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Detection of *Fusarium oxysporum* f.sp. *vasinfectum* in cotton tissue by polymerase chain reaction

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A polymerase chain reaction assay was developed for the detection of *Fusarium oxysporum* f.sp. *vasinfectum* (FOV), a serious wilt pathogen of cotton in many parts of the world. Based on small nucleotide differences in internal transcribed spacer sequences between 18S, 5·8S and 28S ribosomal DNAs, primers Fov1 (5′-CCCCTGTGAACA-TACCTTACT-3′) and Fov 2 (5′-ACCAGTAACGAGGGTTTTACT-3′) were selected. These primers unambiguously amplified a 400-bp DNA fragment of all the FOV isolates tested (from Angola, Brazil, China and the USA) but did not amplify any other isolates of mycoflora associated with cotton, such as *F. moniliforme*, *Verticillium albo-atrum*, *V. dahliae*, *Aspergillus* sp., *F. oxysporum*, *F. sambucinum* or *F. solani*. A control PCR assay was developed employing the universal primer pair ITS1 and ITS2 which amplified a fragment of approximately 220 bp from all isolates tested. This control assay demonstrated that all fungal DNAs were readily amplifiable, thus confirming that the lack of amplification with Fov1 and Fov2 primers was a result of primer specificity and not of other possible causes, such as DNA degradation or the presence of PCR inhibitors. The assay was effective on samples from the stems, leaves, roots and calli, and from plant tissues both with and without symptoms. This detection system proved to be accurate and sensitive and could aid not only diagnosis but also disease monitoring and forecasting.

Introduction

Fusarium oxysporum f.sp. *vasinfectum* (FOV) is a cosmopolitan wilting agent attacking several species of the genus *Gossypium* as well as species of Leguminosae, Malvaceae and Solanaceae. It is highly virulent on cotton (*Gossypium hirsutum*) in America, Asia and Africa. In Angola infections may cause serious yield and quality losses (Ragazzi, 1992). The pathogen, a soil-borne fungus, can survive for several years in a dormant state (chlamydospores) in plant debris or in the soil. It invades the plants through the roots, especially through root wounds caused by nematodes (*Meloidogyne* spp.), and subsequently infects the vascular system, resulting in wilt symptoms (Hillocks, 1992).

There are several pressing reasons for the development of a molecular-based assay for FOV infection. In aetiology and symptom development it is a progressive wilt, very similar to those caused by many other factors such as excesses of chemical substances, drought stress, mature plant senescence, infection with other *Fusarium* species and wilt pathogens of the genus *Verticillium*. Different wilt agents are frequently found together in the same cotton field and it appears that a complex community of *Fusarium* spp., difficult to distinguish morphologically from FOV, often coexist with it on various organs of cotton. A number of *Fusarium* spp. with varying pathogenic capabilities, such as *F. equiseti, F. graminearum*, *F. lateritium, F. moniliforme*, *F. oxysporum*, *F. roseum*, *F. semitectum* and *F. solani* are frequently isolated from mixed infections with FOV from cotton roots, stems, leaves and bolls (Miller & Weindling, 1939; Fulton & Bollenbacher, 1959; Mertley & Snow, 1978; Roy & Bourland, 1982; Batson & Borazjani, 1984; Colyer, 1988; A. Ragazzi, unpublished results). *F. roseum* Link ex Gray is an ambiguous name for *F. sambucinum* Fuckel (Gerlach & Nirenberg, 1982); this fungus is often associated with FOV on cotton, as well as being common on several other malvaceous plants. The similar symptomatology associated with all these *Fusarium* species makes it difficult to identify FOV, which is by far the most serious of these wilt agents. In addition, the symptoms of FOV infection often do not appear until a considerable time after the infection has taken hold, especially in older or moderately resistant plants (Holliday, 1980). For all these reasons, a diagnosis based on visual inspection is not feasible with the FOV-*Gossypium hirsutum* pathosystem.

Current procedures to identify FOV and determine its frequency of occurrence are carried out in the laboratory by isolating the pathogen on nutrient media and

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Table 1 Plant-associated fungi used in the polymerase chain reaction (PCR) assays employing a primer-pair specific for *Fusarium oxysporum* f. sp. *vasinfectum* and a control primer-pair that amplifies a ribosomal DNA fragment from several groups of fungi

Fungus	Isolate	Original host	Country of origin	Source[a]	Tissue	PCR amplification	
						With Fov1-2 specific primers	With ITS1-2 control primers
FOV = *F. oxysporum* f. sp. *vasinfectum*	B4†	Cotton	Angola	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	B7†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	B11†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	B13†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	B19†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CN2†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CN9†	"	"	1	boll	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CN21†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CN22†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CN24†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CS1†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CS3†	"	"	1	boll	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CS6†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CS15†	"	"	1	boll	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	CS17†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	L3†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	L8†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	L10†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	L12†	"	"	1	boll	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	L18†	"	"	1	boll	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	M3†	"	"	1	root	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	M4†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	M9†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	M13†	"	"	1	leaf	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	M16†	"	"	1	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	C1†	"	China	2	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	C2†	"	"	2	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#7808	"	USA	3	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#16421	"	"	3	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#16611	"	"	3	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#16612	"	"	3	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#16613	"	"	3	stem	+	+
FOV = *F. oxysporum* f. sp. *vasinfectum*	ATTC#36198	"	Brazil	3	stem	+	+
FO7 = *F. oxysporum*	_	Spruce	Italy	4	seed	–	+
FMO = *F. moniliforme*	_	Asparagus	"	5	root	–	+
FSA = *F. sambucinum*	_	Cotton	Angola	1	stem	–	+
FSO = *F. solani*	_	"	"	6	stem	–	+
VAA = *V. albo-atrum*	_	Olive	Italy	1	stem	–	+
VDA = *V. dahliae*	_	"	"	1	stem	–	+
ASP = *Aspergillus* sp.	_	Cotton	Angola	1	boll	–	+

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† Ragazzi A., private culture. Numbers designating the individual isolates are printed as subscripts in the text.

inspecting the morphology under a microscope. This approach, besides being time-consuming, is hampered by the fact that cultural characteristics, such as mycelium pigmentation and the formation, shape and size of conidia are very variable, being strongly influenced by the type of substrate and the incubation conditions. Pathogen identification based on infection tests in the field or in growth chambers is also time-consuming and not reliable, since such tests are affected by a range of factors such as the particular isolates selected (should be typical, virulent, sporodochial-type); the proper maintenance of cultures (to prevent variation); the mode of production, type and concentration of the inoculum; inoculation techniques; the choice of cultivars to be tested; plant age, and environmental conditions (temperature, humidity, light conditions, etc.). Additionally, the scoring of symptoms in disease evaluation always retains a subjective element. Together,

these factors make it difficult to standardize pathogenicity tests.

All these difficulties, inherent in traditional identification techniques for FOV, can be overcome by an accurate, rapid and sensitive diagnostic test based on the *in vitro* amplification of fungal DNA by polymerase chain reaction (PCR) (Mullis *et al.*, 1986; Lee & Taylor, 1990; Nazar *et al.*, 1991; Henson & French, 1993; Ward, 1994). PCR-based diagnostic assays have already been developed for *F. oxysporum* f.sp. *ciceris* (Kelly *et al.*, 1994) and *F. oxysporum* f.sp. *gladioli* (Mes *et al.*, 1994), while an assay based on RFLP of total genomic DNA was developed for distinguishing races of the pea vascular wilt pathogen *F. oxysporum* f.sp. *pisi* (Coddington *et al.*, 1987).

In the present study, we looked for specific sequences in the ribosomal DNA of FOV to be used for development of specific detection primers, and then evaluated the PCR assay as a technique to detect the pathogen in infected host tissue and to distinguish it from other *Fusarium* spp. associated with cotton. The PCR assay was also tested on callus to determine whether the pathogen behaved differently in tissue lacking tracheids, the normal diffusion pathways of FOV into the host under natural conditions.

Materials and methods

Fungal isolates

The 40 fungal isolates used in the present study included 37 of *Fusarium*, two of *Verticillium* and one of *Aspergillus* (Table 1). Twenty-eight of the *Fusarium* isolates were used in infection tests on cotton to determine their pathogenicity, and the sensitivity of the PCR detection technique. These were: five FOV isolates each from the five geographic FOV provenances in Angola [Bié (B), Cuanza Norte (CN), Cuanza Sul (CS), Lunda (L) and Malange (M)], one isolate each of *F. sambucinum* (FSA) and *F. solani* (FSO), and a *F. oxysporum* (FO) isolate (specialized form unknown) from Norway spruce seeds (Table 1). Isolates were obtained by surface-sterilizing infected plant organs in 1% sodium hypochlorite for 15 min, rinsing in sterile distilled water, and cutting the organs into pieces (4–5 mm in length) with a sterile scalpel. Individual FOV isolates were designated by a code number indicating the provenance in Angola and a subscript designating the culture number, as shown in Table 1. Prior to the present study, randomly selected FOV isolates from each region had been tested for pathogenicity on cotton cultivars from Angola, and found to be virulent.

Of the remaining twelve isolates, eight, of FOV from Brazil, China and the USA, were used to verify whether the designed pathogen-specific primers also gave positive results with FOV isolates from different continents. Almost all known races of the pathogen were represented: North-American (USA) isolates included races 1, 2, 3 and 4 (culture nos. 16421, 16611, 16612 and 16613, respectively) and the South-American (Brazil) isolate was race 6 (culture no. 36198) (Table 1). Four other isolates, of *Aspergillus* sp (ASP), *F. moniliforme* (FMO), *V. albo-atrum* (VAA) and *V. dahliae* (VDA) (Table 1)] were used to test the specificity of the PCR assay.

Growth of fungal material

To obtain mycelium and conidia, for infection tests on plants and on calli respectively, FOV colonies were grown on Difco PDA (potato–dextrose–agar) at 23°C in the dark. After incubation for about 25 days, conidia were harvested in sterile distilled water. To obtain mycelium for DNA preparation, isolates were grown for 10 days on stationary liquid medium (Norkrans, 1963) at 23°C in darkness. The mycelium was washed with double distilled, autoclaved water, dried on absorbent paper, weighed, and stored at −70 °C until use.

Growth of plant materials

Plants

A total of 200 2-month-old seedlings of the Coker 304 cotton variety (168 seedlings for the tests plus 32 controls) were transplanted to 20 cm high, 15 cm diameter pots containing universal loam and agriperlite, and mantained in a plastic growth chamber with a 12-h day (5000 lux), 28°/24°C, 45/70% RH day/night.

Inoculation was on the stem of sufficiently lignified plants by removing a 2×4 mm patch of the outer bark approximately 10 cm above ground level and placing on the exposed tissue a 2 mm diameter disk of mycelium grown on solid medium. Six seedlings were inoculated with each of the 28 fungal isolates chosen for the infection tests. The inoculation site was covered with cotton wool soaked in sterile water and wrapped with 'M' parafilm (American National Can$_{TM}$). Control plants were inoculated with plugs of medium without the fungus, and the wounds protected as above.

Wilt symptoms began to appear after 2–3 weeks. Root, stem and leaf portions with and without wilt signs were collected for DNA extraction, and for pathogen re-isolation in order to satisfy Koch's postulates. For re-isolation, pieces of tissue (approximately 5×5 mm) were dipped for 30 s in 70% ethanol, sterilized for 20 min with 1% sodium hypochlorite, rinsed for 3×30 s in sterile water, and plated on 9 cm diameter plastic Petri dishes containing PDA medium.

Callus cultures

Similar numbers of callus cultures, obtained from embryos that had been sterilized as described above for plant tissue fragments, were used for infection tests (168 calli plus 32 as controls). Embryos were incubated at 24°C in darkness on MS medium (Murashige & Skoog, 1962) supplemented with 0·5 mg L^{-1} 2,4 dichlorophenoxyacetic acid, 2 mg L^{-1} kinetin, 30 g L^{-1} sucrose and

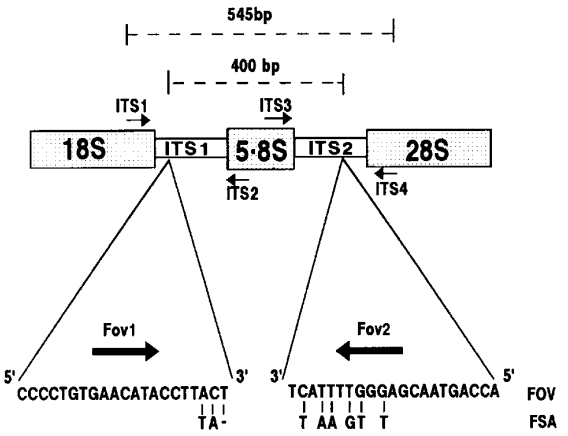

Figure 1 The location of *Fusarium oxysporum* f.sp. *vasinfectum* (FOV)-specific primers Fov1 and Fov2 on the ribosomal gene repeat. Sequence differences within the internal transcribed spacers between FOV and *F. sambucinum* (FSA) (O'Donnell, 1992) are indicated below the sequence of FOV target primers (a dash indicates alignment gaps). The two ITS1 and ITS4 primers (White *et al.*, 1990) produce a 545-bp DNA fragment in FOV isolates.

8 g L^{-1} Difco bacto-agar, and with the pH adjusted to 5·6–5·8. Calli were transferred every 4 weeks. Six calli were inoculated with each FOV isolate in suspensions of $1{\cdot}5 \times 10^7$ conidia per ml of distilled water. The control calli received sterile water. Both infected and uninfected callus tissue was sampled for DNA extraction.

DNA extraction

From fungal mycelium

Total genomic DNA from three FOV isolates (B_7, CN_2 and CS_6), the FMO, VAA, VDA and ASP isolates, and the FOV isolates from Brazil, China and the USA, was prepared using a modified version of the methods of Raeder & Broda (1985) and Lee *et al.* (1990). DNA was extracted from 50 mg of freeze-dried mycelium in 750 μL of extraction buffer (200 mM Tris-HCl pH 8·5, 250 mM NaCl, 25 mM EDTA, 0·5% SDS) at 65°C for 1 h in 1·5 mL Eppendorf tubes. The solution was extracted twice with 600 μL of (1:1) phenol:chloroform and treated with RNAse A. Chloroform:isoamyl alcohol (24:1, 600 μL) was added, mixed and centrifuged at 13000 × ***g*** for 10 min. The supernatant was removed and DNA precipitated by the addition of 0·1 volume of 3 M sodium acetate and 0·6 volume of − 20°C absolute ethanol followed by centrifugation as above for 15 min at 4°C. DNA pellets were washed in 70% ice-cold ethanol and resuspended in 100 μL of TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8). The DNA present in the supernatant was quantified using a TKO-100 Hoefer fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

From plant tissue

Cotton tissue (stem, leaf, root, and callus cells) with and without symptoms was collected from infected and uninfected (control) plants. Roots and stems were sampled by cutting 1-cm segments at various distances from the base of the stem below or above the soil level, respectively. Leaves were sampled entirely and calli were sampled by removing approximately 300–500 mg of tissue with scalpels and forceps. Samples were put in plastic bags and stored at −20°C until use. Cotton is rich in phenolic terpenoids, which bind to the DNA and RNA, making their extraction difficult by conventional means. This problem can be overcome by using an efficient extraction buffer developed for isolation of nucleic acids from plants containing polyphenolics (Maliyakal, 1992). Frozen cotton tissue was homogenized in a buffer containing 5 M guanidine isothiocyanate, 0·2 M Tris-acetate (pH 8·5), 0·7% β-mercaptoethanol, 1% polyvinyl pyrrolidone (soluble PVP, MW 40 kDa), and 0·62% sodium lauroyl sarcosine. The DNA preparation steps subsequent to tissue homogenization were as described by Dellaporta *et al.* (1983).

PCR amplification

PCR amplification was carried out in 25 μL volumes containing: 50 pmol of each primer; 2·5 μL of *Taq* DNA polymerase buffer (10 mM Tris-HCl pH 8·3, 1·5 mM $MgCl_2$, 50 mM KCl and 0·1 mg gelatin); 100 μM each of dATP, dCTP, dGTP and dTTP; 0·1–10 ng of template DNA, and 0·5 unit of *Taq* DNA polymerase (Boehringer). A layer of mineral oil (30 μL) was placed over the reaction mixture to prevent evaporation. Incubation was performed in a Techne PHC-3 Thermal Cycler using 30 reaction cycles, each consisting of 1 min at 94°C, 1 min at 50°C and 3 min at 72°C.

Sequencing the ITS region

The entire internal transcribed spacer located between the 18S and 28S ribosomal genes was first amplified from mycelium of the FOV B_7, CN_2 and CS_6 isolates by means of the universal primers ITS1 and ITS4 (White *et al.*, 1990) (Fig. 1), and the sequence determined. The PCR products were purified with a Prep-A-Gene kit (BioRad, Richmond CA), and sequenced by the dideoxy method using a Sequenase 2.0 kit (USB, United States Biochemical Corporation, Cleveland, OH) and the primers ITS1, −2, −3, −4 (White *et al.*, 1990) for sequencing both strands of the ITS region. The PCR products were radiolabelled with ^{35}S following the supplier's instructions. Sequencing reactions were run for approximately 3 h on 5% polyacrylamide wedge gels in a TBE buffer. Gels were fixed, vacuum dried and exposed to Kodak SB X-ray film for varying periods.

Synthesis of FOV-specific primers and amplification reactions

Based on sequence analysis of the ITS region, two

primers, Fov1 and Fov2, homologous to two short variable regions of Angolan FOV sequences within the internal transcribed spacers (Fig. 1), were synthesized. The Fov1 oligonucleotide was designed as a 21-mer forward primer with a G+C content of 47·6% and had the sequence 5′-CCCCTGTGAACATACCTTACT-3′; the Fov2 oligonucleotide was constructed as a 21-mer reverse primer with a G+C content of 42·8% and had the sequence 5′-ACCAGTAACGAGGGTTTTACT-3′. To ensure primer specificity, the Fov1 primer was designed with three mismatches to *F. sambucinum* at the 3′ end, where the elongation of the complementary strand is initiated. The Fov1 and Fov2 primers annealed to specific FOV nucleotide sequences within the non-coding ITS1 and ITS2 regions (Fig. 1), amplifying the target fungal DNA from purified mycelial samples and from infected cotton tissue. In order to improve primer specificity in mixed template reactions, the FOV-specific PCR reaction was performed at an annealing temperature of 60°C, rather than 50°C, while the other PCR conditions remained as described above. Various amounts of template DNA ranging from 100 ng to 50 fg were tested. Aliquots (3 μL) of the PCR reaction mixture were fractionated on 0·8% horizontal agarose gels (Sigma) stained with ethidium bromide.

Results

The 25 FOV isolates used in this study were randomly selected from a collection of over 100 isolates from local cotton cultivars in Angola. They included isolates from the leaves, roots, vascular tissue and bolls of plants, with and without symptoms, growing on sites where there had been serious outbreaks in the past, and where epidemiological surveys revealed a disease incidence in plantations ranging from 50 to 90% (A. Ragazzi, unpublished results). The other *Fusarium* spp. used in the infection tests were chosen on the basis of some of their peculiarities, such as the frequency of occurrence on the host (*F. oxysporum* and *F. sambucinum*), and the pathogenic role, i.e. the ability to induce symptoms that might be confused with symptoms caused by FOV (*F. solani*).

Infection tests confirmed the virulence of all 25 FOV isolates, the mild pathogenicity of FSA and FSO isolates, which produced few and limited wilt symptoms (mainly sparse foliar spots) and the weak pathogenicity of the FO isolate. Typical tiger stripe lesions were produced by the FOV isolates 4–6 weeks after inoculation, confirming the great sensitivity of the cultivar. Infection tests on calli paralleled the *in planta* results, with the FOV colonies, which exhibited a rapid growth, a dense appearance and abundant hyphae formation.

FOV was successfully re-isolated on PDA. Seventy-two hours after plating, the isolates produced fast growing colonies with an average diameter of 3 cm. FSA and FSO were also re-isolated from cotton tissues, producing smaller colonies (3–5 mm diameter) after the same incubation period. Re-isolation from tissues infected with the FO isolate was successful in only one case, confirming the limited ability of this isolate to colonize cotton. Re-isolation was normally successful from both symptomatic and asymptomatic plant organs, though in a few cases it was unsuccessful from tissues wihout symptoms.

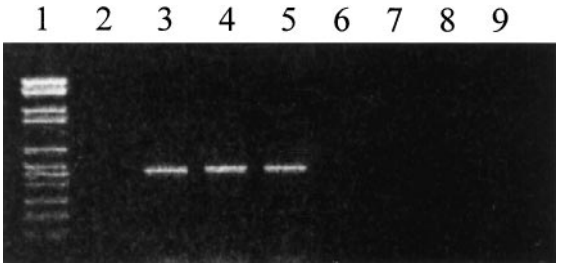

Figure 2 PCR using primers Fov1 and Fov2 on DNA from purified mycelium of FOV and other fungi. Lane 1, molecular weight marker (marker VI, Boehringer; fragment sizes are (bp): 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 298, 234, 234, 220, 154, 154); lane 2, *F. moniliforme*; lanes 3–5, FOV isolates from the USA (ATTC#16611), China (C_1) and Brazil (ATTC#36198), respectively; lanes 6–8, *V. albo-atrum*, *V. dahliae* and *Aspergillus* sp., respectively; lane 9, water control.

The rRNA genes, commonly used in identification and taxonomic studies, were confirmed in the present study to be particularly appropriate for the purpose of providing target sequences for molecular detection. Differences in the nucleotide composition of the variable ITS region have been successfully employed for several groups of plant pathogens to design specific primer sets that amplify DNA selectively among and within species (Nazar *et al*., 1991; Mills *et al*., 1994; Moukhamedov *et al*., 1994; Robb *et al*., 1994; Stammler & Seemüller, 1994; Schilling *et al*., 1996).

To identify suitable sequences for the design of pathogen-specific primers, DNA of the Angolan FOV isolates was first amplified with universal fungal ribosomal primers ITS1 and ITS4. The resulting 545 bp ITS fragment was sequenced (Moricca *et al*., 1995) (EMBL accession numbers X78258, X78259 and X78260) and compared with sequences from the most closely related organisms deposited in the data banks. A comparison of FOV sequences with those from other vascular parasites of cotton, such as *Verticillium* spp., revealed substantial sequence heterogeneity. For example, *Verticillium tricorpus* (Moukhamedov *et al*., 1994) (EMBL accession number L28679) displayed 71·3% identity in a 373 bp overlap within the ribosomal ITS1 and ITS2 regions. Greater homology with FOV ITS sequences was found in *F. sambucinum* (O'Donnell, 1992) (EMBL accession number X65482), with 93·3% identity in a 526 bp overlap within the ribosomal ITS sequences. Two variable regions were found between bases 38–142 in the ITS1 and between bases 339–411 in the ITS2. These variable regions allowed identification of two short sequence traits with clusters of three and six nucleotides that were mismatches between FOV and

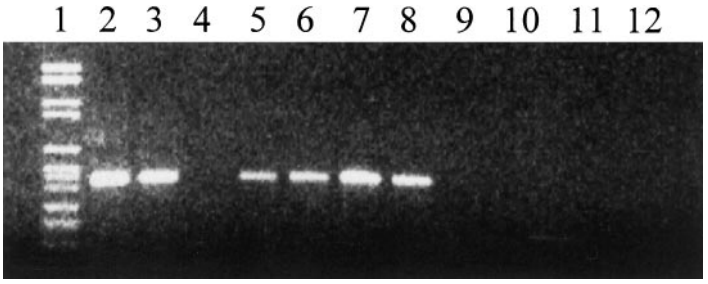

Figure 3 PCR using primers Fov 1 and Fov 2 on DNA from tissue of the Coker 304 cotton variety infected with the FOV L_{10} isolate and other fungi. Lane 1, molecular weight marker (marker VI, Boehringer); lanes 2–3, extracts from stems of FOV-infected symptomatic and asymptomatic cotton plants, respectively; lane 4, extracts from stem of a cotton plant inoculated with a *F. oxysporum* isolate; lanes 5–6, extracts from FOV-infected asymptomatic and symptomatic cotton leaves respectively; lanes 7–8, extracts from FOV-infected symptomatic and asymptomatic cotton roots respectively; lanes 9–10, extracts from cotton stems inoculated with *F. solani* and *F. sambucinum,* respectively; lane 11, extract from uninfected cotton tissue; lane 12, no added template.

F. sambucinum in the ITS1 (bases 20–41) and the ITS2 (bases 400–421), respectively. This made it possible to synthesize two complementary oligonucleotide primers that annealed unambiguously to the rDNA of FOV. Primer specificity was tested using purified fungal genomic DNA and infected plant tissue. Primers Fov1 and Fov2 specifically amplified a 400 bp fragment from all Angolan FOV isolates. No cross-reaction was detected with genomic DNA from any isolates of other fungi that are occasionally associated with cotton, such as *V. albo-atrum*, *V. dahliae* or the related *F. moniliforme* (a member of the Section Liseola), or isolates that are found saprophytically on cotton organs, such as *Aspergillus* sp., all of which were tested for non-specific amplification (Fig. 2). A PCR control assay with universal primers ITS1 and ITS2 was employed to verify whether the negative results obtained in the attempts to amplify the DNA of associated mycoflora were possibly due to mycelial DNA degradation or to the presence of PCR inhibitors. In all these control assays a 220 bp fragment was produced, demonstrating that the lack of amplification with the FOV-specific primers resulted from the specificity of the primers.

Primers Fov1 and Fov2 amplified a band from purified genomic DNA samples of FOV isolates from Brazil, China and the USA, indicating that they are not merely specific to a clonal population of the pathogen occurring in Angola (Fig. 2).

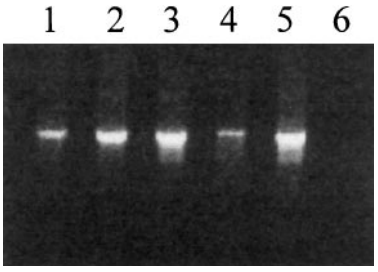

Figure 4 FOV-specific PCR products from tissue of the Coker 304 cotton variety infected with the FOV B_7 isolate. DNA extracts from root (lane 1), callus (lane 2), stem (lane 3) and FOV-infected asymptomatic and symptomatic leaf tissue (lanes 4–5), respectively. Lane 6, no added template.

The assay was effective with DNA extracted from infected symptomatic and asymptomatic cotton tissue (Fig. 3). The signal was strongest in stem tissue, somewhat less so in the root and leaf portions, indicating a higher inoculum density in the large vessels of the stem. No amplification signal was detected from cotton plants inoculated with the FSA, FSO or FO isolates, though the first two isolates occasionally produced wilting symptoms after an equal incubation period. No signal was observed in negative controls containing nucleic acid from uninfected tissue, or with the reaction mixture from vials without added template DNA. Control PCR assays using the ITS1 and ITS2 primers produced a 220-bp fragment from all infected plant tissues, but not from healthy plant tissue. These results clearly indicate that there was no inhibitory effect caused by residues of phenolic compounds, pigments or other substances possibly present in the reaction mixture, but that negative PCR results were unambiguously attributable to the specificity of the FOV primers themselves.

The efficacy of the assay was high on plant tissue exhibiting wilt symptoms, but somewhat lower on tissue of plants infected but without visible symptoms (Figs. 3 and 4). The amplification from calli, roots, stems and leaves gave a strong signal with or without signs (on the callus) or symptoms (on the roots, stems and leaves) of the pathogen. In a very few cases the polymerase chain reaction failed to detect target fungal DNA in asymptomatic leaves, although FOV was always detected on asymptomatic stems and calli. Primers detected fungal DNA from plants and from unorganized callus tissue with approximately equal effectiveness (Fig. 4). In many cases the assay detected FOV in asymptomatic plant tissues 1–3 weeks before symptoms became noticeable.

Sequencing of the 400-bp fragment resulting from PCR amplification proved conclusively that it was the sequence predicted by the sequence data.

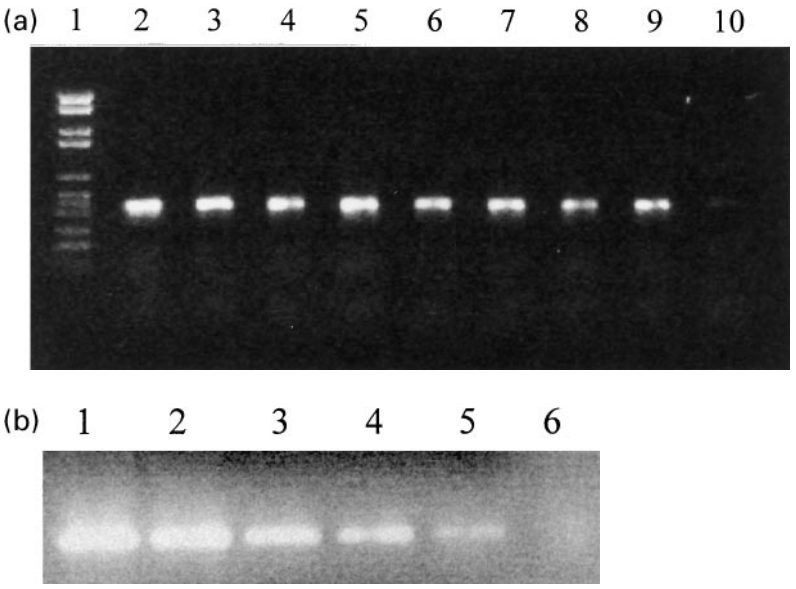

Figure 5 PCR detection of FOV in tissue of the Coker 304 cotton variety. (a) Genomic DNA from the FOV M_{16} isolate. Lane 1 is molecular weight marker (marker VI, Boehringer); lanes 2–10 are: 100 ng, 50 ng, 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 50 fg, respectively. (b) Amplification products from: 1, mycelial DNA from the FOV M_{16} isolate; lanes 2–6, DNA from cotton tissue infected with the FOV M_{16} isolate: 50 ng, 10 ng, 5 ng, 2·5 ng, 1 ng, respectively.

A range of concentrations of DNA from mycelium and infected plants was tested to determine the sensitivity of the assay. For DNA from fungal mycelium, 100 ng to 50 fg was found to be suitable (Fig. 5a), whilst the range for infected cotton was 50 ng to 1 ng (Fig. 5b).

Discussion

The sequence heterogeneity of the internal transcribed spacers constituted the basis of our PCR-based assay for the detection of FOV in cotton. The method is a rapid and accurate means to identify FOV on infected plant tissue. The PCR assay was reliable in detecting the pathogen from plant extracts exhibiting different degrees of disease severity, with positive amplification resulting from both symptomatic and asymptomatic plant organs. The detection of the target fungal DNA from heavily infected, as well as from weakly infected tissue without symptoms or with barely visible symptoms, indicate that the assay is robust and sensitive.

Amplifications from the stem tissue always gave a strong band. This is not particularly surprising considering that inoculation of the fungus was into the stem, where a higher amount of fungal inoculum is generally present near the inoculation site. Such a result might also be attributable to the ability of the cultivar to stop or slow down the fungal spread, probably by activating defence mechanisms, such as accumulation of polysaccharides and/or tylose formation. Vascular occlusions in response to wounding or the presence in the xylem of foreign chemicals or microrganisms is a general phenomenon in plants. It has already been shown that during the early stages of infection by *Fusarium oxysporum* f.sp. *vasinfectum*, extensive vascular occlusions occur in wilt-resistant cotton plants (Bugbee, 1970).

The fact that the designed primers worked with FOV isolates from all geographic provenances, together with their demonstrated specificity, suggests that the diagnostic test developed here would assist in detecting FOV in fields from geographically distant cotton-growing areas and that the assay is able to detect the pathogen also when it occurs in complexes with related or unrelated species. Nazar *et al.* (1991) and Moukhamedov *et al.* (1994) amplified rDNA differentially from *V. albo-atrum*, *V. dahliae* and *V. tricorpus*, using primer-pairs that differed in only two or three bases among the three species. If *Fusarium* species could be detected and differentiated in the same way, it would provide a valuable diagnostic tool to investigate the *Fusarium*/cotton pathosystem.

A similar PCR assay should also assist in the detection of non-pathogenic isolates of FOV, using samples obtained directly from the soil. The classification of *F. oxysporum* into isolates, specialized forms and races has traditionally been based on the criterion of virulence against a given set of cultivars of varying resistance. This approach is useful, but it does not take into account the non-pathogenic isolates, even though these are always the majority in any fungal mycoflora. A knowledge of such isolates is necessary to understand the diversity found among virulent isolates (Correll, 1991).

The use of healthy plant material as a preventive measure has taken centre stage in modern disease control, but this is only possible if infections can be detected at their earliest or latent stages. One of the main causes of the worldwide spread of FOV is precisely the inability to detect infections at an early enough stage. This has been the main cause of the destructive outbreaks that have caused huge yield losses in Angola, and the present study is part of a monitoring programme aimed at controlling cotton wilt in that country. The wide-scale use of rapid and specific PCR-

based methods promises to be an important step in this direction.

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