity scale, in which 1 = germination, no symptoms of disease or colonization on seedling; 2 = germination, little colonization of the seedling, 1 to 19% of the root with lesions; 3 = germination, some colonization of seedling, and 20 to 74% of the root with lesions; 4 = germination, complete colonization of the seedling, and 75% or more of the root with lesions; and 5 = no germination, complete colonization of the seed (Fig. 1). Root length, root weight, and plant length were also measured.

Petri dish pathogenicity assay. A Petri-dish assay was performed to test the aggressiveness of 14 *F. oxysporum* isolates and 11 soybean cultivars following a methodology similar to what has been previously published (Broders et al. 2007b; Muyolo et al. 1993; Zhang and Yang 2000). A 3-mm-diameter plug from a 7-day-old PDA culture of an isolate of *F. oxysporum* was transferred to the center of a Petri dish containing 2% water agar, which was incubated for 4 days under a 12-h photoperiod at 25°C. Eight soybean seeds were then surface disinfested in a 0.5% NaOCl solution for 2 min and rinsed twice with SDW for 2 min. Seed were placed evenly spaced in the dish, approximately 1 cm from the edge of the fungal colony. Plates were incubated at 25°C for 7 days with a 12-h photoperiod. Disease severity was rated using a 0-to-3 scale, as outlined by Broders et al. (2007b): 0 = 100% germination, with no symptoms of root infection; 1 = 70 to 99% germination, with lesion formation on the roots; 2 = 30 to 69% germination, with coalesced lesions; and 3 = 0 to 29% germination and all seed tissues colonized (Fig. 2). The experimental design was arranged in incomplete blocks with three soybean cultivars each, and cultivars Jack and MN1805 as controls on each of the blocks. Therefore, there were three incomplete blocks, each made up of three cultivars and the two check cultivars, Jack and

MN1805. For each treatment combination of cultivar and isolate, there were three Petri dishes, and the experiment was conducted twice. Blocks were considered to be a random effect for the statistical analysis.

Radial growth on PDA. To compare the growth rate of the 14 *F. oxysporum* isolates, 5-mm-diameter mycelial plugs of each isolate were aseptically transferred from a 7-day-old PDA culture to the center of 90-mm-diameter PDA Petri dishes and incubated for 4 days at 25°C, under a 12-h photoperiod (Balasu et al. 2015; Groenewald et al. 2006). Colony diameter was measured in two perpendicular directions on each plate, and an average was calculated after subtracting 5 mm (representing the diameter of the colonized plug) from the final values. The experiment was organized in a completely randomized design with four replicate plates per isolate, and the experiment was conducted twice.

Fungicide sensitivity assays. The 14 isolates were tested for sensitivity to growth inhibition by the fungicides ipconazole and fludioxonil. Technical-grade active ingredients (a.i.) ipconazole (Bayer CropScience, Research Triangle Park, NC) and fludioxonil (Syngenta Crop Protection, Greensboro, NC) were dissolved in

Fig. 1. Fusarium root rot disease intensity evaluated by an ordinal severity scale: 1 = germination, no symptoms of disease or colonization on seedling; 2 = germination, little colonization of the seedling, 1 to 19% of the root with lesions; 3 = germination, some colonization of seedling, and 20 to 74% of the root with lesions; 4 = germination, complete colonization of the seedling, and 75% or more of the seedling root with lesions; and 5 = no germination, complete colonization of the seed.

Fig. 2. Fusarium root rot disease intensity evaluated by a visual severity scale: 0 = 100% germination, with no symptoms of root infection; 1 = 70 to 99% germination, with lesion formation on the roots; 2 = 30 to 69% germination, with coalesced lesions; and 3 = 0 to 29% germination and all seed tissues colonized.

acetone and added to PDA to obtain final concentrations of 0.01, 0.1, 1, 10, and 100 μg a.i./ml. The nonamended control contained acetone at 0.1% (vol/vol) only. A colonized 5-mm plug was transferred from the original PDA cultures to 90-mm-diameter Petri dishes with the amended PDA. Colony diameter was measured after 4 days of incubation at 25°C with a 12-h photoperiod, as described above. Percent growth inhibition was obtained by dividing the diameter of the colonies in the fungicide treatments by the mean colony diameter of the nonamended control. The experiment was organized in a completely randomized design with a factorial arrangement and two replicate Petri dishes per fungicide–concentration–isolate combination. The experiment was repeated three times.

Isolates were also tested for sensitivity to conidial germination inhibition by the QoI fungicides pyraclostrobin and trifloxystrobin. Technical-grade active ingredients pyraclostrobin (BASF Corp., Florham Park, NJ) and trifloxystrobin (Bayer CropScience) were dissolved in acetone to prepare stock solutions of 1×10^4 μg a.i./ml. PDA was amended with each active ingredient to obtain final concentrations of 0.01, 0.1, 1, 10, and 100 μg a.i./ml. Salicylhydroxamic acid (SHAM, Sigma-Aldrich, St. Louis) was dissolved in methanol at a rate of 100 μg a.i./ml to inhibit the alternative oxidase respiratory pathway. All dilutions, including the nonamended control, contained both acetone and methanol at 0.1% (vol/vol) and SHAM at 100 μg a.i./ml.

Table 3. Analysis of variance indicating the effects of soybean cultivar and *Fusarium oxysporum* isolates on disease severity index (DSI), visual severity rating, plant length (PL), root length (RL), and root weight (RW)^a

Source	df	Rolled towel										Petri dish	
		DSI		Severity		PL		RL		RW		Severity	
		F	P > F	F	P > F	F	P > F	F	P > F	F	P > F	F	P > F
Cultivar (C)	10	30.98	<0.0001	23.05	<0.0001	15.27	<0.0001	46.95	<0.0001	26.73	<0.0001	30.70	<0.0001
Isolates (I)	13	65.27	<0.0001	126.84	<0.0001	35.67	<0.0001	13.14	<0.0001	9.72	<0.0001	7.27	0.0001
(C × I)	130	1.32	0.0140	1.36	0.0083	1.21	0.0717	1.64	<0.0001	1.14	0.1587	1.24	0.0455

^a DSI was arcsin transformed. Visual scales of severity, PL, RW, and RL were log transformed. Experiments consisted of three replications (towels) for each treatment combination and two experiments for the rolled-towel assay. Petri-dish assay experiments consisted of three Petri dishes per treatment combination in an incomplete block design, and using soybean cultivars Ripley and Jack as a control for each block. Data were from 11 soybean cultivars and 14 *F. oxysporum* isolates.

Fig. 3. A and B, Mean disease severity index (DSI%) (rolled-towel assay) and C and D, visual severity rating (Petri-dish assay) for the main effects of isolate and soybean cultivar for *Fusarium oxysporum* isolates from Iowa. Means with the same letter are not significantly different. Fisher's protected least significant difference ($P = 0.05$), arcsin transformed data for the rolled-towel assay. Data from the mock-inoculated seed were not included in the analysis.

F. oxysporum conidia (macro- and microconidia) were collected from 7-day-old PDA pure cultures of each isolate by adding 10 ml of SDW per plate and dislodging conidia from the surface with a sterile glass rod. Culture suspensions were passed through three layers of cheesecloth in order to avoid mycelial fragments, and conidia concentrations were adjusted to 2.0×10^5 conidia/ml using a hemacytometer. The adjusted conidia suspension (100 μ l) was added to a single Petri dish (60 by 15 mm) containing PDA. Plates were incubated at 25°C under continuous light for 18 h, then placed at 5°C and immediately scored for germination rate by direct observation under a light microscope at $\times 100$ magnification. One hundred arbitrarily selected conidia per plate were examined and considered germinated if the germ tube was twice the length of the conidium. The experimental design was completely randomized, with a factorial arrangement of fungicide, concentration, and isolate. There were three replicate Petri dishes per treatment combination.

Data analysis. For the rolled-towel assay, the percentage of DSI was arcsin transformed, and root length, plant length, and root weight were log transformed to meet the assumptions of the analysis of variance (ANOVA). Levene's test of homogeneity of variance was performed on the transformed data to compare the two experimental runs for each isolate separately using PROC GLM in SAS (version 9.3; SAS Institute Inc., Cary, NC). Because there were no significant differences between the two experimental runs, all data were combined and transformed, and ANOVA was performed using PROC GLIMMIX. In addition, the average of DSI and severity from each of the three replicate inoculated rolls per cultivar and *F. oxysporum* isolate were used to calculate linear correlation coefficients using the PROC CORR statement. For the Petri-dish assay, the nonparametric disease data from the response variable severity were analyzed using the PROC GLIMMIX statement in SAS. For the radial growth on PDA, Levene's tests of homogeneity and ANOVA were performed using PROC GLM. Data for the two experimental runs were combined because there were no significant differences between the variances. For all experiments, mean separation analyses were performed using a Fisher's protected least significant difference test at $P = 0.05$.

The 50% effective concentration (EC_{50}) values for the growth inhibitors ipconazole and fludioxonil were calculated in a probit regression analysis. For each *F. oxysporum* isolate, the averaged percentage of growth inhibition on each dose was converted to a numerical equivalent of the probit function by using the normal inverse distribution function "NORMINV" in Excel (Microsoft). A linear equation was obtained from the regression between the logarithm of the doses and their respective probit values, and the EC_{50} was calculated to the corresponding dose value where probit was equal to

0.5. For the conidial germination inhibitors pyraclostrobin and trifloxystrobin, EC_{50} calculations were performed using the PROC PROBIT statement for each *F. oxysporum* isolate. Data for this analysis were based on the average of the percent inhibition as compared with the nonamended control.

Results

Rolled-towel pathogenicity assay. The ANOVA indicated a significant effect for the main factors soybean cultivar and *F. oxysporum* isolate, as well as a significant cultivar-isolate interaction for DSI ($P = 0.0140$) and the visual severity scale ($P = 0.0083$). These significant effects indicate that susceptibility varied among all cultivars and aggressiveness differed among the *F. oxysporum* isolates (Table 3).

Mean separation analysis for the main factors indicated a wide range of aggressiveness among isolates and susceptibility among cultivars. Not all isolates caused disease; in particular, FO36, FO42, and FO46 colonized the seed coat but no lesions or fungal colonization were observed in the radicle or hypocotyls. In contrast, isolates FO38, FO41, and FO40 predominantly produced seed rot symptoms (DSI = 100%), causing poor germination (Fig. 3A).

Soybean cultivars used in this experiment differed in susceptibility when inoculated with *F. oxysporum*. Cultivars Ripley and 299 N had few symptoms of root rot regardless of isolate. Conversely, cultivars MN1805 and Jack displayed the greatest disease between isolates (Fig. 3B). The DSI ranged from 2.6% on Ripley soybean when inoculated with isolate FO46 and 60.8% on cultivar MN1805 when inoculated with isolate FO40 (data not shown).

The visual scale of severity presented results similar to those of the DSI. Visual severity ratings in the rolled-towel assay also indicated a significant interaction ($P = 0.0455$) between cultivars and isolates (Table 3). Furthermore, the two methods for root rot assessment (DSI and visual rating scale) were linearly correlated ($r = 0.915$, $P < 0.0001$) (Fig. 4). Pearson's correlation coefficients for the two disease evaluation methods were significant for all cultivars (Table 4).

Pathogenic isolates negatively affected plant development at different magnitudes. Plant length and root weight were significantly reduced for the plants inoculated with the highly aggressive isolates (FO38, FO41, and FO40) when compared with the noninoculated controls. Conversely, nonpathogenic isolates (FO42, FO46, FO36,

Fig. 4. Relationship between the visual scale for severity and disease severity index (DSI) for 11 soybean cultivars and 14 *Fusarium oxysporum* isolates for the rolled-towel assay. Each point represents an average of three towels containing 15 soybean seeds each for a particular isolate-cultivar combination.

Table 4. Pearson correlation coefficients performed by soybean cultivar on disease severity index (DSI), plant length (PL), root length (RL), root weight (RW), and visual scale for severity

Cultivar	Pearson correlations between DSI (%) and plant variables			
	PL	RL	RW	Severity
Ripley	-0.71	-0.44	-0.49	0.88
Prob > r	<0.0001	0.0160	0.0070	<0.0001
299N	-0.61	-0.39	-0.25	0.86
Prob > r	0.0005	0.0380	0.1890	<0.0001
Forrest	-0.72	-0.42	-0.32	0.87
Prob > r	<0.0001	0.0230	0.0920	<0.0001
MAC02	-0.69	-0.50	-0.36	0.94
Prob > r	<0.0001	0.0060	0.0560	<0.0001
MN1606	-0.72	-0.63	-0.08	0.95
Prob > r	<0.0001	0.0003	0.6540	<0.0001
CM396	-0.73	-0.31	-0.21	0.94
Prob > r	<0.0001	0.0980	0.2720	<0.0001
MYC5171	-0.41	-0.22	-0.13	0.93
Prob > r	0.027	0.2580	0.4840	<0.0001
Williams82	-0.68	0.02	-0.08	0.97
Prob > r	<0.0001	0.8810	0.6590	<0.0001
H2494	-0.79	-0.59	-0.64	0.97
Prob > r	<0.0001	0.0008	0.0002	<0.0001
MN1805	-0.56	-0.48	-0.24	0.87
Prob > r	0.001	0.0080	0.2180	<0.0001
Jack	-0.59	-0.26	-0.08	0.93
Prob > r	0.0008	0.1780	0.6690	<0.0001

Fig. 5. Differences in plant characteristics for the main effects of *Fusarium oxysporum* and soybean cultivar. **A** and **B**, Plant length (cm); **C** and **D**, root weight (g); and **E** and **F**, root length (cm). An asterisk (*) indicates significant differences from the noninoculated control ($P = 0.05$) with a t test for mean separation. Comparisons were made individually by isolate and soybean cultivar.

and FO48), which showed the lowest levels of DSI, did not significantly affect the total plant length or root weight (Fig. 5A and C).

Cultivars CM396 and MN1805 did not display significant plant length reductions when inoculated with *F. oxysporum* (Fig. 5B). In addition, only 3 of the 11 cultivars showed reductions in root weight, and 2 displayed reductions in root length compared with noninoculated controls; only highly susceptible cultivars H2494 and Jack exhibited significant reductions in root length (Fig. 5D and F).

Pearson coefficients indicated a significant negative linear correlation between DSI and plant length ($r > 0.05$, $P < 0.0001$). In contrast, plant variables such as root length and root weight were not strongly correlated with DSI for most of the cultivars (Table 4).

Petri-dish pathogenicity assay. Based on the visual severity scale for the Petri-dish assay (Fig. 2), cultivars varied in their susceptibility, and there was a significant interaction between the main factors cultivars and isolates ($P = 0.0455$) (Table 3). Cultivars MN1805 and MAC02 were the most susceptible, showing poor germination and completely colonized seed. In contrast, cultivars 299 N and MYC5171 presented the lowest disease severity, with no symptoms or very small, dark-brown discoloration on seedling roots (Fig. 3D).

Aggressiveness and type of symptoms varied widely among isolates. Isolates such as FO36 and FO42 tended to be nearly

nonpathogenic, producing small brown lesions on roots or no symptoms, such as in the cultivars MYC5171 and 299 N. In contrast, isolates FO40, FO41, and FO43 were highly aggressive, rapidly colonizing seed and causing preemergence damping-off. MN1805 was the most susceptible of the cultivars tested, with an average DSI of 2.8 and 2.7 when inoculated with FO40 and FO41, respectively. In addition, MN1805 was the most susceptible soybean cultivar when inoculated with 12 of the 14 *F. oxysporum* isolates.

Radial growth on PDA. Radial growth at 25°C differed significantly ($P < 0.001$) among the 14 *F. oxysporum* isolates. Radial growth after 4 days varied from 2.3 cm (FO46) to 4.3 cm (FO47). Isolates FO47, FO42, and FO36 had a significantly greater rate of growth than the rest of the isolates. Average growth of all isolates after 4 days was 3.34 cm (Fig. 6).

Fungicide sensitivity assays. Inhibition in radial growth of *F. oxysporum* isolates by fludioxonil was variable and did not appear to be affected by dose. As a result, an EC_{50} value for fludioxonil could not be calculated. Growth inhibition did not exceed 40% compared with the control (Fig. 7). In contrast, ipconazole effectively reduced the growth of all isolates; at the highest two concentrations (10 and 100 µg a.i./ml), ipconazole inhibited growth by 97.3 and 99.3%, respectively (Fig. 7). Ipconazole EC_{50} values varied among isolates; however, isolates FO38 and FO41 were least sensitive (EC_{50} values of 0.747 and 1.667 µg a.i./ml, respectively), while isolate FO40 was most sensitive to ipconazole (EC_{50} value of 0.007 µg a.i./ml) (Table 5).

QoI effectively inhibited conidial germination for all *F. oxysporum* isolates. At the highest concentrations (10 and 100 µg a.i./ml), trifloxystrobin inhibited germination by 90 and 99%, respectively. Pyraclostrobin performed better than trifloxystrobin for the 10 µg/ml concentration (94.6% inhibition) (Fig. 7). Even though QoI displayed similar results, pyraclostrobin was more effective and had a mean EC_{50} value lower than that of trifloxystrobin (Table 5). Interestingly, pyraclostrobin EC_{50} values were high for individual isolates FO38 and FO41 (0.481, and 0.358 mg a.i./liter, respectively), compared with the rest of the isolates. In addition, FO38 also showed a high EC_{50} for trifloxystrobin (1.479 µg a.i./ml) (Table 5).

Discussion

The current study provides information about the interaction of *F. oxysporum* with soybean cultivars and describes phenotypic diversity of *F. oxysporum* that may clarify its role in the Fusarium root rot complex. *F. oxysporum* isolates collected in Iowa displayed a high degree of variability in aggressiveness, ranging from almost nonpathogenic to highly aggressive. Similarly, soybean cultivars displayed a wide range of susceptibility, with some cultivars showing only small lesions on seedling tissues and a few other cultivars experiencing extensive colonization leading to low germination.

F. oxysporum is an important component of the Fusarium root rot complex in soybean, usually observed at a significantly greater frequency than other species of *Fusarium* in soybean roots (Díaz Arias et al. 2013b; Leslie et al. 1990; Nelson and Windels 1992). However, the role of *F. oxysporum* as a pathogen has been elusive due to the high degree of variability between isolates of the fungus. Although previous studies have reported significant differences in susceptibility among cultivars or aggressiveness among *F. oxysporum* isolates, each study has included only one isolate across several cultivars or vice versa. Therefore, conclusions regarding the previously published studies have proven to be contradictory, and suggest that *F. oxysporum* is either a primary pathogen or a secondary weak pathogen of soybean (Armstrong and Armstrong 1950, 1965; Ferrant and Carroll 1981; French and Kennedy 1963; Jester 1973; Killebrew et al. 1993; Zhang et al. 2010). The current study provides a more complete overview from the FOSC, including interactions among several isolates and soybean cultivars.

Using both rolled towel and Petri-dish assays, we were able to identify isolates that exhibited consistent levels of aggressiveness in causing both root rot and damping-off. Other isolates were aggressive at causing either damping-off or root rot but not both. This finding emphasizes the value of using assays that measure both root rot

Fig. 6. Radial growth of 14 *Fusarium oxysporum* isolates on potato dextrose agar after 4 days at 24°C in the dark.

Fig. 7. Inhibitory effects of fungicides on radial growth (fludioxonil and ipconazole) and conidial germination (trifloxystrobin and pyraclostrobin) with respect to dose concentration on 14 *Fusarium oxysporum* isolates.

and damping-off to fully understand the impact of specific isolates on different soybean genotypes. The significant cultivar–isolate interactions suggests that the pattern of resistance for each soybean cultivar differs among isolates. Despite this interaction, we found that cultivars Ripley, Forrest, and 299 N were consistently among the most resistant to *F. oxysporum* in both assays. These cultivars may be useful sources of resistance for plant breeding.

A significant linear relationship ($P < 0.001$) between DSI and the visual severity scale in the rolled-towel assay suggests that these variables provide similar indications of susceptibility of soybean cultivars to infection by *F. oxysporum*. The visual severity scale is a more rapid technique to evaluate multiple cultivars compared with DSI. Although the visual scale is not as precise as the index based on lesion measurement, it was accurate enough to present similar ranking of the less susceptible cultivars and the most aggressive isolates to those obtained with DSI.

Assessments of plant growth characteristics were generally less useful in detecting the negative impact of *F. oxysporum* infection on seedling health. Significant reductions in plant length occurred due to *F. oxysporum* inoculation and plant length was negatively correlated with DSI. However, plant length was not a consistent indicator of susceptibility because some soybean cultivars that exhibited resistance based on DSI or visual severity still experienced a significant reduction in plant length. In addition, root weight and root length were highly variable within and among cultivars and were not correlated with DSI for most cultivars. These results are consistent with those of Zhang et al. (2010), who reported no significant correlations between plant growth variables and root rot severity caused by *Fusarium* spp. in soybean. Zhang et al. (2010) also reported that resistant soybean cultivars could display reductions in root dry weight and plant height as a result of inoculation with *Fusarium* spp. However, analysis of root weight reductions was more useful when it was performed by isolate, because it pinpointed the three most aggressive isolates (FO38, FO41, and FO40) compared with the noninoculated controls.

F. oxysporum isolates differed in colony morphology and radial growth when cultured on PDA media at 25°C. This temperature has been widely reported as the optimum for growth of a majority of *Fusarium* spp. and, more specifically, for formae speciales of *F. oxysporum* from broad bean (*Vicia faba* L.), spinach, and banana (Groenewald et al. 2006; Ivanovic et al. 1987; Naiki and Morita 1983; Nelson et al. 1990). In the current study, differences in radial growth were not significantly correlated with isolate aggressiveness. For example, isolates FO41 and FO40 differed significantly in relative radial growth at 25°C but both isolates were judged to be aggressive; therefore, vegetative radial growth at 25°C was not an indicator of aggressiveness or clade placement within the FOSC.

It would be interesting to observe radial growth on these isolates at different temperatures and determine potential correlations to isolate pathogenicity.

Inhibition of spore germination by trifloxystrobin and pyraclostrobin indicated little variability in sensitivity to members of the QoI class of fungicides across *F. oxysporum* isolates with EC_{50} values ranging from 0.005 to 1.49 $\mu\text{g a.i./ml}$. These findings contrast with previous research reporting that *F. graminearum* isolates collected from corn and soybean in Ohio exhibited reduced sensitivity to trifloxystrobin (Broders et al. 2007a). In that study, trifloxystrobin at the highest rate (100 $\mu\text{g a.i./ml}$) inhibited growth by less than 35% of the untreated control. Additionally, in the current study, ipconazole significantly reduced the growth of all *F. oxysporum* isolates. These findings agree with a previous report in which ipconazole significantly reduced Fusarium wilt caused by *F. oxysporum* f. sp. *niveum* in watermelon field trials (Everts et al. 2014). In addition, research suggests that ipconazole used in combination with QoI have positive effects on the emergence of chickpea infected with *Aschchyta rabiei* (Wise et al. 2009). Conversely, *F. oxysporum* isolates were observed to be insensitive to fludioxonil in the current study. These results indicate that fludioxonil activity may vary widely among species of *Fusarium*. For example, fludioxonil-resistant isolates in the genus *Fusarium* isolated from potato tubers have been reported in Canada and the United States (Gachango et al. 2012; Peters et al. 2008). Although the fludioxonil mode of action has not been elucidated, evidence suggests that the chemical targets the osmotic-regulatory signal transduction pathway (Furukawa et al. 2012). However, resistance to fludioxonil may be conferred by multiple genetic and biochemical mechanisms (Kanetis et al. 2008; Ren et al. 2016). Conversely, fludioxonil has been reported to be effective against *F. graminearum* from soybean and maize when used as a seed treatment (Broders et al. 2007a; Ellis et al. 2011; Munkvold and O'Mara 2002). In other plant systems, fludioxonil has been reported to significantly reduce plant death in asparagus inoculated with *F. oxysporum* f. sp. *asparagi* and *F. proliferatum* (Reid et al. 2002), and improved stands of wheat under field conditions (Mueller et al. 1997).

Even though it would be difficult to determine whether the isolates used in the current research were exposed to the fungicides in the field prior to isolation, we believe this to be the case. The particular fungicides chosen are widely used active ingredients, and have been used for a considerable period of time as stand-alone seed treatment products, as well as in combination with additional active ingredients, for the control of seedling disease in soybean and maize (rotational hosts in the Iowa production system). Fludioxonil was introduced as a seed treatment for several crops, including soybean and maize, in 1994, and trifloxystrobin and pyraclostrobin were approved for the same use in 1999 and 2008, respectively (Munkvold

Table 5. Fungicide sensitivity analysis (50% effective concentration [EC_{50}]) for 14 *Fusarium oxysporum* isolates recovered from soybean^a

Isolates	Pyraclostrobin		Trifloxystrobin		Ipconazole	
	EC_{50} $\log_{10}$ (dose)	EC_{50} ($\mu\text{g a.i./ml}$)	EC_{50} $\log_{10}$ (dose)	EC_{50} ($\mu\text{g a.i./ml}$)	EC_{50} $\log_{10}$ (dose)	EC_{50} ($\mu\text{g a.i./ml}$)
FO36	−0.777	0.166	−1.193	0.063	−1.125	0.056
FO37	−0.991	0.102	−0.717	0.191	−1.120	0.075
FO38	−0.317	0.481	0.170	1.479	−0.747	0.747
FO39	−1.025	0.094	−1.040	0.091	−1.095	0.080
FO40	−1.392	0.040	−1.331	0.046	−2.109	0.007
FO41	−0.445	0.358	−1.560	0.027	0.222	1.667
FO42	−1.222	0.059	−1.661	0.021	−0.972	0.106
FO43	−1.164	0.068	−1.238	0.057	−1.107	0.078
FO44	−0.758	0.174	−0.519	0.302	−1.099	0.079
FO45	−1.156	0.069	−2.236	0.005	−1.271	0.053
FO46	−0.641	0.228	−1.126	0.074	−1.165	0.068
FO47	−0.990	0.102	−0.521	0.300	−1.248	0.056
FO48	−0.949	0.112	−1.469	0.033	−1.018	0.095
FO49	−0.905	0.124	−0.761	0.173	−1.109	0.077
Average	−0.909	0.155	−1.085	0.204	−1.068	0.231

^a EC_{50} values for the active ingredient (a.i.) fludioxonil were not calculated because there was no apparent inhibition of mycelial radial growth with respect to the nonamended control.

2009). To the best of our knowledge, there are no previous reports of sensitivity profiles on *F. oxysporum* isolates associated with soybean roots, and there is a lack of true baseline sensitivity data. These results serve as a reference for future studies and emphasize the need to include *F. oxysporum* isolates from other locations and to test these active ingredients in in vivo trials.

FOSC is a genetically diverse group with polyphyletic origins (Ellis et al. 2014). Not surprisingly, a great deal of variability was observed regarding genotypic and phenotypic characteristics among the isolates used in the current study. For example, there were highly aggressive isolates representing three different clades (clades 3, 5, and 6), and one clade (clade 5) contained isolates representing both high and low aggressiveness according to the DSI. These findings suggest that there is no clear association between aggressiveness toward soybean and genotypic clade defined by multilocus sequence analysis. Our results contrast with those of Ellis et al. (2014), in which isolates from clade 5 were more aggressive than isolates from clades 2 and 3. The discrepancies between the two studies may have resulted from a greater range of disease levels exhibited across the 11 soybean cultivars tested in the current study compared with the single susceptible cultivar used by Ellis et al. (2014), and a greater number of isolates ($n = 170$) used in the phylogenetic studies compared with the 14 isolates used in the current research.

The most effective strategies to manage Fusarium root rot are crop rotation, high-quality seed, delayed planting, and fungicide seed treatments (Nelson 1999). However, it is important to remember that soybean seedling diseases and soybean root rot appear as a disease complex and, as a consequence, the interactions of the FOSC with other pathogens that reside in the soil outside the *Fusarium* genus may influence the effectiveness of these management strategies. In the future, generating information to address this particular topic may aid in the management of complex soil pathosystems, especially when choosing a seed treatment for a particular crop based on field history. In addition, cultivar resistance and the differences in isolate pathogenicity are a key point to explore possibilities in developing long-term plant breeding strategies (Zhang et al. 2013). For example, host transcriptome profiles have been developed for soybean plants inoculated with *F. oxysporum* isolates FO40 and FO36, identified to be pathogenic and nonpathogenic, respectively (Lanubile et al. 2015). Substantial differences were observed in the gene expression profiles in which FO40 activated stronger expression of defense-related genes compared with the nonpathogenic FO36 (Lanubile et al. 2015). Finally, pathogenicity information generated in this study could be used to select nonpathogenic isolates that may serve as biocontrol agents for *F. oxysporum*.

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