Effect of Soil Inoculum Density of *Fusarium oxysporum* f. sp. *vasinfectum* Race 4 on Disease Development in Cotton

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ABSTRACT

Hao, J. J., Yang, M. E., and Davis, R. M. 2009. Effect of soil inoculum density of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 on disease development in cotton. Plant Dis. 93:1324-1328.

The effect of soil inoculum density (0 to 10^6 conidia/g of potting mix) of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 on plant growth (weight, height, and number of nodes), Fusarium wilt symptoms, vascular discoloration, and number of CFU per gram of stem tissue was determined on five cotton cultivars grown in a greenhouse. In the susceptible cv. DP744, symptoms of wilt and reductions in plant growth occurred at inoculum levels of 10^3 conidia/g of potting mix and higher, whereas plant growth of the resistant Pima cv. Ph800 was not affected by any soil inoculum densities. Cvs. DP340, Ph72, and UltEF all responded similarly, with a relatively moderate negative growth response to soil inoculum densities generally beginning at 10^4 conidia/g of potting mix. *F. oxysporum* f. sp. *vasinfectum* race 4 was recovered from the stems of all cultivars by plating macerated stem tissue on a semiselective medium. There was a significant positive correlation between CFU and inoculum density above 10^4 conidia/g of potting mix for DP744 and UltEF. The fungus was recovered relatively infrequently from stems of Ph 800 plants inoculated with any inoculum density of *F. oxysporum* f. sp. *vasinfectum*. Fusarium wilt caused by *F. oxysporum* f. sp. *vasinfectum* race 4 is an inoculum density-dependent disease; the disease was generally mild and the fungus was rarely recovered from stems at inoculum levels less than 10^4 conidia/g of potting mix.

Fusarium wilt of cotton, caused by *Fusarium oxysporum* f. sp. *vasinfectum* W.C. Snyder & H.N. Hansen, causes variable losses in the field depending on the virulence of the pathogen, susceptibility of the cultivar, climatic conditions, soil type and fertility, and interactions with nematodes (1,2,4,7,12,15,17). The disease has become a major concern in California cotton production since the recent discovery of race 4, which was originally reported in India in 1960 (3,5). Because the pathogen survives in soil indefinitely and management options are limited (13,18), plant resistance is being sought; to date, there are few resistant breeding lines to *F. oxysporum* f. sp. *vasinfectum* race 4 (16). *F. oxysporum* f. sp. *vasinfectum* race 4 is most virulent on some Pima cultivars, although the pathogen can also cause severe symptoms and losses in some Upland cultivars (8). Phytogen 800, the only currently available commercial Pima cultivar with good resistance to race 4, is occasionally affected by *F. oxysporum* f. sp. *vasinfectum* (16).

The relationship between soil inoculum density of *F. oxysporum* f. sp. *vasinfectum*

and cotton yield losses was recognized in 1979 by Garber et al. (7), who demonstrated that *F. oxysporum* f. sp. *vasinfectum* race 1, which usually damaged cotton only when plants were co-infected with root-knot nematodes (*Meloidogyne incognita* (Kofoed & White) Chitwood), caused disease in the absence of nematodes at high levels of *F. oxysporum* f. sp. *vasinfectum* soil inoculum. In the presence of nematodes, leaf symptoms appeared earlier because the inoculum density of either *F. oxysporum* f. sp. *vasinfectum* or *M. incognita* or both increased. DeVay et al. (6) also reported a positive correlation between populations of *F. oxysporum* f. sp. *vasinfectum* race 1 and disease incidence in the field, especially in the presence of *M. incognita*. In artificially infested microplots, Starr et al. (14) found that the population densities of *F. oxysporum* f. sp. *vasinfectum* race 1 and *M. incognita* were both positively correlated with cotton plant mortality and yield loss. In controlled experiments, virulent Australian *F. oxysporum* f. sp. *vasinfectum* isolates, which, like *F. oxysporum* f. sp. *vasinfectum* race 4, do not require an interaction with nematodes to cause disease (8), caused increasingly severe symptoms at higher aqueous suspensions of conidia (17). However, the effect of conidial concentration varied with the resistance of the cotton cultivar.

The relationship between inoculum density and severity of Fusarium wilt is important for the development of management

strategies because populations of *F. oxysporum* f. sp. *vasinfectum* propagules in the soil may be affected by cotton cultivars, alternate hosts, and soil treatments. This information is critical for a thorough understanding of disease epidemiology, especially when highly resistant cultivars are not available. The purpose of this study was to characterize the effect of soil inoculum density of *F. oxysporum* f. sp. *vasinfectum* race 4 on disease development and to assess the ability of the fungus to propagate within cotton tissue of five cotton cultivars.

MATERIALS AND METHODS

A greenhouse trial included three Pima (*Gossypium barbadense* L.) cultivars: Deltapine 744 (DP744), Deltapine 340 (DP340), and Phytogen 800 (Ph800); and two Upland (*G. hirsutum* L.) cultivars: Phytogen 72 (Ph72) and Ultima EF (UltEF). Cotton seed were individually planted in 15-cm-diameter pots in UC potting mix (50% sphagnum peat moss; 50% washed sand; and 2.43 kg of dolomite lime, 0.872 kg of oyster shell lime, 0.872 kg of superphosphate, 0.344 kg of calcium nitrate, 135 ml of potassium nitrate, and 90 ml of potassium sulfate per cubic meter) with seven different inoculum densities: 0, 10^1 , 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 conidia/g of potting mix.

Inoculum was prepared from 3-week-old potato dextrose agar cultures of *F. oxysporum* f. sp. *vasinfectum* race 4 isolate CA-14. The plates were flooded with water and conidia were scraped off the culture with an inoculation loop. The conidial suspension was then filtered through four layers of cheesecloth, quantified with the aid of a hemacytometer, and diluted with water. Suspensions were mixed uniformly into UC potting mix with a flame-sterilized cement mixer to give the appropriate final concentration of conidia per gram of potting mix, which was then distributed into the pots.

The experiment was designed with four randomized complete blocks. Each cotton cultivar was planted in potting mix of each inoculum density, with one replicate per block. Plant height and weight, number of plant nodes, severity of leaf symptoms, vascular discoloration, and number of CFU recovered per gram of stem tissue were measured 6 weeks after planting. Plant symptoms were rated on the following scale: 1, no symptoms; 2, chlorosis or wilt

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Accepted for publication 10 August 2009.

restricted to cotyledons or first leaf; 3, similar symptoms extended beyond first leaf; 4, severe symptoms affecting the whole plant; and 5, plant dead. Vascular discoloration was measured from cross and longitudinal sections of the stem at the soil line and rated as follows: 1, no discoloration; 2, light brown, streaky discoloration; 3, continuous dark brown discoloration; and 4, plant dead.

In order to quantify the number of CFU per gram of fresh stem tissue, a piece of stem, approximately 5 cm in length, between the soil line and first leaf was weighed, surface sterilized in 0.5% sodium hypochlorite for 2 min, and homogenized for 2 min in a blender with 100 ml of distilled water. Aliquots of 100 μ l of the suspension at 1:1, 1:10, and 1:100 dilutions were plated onto each of three plates of Komada's *Fusarium* selective medium (9). After 4 to 5 days, individual colonies were counted. Representative colonies were identified as *F. oxysporum* f. sp. *vasinfectum* race 4 by morphological characteristics and confirmed by DNA sequencing of part of the elongation factor gene using primers EF1 and EF2 (11). Plant growth was scored by measuring height, fresh weight of the plant above the soil line, and the number of nodes. All measurements were taken within a 2-day period. The experiment was repeated once.

Significant effects of inoculum treatment on height, weight, node, and CFU were tested by analysis of variance (ANOVA). Data collected for the response variable CFU were transformed to the 0.00161 power in order to meet the ANOVA assumption of normally distributed residuals. Logistic regressions were performed on the categorical response variables (i.e., severity of symptoms and vascular discoloration). Logistic regression calculated the probability of a given dichotomous outcome as a function of the independent variables. In the case of symptoms, the dichotomous outcome variable was a score > 1 (any symptoms) versus score = 1 (no symptoms). If higher scores were available, the variable was also calculated as score > 2, or score > 3 versus all other scores. In the case of vascular discoloration, the dichotomous outcome variable was a score > 1 (any vascular discoloration) versus score = 1 (no vascular discoloration). If higher scores were available, the variable was also calculated as score > 2, or score > 3 versus all other scores. Regressions and ANOVAs were performed using SAS statistical software (SAS Institute Inc., Cary, NC).

RESULTS

Because results of the two trials were similar and there was no significant interaction between inoculum density and trial, data from the two trials were combined for the final analysis. A significant interaction between inoculum density and cultivar

occurred for all response variables ($P < 0.0001$); therefore, the main effects of inoculum density were analyzed separately for each cultivar.

Three cultivars (Pima cv. DP340 and Upland cvs. Ph72 and UltEF) behaved similarly in response to inoculum density. Their response trend was intermediate to those of the resistant Pima Ph800 and susceptible Pima DP744. For this reason, results are presented only for cvs. DP744, Ph800, and UltEF.

At inoculum levels of 10^2 conidia/g of potting mix and lower, plants of all five cultivars were asymptomatic or symptoms were rare (Fig. 1). In cvs. DP744 and UltEF, symptom expression was progressively more severe from 10^3 to 10^6 conidia/g of potting mix. Based on symptoms, DP744 was the most susceptible cultivar. Symptom expression in Ph800 was light and erratic (Fig. 1).

The effect of inoculum density on symptom development was determined by logistic regression. For cv. DP744, the probability of developing symptoms ($S > 1$) was plotted against the range of inoculum densities, resulting in a logistic curve where symptoms developed exponentially at lower inoculum levels, then more slowly at higher densities (Fig. 2). The fulcrum of the curve, where probability = 0.5, occurred between 10^2 and 10^3 conidia/g of potting mix. At 10^4 conidia/g of potting mix or higher, the probability reached 1, indicating that almost all DP744 plants exhibited symptoms. The logistic regres-

sion was recalculated to capture the probability of developing symptoms of higher scores ($S > 2$ and $S > 3$). The resulting curves each show a more delayed exponential development of symptoms and fulcrums at higher inocula. For the regression of $S > 2$, the fulcrum fell between 10^3 and 10^4 conidia/g of potting mix and reached a probability of 1 at 10^5 conidia/g of potting mix. For the regression of $S > 3$, the fulcrum occurred between 10^4 and 10^5 conidia/g of potting mix and the maximum probability of 0.8 was plotted at 10^6 conidia/g of potting mix.

The logistic regressions for symptom development in cvs. UltEF and Ph800 were different from that of the susceptible cultivar DP744 (Fig. 2). Both regressions were significant only when the probability of developing symptoms was $S > 1$ and both had less dramatic exponential development of symptoms than DP744. UltEF showed an intermediate response to symptom development with a steady slope, a fulcrum between 10^3 and 10^4 conidia/g of potting mix, and a maximum probability of 0.9 at 10^6 conidia/g of potting mix. The logistic regression of symptom development for Ph800, although significant, was not a typical logistic curve, and reached a maximum probability of 0.5 at 10^6 conidia/g of potting mix.

The effect of inoculum density on vascular discoloration was also determined by logistic regression for each cultivar (Fig. 3). For DP744, the probability of developing vascular discoloration ($V > 1$) was

Fig. 1. Mean symptom responses of cotton cvs. DP744, Ph800, and UltEF to inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* race 4. Plant symptoms were rated on the following scale: 1, no symptoms; 2, chlorosis or wilt restricted to cotyledons or first leaf; 3, similar symptoms extended beyond first leaf; 4, severe symptoms affecting the whole plant; and 5, plant dead.

Fig. 2. Predicted probabilities (P_i) of scoring wilt symptoms (S) for cotton cvs. DP744, Ph800, and UltEF. Plant symptoms were rated on the following scale: 1, no symptoms; 2, chlorosis or wilt restricted to cotyledons or first leaf; 3, similar symptoms extended beyond first leaf; 4, severe symptoms affecting the whole plant; and 5, plant dead. In the legend, $S > 1$ plots the P_i of symptoms at any level above 1; $S > 2$ plots the P_i of symptoms at levels above 2; and $S > 3$ plots the P_i of symptoms at levels above 3. For DP744, $P_i = 1/(1 + e^{8.3890 - 3.044x})$ when $S > 1$, $P_i = 1/(1 + e^{7.5474 - 2.3233x})$ when $S > 2$, and $P_i = 1/(1 + e^{12.2450 - 3.3706x})$ when $S > 3$. For UltEF, $P_i = 1/(1 + e^{3.1153 - 0.9275x})$ when $S > 1$ and $P_i = 1/(1 + e^{6.0426 - 0.12495x})$ when $S > 2$. For Ph800, $P_i = 1/(1 + e^{4.9300 - 0.7923x})$ when $S > 1$. In the equations, x = inoculum density ($\log_{10}$ conidia/g of potting mix).

Fig. 3. Predicted probabilities (P_i) scoring vascular discoloration (V) for cvs. DP744, Ph800, and UltEF. Vascular discoloration was measured from cross and longitudinal sections of the stem at the soil line and rated as follows: 1, no discoloration; 2, light brown, streaky discoloration; 3, continuous dark brown discoloration; and 4, plant dead. In the legend, $V > 1$ plots the P_i of vascular discoloration at any level above 1; $V > 2$ plots the P_i of vascular discoloration at levels above 2; and $V > 3$ plots the P_i of vascular discoloration at levels above 3. For DP744, $P_i = 1/(1 + e^{11.3492 - 3.3076x})$ when $V > 1$, $P_i = 1/(1 + e^{5.4263 - 1.2256x})$ when $V > 2$, and $P_i = 1/(1 + e^{8.389 - 3.044x})$ when $V > 3$. For UltEF, $P_i = 1/(1 + e^{4.6263 - 1.2744x})$ when $V > 1$ and $P_i = 1/(1 + e^{4.889 - 0.8209x})$ when $V > 2$. For Ph800, $P_i = 1/(1 + e^{4.5186 - 0.1784x})$ when $V > 2$. In the equations, x = inoculum density ($\log_{10}$ conidia/g of potting mix).

plotted against the range of tested inoculum densities, resulting in a typical S-shaped logistic curve with a fulcrum between 10^3 and 10^4 conidia/g of potting mix and a probability of 1 at 10^5 conidia/g of potting mix. Two more significant logistic regressions were calculated for DP744, one plotting the probability of developing vascular discoloration above score 2 and another for discoloration above score 3 ($V > 2$ and $V > 3$, respectively). The resulting graphs were each less dramatic than the $V > 1$ regression plot, with fulcrums between 10^4 and 10^5 conidia/g of potting mix and maximum probabilities less than 1 at the highest inoculum density.

For cv. UltEF, two significant logistic regressions were calculated for the probability of developing vascular discoloration ($V > 1$ and $V > 2$; Fig. 3). Like the curve of $V > 1$ for DP744, the curve of $V > 1$ for UltEF had a steady slope and fulcrum between 10^3 and 10^4 conidia/g but, unlike the curve for DP744, the curve did not level off at a probability of 1 until 10^6 conidia/g of potting mix. The UltEF curve of $V > 2$ was more similar to the DP744 curve of $V > 3$, with a fulcrum between 10^4 and 10^5 conidia/g of potting mix and a maximum probability of approximately 0.4 at the highest inoculum density. The probability of vascular discoloration for cv. Ph800 was almost 0 at all inoculum densities; therefore, the logistic regression was not a typical logistic curve (Fig. 3).

The relationship between inoculum density and plant growth responses was also measured. There were significant negative correlations between inoculum density and fresh weight for cv. DP744 (weight [g] = $33.444 - 5.2592[\log_{10}(\text{inocula})]$, $R^2 = 0.4498$, $P < 0.0001$) and UltEF (weight (g) = $21.625 - 0.085[\log_{10}(\text{inocula})]$, $R^2 = 0.087$, $P = 0.028$). In contrast, plant weights of Ph800 were not affected by any inoculum density Ph800 ($R^2 = 0.0001$, $P = 0.94$; Fig. 4A). There was a negative correlation between plant height and inoculum density for DP744 relative to the non-inoculated plants, with height (cm) = $44.60625 - 4.66250(\log_{10}(\text{inocula}))$, $R^2 = 0.32$, and $P = 0.001$ (Fig. 4B), but there was no significant correlation between plant height and inoculum density for Ph800 ($R^2 = 0.02$, $P = 0.34$) and UltEF ($R^2 = 0.01$, $P = 0.48$). There was a significant negative correlation between number of plant nodes and inoculum density for DP744 with node = $6.7857 - 0.3036(\log_{10}(\text{inocula}))$, $R^2 = 0.17$, and $P = 0.002$, but there was no correlation between number of plant nodes and inoculum density for UltEF ($R^2 = 0.0001$, $P = 0.95$) and Ph800 ($R^2 = 0.0005$, $P = 0.87$) (Fig. 4C).

At inoculum levels between 10^3 and 10^6 CFU/g of potting mix, there was a positive correlation between inoculum density and CFU of *F. oxysporum* f. sp. *vasinfectum* race 4 recovered in stem tissue of cvs. DP744

(CFU = $-3100.7 + 1553.9[\log_{10}(\text{inocula})]$, $R^2 = 0.2337$, $P = 0.01$) and UltEF (CFU = $0.078 + 0.041[\log_{10}(\text{inocula})]$, $R^2 = 0.1628$, $P = 0.002$) (Fig. 5). No significant effect of inoculum density on recoverable CFU in Ph800 was observed ($R^2 = 0.004$, $P = 0.69$); however, a few CFU were recovered from stems of plants grown in pots with 10^2 CFU/g of potting mix (Fig. 5).

DISCUSSION

Fusarium wilt of cotton is an inoculum-density-dependent disease. A general threshold for disease development was observed between 10^3 and 10^4 conidia of *F. oxysporum* f. sp. *vasinfectum* race 4 per gram of potting mix for all cotton cultivars except the resistant Pima cv. Ph800. This threshold is similar to the threshold for disease development caused by *F. oxysporum* f. sp. *vasinfectum* race 1 in the presence of nematodes but nearly 10 times lower than that observed for disease development caused by *F. oxysporum* f. sp. *vasinfectum* race 1 in the absence of nematodes (6,7). Symptom and vascular discoloration expression were affected by the susceptibility of cultivars and also associated with the inoculum density.

Plant growth variables, including height, weight, and number of nodes, of the susceptible cv. DP744 were negatively correlated with *F. oxysporum* f. sp. *vasinfectum* inoculum density. The growth of the resistant cv. Ph800 did not respond to inoculum density for any plant growth variable. Plant weight but not plant height or number of nodes of the moderately susceptible cultivar, UltEF, was negatively affected by *F. oxysporum* f. sp. *vasinfectum*, suggesting that plant weight is more sensitive to the pathogen than the other growth variables.

The number of *F. oxysporum* f. sp. *vasinfectum* CFU recovered per gram of stem tissue of cotton plants was greatest in cv. DP744, supporting the contention that this cultivar is a highly susceptible commercial cultivar to *F. oxysporum* f. sp. *vasinfectum* race 4. Very few propagules of *F. oxysporum* f. sp. *vasinfectum* were recovered in the stems of PH800 and there was no correlation between CFU and inoculum density, confirming resistance to the pathogen. A moderate number of propagules were recovered in stems of cv. UltEF, suggesting a moderate level of susceptibility to *F. oxysporum* f. sp. *vasinfectum*.

Although plant growth of the resistant Pima cv. Ph800 was not significantly affected by any soil inoculum density, mild symptoms were observed at inoculum densities of 10^4 conidia/g of soil and higher. In addition, a few *F. oxysporum* f. sp. *vasinfectum* colonies were recovered from symptomatic Ph800 plants twice during the CFU assay from the stems of plants, indicating that inoculum of *F. oxysporum* f. sp. *vasinfectum* race 4 may increase in the field even when Ph800 is

Fig. 4. Effect of inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 on A, plant weight, B, plant height, and C, number of plant nodes of cotton cvs. DP744, Ph800, and UltEF. Vertical bars at each data point are standard errors.

Fig. 5. Effect of inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 on CFU of the fungus per gram of stem tissue from cotton cvs. DP744, Ph800, and UltEF. Vertical bars at each data point are standard errors.

grown. In California, yields of Ph800 have been negatively impacted by *F. oxysporum* f. sp. *vasinfectum* race 4 in commercial fields on several occasions (R. M. Davis, unpublished). In this greenhouse study, however, growth of Ph800 was not affected at inoculum densities as high as 10^6 conidia/g of soil. This may suggest that field soil inoculum levels in certain cases are greater than 10^6 conidia/g of soil or that other factors are influencing the incidence of disease on commercial farms. Indeed, results of this greenhouse study may not reflect field responses of cotton cultivars because field-grown plants are frequently under environmental or biotic stresses not present in the greenhouse. Another inherent difference between the conditions of this greenhouse study and those of the field is the inoculum itself. *F. oxysporum* f. sp. *vasinfectum* overwinters in field soil primarily as chlamydospores (10), but micro- and macroconidia were used in this study to infest the soil. Conidia are not as well suited for long-term survival in the soil as are the thick-walled chlamydospores but may germinate readily in pot

cultures (10). Despite the differences between this experiment and field conditions, disease development in the field likely follows the general trends observed here. Further research is necessary in order to determine the soil inoculum threshold for disease development in the field.

ACKNOWLEDGMENTS

We thank the Cotton Incorporated California State Support Committee for providing partial financial support and K. Perez for technical help.

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