EVALUATION OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM* RACE 4 AS A SEEDLING PATHOGEN AND IN CO-INOCULATION ASSAYS WITH *RHIZOCTONIA SOLANI*

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Abstract

Fusarium oxysporum f. sp. *vasinfectum* (Fov) race 4 is an important wilt pathogen of cotton that has also been known to cause damping off and seedling mortality in infested fields. Fov race 4 is geographically limited, first identified in California in 2001 and confirmed in Texas in 2017. Since the introduction of Fov race 4 in California, it has been a recurring and expanding threat to California's cotton production. It has also been observed in California that more disease often develops when Fov race 4 and *Rhizoctonia solani* are present in the same field, thus suggesting a potential interaction between the two fungi. Therefore, the objectives of this study included (1) collecting isolates of Fov and *R. solani* from commercial and grower fields in California for genotypic and phenotypic evaluations; and (2) evaluating the interaction between Fov race 4 and *R. solani* in co-inoculation assays. Isolates of Fov were genotyped using two sets of Fov race 4 specific primers. Sixteen isolates were phenotypically characterized for their ability to cause seedling infections using a rolled towel assay. The results found genotypic variations between Fov race 4 isolates. Based on the phenotypic evaluation of Fov race 4 isolates, all isolates produced seedling symptoms on cotton. In greenhouse assay the interaction between Fov race 4 and *R. solani* was evaluated. There was a significant difference between fungal treatments ($P < 0.0001$) with more disease development in the co-inoculation treatment, which confirm field observations when both fungi are present in the same field.

Introduction

The fungus *Fusarium oxysporum f.* sp. *vasinfectum* (Fov) is the cause of an important wilt disease in cotton producing regions. In the United States, there are five nominal races of Fov, races 1, 2, 3, 4, and 8, all of which have been confirmed in California (Kim et al., 2005; Cianchetta et al., 2015). Fov race 4 is a particularly virulent wilt pathogen to susceptible cotton cultivars, even in the absence of nematodes. Since 2001, Fov race 4 was geographically limited to the state of California, until its confirmation in Texas in 2017 (Halpern et al., 2017). The severity and symptoms induced by Fov race 4 are dependent on several factors including, susceptibility of cotton cultivar, pathogen genotype, as well as environmental factors and inoculum density (Araujo et al., 2016; Hillocks, 1992; Hutmacher et al., 2005; Ulloa et al., 2006). Susceptible Pima (*Gossypium barbadense* L.) varieties, when infected often develop more severe symptoms than Upland (*G. hirsutum* L.) varieties. Foliar symptoms in Pima develop as marginal chlorosis and necrosis. In Upland varieties, marginal foliar symptoms do not develop and often times no foliar symptoms develop even when vascular symptoms are severe (Hutmacher et al., 2005). Under favorable environmental conditions and when high levels of Fov race 4 inoculum is present in the field, damping off and seedling mortality can occur early in the growing season. Another factor that can impact disease development caused by Fov race 4 is the interaction with other soilborne fungi. It has been observed in California that when Fov race 4 and *Rhizoctonia solani* are present in the same field there seems to be an increase in the development of wilt symptoms caused by Fov race 4 infections (Hutmacher, *personal communication*).

The last Fov survey conducted in California was in 2012 and 2013, and only focused on the identification of Fov race 4 (Cianchetta et al., 2015). The Fov race 4 specific primers (Yang et al., 2006) that were used in the survey, have since been found not to be able to differentiate race 4 from races 3 and 7 (Crutcher et al., 2016). More recently, a new set of primers have been developed and can separate Fov race 4 from other Fov races, based on the Tfo1 transposon insertion in the phosphate: H+ symporter gene unique to some Fov race 4 isolates (Ortiz et al. 2017). Therefore, the objectives of this study were to (1) collect isolates of Fov and *R. solani* from commercial and grower

fields in CA for genotypic and phenotypic evaluations; (2) to evaluate the interaction between Fov race 4 and *R. solani* in co-inoculation assays.

Materials and Methods

Isolate Collection and Identification

Symptomatic plants were collected from seven commercial or grower fields in California during the 2017 and 2018 growing season. Plant tissue was surface sterilized with 10% commercial bleach (5.25% sodium hypochlorite) and plated onto Nash Snyder medium (Nash and Snyder, 1962) or a semi-selective medium for the isolation of *Rhizoctonia* (Gutierrez et al., 1997). Single spore isolations were completed for all Fov isolates and hyphal tip transfers for *R. solani* isolates. Isolates were identified using morphological characteristics. To determine if the Fov isolates were Fov race 4, two sets of race specific primers were used (Ortiz et al. 2017; Yang et al. 2006). DNA for each Fov isolate was extracted using the PrepMan ® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) following manufacturer's instructions. For all assays a 25 µl PCR reaction mix was prepared using 2 µl genomic DNA (20-50 ng), 5 µl 5X Color GoTaq reaction buffer (Promega Corp., Madison, WI), 2.5 µl of 25 Mm $MgCl_2$, (25mM), 1 µl of 2 mm each dNTP (Fermentas/Thermo Fisher Scientific, Inc., Pittsburg, PA), 0.5 µl GoTaq Taq polymerase (Promega Corp.), 2.5 µl of each of a 5 pmol concentration of primers. The thermocycler conditions were as described by Yang et al. (2006) and Ortiz et al. (2017). PCR product was analyzed using gel electrophoresis on a 2% agarose gel containing 1% GelRed (Biotium inc., Fremont, CA).

Pathogenicity Assays

For the evaluation of seedling pathogenicity, 16 isolates of Fov were used in a rolled towel assay as described by Ellis et al., (2011, 2012). All 16 isolates produced the 208 b.p. amplicon using the primers by Yang et al. (2006), while only 12 of the Fov isolates used produced the 583 b.p. amplicon unique to Fov race 4 using the Ortiz et al. (2017) primers. For each isolate, ten seeds of either the moderately resistant Fov race 4 Upland cultivar FM 2334 or seeds of the moderately susceptible Pima cultivar PHY-830 were placed on a moistened germination towel and each seed was inoculated with 100 µl of a 1×10^6 conidial suspension. Non-inoculated seeds were used as checks to ensure seed quality. The inoculated seed was covered with another moistened towel, rolled up, and placed in a 25-liter bucket. Each bucket was covered with a black plastic bag and kept at 25°C for ten days. The experimental design was a randomized complete block, with one towel of each isolate and plant cultivar combination being randomly assigned to one of three buckets. The experiment was repeated for a total of two times. At ten days, seedlings were rated using a disease severity index (DSI) and ordinal rating scale (Ellis et al., 2011). Briefly, DSI is the lesion length divided by the total plant length and multiplied by 100. The ordinal rating scale was from 1-to-5, where: 5 = no germination, complete colonization of the seed; and 1 = germination, healthy seedling with no visible signs of colonization (Ellis et al., 2011). The DSI data was analyzed using the procedure PROC GLIMMIX of SAS (SAS Institute Inc., Cary, NC) and the ordinal data was analyzed using a nonparametric approach as described by Shah and Madden (2004) using PROC MIXED of SAS.

The Fov race 4 isolate TM13 and *R. solani* isolate RS1 were used to examine the potential interaction between these two fungi. Briefly sterilized oat seed was inoculated with isolate TM13 or RS1. Next, 15 ml of a conidial suspension of Fov race 4 isolate TM13 was prepared at 1 x 10^6 conidia/ml and used to inoculate 1 kg of autoclaved oat seed. For *R. solani*, a 10-day old culture was cut into small pieces and mixed with 1 kg of autoclaved oat seed. The Fov and *R. solani* inoculated oat seeds were incubated for 3 weeks and then air dried in a laminar flow hood (Beccera Lopez-Lavalle et al. 2012). Treatments included Fov race 4 alone, *R. solani* alone, Fov race 4 and *R. solani* in co-inoculation, and a non-inoculated control. At planting, a 1:1 potting mixture of peat moss and vermiculite was used and mixed with fungal inoculum at a ratio by volume of 30% fungal inoculum and 70% potting soil. For the co-inoculation mixture, the ratio was 1 part Fov (15%) and 1 part *R. solani* (15%). Three seeds of a variety were planted into 4-inch pots containing 500 ml of the peat moss-vermiculite mixture. Non-inoculated control plants were grown in the potting mix amended with sterile oats. The experiment was conducted in a greenhouse at the University of California Kearney Agricultural Research and Extension Center in Parlier, CA. The experimental design was a randomized complete block design, with replication as the blocking factor. After five weeks, plants were rated for foliar and vascular symptoms using two qualitative scales of 0-5 (0 = no symptoms and 5 = severe symptoms, dead) (Ulloa et al., 2006). Stand counts and plant height were also recorded. There were four replications and the experiment was conducted three times. Data was analyzed using the PROC GLIMMIX and PROC MIXED procedures in SAS.

Results and Discussion

A total of 138 Fov and eight *R. solani* isolates were collected from symptomatic plants from seven commercial or grower fields in the San Joaquin Valley during the 2017 and 2018 growing season. All Fov isolates collected were positive using the primers designed by Yang et al. 2006, producing the 208 bp amplicon. For the Ortiz et al. (2017) primers, 85 isolates produced the 583 bp amplicon unique to Fov race 4, while 53 isolates produced the 396 bp that is produced by other Fov races. For the isolates tested that did not amplify the 583 bp amplicon specific for Fov race 4, this may suggest the presence of another Fov race or Fov race 4 genotype that is contributing to disease loss in California cotton fields.

In the rolled towel assays, all isolates were pathogenic and there was a significant difference among isolate and plant cultivar ($P<0.0001$) for DSI and ordinal ratings. DSI results were similar to those of the ordinal rating scale. Average DSI for isolates ranged from 34.8-43.8% and 55.7-73.9% for the FM-2334 and PHY-830, respectively (Figure 1). Average ordinal rating for isolates ranged from 2.1-3.4 and 3.7-4.3 for the FM-2334 and PHY-830, respectively (Figure 2). Based on our initial results, the rolled towel assay shows promise and should be further evaluated as an early detection method for resistance/tolerance at the seedling stage. If the rolled towel assay is a reliable screening method, this would greatly cut down on the time, space, and work currently necessary to screen cotton germplasm for resistance/tolerance to Fov race 4.

For the co-inoculation assays the data from all three experiments were analyzed together since there was no significant difference among experiment and the interaction among factors ($P = 0.05$). There was no significant difference among the varieties ($P = 0.05$), however there was a significant difference for treatment ($P < 0.0001$) for stand count, height, vascular and foliar symptoms (Table 1). There was also more disease development in the co-inoculation treatment for stand count, height, vascular and foliar symptoms compared to other treatments (Table 1). These results suggest that there is an interaction when these two pathogens are present in the same field, causing a significant increase in disease development. For all of the varieties tested there was a tendency for higher disease development when co-inoculated. These results might suggest that when Fov race 4 resistant cultivars are infected by *R. solani*, the resistance is compromised, thereby increasing Fov race 4 infections.

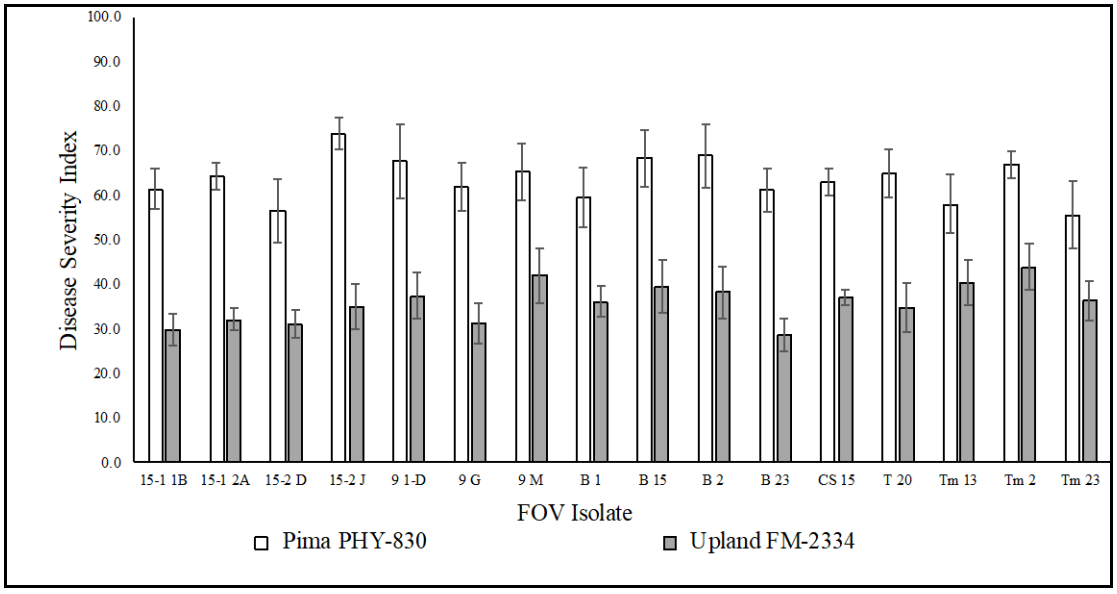

Figure 1. Aggressiveness of 16 Fov isolates as a seedling pathogen towards cotton using Pima cultivar PHY-830 and Upland cultivar FM-2334. The disease severity index is a measurement of the lesion length/total plant length and multiplied by 100.

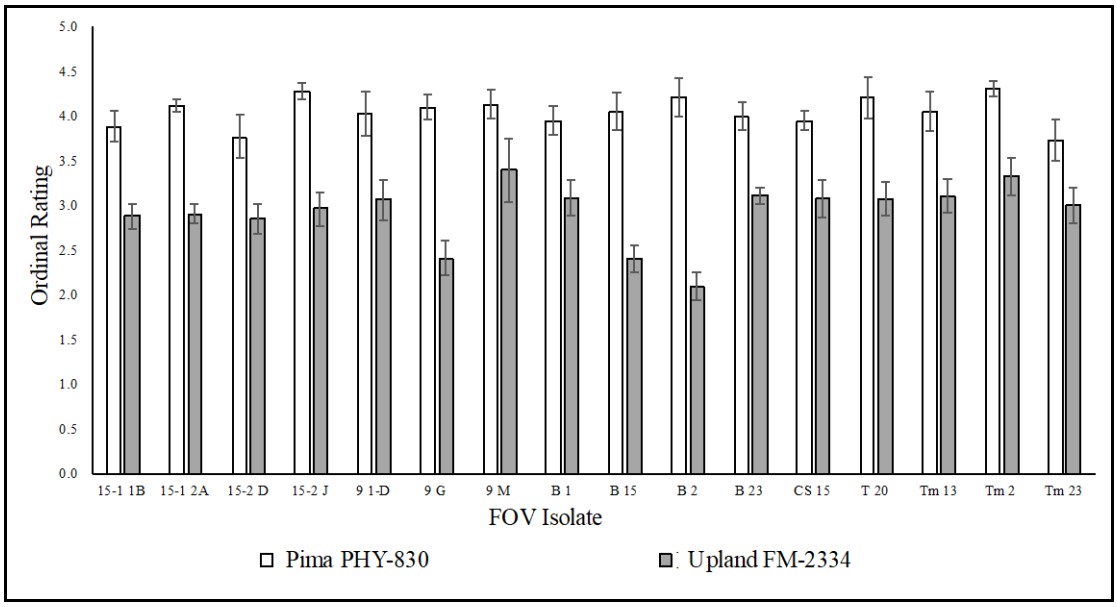

Figure 2. Aggressiveness of 16 Fov isolates as a seedling pathogen towards cotton using Pima cultivar PHY-830 and Upland cultivar FM-2334. The ordinal rating scale was from 1-to-5, where: 5 = no germination, complete colonization of the seed; and 1 = germination, healthy seedling with no visible signs of colonization (Ellis et al., 2011).

Table 1. Combined averages for the five varieties of cotton for the final stand count, height, foliar and vascular ratings when inoculated with Fov, *R. solani*, and in co-inoculations with Fov and *R. solani*.

Treatment	Stand	Height	Foliar Rating	Vascular Rating
Control	2.70a	5.32a	0.16a	0.00a
Fov	2.57a	4.88b	1.57b	2.16b
R. solani	1.90b	4.65b	1.40b	0.00a
Fov + *R. solani*	1.10c	3.78c	3.60c	3.82c

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References

Araujo, D.V., Machado, J.C., Pedrozo, R., Pfenning, L.H., Kawasaki, V.H., Neto, A.M., and J.A. Pizatto. 2016. Transmission and effects of *Fusarium oxysporum f.sp. vasinfectum* on cotton seeds. African Journal of Agricultural Research, 11:1815-1823

Becerra Lopez-Lavalle, L. A., Potter, N., and Brubaker, C. L. 2012. Development of a rapid, accurate glasshouse bioassay for assessing Fusarium wilt disease responses in cultivated *Gossypium* species. Plant Pathol. 61:1112-1120.

Cianchetta, A. N., Allen, T. W., Hutmacher, R. B., Kemerait, R. C., Kirkpatrick, T. L., Lawrence, G. W., Lawrence, K. S., Mueller, J. D., Nichols, R. L., Olsen, M. W., Overstreet, C., Woodward, J. E., and R.M. Davis. 2015. Distribution of *Fusarium oxysporum* f. sp. *vasinfectum* in the United States. Journal of Cotton Science, 19: *328-336.*

Crutcher, F.K., Doan, H.K., Bell, A.A., Davis, R.M., Stipanovic, R.D., Nichols, R.L., and J. Liu. 2016. Evaluation of methods to detect the cotton wilt pathogen *Fusarium oxysporum* f. sp. *vasinfectum* race 4. Eur. J. Plant Pathol. 144:225-230.

Ellis, M.L., Broders, K.D., Paul, P.A., and A.E. Dorrance. 2011. Infection of soybean seed by *Fusarium graminearum* and effect of seed treatments on disease under controlled conditions Plant Dis. 95: 401-407

Ellis, M. L., Cruz, D., Leandro, L. F., and G.P. Munkvold. 2014. Genotypic and phenotypic characterization of fungi in the *Fusarium oxysporum* species complex from soybean roots. Phytopathology 104:1329-1339.

Guttierez, W. A., Shew, H. D., and T.A. Melton. 1997. Sources of inoculum and management for *Rhizoctonia solani* damping-off on tobacco transplants under greenhouse conditions. Plant Dis. 81:604-606.

Halpern, H., Bell, A., Wagner, T., Liu, J., Nichols, R., Olvey, J., Woodward, J., Sanogo, S., Jones, C., Chan, C. and M. Brewer. 2018. First Report of Fusarium Wilt of Cotton Caused by *Fusarium oxysporum* f. sp. *vasinfectum* Race 4 in Texas, U.S.A. Plant Dis. 102:446-446.

Hillocks, R.J. 1992 Fusarium wilt. In: Hillocks RJ (ed) Cotton diseases. CAB International, Wallingford, pp 127-160.

Hutmacher, B., Keeley, M.P., Davis, M.R., Kim, Y., Ulloa, M., Wright, S., Munk, D.S., Vargas, R.N., Roberts, B.A., Marsh, B.H., and R. Percy. 2005. Fusarium in Acala and Pima cotton: Symptoms and disease development. In: Proceedings of Beltwide Cotton Conference. National Cotton Council of America, New Orleans, pp. 245-246.

Kim, Y., Hutmacher, R. B., and R. M. Davis. 2005. Characterization of California isolates of *Fusarium oxysporum f. sp. vasinfectum*. Plant Dis. 89:366-372.

Nash, S. M., and W. C. Snyder. 1962. Quantitative estimations by plate counts of propagules of the bean root rot Fusarium in field soils. Phytopathology 52: 567-572.

Ortiz, C. S., Bell, A. A., Magill, C. W., and J. Liu. 2017. Specific PCR detection of *Fusarium oxysporum* f. sp. *vasinfectum* California race 4 based on a unique *Tfo1* insertion event in the PHO gene. Plant Dis. 101:34-44.

Shah, D. A., and Madden, L. V. 2004. Nonparametric analysis of ordinal data in designed factorial experiments. Phytopathology 94:33-43.

Ulloa, M., Hutmacher, R.B., Davis, R.M., Wright, S.D., Percy, R., and B. Marsh. 2006. Breeding for Fusarium wilt race 4 resistance in cotton under field and greenhouse conditions. J. Cotton Sci. 10:114-127.

Yang, M. E., Davis, R. D., and Hutmacher, R. B. 2006. Fusarium wilt cotton in California: characterization and PCR-based detection of race 4. In: Proceedings of Beltwide Cotton Conference. National Cotton Council of America, Memphis. pp 93-96.