The *Journal of Cotton Science 12:165–170 (2008)*
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PLANT PATHOLOGY AND NEMATOLOGY

Development of a DNA-based Macroarray for the Detection and Identification of *Fusarium oxysporum* f. sp. *vasinfectum* in Cotton Tissue

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ABSTRACT

A DNA-based macroarray was developed to quickly and accurately identify all known races and the two Australian biotypes of *Fusarium oxysporum* f. sp. *vasinfectum*, the causal agent of Fusarium wilt of cotton. The macroarray utilized oligonucleotide probes designed from sequences of the elongation factor gene unique to Races 1, 3, 4, 8 or the Australian biotypes. Starting with diseased tissue, the entire detection process from DNA extraction to race identification could be completed within 8 hours. The assay reproducibly identified all *F. oxysporum* f. sp. *vasinfectum* races and biotypes from cultures, and greenhouse- or field-grown infected cotton plants.

Fusarium wilt of cotton, caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans, is a persistent and widespread disease in most cotton-growing areas in the world (Davis et al., 2006, Smith and Snyder, 1975). In California, the disease was first identified in 1960 on upland cotton cultivars (*Gossypium hirsutum* L.) (Garber and Paxman, 1963). Kim et al. (2005) characterized four biotypes, called races, in a population of *F. oxysporum* f. sp. *vasinfectum* (FOV) strains from upland and Pima (*Gossypium barbadense* L.) cultivars in California. Although the use of the term race to describe each of these biotypes is common in the literature, a clear relationship between resistance genes in cotton and individual strains of FOV has not been elucidated. However, several genetic approaches recently applied to large collections of FOV isolates confirmed distinct lineages of most of the biotypes and the term race will be retained here (Bridge et al., 1993, Fernandez et al., 1994, Hering et al., 1999; Kim et al., 2005; Nirenberg et al., 1994; Skovgaard et al., 2001).

Worldwide, eight races plus two unique but closely related biotypes in Australia have been described (Kim et al., 2005). No race designation was made for the Australian biotypes since by the time of their discovery in 1995, the term race for FOV biotypes was deemed invalid (Davis et al., 1996; Davis et al., 2006; Kochman, 1995). Some of the races previously described are apparent synonyms. For example, race 3 and 5 are undoubtedly identical (Nirenberg et al., 1994), as are 4 and 7 (Skovgaard et al., 2001). In addition, races 1, 2, and 6 are so closely related genetically that a new group, called race A for convenience, has been proposed for these three races (Assigbetse et al, 1994).

In California, management strategies vary among the four races of FOV. Race 4, which has caused severe economic losses in the San Joaquin Valley, is managed by resistance available in at least one commercial cultivar (Ulloa et al., 2006) while race 1 is managed by reduction of numbers of root knot nematodes (*Meloidogyne incognita* Kofoid and White, Chitwood) since race 1 causes economic losses only when cotton is infected by both FOV and nematodes (DeVay et al., 1997, Garber et al., 1979). Seed certification also requires the accurate identification of FOV to the level of race; cotton seed from California fields infested with FOV race 4 cannot receive certification. Therefore, it is important to accurately identify individual races of FOV. To that end, we have developed an oligonucleotide-based macroarray for the rapid and accurate detection of all known races or biotypes of FOV.

MATERIALS AND METHODS

Isolates. FOV isolates used in this study included CA-10 (race 1), CA-11 (race 3), CA-14 (race 4), CA-1 (race 8), Aust-16 (one of two biotypes from Australia), and Aust-19 (the other Australian biotype). All isolates were representatives of a larger collection originally isolated from cotton plants in commercial fields in California or Australia (Kim et al., 2005).

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All isolates were fully characterized in the previous study by partial sequences of translational elongation factor (EF-1α), phosphate permase (PHO), and β-tubulin (BT) genes, as well as restriction digests of the intergenic spacer (IGS) region of nuclear r-DNA, and pathogenicity tests. The Australian biotypes were distinguished from one another by a single base difference in the beta-tubulin gene and unique restriction digestion polymorphisms of the intergenic region of nuclear rDNA, but sequences of the EF-1α gene were identical (Kim et al., 2005).

In addition to FOV, non-target *F. oxysporum* strains, including *F. oxysporum* f. sp. *cepae* D12 (originally isolated from onions grown on a commercial farm in California), *F. oxysporum* f. sp. *lycopersici* D24 (from tomatoes in California), *F. oxysporum* f. sp. *melonis* D03 (from cantaloupe in California), and two saprophytic strains of *F. oxysporum*, isolates 1502 and 377, were included in the study.

Oligonucleotide Probe Design and Array Development. Because EF-1α sequences separated all North American races of FOV and the Australian biotypes (Kim et al., 2005), they were used in the current study. Genbank accession numbers of the EF-1α gene sequences used to develop race-specific oligonucleotides are; CA-1 (AY714098), CA-10 (AY714099), CA-11 (AY714100), CA-14 (AY714101), Aust-16 (AY714096), and Aust-19 (AY714097)(Kim et al., 2005). Sequences were aligned with the CLUSTAL X program (Thompson et al., 1997) and the polymorphic sites were visually identified. No polymorphic sites were identified between the two Australian biotypes. Race-specific probes were designed and screened based on two criteria: a length of 17 to 27 nucleotides and an estimated melting temperature (Tm) range of 50 to 60 ˚C. The Tm was estimated using the formula Tm= 64.9 +41 × (y+z-16.4)/(w+x+y+z), where w, x, y, z are the number of the bases A, T, G, C in the sequence, respectively (Howley et al, 1979). Twenty-six probes were generated for the detection of the four FOV races and Australian biotypes (Table 1).

Macroarray development followed the protocol described in Zhang et al. (2007). Briefly, 20 fmol of each of the detector oligomer probes were spotted onto Hybond N+ nylon membranes (Amersham Biosciences, Piscataway NJ) with a 96-pin replicator (model 250520, Nalge Nunc International Corp., Rochester, NY) in quadruplicate. The positive controls included three primers, EF1, EF2, and EF22R (the reverse complement sequence of primer EF22), known to hybridize with DNA of all known *Fusarium* spp. (O'Donnell et al. 1998) (Table 1). Negative controls on the array included the spotting buffer (4 μM sodium carbonate buffer pH 8.4, 3×SSC, 0.01% N-lauroyl sarcosine, and 0.004% bromophenol blue), cotton EF1 oligonucleotide, and cotton EF1R, which were designed based on the cotton (*Gossypium hirsutum* and G. *barbadense*) EF-1α gene sequence (Table 1). Oligomers were spotted at a concentration of 50 μM in spotting buffer. Spotted arrays were air-dried for 10 min and fixed by UV at 240 mJ/cm^2.

The macroarray was tested with DNA extracted from fresh tissue of pure cultures of the individual races and biotypes, DNA extracted from fresh tissue of plants inoculated with conidial suspensions of FOV, and DNA extracted from fresh tissue of plants naturally infected with FOV from commercial fields in California. Cultures of the four known races of FOV were originally obtained from cotton plants in commercial fields in California (Kim et al., 2005), and cultures from Australia were obtained under USDA-APHIS permit no. 53311. Cultures were grown for 6 or 7 days at room temperature on a shaker in 250 ml Erlenmeyer flasks containing 100 ml of sterile medium consisting of (per liter) D-glucose (20.7 g), DL-asparagine (1.2 g), $K_2HPO_4 \cdot 3H_20$ (1.2 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), yeast extract (0.5 g), and NaCl (0.1 g). After the mycelium was harvested by filtration through Miracloth (Calbiochem; San Diego, CA), DNA was extracted from cultures using DNeasy Plant Mini kits, (Qiagen Inc; Valencia, CA) according to the manufacturer's protocol.

In the greenhouse, 2-week-old seedlings of Phytogen-72, an upland cultivar, and DP-744, a Pima cultivar, were inoculated with races 1, 3, 4, or 8 at the one-true-leaf stage of growth by submerging their roots in spore suspensions of 1 x 10^5 conidia per ml for 2 min. The spore suspensions were made by flooding colonized cultures on potato-dextrose agar with water and scraping off the spores and filtering the spore suspension through four layers of cheesecloth. Due to strict quarantine regulations, pathogenicity tests with the Australian biotypes could not be conducted. After 4 weeks in a greenhouse, foliar symptoms (interveinal chlorosis and necrosis and leaf wilting) were visible on inoculated plants. After the lower parts of stems were cut into small pieces about 1 cm long, DNA was extracted using MoBio Ultraclean Soil DNA Isolation kit, (MoBio Laboratories, Inc; Carlsbad, CA) following the manufacturer's instructions. Stems of symptomatic plants from three commercial fields in Fresno County, California, were also included in the macroarray tests.

Table 1. Sequence, target race, array detection results, and the position of the oligonucleotide probes on the *Fusarium oxysporum* f. sp. *vasinfectum* macroarray.

Position	Probe Name	Target race	Array Detection [z]	Sequence (5'-3')
B2	Fov1348_6_EF80	1,3,4,8	1,3,4,8	GGTATTTCTCAAAGTCAACATACT
C2	Fov148_1_EF30	1,4,8	Au*,1,3*,4,8	CGACAATGAGCATATCTGCCATCG
D2	Fov148_2_EF30	1,4,8	1,4,8	CGACAATGAGCATATCTGCCAT
E2	Fov148_2_EF30R	1,4,8	1,4,8	ATGGCAGATATGCTCATTGTCG
F2	Fov138_10_EF370	1,3,8	1,3,8	CCATTCTCACAACCTCAATGAG
G2	Fov138_10_EF370R	1,3,8	1,3,8	CTCATTGAGGTTGTGAGAATGG
H2	Fov348_9_EF340	3,4,8	3,4,8	ACTTGAGCGACGGGAGCGTTTG
B3	Fov1_7_EF340	1	1	ACTTGAGCGAAGGGAGCGTTTG
C3	Fov1_6_EF340R	1	1	ACTTGAGCGAAGGGAGCGTTTG
D3	Fov1_7_EF400R	1	1	TTAGTGACTGCTTCACACGTGACG
E3	Fov3_19_EF60	3	3	AAGACCTGGTGGGGTATTTCTC
F3	Fov3_19_EF60R	3	3	GAGAAATACCCCACCAGGTCTT
G3	Fov3_17_EF60	3	3 (weak)	AAGACCTGGTGGGGTATTTCT
H3	Fov3_17_EF60R	3	3 (weak)	AGAAATACCCCACCAGGTCTT
B4	Fov3_18_EF60	3	negative	AGACCTGGTGGGGTATTTCT
C4	Fov3_18_EF60R	3	negative	AGAAATACCCCACCAGGTCT
D4	Fov4_11_EF370	4	4	CCATTCTCAGAACCTCAATGAGT
E4	Fov4_12_EF370R	4	4	CACTCATTGAGGTTCTGAGAATGG
F4	Fov8_14_EF410	8	8	GTCACTAACCACTCAACAATAGGA
G4	Fov8_14_EF410R	8	8	TCCTATTGTTGAGTGGTTAGTGAC
H4	Fov8_13_EF410	8	8	GTCACTAACCACTCAACAATAGG
B5	FovAu_3_EF80	Au	Au	GGTACTTCTCAAAGGCAACATACT
C5	FovAu_3_EF80R	Au	Au	AGTATGTTGCCTTTGAGAAGTACC
D5	FovAu_4_EF80	Au	Au	GTACTTCTCAAAGGCAACATACT
E5	FovAu_5_EF340	Au	Au	ACTTGAGCGACGGGGCGCGTT
F5	FovAu_9_EF340R	Au	Au	AACGCGCCCCGTCGCTCAAGT
A1,A2,A3	EF1	*Fusarium* spp.	1,3,4,8,Au	ATGGGTAAGGAA/GGACAAGAC
B1,C1,D1	EF2	*Fusarium* spp.	1,3,4,8,Au	GGAG/AGTACCAGTG/CATCATGTT
E1,F1	EF22R	*Fusarium* spp.	1,3,4,8,Au	GAGCTCGGTAAGGGTTCCT
A4,A5	CottonEF1	*Gossypium hirsutum*	negative	AGACCCTCTGACAAGCCCCT
G1,H1	CottonEF1R	*Gossypium hirsutum*	negative	AGGGGCTTGTCAGAGGGTCT
G5,H5	Buffer	none	negative	

[z] An * indicates cross hybridization.

To confirm that the plants from the greenhouse or field were infected with FOV, stems were washed in soapy water, surface sterilized in 0.525 % sodium hypochlorite for 2 minute, and plated onto acidified potato dextrose agar. Seven days after hyphal tips were transferred to the liquid medium described above, DNA was extracted using DNeasy Plant Mini kits, (Qiagen Inc) according to the manufacturer's protocol. The identity of the races was confirmed by sequencing the 654 bp fragment of the translation elongation factor-1α gene following amplification with primers EF1 and EF2 as described by Kim et al., 2005.

Hybridization. The 654 bp fragment of the EF gene of the extracted DNA was amplified with primers EF1 and EF2 (O'Donnell et al., 1998) in 50 µl reactions containing 1 × ThermoPol reaction buffer (New England BioLabs; Ipswich, MA), 1mM dNTPs,

0.2 μM each primer, 1 unit *Taq* DNA polymerase (New England BioLabs), and 15 ng of genomic DNA. The following PCR cycling conditions were used: 95 ˚C for 5 min., 35 cycles of 95 ˚C 1 min., 56 ˚C 1 min., and 72 ˚C 1 min., then followed by 10 min. at 72 ˚C. PCR products were purified according to the manufacturer's protocol using the QIAquick PCR Purification Kit (Qiagen Inc). Negative controls (no template DNA) were included with each assay. Amplicons were visualized on a 1.5% agarose gel by staining with ethidium bromide.

Amplicons were labeled and hybridized using Gene Images AlkPhos Direct Labeling and Detection System with CDP-*Star* (GE Healthcare; Buckinghamshire, UK) following the manufacture's protocol. The experimental conditions were as follows: prehybridization at 55 ˚C for 15 min, hybridization at 55 ˚C for 2 h, two primary washes at 55 ˚C for 10 min, two secondary washes at room temperature (22 ˚C) 5 min, followed by 30 min film exposure after one hour's reaction with the detection reagent. Solutions and protocol details followed the manufacturer's instructions (GE Healthcare). Chemiluminescence was detected with Kodak Biomax Light film. Each assay of DNA from culture, plant tissue from the greenhouse, and plant tissue from commercial fields was conducted twice.

RESULTS AND DISCUSSION

The hybridization between the FOV DNA array and the FOV isolates produced five different patterns, corresponding to the Australia biotypes, Race 1, Race 3, Race 4, and Race 8 (Fig. 1). These results were highly reproducible. The array was able to discriminate between all four FOV races and the Australian isolates in culture and all available FOV from cotton tissue, including all four of the races in colonized plants in the greenhouse and the two races (1 and 4) in naturally infected field plants. Each race produced a unique hybridization pattern that distinguished it from other races (Fig.1). The two Australian biotypes had identical EF-1α sequences and therefore could not be differentiated with this array. When races 1, 3, 4, and 8 were combined and used in a hybridization of a single array, all race-specific hybridization patterns were observed.

As expected, the positive control probes (EF1, EF2, and EF22R) hybridized with all isolates, while the negative controls (cotton EF1, cotton EF1R, and buffer) did not hybridize. Fifteen of the 26 FOV-specific oligomers were single-race specific and hybridized exclusively with one race. Each race had 2 to 5 specific probes (Fig. 1). Four oligomers (Fov3_17_EF60, Fov3_17_EF60R, Fov3_18_EF60, and Fov3_18_EF60R) did not hybridize or the signals were very weak. The remaining seven oligomers were designed as multi-race specific and hybridized with three or more of the races. Cross hybridization occurred in only one of the 26 FOV-specific oligomers. This was probe Fov148_1_EF30, which hybridized with its target isolates (races 1, 4, and 8), as well as the Australian and race 3 isolates.

Of the five nontarget *Fusarium* spp. tested against the FOV array, the two saprophytic *F. oxysporum* isolates did not hybridize with any of the FOV-specific probes. DNA of *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *lycopersici* hybridized to all the FOV race 3-specific oligomers, while

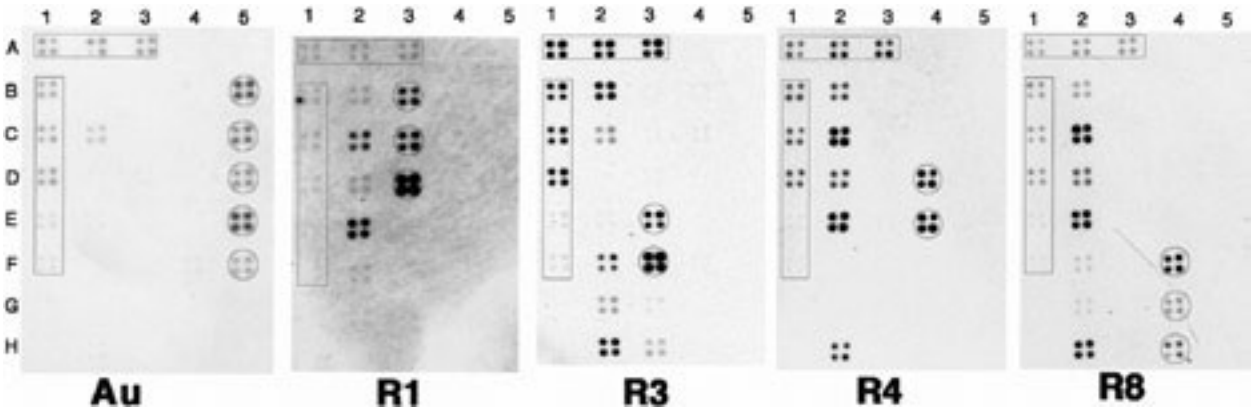

Figure 1. Macroarray differentiating Australian biotypes (Au), Race 1 (R1), Race 3 (R3), Race 4 (R4), and Race 8 (R8) isolates of *Fusarium oxysporum* f. sp. *vasinfectum*. Positive controls are in rectangles and single-race specific probes are in circles. Each oligonucleotide probe is spotted 4 times on the array.

F. oxysporum f. sp. *melonis* hybridized with all the FOV race 4-specific probes. Upon comparison of the EF sequences of these species, we found that EF-1α sequences of *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *lycopersici* were identical to FOV race 3 at the locations where race-specific probes were designed, and that *F. oxysporum* f. sp. *melonis* has an EF-1α sequence identical to that of FOV race 4. Because none of the non-target formae speciales were able to infect cotton upon greenhouse inoculation (R. M. Davis, unpublished), and cotton has never been reported as a host for any of these strains, we are confident that this macroarray provides a useful tool for race identification of FOV. In addition, each of the race-specific probes was checked against all sequences in GenBank, and while several sequences were identical to other *F. oxysporum* formae speciales (as described above), none of the probes were identical to any other pathogen of cotton, to any saprophytic fungi, or to any other organisms that may be found in or on a cotton plant.

This study identified 25 probes in the EF region of the genome of *Fusarium* to identify FOV to the level of race. This region has the added advantage of utilizing a gene region commonly used in *Fusarium* phylogenetic study and taxonomy. Use of the macroarray technology will assist cotton growers by providing a rapid (the entire assay was completed in about 8 hours) and accurate means to identify FOV to the level of race. Because race 4 and the Australian biotypes, unlike the other known races, are highly virulent on certain cultivars of cotton, the rapid identification of specific races is needed for management of the disease. For example, avoidance of cotton altogether in infested fields, long rotation out of cotton, or the use of resistant cultivars are all options if the virulent races are detected. Furthermore, the macroarray aids in the certification of cotton seed fields since the detection of race 4, but not the more common race 1, in a seed field prevents certification in California.

ACKNOWLEDGEMENTS

This project was supported by the National Research Initiative of the United States Department of Agriculture (USDA) Cooperative State Research, Education, and Extension Service, grant number 2004-35605-14325 and Cotton Incorporated grant number 02-238CA.

REFERENCES

Assigbetse, K.B., D. Fernandez, M.P. Dubois, and J.-P. Geiger. 1994. Differentiation of *Fusarium oxysporum* f. sp. *vasinfectum* races on cotton by random amplified polymorphic DNA (RAPD) analysis. Phytopathology 84:622-626.

Bridge, P.D., M.A. Ismail, and M.A. Rutherford. 1993. An assessment of aesculin hydrolysis, vegetative compatibility and DNA polymorphism as criteria for characterizing pathogenic races within *Fusarium oxysporum* f. sp. *vasinfectum*. Plant Pathol. 42: 264-269.

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

Davis, R.M., P.D. Colyer, C.S. Rothrock, and J.K. Kochman. 2006. Fusarium wilt of cotton: Population diversity and implications for management. Plant Dis.90: 692-703.

DeVay, J.E., A.P. Gutierrez, G.S. Pullman, R.J. Wakeman, R.H. Garber, D.P. Jeffers, S.N. Smith, P.B. Goodell, and P.A. Roberts. 1997. Inoculum densities of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* in relation to the development of Fusarium wilt and the phenology of cotton plants (*Gossypium hirsutum*). Phytopathology 87:341-346.

Fernandez, D., K. Assigbetse, M.-P. Dubois, and J.-P. Geiger. 1994. Molecular characterization of races and vegetative compatibility groups in *Fusarium oxysporum* f. sp. *vasinfectum*. Appl. Environ. Microbiol. 60:4039-4046.

Garber, R.H., E.C. Jorgenson, S. Smith, and A.H. Hyer. 1979. Interaction of population levels of *Fusarium oxysporum* f. sp. *vasinfectum* and *Meloidogyne incognita* on cotton. J. Nematol. 11:133-137.

Garber, R. H., and G.A. Paxman. 1963. Fusarium wilt of cotton in California. Plant Dis. Rep. 47:398-400.

Hering, O., H.I. Nirenberg, S. Koehn, and G. Deml. 1999. Characterization of isolates of *Fusarium oxysporum* Schlecht. f. sp. *vasinfectum* (Atk.) Snyd. & Hans., races 1-6, by cellular fatty acid analysis. J. Phytopathol. 147:509-514.

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

Kochman, J. K. 1995. Fusarium wilt in cotton, a new record in Australia. Autralasian Plant Pathol. 24:74.

Nirenberg, H.I., G. Ibrahim, and S.H. Michail. 1994. Race identity of three isolates of *Fusarium oxysporum* Schlect. f. sp. *vasinfectum* (Atk.) Snyd. & Hans. from Egypt and the Sudan. Z. Pflanzenkr. Pflanzenschutz 101:594-597.

O'Donnell, K., H.C. Kistler, E. Cigelnik, and R.C. Ploetz. 1998. Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. Proc. Natl. Acad. Sci. USA, 95: 2044-2049.

Skovgaard, K., H.I. Nirenberg, K. O'Donnell, and S. Rosendahl. 2001. Evolution of *Fusarium oxysporum* f. sp. *vasinfectum* races inferred from multigene genealogies. Phytopathology 91:1231-1237.

Smith, S. N., and W.C. Snyder. 1975. Persistence of *Fusarium oxysporum* f. sp. *vasinfectum* in fields in the absence of cotton. Phytopathology 65:190-196.

Thompson, J. D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins, 1997. The Clustal X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids. Res. 24:4876-4882.

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

Zhang, N., D.M. Geiser, and C.D. Smart. 2007. Macroarray detection of solanaceous plant pathogens in the *Fusarium solani* species complex. Plant Dis. 91:1612-1620.