Effects of Temperature and pH on *Fusarium oxysporum* and Soybean Seedling Disease

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Abstract

Fusarium oxysporum (*Fo*) is an important pathogen that reduces soybean yield by causing seedling disease and root rot. This study assessed the effects of pH and temperature on *Fo* fungal growth and seedling disease. In an in vitro assay, 14 *Fo* isolates collected from symptomatic soybean roots across Iowa in 2007 were grown on artificial culture media at five pH levels (4, 5, 6, 7, and 8) and incubated at four temperatures (15, 20, 25, or 30°C). In a rolled-towel assay, soybean seeds from *Fo*-susceptible cultivar Jack were inoculated with a suspension of a pathogenic or a non-pathogenic *Fo* isolate; both isolates were previously designated for their relative aggressiveness in causing root rot at 25°C. The seeds were placed in rolled germination paper, and the rolls were incubated in all combinations of buffer solutions at four pH levels (4, 5, 6, and 7), and four temperatures (15, 20, 25, or 30°C). There was a significant interaction

between temperature and pH ($P < 0.05$) for in vitro radial growth and root rot severity. Isolates showed the most in vitro radial growth after incubation at pH 6 and 25°C. For the rolled-towel assay, the pathogenic isolate caused the most severe root rot at pH 6 and 30°C. Gaussian regression analysis estimates for optimal conditions were pH 6.3 at 27.1°C for maximal fungal growth and pH 5.9 at 30°C for maximal root rot severity. These results indicate that optimal pH and temperature conditions are similar for *Fo* growth and disease in soybean seedlings and suggest that *Fo* may be a more important seedling pathogen when soybeans are planted under warm conditions in moderately acidic soils.

Keywords: fungi, field crops, oilseeds and legumes, epidemiology, climate/weather effects

Fusarium oxysporum Schltdl. (*Fo*) is one of the fungal species most commonly isolated from soybean roots in the soybean producing regions of North America (Díaz Arias et al. 2013a; Ellis et al. 2014; Zhang et al. 2010). *Fo* has been associated with soybean damping-off, seed and seedling root rot, and vascular wilt (Armstrong and Armstrong 1965; Datnoff and Sinclair 1988; Díaz Arias et al. 2013a, 2013b; Leath and Carroll 1982). Although there is some understanding of *Fo* genetic and phenotypic diversity, including the existence of pathogenic and nonpathogenic isolates and variable levels of resistance among soybean cultivars (Cruz Jimenez et al. 2018; Díaz Arias et al. 2013a; Ellis et al. 2014; Lanubile et al. 2015), the effects of temperature and pH in the soybean-*Fo* interaction are unknown. These variations in disease severity may be influenced by abiotic factors such as temperature and pH that alter host-pathogen dynamics, making it difficult to understand the processes involved in soybean root rot development.

The most suitable pH for growth for plant pathogenic isolates in the *Fusarium* genus has been reported in the 3.5 to 7 range (Cochrane 1958; Groenewald et al. 2006; Gupta et al. 2010; Srobar 1978).

Maximal in vitro radial growth in many *Fusarium* spp. such as *F. beomiforme*, *F. proliferatum*, and *F. verticillioides* has been found at temperatures close to 30°C (Marin et al. 1995; Miller and Burke 1985; Nelson et al. 1990). In addition, plant pathogenic *Fo* isolates from other host species have been found to have a maximal radial growth at temperatures between 25 and 28°C (Brownell and Schneider 1985; Gupta et al. 2010; Nelson et al. 1990; Webb et al. 2015).

There is evidence of significant interactions between pH and temperature on fungal radial growth in *Fusarium* spp. (Chen et al. 2013;

Marin et al. 1995; Schuerger and Mitchell 1992). In addition, temperature alters the expression of host defense mechanisms favoring resistance or susceptibility reactions (Harling et al. 1988; Stuthman et al. 2007). For example, moderately resistant or highly resistant cultivars of banana, carnation, chickpea, or lettuce become susceptible under high temperatures (>25°C) after inoculation with their respective *Fo* f. sp. (Brake et al. 1995; Harling et al. 1988; Landa et al. 2006; Scott et al. 2010). Differences in soil pH might alter *Fusarium* spp. growth rate, spore attachment on plant roots, and inoculum density, contributing to the expression of wilt symptoms on plants exposed to different temperatures (Chen et al. 2013; Gatch and du Toit 2017; Navas-Cortés et al. 2007; Schuerger and Mitchell 1992).

Mathematical models help to understand the interactions of abiotic factors on pathogens and disease development. Polynomial models have been used to describe temperature and pH effects on *Fusarium* wilts under greenhouse and hydroponic conditions in cucumber and mung bean (Chen et al. 2013; Schuerger and Mitchell 1992). Gaussian and quasi-Gaussian models are used to describe spatial dispersion over long distances of spore-bearing vascular plants and rust spores (Skelsey et al. 2008; Soubeyrand et al. 2008). Gaussian models have been used in plant pathology to simulate spore wind dispersal of *Phytophthora infestans* in potatoes (Spijkerboer et al. 2002) and describe spatial movement of wind-dispersed spores of *Gibberella zeae* foci on wheat plots (Paulitz et al. 1999; Prussin et al. 2015). Development of a Gaussian model may be useful to describe how these environmental factors influence the development of soybean root rot caused by *Fo*.

The goal of this research was to evaluate the importance of pH and temperature in the development of soybean root rot caused by *Fo*. Our objectives were to determine the effects of pH, temperature, and their potential interaction on (i) in vitro radial growth, (ii) soybean root rot under growth chamber conditions, and (iii) characterization of *Fo* isolates as pathogenic or nonpathogenic.

Materials and Methods

Effects of pH and temperature on in vitro radial growth. Fourteen *Fo* isolates collected from symptomatic roots across Iowa were single-spore isolated, identified to species microscopically and by DNA sequencing (Díaz Arias et al. 2013a), phenotypically and

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genotypically characterized (Cruz Jimenez et al. 2018; Ellis et al. 2014), and maintained on dry silica beads at 4°C until use. To prepare inoculum for the experiments, the *Fo* isolates were grown on potato dextrose agar (PDA) at 39 g/liter (Difco, Becton, Dickinson and Co., Sparks, MD) at 25°C for 4 days, with a 12-h photoperiod. A 5-mm plug was punched from the PDA culture and transferred to the center of a 90-mm-diameter Petri dish containing differential media with one of five pH levels (4, 5, 6, 7, and 8) and additionally full-strength PDA medium (pH ~6.5).

The media differing in pH levels were prepared as described by Groenewald et al. (2006). Citrate/phosphate buffer was prepared by titrating 0.1 M citric acid (Amresco, Solon, OH) with 0.2 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (Acros, Springfield Township, NJ) to reach a pH of 4 or 5. Phosphate buffer was prepared by titrating 0.2 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (Acros) with 0.2 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (Acros) to reach a pH of 6 or 7. Boric acid buffer was prepared by titrating 0.2 M boric acid (Fisher, Chicago, IL) with 0.05 M sodium tetraborate $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ (Fisher) to reach a pH of 8. Once the buffers for pH 4, 5, 6, 7, and 8 were prepared, a basal medium was added to each liter of buffer. The basal medium consisted of 45 g of sucrose (C&H, Crockett, CA), 3 g of NaNO_3 (Sigma-Aldrich), 1.5 g of K_2HPO_4 (Fisher), 0.75 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich), 0.75 g of KCl (Fisher), and 0.015 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Fisher). Agar (Difco, Becton, Dickinson and Co.) was added at a rate of 15 g/liter for pH buffers 5, 6, 7, and 8, with a higher rate of agar of 18 g/liter for pH 4 owing to the reduced gelling capacity of agar at low pH (Wetzstein et al. 1994; Yamamoto and Cunha 2007). In addition to the pH differential media, a full-strength PDA medium at 39 g/liter (pH ~6.5) was included in the experiments.

Fo cultures on the pH differential media and full-strength PDA medium (pH ~6.5) were incubated at four different temperatures (15, 20, 25, and 30°C) for 5 days in an incubator (Hoffman Manufacturing, Corvallis, OR) at 65% relative humidity and in the dark. Diameter of each colony was then measured in two perpendicular directions, and an average of the two measurements was calculated after subtracting the 5-mm diameter of the colonized plug. The experiment was organized in a split-plot in a completely randomized design, in which temperature was the main plot treatment and the pH differential medium/isolate combination randomized within each chamber was considered as the subplot treatment. There were four replicate Petri dishes per temperature/pH differential medium/isolate combination, and the experiment was conducted two times.

Effects of pH and temperature on seedling disease. *Fo* isolates FO38 and FO42, previously identified as pathogenic and nonpathogenic to soybeans at 25°C, respectively (Cruz Jimenez et al. 2018; Díaz Arias et al. 2013a; Ellis et al. 2014), were grown on PDA for 7 days at 25°C, with a 12-h photoperiod, to promote conidial formation. Macro- and microconidia were collected by rinsing the plates with sterile distilled water and scraping the surface of the cultures with a sterile stick. The suspension was filtered 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 1×10^6 conidia/ml.

Fifteen seeds of *Fo*-susceptible cultivar Jack (Cruz Jimenez et al. 2018) were placed on moist towels and individually inoculated with 200 μl of the spore solution of FO38 or FO42. Paper towels were moistened with a buffer solution adjusted to pH levels 4, 5, 6, or 7 using citric acid (Amresco) and sodium hydroxide (NaOH) (Fisher). Each towel was rolled up and placed in a 1,500-ml glass jar containing 250 ml of the respective buffer and incubated for 8 days in growth chambers (Hoffman Manufacturing) at four different temperatures (15, 20, 25, and 30°C) at 65% relative humidity and with a 12-h photoperiod.

Each roll was considered as an experimental unit, placing one roll per glass jar with 15 seeds or subsamples on each roll. There were three inoculated rolls for each of the *Fo* isolates and three noninoculated rolls for each pH and temperature treatment combination (4 temperatures $\times$ 4 pH $\times$ 3 inoculation treatments $\times$ 3 reps = total

144 experimental units). The experiment was conducted in a split-plot design. Temperature was the main plot treatment, and the combination of differential pH buffer and isolate randomized in each growth chamber was the subplot treatment. There were three replicate rolls per temperature/pH/*Fo* isolate combination. The experiment was conducted two times.

Seedlings were rated for root rot using two methods: a disease severity index (DSI) and a visual disease severity scale (1 to 5), as described by Ellis et al. (2011). DSI was calculated as the ratio between the lesion length (discolored or colonized hypocotyl and radicle) and the total plant length, multiplied by 100. When seeds did not germinate and were completely colonized, they were assigned a DSI value of 100%. The visual 1 to 5 disease severity scale was assessed according to the following ratings: 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.

Reductions in seedling growth owing to inoculation were calculated by measuring the root weight, root length, and shoot length on each *Fo*-inoculated plant, dividing by the average measure of the noninoculated control within each temperature and pH combination treatment and then multiplying by 100.

Data analysis. Variances of the raw data for the two experiments of radial growth were compared by using Levene's test for homogeneity under the general linear model procedure (PROC GLM) of SAS version 9.3 (SAS Institute, Cary, NC). Variances of the two experiments were not statistically different according to the test ($P = 0.95$); therefore, the raw data for the two experiments were combined for subsequent analysis. In addition, normal distribution of the residuals was tested under the PROC UNIVARIATE statement. Analysis of variance was performed under PROC GLIMMIX, in which experimental replication, temperature, pH differential media or full-strength PDA medium, and *Fo* isolates were considered as fixed factors, and the two experimental replications and their interaction with temperature were considered as random effects. In addition, a four-way interaction term between experimental replication, temperature, pH differential media, and *Fo* isolates was added as a random effect for the statistical analysis. Mean separation analyses were performed using a Tukey's multiple comparison test ($P = 0.05$).

For the pathogenicity assay, the average of DSI was arcsine transformed, and the disease severity scale was square-root transformed. Analysis of variance was performed under the PROC GLIMMIX statement, in which experimental replication, temperature, pH buffers, and *Fo* isolates were considered as fixed factors. The two experimental replications and their interaction with temperature were considered as random effects. In addition, a four-way interaction term between experimental replication, temperature, pH buffers, and *Fo* isolates was added as a random effect for the statistical analysis. Denominator degrees of freedom were calculated by the Satterthwaite method, and mean separation analyses on the transformed data were performed using a Tukey's multiple comparison test ($P = 0.05$). The average raw data of DSI and the visual scale for severity for each of the *Fo*-inoculated rolls were used to calculate linear correlation coefficients using the PROC CORR statement.

Exponential, polynomial, logistic, Gompertz, and Gaussian models were tested to describe the radial growth and root rot of *Fo* under the pH and temperature levels selected for the experiments. Model selection was based on the following criteria described by Byamukama et al. (2011): low standard error of the estimate, significant F statistics ($P \leq 0.05$), and high coefficients of determination (R^2). The model that best described and fit the data was selected to obtain parameter estimates.

Gaussian regression analyses for fungal radial growth and DSI were performed for each individual *Fo* isolate using SigmaPlot version 13 (Systat Software, San Jose, CA) under the following function:

$$f = a \exp \left\{ -5 \left[\left(\frac{x - X_0}{b} \right)^2 + \left(\frac{y - Y_0}{c} \right)^2 \right] \right\}$$

where a is the largest value of the response variable (DSI or fungal radial growth), X_0 is the temperature value for the maximum a , Y_0 is the pH value for the maximum a , and b and c are the pH and temperature ranges for the peak shaped function. Estimated values of temperature and pH for maximal fungal radial growth and disease severity were obtained by using a nonlinear optimization procedure (solver add-in) in Excel (Microsoft) under the following constraints: pH range between 4 and 7 and temperature range between 15 and 30°C.

Results

Effects of pH and temperature on in vitro radial growth. Analysis of variance indicated that main effects of temperature, pH, and *Fo* isolate and their interactions were highly significant ($P < 0.0001$) for fungal radial growth. Maximal radial growth was observed at pH 6 at 25 and 30°C, for isolates FO41 and FO49 (6.39 and 6.3 cm,

respectively). Minimal radial growth was observed at 15°C at pH 4 for isolate FO38 (0.3 cm) (Fig. 1). No growth was observed at pH 8 for any of the *Fo* isolates evaluated in this study.

On full-strength PDA there was a strong effect of temperature and isolate on radial growth with a significant interaction between these two factors ($P < 0.0001$). Individual isolates had significant differences in their final radial growth. Maximal radial growth was observed at 25°C for isolates FO47 and FO36 (4.38 and 4.15 cm, respectively). Minimal radial growth was observed at 15°C across all isolates, with FO41 growing the least (1.11 cm) (data not shown).

The Gaussian model best described the changes in radial growth across levels of pH and temperature for all *Fo* isolates, with significant F statistics ranging from 6.8012 to 37.3336, indicating there was a strong relationship between temperature, pH, and radial growth (Table 1). Coefficients of determination (R^2) from the Gaussian regression analyses varied among isolates, explaining up to 93.1% of the variation in fungal radial growth. In addition, the Gaussian model

Fig. 1. Radial growth of 14 *Fusarium oxysporum* isolates with respect to temperature and pH of the growth medium. **A, B, C, and D,** Isolates growing in citrate/phosphate buffer set at pH 4, 5, 6, and 7, respectively. Data represent the mean values of two experiments with four replicate Petri dishes for each treatment combination.

had lower standard error of the estimates, with values ranging from 0.3907 to 0.9157 (Table 1), compared with exponential, logistic, and Gompertz models (data not shown).

Estimated values of optimal pH and temperature for maximal radial growth obtained from the Gaussian regression analysis indicated variation among isolates. pH estimated values varied between 5.6 and 6.8, and temperatures varied between 25.4 and 30°C. A separate regression was performed on the average data of fungal radial growth across *Fo* isolates; the average estimate of pH and temperature for maximal radial growth over all *Fo* isolates was 6.3 and 27.1°C, respectively (Table 1, Fig. 2).

Effects of pH and temperature on seedling disease. According to the analysis of variance, there was a significant effect of isolate ($P < 0.0001$), and the interaction between temperature and isolate was significant ($P < 0.0001$) on DSI, indicating that temperature differentially affected the severity of each particular isolate (Table 2, Fig. 3).

DSI varied across levels of pH and temperature. For the pathogenic isolate, the highest level of root rot was observed at pH 6 and 30°C (67%), and the lowest levels of disease severity were observed at 15°C at pH 4 and 5 (4.2%) (Fig. 3A). Interestingly, when the mean separation analyses were performed to test the effect of temperature on the DSI of FO38 isolate at individual pH levels, 30 and 25°C had significantly higher levels of disease in all pH levels. Root rot severity at 15 and 20°C displayed the lowest levels of disease for all pH levels (Fig. 3A).

Disease levels for the nonpathogenic FO42 isolate were significantly lower than the pathogenic isolate but showed similar trends. The highest disease severity (17.2%) was observed at the same level of pH and temperature as the pathogenic isolate: pH 6 at 30°C (Fig. 3B). Mean separation analysis indicated no differences in disease severity between 15, 20, and 25°C for all pH levels (Fig. 3B).

Root rot symptoms differed between the pathogenic and nonpathogenic *Fo* isolates. Soybean seed inoculations performed with the pathogenic isolate FO38 caused extended colonization of radicles, hypocotyls, cotyledons, and tissue maceration. Subsequently, germination was lower, and soybean seedlings had poor root and shoot development (Fig. 4A to D). Conversely, inoculations performed with the nonpathogenic isolate FO42 caused a few small spots of tissue

discoloration on the hypocotyls and radicles, but tissue structure and consistency were intact with no maceration or softening (Fig. 4E to H). DSI on the noninoculated control seedlings was less than 5%, with a few seedlings showing small spots of discoloration on the hypocotyls.

Analysis of variance also revealed significant effects of isolate for the percentage of reduction on seedling growth variables (Table 2, Fig. 5).

The highest root weight reductions were observed at 30°C, ranging from 47 to 35%, and the lowest reductions at 15°C, ranging from 22 to 8.2%, when seeds were inoculated with the pathogenic isolate FO38. Mean separation analysis indicated that the pathogenic isolate FO38 produced significant reductions on root weight at 30°C at pH 4 and 5 (Fig. 5A). Conversely, seeds inoculated with the nonpathogenic FO42 isolate presented significantly lower reductions in root weight (<20%) (Fig. 5B).

Fig. 2. Estimates of fungal radial growth (cm) from the Gaussian regression analysis across the main factors pH at 4, 5, 6, and 7 and temperature at 15, 20, 25, and 30°C for the average growth of 14 *Fusarium oxysporum* isolates. Data represent the mean values of two experiments with four replicate Petri dishes for each treatment combination.

Table 1. Estimates of pH and temperature obtained from the nonlinear optimization procedure from the Gaussian model for in vitro fungal radial growth and root rot on *Fusarium oxysporum* isolates

Gaussian model parameters and statistics for in vitro radial growth							
Isolates	pH	Temperature (°C)	Radial growth (cm) ^a	SEE ^b	F statistic	P > F	Gaussian regression R ² ^c
FO36	6.2	27.8	5.2	0.6540	15.8475	0.0002	0.8521
FO37	6.7	28.1	5.5	0.6252	16.3469	0.0001	0.8560
FO38	5.8	28.1	3.0	0.4427	9.9208	0.0001	0.7830
FO39	6.7	27.6	4.5	0.7199	9.1845	0.0016	0.7696
FO40	6.1	27.3	5.3	0.3907	37.3336	<0.0001	0.9314
FO41	6.0	25.4	5.8	0.8204	11.8469	0.0006	0.8116
FO42	6.4	27.1	4.9	0.5791	17.1491	0.0001	0.8618
FO43	6.8	27.0	5.0	0.9150	6.8012	0.0052	0.7121
FO44	6.4	26.6	5.1	0.7169	13.9697	0.0003	0.8355
FO45	6.4	28.9	5.2	0.6895	16.4253	0.0001	0.8566
FO46	6.7	30.0	4.5	0.8193	7.2480	0.0041	0.7249
FO47	6.5	26.7	5.4	0.5334	23.8329	<0.0001	0.8966
FO48	6.5	27.4	4.9	0.6307	15.3397	0.0002	0.8480
FO49	6.4	26.0	6.0	0.8846	11.1288	0.0007	0.8019
Average	6.3	27.1	4.9	0.5792	17.1491	0.0001	0.8618
Gaussian model parameters and statistics for root rot seedling disease							
Isolates	pH	Temperature (°C)	DSI (%) ^a	SEE ^b	F statistic	P > F	Gaussian regression R ² ^c
FO38	5.9	30	64.0	2.9389	168.7979	<0.0001	0.9840
FO42	5.9	30	17.1	1.0859	70.2363	<0.0001	0.9623

^a Maximal radial growth and maximal disease severity index (DSI) estimates were obtained by using a nonlinear optimization procedure including pH range from 4 to 7 and temperature range from 15 to 30°C for each isolate.

^b SEE = standard error of estimate.

^c R² = coefficient of determination.

The highest root length reductions were observed when seeds were inoculated with the pathogenic isolate FO38 and incubated at 25°C and pH 5, 6, and 7, ranging from 21 to 16%. Similarly, seeds incubated at 30°C and pH 4 displayed a root length reduction of 20%. The lowest root length reductions were observed at 15°C, fluctuating from 8 to 6% (Fig. 5C). In contrast, seeds inoculated with the nonpathogenic FO42 isolate showed significantly lower reductions in root length (<13%) (Fig. 5D).

Plant length reductions were the highest when seeds were inoculated with the pathogenic isolate FO38 and incubated at 30°C and pH 4 and 6 (25%). The lowest plant length reductions were observed at 15°C, ranging from 9.3 to 4.1% (Fig. 5E). Reductions in plant length were <9% when seeds were inoculated with the nonpathogenic FO42 isolate, with a few observations presenting no reductions (Fig. 5F).

The visual scale of severity presented similar results to DSI. The main effects of temperature and isolate were highly significant ($P < 0.0001$) (Table 2), indicating that the effect of the isolates on severity depends on the temperature. DSI and the visual scale for severity were linearly related. Pearson's correlation coefficients performed separately by *Fo* isolates were highly significant ($P < 0.0001$), indicating a strong positive linear relationship (Fig. 6).

Although there were highly significant differences in the disease intensity caused by the *Fo* isolates, the Gaussian regression analysis

described with high precision the variation in disease across pH levels and temperatures. Gaussian regressions performed separately on each *Fo* isolate had low standard errors of the estimates, indicating higher precision for predicted values of disease, compared with other standard models (data not shown). In addition, regression analysis showed significant *F* statistics ($P < 0.0001$) and high coefficients of determination (R^2), explaining 98 and 96% of the variation in disease for the pathogenic and nonpathogenic isolate, respectively (Table 1).

Estimated optimal values of pH and temperature for maximal root rot obtained from the Gaussian regression analysis were equivalent for the pathogenic and nonpathogenic *Fo* isolates. Interestingly, estimated values of pH at 5.9 and 30°C produced the highest level of root rot for both isolates. Under these optimal pH and temperature conditions, the pathogenic *Fo* isolate FO38 had a maximal estimated root rot of 64%, and the nonpathogenic FO42 had a maximal estimated root rot of 17%. (Table 1, Fig. 7).

Discussion

This study provides evidence that pH and temperature have significant effects on *Fo* fungal radial growth and severity of Fusarium root rot of soybean seedlings, increasing our understanding of the epidemiology of the disease. For most of the isolates, the combination of pH 6 and 25°C resulted in the fastest in vitro radial growth, and the

Table 2. Analysis of variance indicating the effects of pH and temperature on seedling disease after inoculation of soybean seeds with pathogenic (FO38) and nonpathogenic (FO42) *Fusarium oxysporum* isolates in a rolled-towel assay

Source	df	DSI ^a		Severity		Root weight reduction (%)		Root length reduction (%)		Plant length reduction (%)	
		<i>F</i> value	<i>P</i> > <i>F</i>	<i>F</i> value	<i>P</i> > <i>F</i>	<i>F</i> value	<i>P</i> > <i>F</i>	<i>F</i> value	<i>P</i> > <i>F</i>	<i>F</i> value	<i>P</i> > <i>F</i>
Replicate	1	29.44	0.0122	5.87	0.0940	0.20	0.6818	1.99	0.1606	0.04	0.8614
Temperature (T)	3	57.99	0.0037	25.38	0.0124	3.29	0.1769	1.30	0.2768	0.82	0.5643
pH	3	13.06	<0.0001	8.78	0.0003	0.38	0.7708	2.04	0.1111	0.45	0.7185
pH × T	9	1.90	0.0937	1.08	0.4048	2.10	0.0330	1.23	0.2799	0.70	0.7004
Isolate (I)	1	326.64	<0.0001	188.85	<0.0001	42.60	<0.0001	12.65	0.0005	9.88	0.0039
T × I	3	21.88	<0.0001	23.85	<0.0001	7.97	<0.0001	1.98	0.1195	2.98	0.0482
pH × I	3	0.10	0.9574	0.44	0.7252	0.04	0.9909	0.40	0.7551	0.05	0.9870
pH × T × I	9	0.29	0.9716	0.57	0.8076	0.18	0.9960	1.04	0.4110	0.32	0.9625

^a Disease severity index (DSI) was arcsine transformed. Visual scale of severity was square-root transformed. Percentage of reduction on seedling growth variables was calculated relative to the noninoculated control. Experiments consisted of three replications (towels) for each treatment combination and two experiments for the rolled towel assay.

Fig. 3. Disease severity index (DSI) with respect to pH buffer and incubation temperature for **A**, FO38 pathogenic *Fusarium oxysporum* (*Fo*) isolate and **B**, FO42 nonpathogenic *Fo* isolate. Comparisons were made separately by pH buffer. Means with the same letter are not significantly different according to Tukey's multiple comparison test ($P = 0.05$).

most severe root rot development occurred at pH 6 and 30°C. Using Gaussian models, the optimal estimated conditions were pH 6.3 at 27.1°C for maximal fungal growth and pH 5.9 at 30°C for maximal root rot severity, showing that *Fo* growth and seedling disease are favored by similar conditions.

The results from this study are similar to the findings of earlier reports for in vitro radial growth of other *Fusarium* species and *formae speciales*. For example, *Fo* f. sp. *cubense* displayed a maximum radial growth at pH 6 at 25°C, with a minimum growth at pH 4, using the same citrate/phosphate buffer medium (Groenewald et al. 2006). Similar results for optimal fungal growth at 25°C were reported for *Fo* f. sp. *lactucae* (Scott et al. 2010), *Fo* f. sp. *fabae* (Ivanovic et al. 1987), and *Fo* f. sp. *spinaciae* (Naiki and Morita 1983).

Growth of fungi affects the pH of the medium owing to the production of secondary metabolites, pigments, absorption of anions, and production of ammonia (Cochrane 1958). These metabolic activities tend to acidify the medium, as has been demonstrated in *Fo* (Srivastava et al. 2011), making it complex to draw conclusions when poorly buffered growth medium such as PDA is used. These results suggest that studies to determine optimal temperature for fungal growth must be viewed with some caution when PDA is used. If the fungus is able to change the pH of the medium to favor its growth, then conclusions about the optimal pH for growth could be misleading and a buffered medium must be used.

Our findings of the effect of pH on fungal growth and disease severity agreed with a number of studies on *Fusarium* spp. in which slightly acidic growth medium at around pH 6 promotes fungal growth and sporulation (Agarwal and Sarbhoy 1978; Cochrane 1958; Groenewald et al. 2006; Gupta et al. 2010; Wu et al. 2009). However, pH is not a unitary factor influencing the mechanisms of growth; other physical parameters such as temperature, water potential, and relative humidity may interact to a certain degree (Cochrane 1958; Pehrson 1948). Optimal pH for fungal growth can change

along with increasing or decreasing temperatures. For example, optimal pH for growth of *Phacidium infestans* on liquid buffered medium decreased with temperature (Pehrson 1948). This contrasts with our findings on *Fo* in which the optimal pH remained at 6.0 for growth across all temperatures.

Fungal growth also depends on genetic background and physiological adaptations to predominant temperatures of geographical areas (Burgess et al. 1988; Manshor et al. 2012; Walker and White 2005). Isolates from tropical regions may have higher optimal growth temperature than isolates from temperate and subtropical regions (Cochrane 1958; Nelson et al. 1990). For example, the greatest fungal radial growth tends to occur at 25°C for *Fo* f. sp. *betulae* isolates collected from temperate regions in the United States (Webb et al. 2015), as well for *Fo* f. sp. *psidii* and *F. solani* isolates collected from subtropical regions in India (Gupta et al. 2010). In contrast, isolates of different *Fusarium* species occurring in arid and tropical regions of Australia grow the best at 30°C (Nelson et al. 1990; Sangalang et al. 1995). It would be interesting to include *Fo* isolates from tropical regions and compare them to those from Iowa in future pH and temperature studies.

Our results on root rot severity are similar to those reported for *Fusarium* root rot in other *Fusarium* species, such as *Fo* f. sp. *dianthi*, *cucumerinum*, *cubense*, *ciceris*, *spinaciae*, *phaseoli*, and *lactucae* along with *F. graminearum* (Ben-Yephet and Shtienberg 1994; Chen et al. 2013; Ellis et al. 2011; Groenewald et al. 2006; Landa et al. 2006; Naiki and Morita 1983; Schuenger and Mitchell 1992; Scott et al. 2010), in which the highest disease severity was observed at temperatures between 25 to 30°C. Interestingly, the majority of these species were predominantly from humid subtropical climatic zones. Gaussian regression analysis indicates that pathogen fungal growth and root rot severity temperature curves have similar trends and follow each other closely, suggesting that pH and temperature have similar effects on *Fo* growth and soybean root rot severity caused by *Fo*.

Fig. 4. Soybean seedlings incubated at 30°C for 8 days after seed inoculation with *Fusarium oxysporum* isolates with a spore suspension of 1×10^6 conidia/ml. **A, B, C, and D,** Seedlings inoculated with pathogenic isolate FO38 growing in citric acid and NaOH buffer set at pH 4, 5, 6, and 7, respectively. **E, F, G, and H,** Seedlings inoculated with the nonpathogenic isolate FO42 growing in citric acid and NaOH buffer set at pH 4, 5, 6, and 7, respectively.

Similarities in these curves might indicate that temperature influences the level of susceptibility in the host (Dickson 1923; Jones 1924).

Low temperatures have been reported to favor plant defense mechanisms making soybean seedlings more resistant to *Fo* (Koretsky 2001). There is evidence of overexpression of resistance-related proteins (esterase and PR-2) after soybean cultivars were inoculated with pathogenic *Fo* isolates at low temperatures. *Fo*-inoculated seedlings

incubated at 8°C had higher 1,3- β -glucanase activity compared with seedlings incubated at 24°C (Koretsky 2001). Therefore, low temperatures may favor resistance to *Fo* on soybeans, agreeing with our observations of low root rot severity and low percent reduction in seedling growth variables, such as root weight, root length, and plant length, at 15 and 20°C under growth chamber conditions. Root rot at temperatures between 25 and 30°C may be influenced by a lack

Fig. 5. Percentage of reduction on seedling growth variables relative to the noninoculated control with respect to pH buffer and incubation temperature for **A, C, and E**, plants inoculated with F038 pathogenic *Fusarium oxysporum* (*Fo*) isolate and **B, D, and F**, plants inoculated with F042 nonpathogenic *Fo* isolate. Comparisons were made separately by pH buffer. Means with the same letter are not significantly different according to Tukey's multiple comparison test ($P = 0.05$).

of soybean resistance, which, combined with faster growth and colonization of *Fo* at its optimal growth temperature, resulted in more severe root rot. Nevertheless, these temperature-dependent defense mechanisms need further study.

Our findings regarding optimal temperature for disease development conflict with some greenhouse and field observations in which soybean root rot caused by *Fo* occurred at low temperatures (<18°C) and disease severity was reduced when temperature increased from 26 to 32°C in *Fo*-infested saturated soils (Carson et al. 1991; Farias and Griffin 1990; French 1963). A possible reason for these conflicting results is the variability in soil temperature and soil moisture under field conditions. It is important to observe the effect of various temperatures on disease development at early soybean stages. The ideal soybean germination and emergence temperatures are between 25 and 30°C (Hatfield and Egli 1974). Soybean planting in Iowa rarely takes place when soils are in this temperature range. However, in other regions, especially where soybean is double cropped with winter cereals, temperatures in this range may be common at planting (Dillon 2014). In Iowa, soil temperatures of 25 to 30°C occur during late May or early June (4 or 5 weeks after planting) (Licht et al. 2013). The usual soil

temperature at planting in Iowa for late April or early May is 10°C (De Bruin and Pedersen 2008), and emergence takes between 7 and 14 days, increasing the risk for seedling disease. Seedling disease in soybean is usually associated with early planting and low soil temperature; under those conditions, *Fo* seems unlikely to be the primary pathogen.

Disease severity and root rot symptoms varied significantly between the pathogenic and nonpathogenic *Fo* isolates. Lanubile et al. (2015) demonstrated significant differences in soybean defense gene expression profiles in response to pathogenic and nonpathogenic *Fo* isolates. Pathogenic *Fo* isolates induced a strong defense reaction compared with nonpathogenic, which induced relatively little host response to inoculation. These descriptions agreed with our observations on the nonpathogenic FO42 isolate, which included no symptoms of root rot, or very small black-brown discolorations on seedling roots, or hypocotyls.

The nonpathogenic isolate FO42 showed a notable ability to promote plant growth; seedlings inoculated with this isolate showed significantly higher root weight and total plant length compared with the noninoculated control. Plant growth stimulation by nonpathogenic *Fo* isolates has also been observed in other hosts (Fracchia et al.

Fig. 6. Linear relationship between disease severity index (DSI) and visual scale for severity. Each data point represents the average disease of one towel containing 15 soybean seeds inoculated with **A**, FO38 pathogenic *Fusarium oxysporum* (*Fo*) isolate and **B**, FO42 nonpathogenic *Fo* isolate. Data from the noninoculated seeds were not included in the analysis. r = Pearson's linear correlation coefficient.

Fig. 7. Estimates of disease severity index (DSI) from the Gaussian regression analysis across the main factors pH at 4, 5, 6, and 7 and temperature at 15, 20, 25, and 30°C for **A**, FO38 pathogenic *Fusarium oxysporum* (*Fo*) isolate and **B**, FO42 nonpathogenic *Fo* isolate. Data represent the disease mean values of two experimental replicates for each *Fo* isolate.

2000; Thongkamngam and Jaenakorn 2017). This effect has been associated with the interaction of certain fungal metabolites and volatiles with the host hormone transport and signaling of auxins (Bitas et al. 2015). Additional research on the interaction between pathogenic and nonpathogenic *Fo* isolates must be done to identify potential for biocontrol and how biocontrol efficacy might be influenced by abiotic factors such as pH and temperature in soybeans.

Although the disease severity produced by the isolates FO38 and FO42 had similar trends in response to environmental factors, severity and estimates of percent reduction in seedling growth variables for the nonpathogenic FO42 isolate were significantly lower in all combinations of pH and temperature compared with the pathogenic FO38 isolate. These results suggest that it is unlikely that environmental factors such as pH and temperature determine the potential pathogenicity of a particular *Fo* isolate. Abiotic factors influence the level of disease caused by *Fo* pathogenic isolates, but pathogenicity may be conferred by horizontal gene transfer of genomic regions rich in transposons or pathogenicity-related genes that contribute to the origin of different f. sp. or pathogenic genotypes (Ma et al. 2010).

This is the first report quantifying and modeling the effects of abiotic factors on *Fo* in soybeans. We found that the Gaussian model best explained the effects of temperature and pH on fungal radial growth and disease severity. Previous studies on *Fusarium* wilts in other pathosystems have used polynomial models to describe the interaction between temperature and pH for fungal growth in soil (Chen et al. 2013) and spore attachment under hydroponic conditions (Schuenger and Mitchell 1992). In addition, Navas-Cortés et al. (2007) developed exponential models to describe effects of soil temperature and inoculum density on *Fusarium* wilt of chickpea. Interestingly, these polynomial and exponential models describe trends and optimal temperatures similar to our studies of *Fo* on soybeans. The models developed for *Fo* growth and root rot will give insights on high- and low-risk scenarios to growers and farmers. This simple quantitative model captures crucial features of host-pathogen interactions on key abiotic variables such as pH and temperature. Additional variables could be added to create more complex and representative models.

The information generated in this research will increase knowledge about the epidemiology of *Fo* and may help to predict the risk of *Fusarium* root rot. This knowledge may assist breeders to better identify resistant soybean cultivars, taking into account that temperature significantly influences root rot development and *Fo* aggressiveness. Quantifying the impact of environmental factors on root rot disease development by the use of quantitative models may help to design more efficient management strategies by finding environmental conditions to limit disease development through the adjustment of soil pH, planting dates, and the use of cultivars that may show a temperature-dependent reaction.

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