SURVEY OF *FUSARIUM OXYSPORUM* F. SP. *VASINFECTUM* RACE 4 IN ARIZONA COTTON

Yizhou Mao
Jiahuai Hu
UA School of Plant Sciences
Tucson, Arizona

Abstract

Fusarium species are implicated in several diseases of cotton including root rot, the seedling damping off disease complex, wilt, and boll rot. A survey was conducted to identify *Fusarium* species associated with cotton roots and rhizosphere soils in Arizona. Of particular interest was to determine whether *F. oxysporum* f. sp. *vasinfectum* (FOV) race 4 is present in Arizona cotton fields. A hundred and eighty cotton samples were collected from seven counties in Arizona. *Fusarium* isolates were recovered from roots and rhizosphere soils using Komada and PDA medium. A total of 118 isolates of *Fusarium* species were recovered from roots and soils. Seven *Fusarium* species were recovered including *F. brachygibbosum* (4%), *F. culmorum* (1%), *F. equiseti* (16%), *F. fujikuroi* (2%), *F. oxysporum* (20%), *F. proliferatum* (1%), and *F. solani* (56%). *F. solani* was predominant and was more common in the soil (76%) than from cotton roots (24%). The next prevalent *Fusarium* species was *F. oxysporum*, occurring more in cotton roots (54%) than in the soils. Approximately 79% (19 isolates) of *F. oxysporum* were identified as FOV using FOV-specific primers. However, race 4 was not found in this study. Our survey results will be useful for cotton breeding and selection of suitable cotton cultivars for effective management of Fusarium wilt in Arizona.

Introduction

Fusarium species are ubiquitous saprophyte in soil and cause economically important diseases on cotton including damping off, wilt, boll rot, and root rot. *Fusarium oxysporum* and *Fusarium solani* are the most common species complexes in cotton and most well-known for causing root rot symptoms. Other *Fusarium* species have been associated with cotton roots but their relative aggressiveness and impact in cotton production as root pathogens is unclear. Affected plants may exhibit poor root growth or slow emergence. Root symptoms include dark brown lesions, staining in the lower portion of root vascular system, or decay of the entire taproot (Zhang *et al.*, 1996). Fusarium wilt of cotton (*Gossypium hirsutum* L. and *Gossypium barbadense* L.), caused by *Fusarium oxysporum* f. sp. *vasinfectum* W.C. Snyder & H.N. Hans (FOV), is an important disease affecting nearly all cotton growing regions of the world. In the U.S., it is estimated that 0.02% cotton bales are lost to FOV (Lawrence *et al.*, 2014). FOV colonizes the roots and vascular system of susceptible cotton cultivars, causing root and vascular discoloration, wilting and sometimes death of the plant. Symptom expressions vary with pathogen genotype, inoculum density, cotton cultivar, and plant age (Hao *et al.*, 2009). Six nominal races of FOV (i.e.1, 2, 3, 4, 6, and 8) are reported to affect cotton worldwide, of which all races but 6 are found in the U.S. Of these genotypes, FOV race 4 is of most concern to U.S. cotton growers because it is a particularly virulent genotype and causes economic losses independent of the root-knot nematode (*Meloidogyne incognita*) (Davis *et al.*, 1996).

FOV4 poses the greatest threat to U. S. cotton production. It was first detected in California in 2001 (Kim *et al*., 2005) and has spread extensively in the San Joaquin Valley (SJV). Currently, FOV4 has been confirmed in Pima cultivars in El Paso and Hudspeth counties in the Upper Rio Grande Valley (URGV) in Texas (Halpern *et al.*, 2017). Moreover, symptoms resembling FOV4 have been found in adjacent areas of New Mexico as well. FOV4 is more likely to be found in Pima cotton cultivars, particularly following the planting of Hazeera hybrid cultivars. No fungicides or biocontrol products are known effective on FOV4 control. Meanwhile, over a decade-breeding efforts have not produced any cultivars with true resistance.

Arizona has a long history of Pima cotton breeding and production. In 2017, Arizona planted a little over 13,000 acres extra-long staple Pima varieties. Many Pima cultivars are particularly susceptible to FOV race 4 (Kim *et al.*, 2005). Commercial cultivars resistant to race 4 are limited (Hutmacher *et al.*, 2013); Phytogen 800, a Pima cultivar, is the most resistant cultivar to FOV race 4 (Ulloa *et al.*, 2006). Upland cultivars, while affected by FOV race 4, are not as susceptible to the pathogen as are Pima varieties (Ulloa *et al*., 2006). Due to its proximity to California, New Mexico, and URGV, Arizona is vulnerable to the spread of FOV race 4. A previous survey of FOV4 supported by the Arizona Crop Improvement Association, UA Extension, Arizona Cotton Growers Association, and the Arizona Cotton Research and Protection Council was last completed in 2012 and did not find FOV4 in Arizona. However, recent discovery of FOV4 in URGV has raised great concern about its possible presence in Arizona Pima cotton

cultivars. Therefore, a comprehensive survey with in-depth sampling strategy is urgently needed for Arizona cotton industry to adequately address the real threat by FOV4. The goal of this survey project is to provide the industry with an updated assessment of the distribution of *Fusarium* species, in particular FOV4 in Arizona. Specific objectives are to: i) detect the presence of FOV4 in Arizona cotton grower fields, ii) identify the disease causing organism through culturing and molecular methods, and iii) characterize the diversity and frequency of *Fusarium* species associated with cotton roots in Arizona.

Materials and Methods

Sample collection

In collaboration with 14 field crews of Arizona Cotton Protection and Research Council, cotton roots were sampled in 2018 from each of 10-14 arbitrarily selected fields in 7 Arizona counties. Two to three plants with as much of the root system as possible were collected arbitrarily from each field at various growth stages of emergence, squares, and first flower; plants were dug and soil was collected from roots before shipping. Samples were shipped overnight to the University of Arizona Extension Plant Pathology Lab and stored at 4°C until processed. Some fields were sampled only at one growth stage. A total of 180 samples was collected from 90 fields in 7 counties. In each field, sampling date, location (geographic coordinates), row spacing, tillage type, and cotton growth stage were recorded.

Isolation of *Fusarium* species from cotton roots and rhizosphere soil

Roots were rinsed under running tap water for 5 min to remove soil and debris from the field. Fungi were isolated from symptomatic and asymptomatic roots by excising two 2-3-cm long root pieces from each plant, one from the taproot and one from the lateral root tissue. A total of 3,600 root pieces from both symptomatic and asymptomatic roots were surface disinfected in 0.5% NaClO for 1 min, 70% ethanol for 1 min, rinsed 3 times in sterile distilled water, and blotted dry. The surface-sterilized segments were plated separately on both PDA medium and Komada agar medium, which is selective for *Fusarium* (Komada, 1975). Rhizosphere soil was collected from cotton roots and 1 g of rhizosphere soils were diluted in 100 mL of distilled water and shaken vigorously to make soil suspension. A total of 100 µL of soil suspension were inoculated onto Komada's medium and three plates were inoculated for each soil sample. The inoculated plates had been incubated at 25°C for 5 days in darkness, individual *Fusarium* colonies growing from them were transferred to 2% water agar and pure isolates obtained by transferring hyphal tips from these subcultures to PDA.

Identification of *Fusarium* isolates

Four isolates from each sample were arbitrarily selected and cultured for morphological identification. Out of 617 isolates, 118 isolates were identified as a *Fusarium* species based on cultural and morphological characteristics. Each *Fusarium* isolate was maintained on potato dextrose agar (PDA) and cultures were incubated for 7-20 days at room temperature under fluorescent light. Each isolate was examined microscopically and identified to species according to the system of Leslie and Summerell (Leslie and Summerell, 2006). Species were identified based on the most distinctive morphological characteristics. Main morphological characteristics observed were morphology of macroconidia and microconidia, conidial arrangements (singly, false heads and chains), nature of the conidiogenous cell that include formation of monophialides (e.g. *F. oxysporum*) and polyphialides (e.g. *F. proliferatum* and *F. subglutinans*) and length of the conidiogenous cell (e.g. *F. solani* vs *F. oxysporum*); formation and arrangement of chlamydospores (e.g. *F. equiseti*), and colony appearance and pigment formation in PDA (Fig 1). Species identities were confirmed for a subset of 118 isolates by amplification and sequencing of the ITS gene region. The isolates were selected to represent all the distinguishable morphological groups that were observed. All isolates were grown on PDA at room temperature for 7 days. The mycelia were harvested by scraping and placed into a 1.5 ml centrifuge tube, and then each sample was lyophilized by sonication for 3 sec. Genomic DNA was extracted using a modification of the cetyltrimethylammonium bromide (CTAB) procedure (Innis *et al.*, 1990), and DNA was quantified using a spectrophotometer, and then stored at -20°C until processed. Primer pairs used for amplification and sequencing were ITS4 and ITS5 (Table 1) (White *et al.*, 1990). PCR reactions were conducted in 25-µl volumes. Each reaction tube contained approximately 2 µl of a 10-ng/µl DNA template, 2.5 µl of 10× DreamTaq buffer (35mM $MgCl_2$), 17.3 µl of sterile distilled water, 2 µl of 2 mM dNTPs, 0.5 µl each of forward and reverse primers (10µM), and 0.2 µl of DNA Taq polymerase (DreamTaq). The thermal cycling parameters were initial denaturation at 94°C for 3 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min was done at the end of the amplification (White *et al.*, 1990). Negative controls (no DNA template) were used in every experiment to test for the presence of contamination in reagents. PCR products were purified adding 2 µl of ExoSAP-IT reagent

(Affymetrix Inc., Cleveland, Ohio) in 5 µl of PCR reaction. PCR products were sequenced at the BIO5 Core DNA and Sequencing Facility at the University of Arizona. Sequences were submitted for BLAST searches for comparison to known DNA sequences in the NCBI and FUSARIUM-ID (http://isolate.fusariumdb.org/index.php) databases.

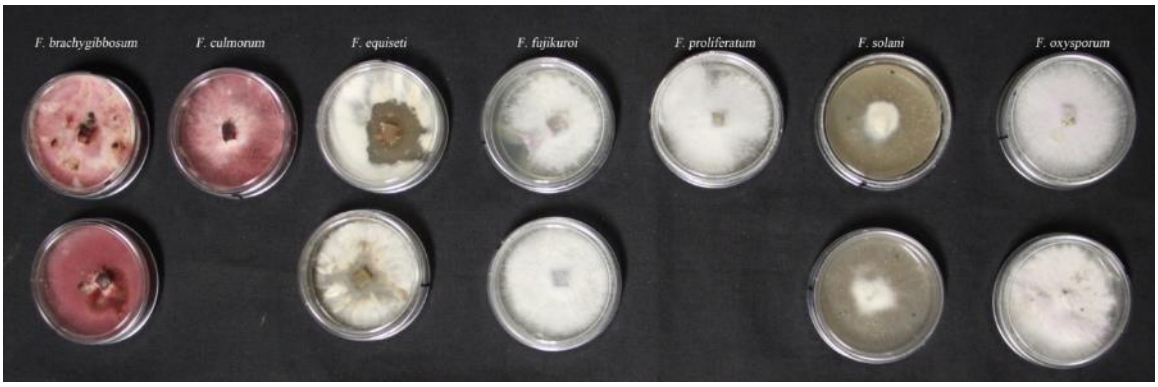

Figure 1. Colony morphology of *Fusarium spp.* isolates on PDA media

PCR detection of *Fusarium oxysporum* f.sp. *vasinfectum* (FOV) and FOV race 4 (FOV4)

FOV specific primers (Fov1-Eg-f & Fov1-Eg-r) (Table 1) were used to determine whether FOV isolates were present in our collection of *F. oxysporum* isolates (Abd-Elsalam *et al.* 2006). Twenty-four *F. oxysporum* isolates were grown on PDA media for a week. Genomic DNA isolation and quantification were performed as described above. PCR reactions were conducted as described above with the following thermocycling parameters: initial denaturation at 94°C for 1 min, followed by 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min was done at the end of the amplification (Abd-Elsalam *et al.* 2006).

FOV4 was detected based on a PCR assay (Table 1) developed by Ortiz *et al* (Ortiz *et al.* 2017). This PCR assay detects and amplifies 583-bp *Tfo1* insertion event in the *PHO* gene. PCR reactions were conducted as described above with the following program: initial denaturation at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 2 min. A final extension at 72°C for 5 min was done at the end of the amplification (Ortiz *et al.* 2017).

g##	*I xvdulxp* #vshflhv		
h	Vht xhqfh	,	Uhihuhqfh
	-WF F WF F J F WWDWWJ DWDWJ F -6Ñ	3 - 3	
	-J J DDJ WDDDDJ WF J WDDF DDJ J -6Ñ		
-Hj -i	-F F DF Wj Wj Dj WDF WF WF F WF j -6Ñ	;	Deg-
-Hj -u	-F F F Dj j F J WDF WWj DDj j DDF -6Ñ		*o*
I r yS-I	-J J F F J DWDWWJ WF J J WF J WD-6Ñ	6	#
I r yW-U	-DWF WJ WF WWWF J WF J J F DDW-6Ñ		

Data Analysis

The relative frequency (F) of *Fusarium* species was computed using the formula F = (n/N)* 100, where n= the number of isolates of each species and N= the total number of isolates of all species. In order to determine differences in frequency of isolation between sample types, analysis of variance was conducted using the general linear model procedure (PROC GLM) of SAS (SAS Institute Inc., Cary, NC). Means were compared using Fisher's protected least significant difference (LSD) at $P = 0.05$.

Results

Typical symptoms of Fusarium root rot were observed in many of the cotton samples. A total of 1,485 isolation plates were established from cotton roots and rhizosphere soils (Fig. 2). Out of 617 isolates obtained from cotton roots and rhizosphere soils, 118 isolates were identified as a *Fusarium* based on cultural and morphological characteristics. Seven species were identified including *F. solani*, *F. oxysporum*, *F. equiseti*, *F. brachygibbosum*, *F. fujikuroi*, *F. proliferatum*, and *F. culmorum* according ITS region sequences. Morphological characteristics varied among isolates belonging to same seven species, particularly for *F. oxysporum* and *F. solani*. Strains belonging to the *F. oxysporum* complex showed relatively large variation in culture morphology on PDA.

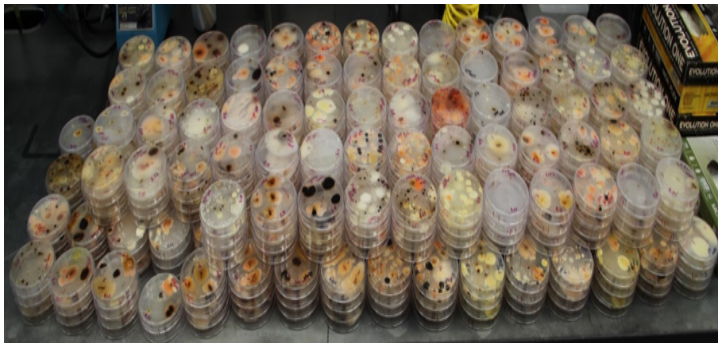

Figure 2. Isolation plates: root tissues were plated on both Komada and PDA media (6 replicate plates), rhizosphere soil were plated on Komada media (3 replicate plates).

Differences in relative frequency of isolation were observed among species. Overall, *F. solani* had the highest frequency of recovery followed by *F. oxysporum*, and *F. equiseti*, each ranging from 16 to 56% of the isolates (Table 2). Other species such as *F. brachygibbosum*, *F. proliferatum* and *F. culmorum* represented 1% to 4% of the isolates. For example, *F. solani* was the most frequently isolated (56%), followed by *F. oxysporum* (20%) (Table 1).

#		*I xvdulxp* #	#	#
I xvdulxp #vs hf lhv				o
l	57			
l		(,	: # ,	
p	8	6# ,	5# ,	
	5	5#	3#	
p	4	4#	3#	
p	4	4#	3#	
	;			

There were not differences in number of species observed between cotton roots and rhizosphere soils. However, significant differences in species frequency were observed between cotton roots and rhizosphere (Table 2). *F. oxysporum* and *F. equiseti* was recovered at a higher frequency at cotton roots (54%, 63%, respectively) compared to rhizosphere soil (46%, 37%, respectively). Conversely, species such as *F. solani* and were more frequently recovered in the rhizosphere soils (76%) than in cotton roots (24%). For other species, such as *F. proliferatum*, there were little differences in frequency of isolation according to sample types (Table 2).

Out of 24 isolates of *F. oxysporum*, 19 isolates were identified as *F. oxysporum* f.sp. *vasinfectum* (FOV) (Fig. 3). FOV race 4 was not detected by race 4-specific primers (Fig. 4).

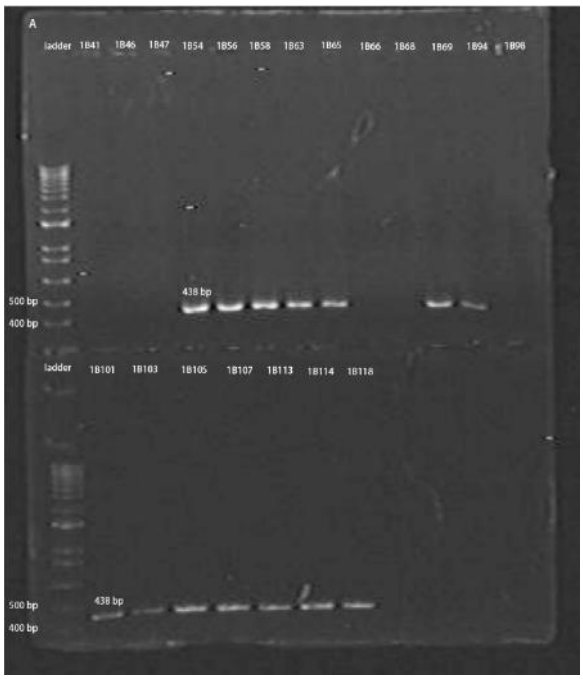

Figure 3. PCR amplification of *Fusarium oxysporum* DNA using FOV-specific primer pairs. PCR products were resolved by 1.5% agarose gel electrophoresis. The first lane: ladder, the other lanes: FOV DNA samples.

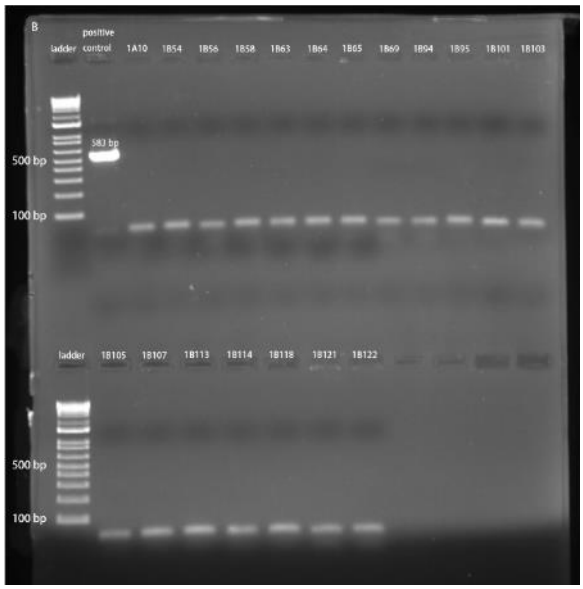

Figure 4. PCR detection of *Fusarium oxysporum* f.sp. *vasinfectum* race 4 using race4-specific primer pairs. PCR products were resolved by 1.5% agarose gel electrophoresis. The first lane: ladder, the second lane: positive control (FOV4 DNA template), and the remaining lanes: FOV DNA samples.

Summary

FOV race 4 was not found in Arizona during 2018 growing season. Using morphological characterization in combination with molecular analysis, we reported 7 *Fusarium* species associated with cotton roots and rhizosphere soils in Arizona cotton fields. Our results confirm that in Arizona soils, Fusarium root rot pathogens are complex and diverse. The new information generated in this study on the frequency of *Fusarium* species associated with cotton roots and soils suggests the need to i) understand the importance of root-infecting *Fusarium* species on cotton productivity in Arizona and ii) test the effectiveness of some management tools, such as seed treatments, to manage Fusarium root rot complex.

Some *Fusarium* species recovered from cotton roots and soils in this study have been reported as causal agents of Fusarium root rot in North America; others have only been reported as species associated with cotton roots and soils but their pathogenicity has not been proven (Zhang *et al.*, 1996). This study provides the first documentation regarding frequency and geographic distribution of Fusarium root rot-related species among the seven Arizona cotton growing areas. Overall, *F. oxysporum* f.sp. *vasinfectum*, *F. solani*, and *F. equiseti* were prevalent and abundant in all seven Arizona Cotton districts, whereas *F. brachygibbosum*, *F. proliferatum* and *F. culmorum*, were less frequently found. It has been noted that climate is a major factor influencing the distribution of *Fusarium* species in soil (Boohan *et al.*, 2003; Saremi *et al.*, 1998). The cool and wet weather pattern in the spring of 2018 might have favored development of damping off in early in the season that caused plant mortality in several Arizona cotton fields. The cool temperatures in 2009 during late spring and early summer may have influenced the abundance and frequency of isolation of some *Fusarium* species. Crop management practices such as row spacing, plant population and tillage are valuable components of integrative disease management. It is unclear that how these crop management practices affect *Fusarium* species and populations in the soils. Previous reports on the effects of tillage practices on *Fusarium* populations showed inconsistent effect on *Fusarium* populations (Swan *et al.*, 2000; Warren and Kommedahl, 1973). This study did not attempt to determine differences in *Fusarium* frequency among crop management practices including tillage and row spacing. Further research may be needed to determine whether there is an effect of tillage practices and row spacing on Fusarium root rot species on cottons and to better understand if root rot disease in cotton could increase under these management practices in Arizona fields.

Acknowledgements

We want to thank Jinggao Liu for providing us FOV4 DNA as positive control in this study. In addition, we thank field crews of Arizona Cotton Research and Protection Council for their cooperation sampling cotton plants around Arizona. This project was funded by the Cotton Incorporated and Arizona Cotton Research and Protection Council.

References

Abd-Elsalam, K.A., Asran-Amal, A., Schnieder, F., Migheli, Q., Verreet, J.-A. 2006. Molecular detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton roots by PCR and real-time PCR assay. Journal of Plant Diseases and Protection 113 (1), S. 14-19.

Davis, R. D., Moore, N. Y., and Kochman, J.K. 1996. Characterisation of a population of *Fusarium oxysporum* f. sp. *vasinfectum* causing wilt of cotton in Australia. Aust. J. Agric. Res. 47:1143-1156.

Halpern, H. C. A. A. Bell, T. A. Wagner, J. Liu, R. L. Nichols, J. Olvey, J. E. Woodward, S. Sanogo, C. A. Jones, C. T. Chan and M. T. Brewer. 201x. First report of Fusarium wilt of cotton caused by *Fusarium oxysporum* f. sp. *vasinfectum* race 4 in Texas, U.S.A. Plant Disease (submitted 7/24/2017).

Hao, J.J., M.E. Yang, and R.M. Davis. 2009. Effect of soil inoculum density of *Fusarium oxysporum* f. sp. *vasinfectum* race 4 on disease development in cotton. Plant Dis. 93:1324-13.

Hutmacher, R.B., M. Ulloa, S.D. Wright, B.T. Campbell, R. Percy, T. Wallace, G. Myers, F. Bourland, D. Weaver, P. Chee, P. Thaxton, J. Zhang, W. Smith, J. Dever, V. Kuraparthy, D. Bowman, D. Jones, and J. Burke. 2013. Elite upland cotton germplasm-pool assessment of Fusarium wilt resistance in California. Agron. J. 105:1635- 1644.

Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., 1990. PCR Protocols: A Guide to Methods and Applications. Academic Press Inc., New York, NY, p. 482.

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

Komada, H. 1975. Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. Review of Plant Protection Research 8: 114-125. (2).

Lawrence, K.S., M.W. Olsen, T.R. Faske, R.B. Hutmacher, J.D. Muller, J. Mario, B. Kemerait, C. Overstreet, G.B. Sciumbato, G.W. Lawrence, S. Atwell, S. Thomas, S. Koenning, R.K. Boman, H.K. Young, J.E. Woodward and H.L. Mehl. 2014. Cotton disease loss estimate committee report – 2014. Poster session presented at: Beltwide Cotton Conferences; 2015 Jan 6-7; San Antonio, TX.

Leslie, J.F. and Summerell, B.A. 2006. The Fusarium Laboratory Manual. Blackwell Publishing, Oxford, UK. 388pp.

Ortiz, C.S., Bell, A.A., Magill, C.W., Liu, J. 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 Disease 101: 34-44.

Saremi, H. Burgees, L.W., Backhouse, D. 1998. Temperature effects on relative abundance of *Fusarium* species in a model plant-soil ecosystem. Soil Biology and Biochemistry 31: 941-94.

Swan, L.J. Backhouse, D., Burges, L.W. 2000. Surface soil moisture and stubble management practice effects on the progress of infection of wheat by *Fusarium pseudograminearum*. Aust. J. Exp. Agric. 40: 693-69.

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

Warren, H.L. and Kommedahl, T. 1973. *Fusarium* species in roots and soil associated with monoculture of soybean in Minnesota. Plant Dis. Rep. 57:912-1914.

White TJ, Bruns TD, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ eds. PCR protocols, a guide to methods and applications. San Diego, California: Academic Press. p315-322.

Zhang, J., Howell, C.R., Starr, J.L., and Wheeler, M.H. 1996. Frequency of isolation and the pathogenicity of *Fusarium* species associated with roots of healthy cotton seedlings. Mycol. Res. 100 (6): 747-752.