ISOLATION AND MOLECULAR IDENTIFICATION OF *FUSARIUM OXYSPORUM* ISOLATES FROM COTTON CROPS IN ALABAMA

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Abstract

Cotton lines submitted to the Fusarium Wilt / Root knot nematode evaluation were examined to determine their response to both pathogens the root-knot nematode (*Meloidogyne incognita* race 3*)* and *Fusarium oxysporium* f. sp *vasinfectum* (FOV). Plants with wilt symptoms were collected and the fungi isolated were cultured on Potato Dextrose Agar (PDA). DNA was extracted to do a PCR amplification, using two sets of primers ITS1 and ITS4 that amplify internal transcribed spacer (ITS) regions 1 and 2, including the 5.8 rRNA, and FOV1 and FOV2 amplify a sequence within the ITS regions. The fragment amplified by the ITS1 and ITS4 primers was in average 540 bp, and identify 91% of the samples as *Fusarium oxysporum* f. sp. *vasinfectum* (98% similar to FOV strain from China) and 9% of the samples as *F. oxysporum.* All isolates of FOV had identical ITS1 region and 5.8S rRNA gene, but there are variations in bases in the ITS2 region forming two different groups of isolates. Primers FOV1 and FOV2 did not amplified because the sequences within the ITS1 and ITS2 are different from the one of the primer. Sequences in other genes need to be studied to identify the race of FOV present in Alabama.

Introduction

***Fusarium oxysporum* f. sp. *vasinfectum* (FOV) was first reported by Atkinson in 1892 in cotton in Alabama causing wilting disease (**Colyer, 2001) **This disease is widespread in most of the cotton-growing areas in the world. FOV produced chlamydospores that allow the fungus survive for several years in plant debris or soil. It invades the plant through the roots, and infection is increased by the wounds caused by root-knot nematode (*Meloidogyne* spp.) (Davis et al., 2006;** Colyer, 2001; **Moricca et al., 1998). Later the vascular system is invaded and consequently wilting symptoms appear. Symptoms of Fusarium wilt can appear at any stage of the plant development, depending on temperature, host susceptibility, and inoculums density (**
. At the present, FOV cause 1%, 0.7%, and 0.5% yield loss in Louisiana, Texas, and Alabama, respectively (Blasingame et al., 2008).

Initially, differential hosts were established to separate six physiological races. Race 1, is considered widely distributed in cotton in Africa, Asia, Europe, North and South America, and is pathogenic on upland (*Gossypium hirsutum*) and Pima (*G. barbadense*) cottons. Race 2, has been isolated in North Carolina and is different from race 1 because it also infects tobacco cv. Gold Dollar, and soybean cv. Yelredo. Race 3, was originally found in Egypt as pathogen in upland and Pima cottons. Race 4, was first identified in India as pathogen on *G. arboreum* and *G. herbaceum.* Race 5, isolated in Africa, is similar to race 3 but is pathogenic on *G. barbadense* cv. Ashmouni, a cultivar that is resistant to race 3. Race 6, is present in Brazil and Paraguay. The hosts alfalfa and okra are susceptible to races 7 and 8 which have been identified in China (Davis et al., 2006; . Additional to the races there two Australian biotypes that have a similar reactions to cotton hosts as races 1, 2 and 6; however, genetic analyses indicate that they are different form races 1, 2 and 6.

The objective of this project is to isolate and identify the wilting agent present in our field by PCR using the primers universal primers ITS1 and ITS4, and the specific primer FOV1, FOV2, for future race identification.

Materials and Methods

Cotton plants of 41 different breeding lines with wilt symptoms were collected from the Plant Breeding Unit of the E. V. Smith Research and Extension Center near Tallassee, AL. Fungi were isolated from the hypotocol and upper tap roots of symptomatic cotton plants and grown on Acidified Potato Dextrose Agar (APDA) at 27°C for 7 days. Fungal mycelia were harvested, placed in a mortar, frozen with liquid nitrogen and transferred to an eppendorf tube without letting the sample thaw. DNA was extracted using the Dneasy Plant Mini Kit® (Quiagen Inc.). The internal transcribed spacer (ITS) regions 1 and 2, including the 5.8 rRNA, were amplified in 50 µL reactions on a Multigene Labnet thermocycler. The primers used were ITS1, ITS4, FOV1 and FOV2 (Table 1). For the PCR reaction for the ITS1 and ITS4 the thermal cycles were initial denaturation: 3 min at 95°C, annealing: 35 cycles of 1 min at 95°C, extension: 40 s at 54°C, denatured: 40 s at 72°C, and final extension: 10 min at 72°C. For the FOV1 and FOV2 were 30 cycles, each consisting of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C.After PCR amplification, the products were purified with QIAquick columns (Quiagen Inc.) according to manufacturer's instructions. The resulting amplified products were sequenced at the Lucigen Genomics facility. Sequence analyses were edited using Chromas Lite 2.01 software (www.technelsyum.com.au). Alignments of the sequences were done in Mega 4.1 software (Tamura *et al.,* 2007), and then were subjected to blast analysis in National Center for Biotechnology Information **(**NCBI**).** Phylogenetic bootstrap tests were performed using maximum parsimony algorithm in Mega 4.1 software (Tamura *et al.,* 2007). In the phylogenetic bootstrap test sequences were compared to an FOV isolate from Angola and *F. sambucinum* was used as an outgroup.

Table 1. DNA sequences of the primer used, size, cycles and temperatures.

Primer	Sequence	Size	Cycles	Temperatures	Reference
ITS 1	5'-TCCGTAGGTGAACCTGCGG-3'			1 min at 95˚C	
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	545 bp	35	40 sec at 54˚C	White *et al.*,1998
				10 min at 72˚C	
FOV1	5'-CCCCTGTGAACATACCTTACT-3'			1 min at 94˚C	
FOV2	5'-ACCAGTAACGAGGGTTTTACT-3'	400 bp	30	1 min at 50˚C	Moricca *et al.*, 1998
				3 min at 72˚C	

Results and Discussion

The fragment amplified by the ITS1 and ITS4 primers was in average 560 bp. Conversely, primers FOV1 and FOV2 did not amplify any of the isolates. The ITS1 and ITS4 primers identify in 95% of the samples as *Fusarium oxysporum* f. sp. *vasinfectum* 98% similar to FOV strain Anyang city from China (NCBI accession number EU849584.1), and 5% of the samples as *F. oxysporum* 99% similar to *F. oxysporum* (NCBI accession number GQ922564.1) (Table 2). All isolates of *Fusarium* spp. had identical ITS1 region of 172 bp. They are very similar to the sequences of FOV from Angola, but they do not have a C in the ITS1 region at nucleotide 47. The sequence of the 5.8S rRNA gene is 100% homologous to all the FOV isolates including the isolate from Angola. The ITS2 region of the isolates shows differences in sequences.

The phylograms built based on the ITS and 5.8S rRNA show two different group of isolates of FOV. One group conformed by the FOV isolated from the cotton lines FB-7, TL-6, PHY-FB3, PHY-FB5, DF-5, MB-3, MS-6, FB-3, DJ-7, CW-2, CW-3, PHY-MM4, NC4, PHY-MM7, CW-4, PHY-MM2, FS-1, PHY-MM5, DF-4, DF-6, DF-8, and LM-8. The other group was composed by the FOV isolates from DF-8, LM-8, LM-6, FB-4, CW-7, FB-8, LM-3, AU-3202, CW-5, LM-7, Rowden, DF-2, PHY-MM6, LM-1, CW-8, M-315, LM-5, LM-2, Lonren, and PHY-FB6. The isolate from FOV from Angola formed a different clade from the rest of the FOV (Figure 1).

Primers FOV1 and FOV2 did not amplified because the sequences within the ITS1 and ITS2 are different from the one of the primers. The next step with the DNA isolated from the breeding lines is necessary to determine the race(s) present in Alabama, therefore is necessary to evaluate the primers Fov1-Eg-f and Fov1-Eg-r that amplify 16S and 23S rRNA and are specific for race 3 present in Egypt (Abd-Elsalam, 2006). Also, further studies need to be

conducted using the sequences of the genes Elongation factor (primers EF-1 and EF-2), Beta-tubulin (primers BT3 and BT5), Phosphate permease (primers PHO1 and PHO6), and the Intergenic spacer region IGS of nuclear rDNA (primers LR12R and CNS1) to establish to which of the lineages proposed by Kim *et al.,* (2005) belong the strains of FOV of Alabama. Other techniques can be necessary to identify the race such as DNA macroarray developed by Gilbert *et al.*, 2008 to confirm the presence of the races 1, 3, 8, and the Australian biotypes. The Alabama FOV population needs to be studied in more detail; recently there have been reported new genotypes of FOV in the southeastern United States (Holmes et al., 2009).

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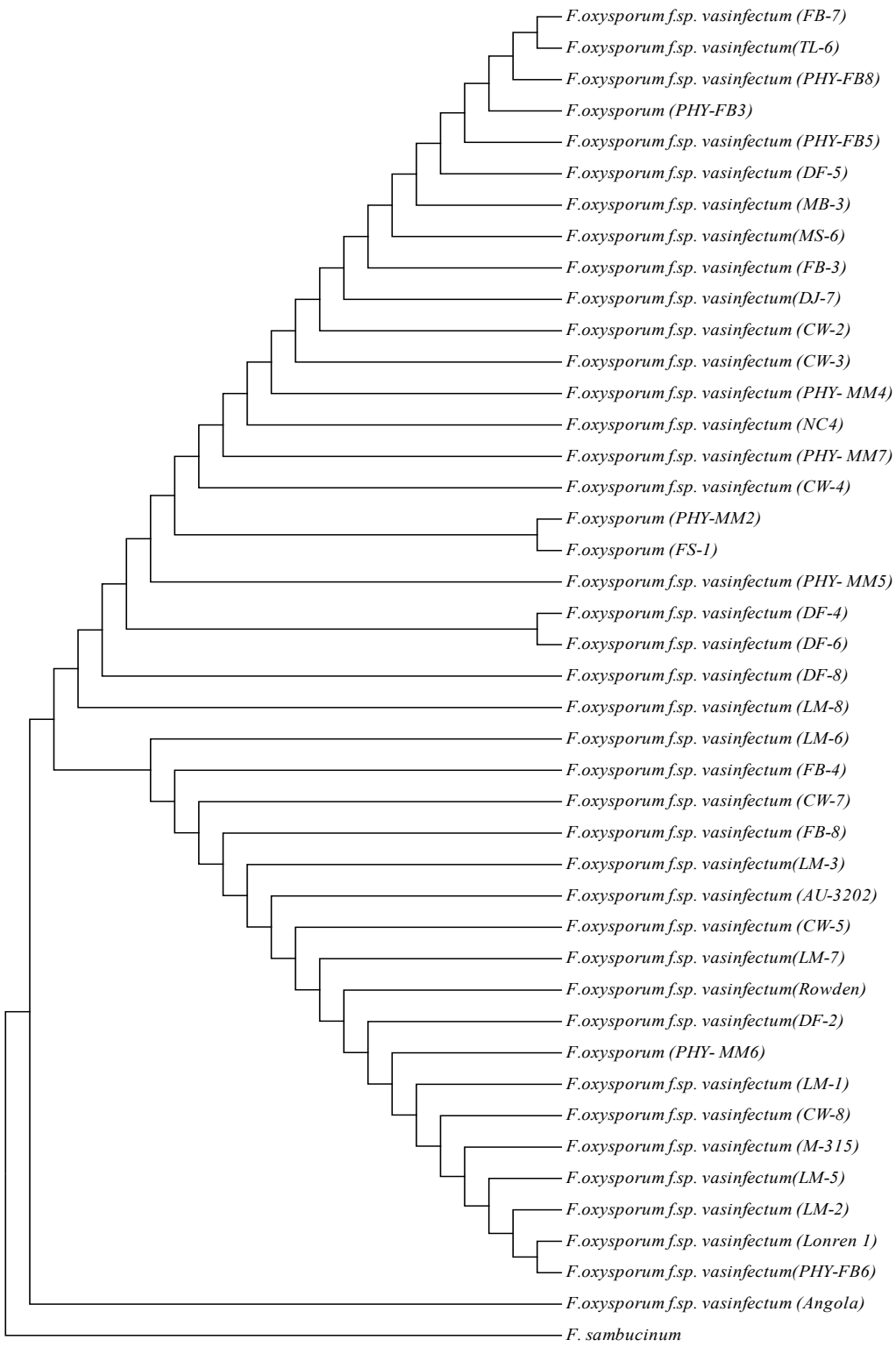

Figure 1. Phylogenetic bootstrap of Maximum parsimony of the sequences of the ITS regions and 5.8 rRNA of the FOV isolates from the PBU of the EVSREC in Alabama.

Table 2. *Fusarium* oxysporum f. sp. v*asinfectum* from cotton lines at the PBU of the EVSREC in Alabama.

Sample	Breeding line	Location	Fragment size (bp)	Fungi	Similarity (%)
1	AU 3202		563	*Fusarium oxysporum* f. sp. *vasinfectum*	99
2	CW-2		546	*Fusarium oxysporum* f. sp. *vasinfectum*	100
3	CW-3		527	*Fusarium oxysporum* f. sp. *vasinfectum*	99
4	CW-4		523	*Fusarium oxysporum* f. sp. *vasinfectum*	99
5	CW-5		532	*Fusarium oxysporum* f. sp. *vasinfectum*	98
6	CW-7		624	*Fusarium oxysporum* f. sp. *vasinfectum*	99
7	CW-8		540	*Fusarium oxysporum* f. sp. *vasinfectum*	99
8	DF-2		529	*Fusarium oxysporum* f. sp. *vasinfectum*	98
9	DF-4		563	*Fusarium oxysporum* f. sp. *vasinfectum*	99
10	DF-5		525	*Fusarium oxysporum* f. sp. *vasinfectum*	98
11	DF-6		525	*Fusarium oxysporum* f. sp. *vasinfectum*	98
12	DF-8		580	*Fusarium oxysporum* f. sp. *vasinfectum*	99
13	FB-3		583	*Fusarium oxysporum* f. sp. *vasinfectum*	99
14	FB-4		592	*Fusarium oxysporum* f. sp. *vasinfectum*	99
15	FB-7		556	*Fusarium oxysporum* f. sp. *vasinfectum*	98
16	FB-8		532	*Fusarium oxysporum* f. sp. *vasinfectum*	99
17	DJ-7		546	*Fusarium oxysporum* f. sp. *vasinfectum*	99
18	LM-1		516	*Fusarium oxysporum* f. sp. *vasinfectum*	99
19	LM-2		528	*Fusarium oxysporum* f. sp. *vasinfectum*	96
20	LM-3		565	*Fusarium oxysporum* f. sp. *vasinfectum*	97
21	LM-5		631	*Fusarium oxysporum* f. sp. *vasinfectum*	99
22	LM-6		584	*Fusarium oxysporum* f. sp. *vasinfectum*	99
23	LM-7		540	*Fusarium oxysporum* f. sp. *vasinfectum*	99
24	LM-8		587	*Fusarium oxysporum* f. sp. *vasinfectum*	99
25	FS-1		540	*Fusarium oxysporum*	100
26	Lonren 1		554	*Fusarium oxysporum* f. sp. *vasinfectum*	99
27	MB-3		649	*Fusarium oxysporum* f. sp. *vasinfectum*	99
28	M-315		575	*Fusarium oxysporum* f. sp. *vasinfectum*	99
29	NC4		650	*Fusarium oxysporum* f. sp. *vasinfectum*	98
30	MS-6		572	*Fusarium oxysporum* f. sp. *vasinfectum*	98
31	PHY-MM2		554	*Fusarium oxysporum*	98
32	PHY-MM4		603	*Fusarium oxysporum* f. sp. *vasinfectum*	99
33	PHY-MM5		536	*Fusarium oxysporum* f. sp. *vasinfectum*	99
34	PHY-MM6		567	*Fusarium oxysporum* f. sp. *vasinfectum*	96
35	PHY-MM7		547	*Fusarium oxysporum* f. sp. *vasinfectum*	99
36	PHY-FB3		561	*Fusarium oxysporum* f. sp. *vasinfectum*	99
37	PHY-FB5		582	*Fusarium oxysporum* f. sp. *vasinfectum*	98
38	PHY-FB6		553	*Fusarium oxysporum* f. sp. *vasinfectum*	99
39	PHY-FB8		539	*Fusarium oxysporum* f. sp. *vasinfectum*	100
40	Rowden		555	*Fusarium oxysporum* f.sp. *vasinfectum*	98
41	TL-6		619	*Fusarium oxysporum* f. sp. *vasinfectum*	99