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Development of a Conventional and Lightcycler PCR Assay for Detection of *Fusarium solani*

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ABSTRACT

Early, precise detection and identification of soil-borne fungi are essential for efficient plant disease management. Three sets of specific primers were designed from the internal transcribed spacer (ITS) regions, ITS-Fs-forward and ITS-Fs-reverse, of the rRNA gene to identify *Fusarium solani* isolates in pure mycelial culture. The specificity and suitability of the PCR techniques has been tested with different cotton fungal pathogens, including various *Fusarium* species and 29 *F. solani* isolates. ITS-Fs7 and ITS-FS8 primers amplified two fragments (480 and 390 bp) from *F. solani* genomic DNA, and the ITS-Fs2 primer set amplified two different sizes (540 and 420 bp) of fragments. The 390 bp- and 420 bp- fragments were specific to *F. solani*. The primer sets amplified none of fragments from *Rhizoctonia solani* and *Macrophomina phaseolina*. In the assay, SYBR Green I was used as fluorescent dye enabling real-time detection of PCR products. Characterization of the amplicons was achieved by melting point analysis (85 ± 0.1°C). In conventional PCR the detection limit of all primers was 100 pg, whereas detection threshold of the LightCycler PCR were 100 fg of genomic DNA from *F. solani* mycelium. Real-time PCR assay allows a more reliable and rapid detection of *F. solani* which is considerably faster than conventional detection assays. The present PCR test represents a rapid and reliable method for genetically based identification of *F. solani* cultures. To the best of our knowledge, this is the first report describing the application of real-time PCR assay for the identification of *F. solani* isolates recovered from cotton.

Keywords: free-gel based technique, *Gossypium*, *Nectria haematococca*, nucleotide sequence
Abbreviations: dNTP, deoxynucleoside triphosphate; **ITS**, internal transcribed spacer, **PCR**, polymerase chain reaction; **PDB**, Potato Dextrose Broth

INTRODUCTION

Fusarium species have frequently been isolated from diseased cotton roots, and have often been reported as pathogens of cotton seedling roots (Johnson *et al.* 1978; Roy and Bourland 1982; Colyer 1988; Abd-Elsalam *et al.* 2006a).

The fungus *Fusarium solani* infects a wide range of crops throughout the world. In cotton (*Gossypium barbadense*) it causes seedling disease and root rots. *F. solani* and *F. oxysporurn* were consistently the most common species in the rhizoplane of healthy and diseased seedlings of cotton in Egypt (Moubasher *et al.* 1984). *F. solani* (sexual stage *Nectria haematococca*) is a pathogenically diverse species found on a wide range of plant and animal hosts. On plant hosts, there are a number of *formae speciales* described with specific host ranges, causing diseases such as seedling damping off, root and hypocotyl rot (Burke and Hall 1991), tuber dry rots (Huguelet and Hooker 1981), wilts (Huguelet and Hooker 1981; Martyn 1996), and fruit rots (Martyn 1996). Data from mating studies (Matuo and Snyder 1973) and molecular phylogeny analysis (O'Donnell 2000) indicate that *F. solani* is a rather large species complex as currently delineated. *F. solani* and *F. equiseti* approximated 60 and 30% of all fungi isolated from diseased seedlings, respectively (Pizzinatto and Menten 1991; Chimbekujwo 2000). It has been known that *F. solani* was more frequent and virulent than *F. equiseti* (Johnson *et al.* 1978; Sharma and Sandhu 1986; Colyer 1988; Solymani *et al.* 1993).

Identification within closely related fungal species, such as *Fusarium* spp., needs considerable skill (Marasas *et al.* 1984). To overcome the problems related to detection and identification, the development of genetically based methods is required.

PCR has several advantages over classical identification based on morphology, which requires taxonomical expertise and is also time consuming. Newly, PCR-based assays for species-specific detection of numerous *Fusarium* species in plants have been reported (Nicholson *et al.* 1998; Edwards *et al.* 2001; Abd-Elsalam *et al.* 2003; Juradoa *et al.* 2006).

O'Donnell and Gray (1995) designed *F. solani* f. sp. *phaseoli*-specific PCR primers based on rDNA sequences and used these primers to detect the fungus on inoculated soybean roots. In a previous study, a pair of *F. solani* f. sp. *glycines* primers, Fsg1 and Fsg2, were developed based on the mitochondrial small subunit rDNA sequences (Li *et al.* 2000).

Real-time PCR assays have been implemented for the detection of fungal plant pathogens in several recent studies (Winton *et al.* 2002; Guo *et al.* 2005, Abd-Elsalam *et al.* 2006b). Applications of the real-time PCR technologies has greatly improved our ability to detect and monitor plant fungal pathogens, including increased sensitivity, reduction of time required for testing, and better chance to differentiate complex infection as well as phytopathogenic isolates (Abd-Elsalam 2003; Dyer *et al.* 2006). Furthermore, real-time-PCR has the potential for a precise quantification of target DNA (Schmittgen 2001). Novel real-time quantitative PCR assays were developed for both absolute and relative quantification of *F. solani* f. sp. *glycines* (Gao *et al.* 2004). In the current research we describe a simple real-time PCR assay with the LightCycler system employing species-specific primers and SYBR Green fluorescent dye for identifica-

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Table 1 Fungal species, isolate number, origin, host and results of PCR amplification using three *Fusarium solani*-specific primers.

Fungal isolates [a]	Origin	Host	PCR specificity [b]					
			ITS-FS8		ITS-FS7		ITS-Fs2	
			480 bp	390 bp	480 bp	390 bp	540 bp	420 bp
Fs1	Egypt	Cotton	●	●	●	●	●	●
Fs2	Egypt	Cotton	●	●	●	●	●	●
Fs3	Egypt	Cotton	●	●	●	●	●	●
Fs4	Egypt	Cotton	●	●	●	●	●	●
Fs5	Egypt	Cotton	●	●	●	●	●	●
Fs6	Egypt	Cotton	●	●	●	●	●	●
Fs7	Egypt	Cotton	●	●	●	●	●	●
Fs8	Egypt	Cotton	●	●	●	●	●	●
Fs9	Egypt	Cotton	●	●	●	●	●	●
Fs10	Egypt	Cotton	●	●	●	●	●	●
Fs11	Egypt	Cotton	●	●	●	●	●	●
Fs12	Egypt	Cotton	●	●	●	●	●	●
Fs13	Egypt	Cotton	●	●	●	●	●	●
Fs14	Egypt	Cotton	●	●	●	●	●	●
Fs15	Egypt	Cotton	●	●	●	●	●	●
Fs16	Egypt	Cotton	●	●	●	●	●	●
Fs17	Egypt	Cotton	●	●	●	●	●	●
Fs18	Egypt	Cotton	●	●	●	●	●	●
Fs19	Egypt	Cotton	●	●	●	●	●	●
Fs20	Egypt	Cotton	●	●	●	●	●	●
Fs21	Egypt	Cotton	●	●	●	●	●	●
Fs22	Egypt	Cotton	●	●	●	●	●	●
Fs23	Egypt	Cotton	●	●	●	●	●	●
Fs24	Egypt	Cotton	●	●	●	●	●	●
Fs25	Egypt	Cotton	●	●	●	●	●	●
Fs26	Egypt	Cotton	●	●	●	●	●	●
Fs27	Egypt	Cotton	●	●	●	●	●	●
Fs28	Egypt	Cotton	●	●	●	●	●	●
Fs29	Egypt	Cotton	●	●	●	●	●	●
Fov	Egypt	Cotton	●	○	●	○	●	○
Fo	Egypt	Cotton	●	○	●	○	●	○
Fv	Egypt	Cotton	●	○	●	○	●	○
Fsa	Egypt	Cotton	●	○	●	○	●	○
Rs (AG2)	Egypt	Cotton	○	○	○	○	○	○
Rs (AG4)	Egypt	Cotton	○	○	○	○	○	○
MP	Egypt	Cotton	○	○	○	○	○	○

[a] *Fusarium solani*= Fs, *Fusarium oxysporum* f. sp. *vasinfectum* (Fov), *Fusarium oxysporum* (Fo), *Fusarium verticillioides* (Fv), *Fusarium sambucinum* (Fsa), *Rhizoctonia solani* *Macrophomina phaseolina* (Mp).

[b] The presence or absence of species-specific amplicon is indicated by a positive (●) or negative (○) for each set of primers.

tion *F. solani* in pure mycelial culture. The detection specificities and sensitivities of the conventional PCR and real-time PCR systems were compared.

MATERIALS AND METHODS

Fungal culture, growth conditions, and DNA isolation

F. solani isolates was obtained from the culture collection of the Cotton Disease Department, Plant Pathology Research Institute (**Table 1**), and was cultured in Potato Dextrose Broth (PDB) at 25°C in a 250-ml conical flask for 10 days without shaking. Mycelia were harvested and pulverized to a fine powder in the presence of liquid nitrogen for DNA extraction as described by Abd-Elsalam *et al.* (2007).

Fungal DNA amplification

The ribosomal internal transcribed spacers were amplified using the following primer pairs: ITS1 (5′-GAAGTAAAAGTCGTAACAAG-3′) and ITS4 (5′-CCTCCGCTTATTGATATGC-3′) (White *et al.* 1990). PCR was performed with a thermal cycler (Techne TC-312, Techne, Stone, UK) with 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM each of the four deoxynucleoside triphosphates (dNTPs), 0.2 pmol (each) primer, and 2.5 U of *Taq* polymerase (ABgene, Epsom, UK) in a total volume of 50 µl. The initial cycle of 95°C for 30 sec was followed by 45 cycles (1 cycle consists of denaturation [15 sec at 94°C], annealing [15 s at 65°C], and extension [15 sec at 72°C] steps), followed by a final extension step (5 min at 72°C).

Gel electrophoresis

Gel electrophoresis with 1.5% agarose gels was conducted with 1× TAE buffer at 4.5 V/cm for 2 h. A 100-bp DNA ladder (Roche, Germany) was run concurrently with amplicons for sizing of the bands. DNA was visualized by UV flourescence after staining with ethidium bromide. UVIsoft analysis (Gel Documentation and Analysis Systems, Uvitec, Cambridge, UK) were used to capture the image and to calculate molecular sizes.

Molecular cloning and sequencing

Amplification products were excised from gels, cloned into pGEM–T Easy Vector Systems (Promega Corp. Madison, Wis.) and transformed into electro-competent *E. coli* (DH5 α), according to the supplier's instructions (Promega). Inserts of eight *F. solani* isolates were sequenced using the SequiTherm EXCEL II DNA Sequencing Kit with fluorescently labeled M13 Forward and Reverse primers (Epicentre, Biozyme, Germany) in a LI-COR Sequencer (LI-COR Inc., Lincoln, Neb.). Double-strand sequencing was performed for each clone. Sequence data were assembled using the DNAStar (Inc., Madison, Wis.).

Primer design

Three sets of species-specific primers were designed from the internal transcribed spacer (ITS) regions, ITS-Fs-forward and ITS-Fs-reverse, of the rRNA gene to identify *F. solani*. Primers were

Table 2 Description of the PCR primer sequences, GC contents, location lengths, *Tm*s, and amplicon sizes used in this study.

Primers code	5′-3′ sequence	TM	GC%	Length (nt[y])	Amplicon size (bp)
ITS-Fs2 -f	TAACggCTgAACTggCAACTT	54.1	48	21	540
ITS-Fs2-r	TggCATTCATCATTTACTg		37	19	420
ITS-Fs7-f	ACggCTgAACTggCAACTTgg	61.8	57	21	480
ITS-Fs7-r	gTgCCTTCgTCCTTCAACgTC		57	21	390
ITS-Fs8-f	ggCTgAACTggCAACTTggAg	61.8	57	21	480
ITS-Fs8-r	CTgTgCCTTCgTCCTTCAACg		57	21	390

[y]nt, nucleotides.

designed using the MegAlign software package (DNASTAR, Lasergene). All primers were synthesized and HPSF-purified by MWG Biotech (Ebersberg, Germany). For PCR, the lyophilized primers were resuspended in sterile molecular biology grade water (Sigma, Deisenhofen, Germany) according to the supplier's recommendations to obtain a concentration of 50 pmol/µl. Annealing temperatures were optimized using a temperature gradient from 50 to 65°C.

Light cycler-based PCR assay

Real-time PCR was performed using the LightCycler System (Roche Diagnostics, Germany). The reaction mixture contained 2 µl LightCycler FastStart DNA MasterPLUS SYBR Green I 1X (Roche Diagnostics, Germany) and 500 nM of each primer, brought to 16 µl with water. After adding 2 µl DNA extract both mixtures were transferred to reaction capillaries (Roche Diagnostics, Germany).

The PCR LightCycler reaction was carried out as follows: one cycle of denaturation at 95°C for 20 min, followed by 45 cycles of amplification at 95°C for 10 sec and 55°C for 15 sec at a programmed temperature transition rate of 20°C/sec, 72°C for 20 sec at a transition rate of 10°C/sec. On completion of amplification, a melting curve was produced by holding the reaction at 95°C for 60 sec, then at 40°C for 60 sec, followed by slow heating at a transition rate of 0.1°C/sec to 85°C.

Specificities of oligonucleotide primers

The specificities of the oligonucleotide primers (**Table 2**) and the target DNA fragments, for the detection of *F. solani* isolates were tested by PCR amplification of the purified genomic DNA of all isolates listed in **Table 1** by using the optimum PCR conditions and cycling parameters described above. The specificity of the real-time PCR primers was checked using 10 ng of DNA extracted from the fungi listed in **Table 1**. All of the selected fungi originated from cotton roots.

Sensitivity of PCR detection for *F. solani*

Purified genomic DNA (10 ng) from *F. solani* isolate was 7-fold diluted in sterile distilled water up to 100 fg. PCR amplification was performed under the determined optimal conditions at an annealing temperature of 54 or 62°C and other parameters as described above. PCR assay was repeated two times to determine the consistency of the level of detection.

Nucleotide sequence accession numbers

The nucleotide sequence data for *F. solani* isolates in the Gene Bank database under association numbers, DQ486874, DQ486875, DQ486876, DQ486877, DQ486878, DQ486879, DQ486880, and DQ486881.

RESULTS

Primer design and testing

The three primers shown in **Table 2** were synthesized and designated ITS-Fs8, ITS-Fs7, and ITS-Fs2. Melting temperature profiles of the PCR products at the end of the cycling reactions were determined with samples containing *F. solani* DNA. ITS-Fs8 and ITS-Fs7 primers were designed to operate at high annealing temperatures (62°C), while ITS-Fs2 is a primer designed to anneal at 54°C. All primers were designed to operate at high annealing temperatures, thereby preventing the co-amplification of nonspecific target DNA, including DNA derived from other fungal species. Species-specific primers were chosen with mismatches located in 3′.

Specificity of *F. solani* primers for real-time and conventional PCR

In order to set up the experimental conditions for the PCR assay, genomic DNAs were prepared from the different fungi and then used as template in PCR experiments**.** Three pairs of primers (ITS-Fs8, ITS-Fs7 and ITS-Fs2) produced PCR products of 390 and 420 bp for *F. solani*, respectively (**Fig. 1**). This distinguished PCR product was seen with all the *F. solani* isolates tested (**Table 2**). PCR performed on the DNA isolates of *Fusarium* species did not result in amplification of the expected 390 and 420-bp DNA fragment. No amplification signal was visible in any of the other fungal species tested (**Fig. 2**). In order to avoid false-negative results, some PCR assays were performed at least twice.

A double dissociation peak of increased fluorescence was obtained for the specific three primer sets at a melting temperature of 83 to 85°C. The species-specific PCR product for *F. solani* generated with primers ITS-Fs2 was 420 bp long and had a melting temperature of 85.0 ± 1°C in the PCR buffer. Experiments with three primer pairs when used separately did not generate any unspecific products even in controls with no template DNA. No melting peak was obtained when DNA from *R. solani* was used as template (**Fig. 3**).

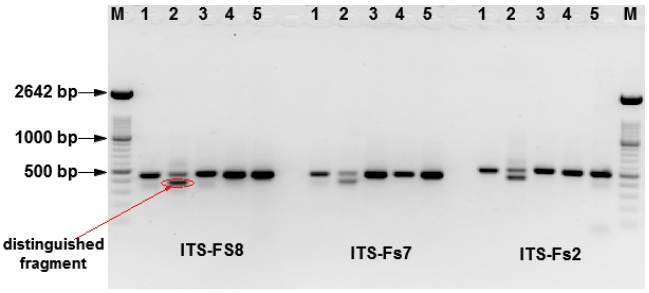

Fig. 1 Specific detection of *F. solani* isolates using PCR with ITS-FS8, ITS-Fs7 and ITS-FS2 primers. Lane M, 100 bp ladder marker; lane 1, Fov; lane 2, Fs; lane 3, Fv ; lane 4, Fsa; lane 5, Fo.

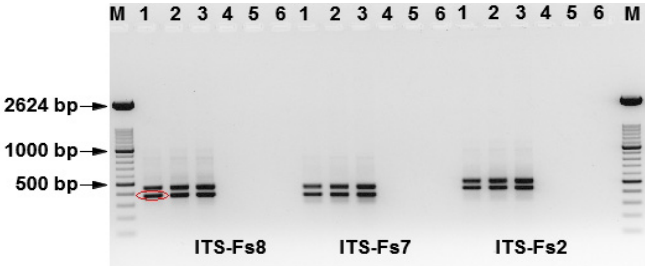

Fig. 2 PCR amplification of genomic DNA from different isolates of *Fusarium solani* using ITS-FS2 primers. Lane M, 100 bp ladder marker; lane 1, Fs1; lane 2, Fs2; lane 3, Fs3; lane 4, Rs (AG2); lane 5, Rs (AG4) and lane 6, MP.

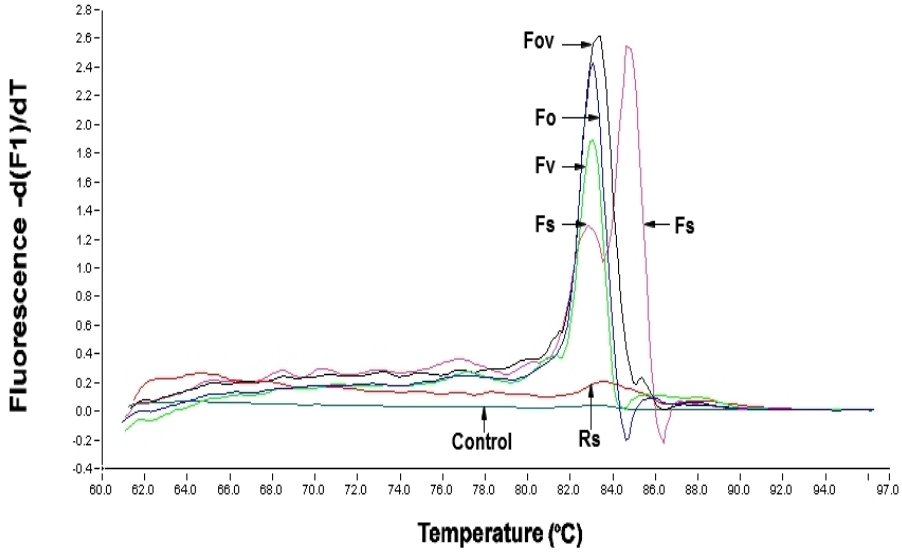

Fig. 3 Progress and melting curves of PCR products used for the detection of *F. solani*. The melting curve is shown for six selected isolates, Fov, Fo, Fv, Fs, Rs and Control. A clear peak at 83°C is visible for all *Fusarium* species, whereas a second peak at 85°C is visible for *Fuarium solani* with the primer sets of ITS-Fs2. No peaks are seen in *R. solani* (Rs) and the negative control.

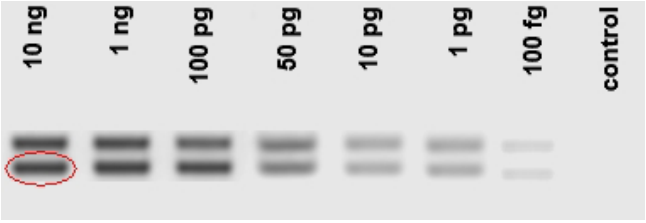

Fig. 4 Sensitivity of LightCycler PCR assessed by using 7-fold dilutions of *F. solani* DNA. Primer pair's ITS-Fs8 generated distinct amplified fragments up to 100 fg. Negative controls with no DNA (control).

Finally, these results indicated that *F. solani* could be detected by the PCR protocol here described using a simple and rapid miniprep procedure for the extraction of the DNA from the fungal mycelium.

Detection sensitivity of conventional PCR and real-time PCR

With serially diluted DNA in water, the sensitivity of the conventional PCR was sufficient to amplify 100 pg of DNA from F. *solani*. For real-time PCR, the lowest amount of DNA consistently amplifiable was 100 fg from *F. solani*. However, at 100 fg the PCR product was strong enough to be clearly recognizable (**Fig. 4**). Real-time PCR 10 times more sensitive than conventional PCR. Negative controls processed simultaneously were consistently negative.

DISCUSSION

Identification and classifications of *Fusarium* spp. based on the morphology of fungal structures, which are typically examined in pure culture, is quite laborious and time consuming.

The recently developed method offers a reliable and sensitive test for *F. solani*, suitable as a screening test, complementary to isolation on media or other methods, and could also be used for fast and specific identification of isolated colonies. These findings prompted us to assess the possibility of detection and identification of this fungus from the mycelia directly grown from infected plant tissues.

Demeke *et al.* (2005) applied species-specific PCR analysis for the identification of nine *Fusarium* species in pure culture. Filion *et al.* (2003) described the use of real-time PCR conjugated with the fluorescent SYBRR Green I dye to quantify and correlate seeded fungal propagule numbers to the amount of genomic DNA extracted from two ecologically different soil-inhabiting fungi, the plant pathogen *Fusarium solani* f. sp. *phaseoli*.

The objective of this study, new PCR primers were designed based on the 18S rDNA sequence of *F. solani*. They were used in a PCR-based assay to rapidly identify this pathogenic fungus.

The amplification of a 390 and 420 bp PCR products from 29 field isolates of *F. solani*, including isolates collected from cotton growing areas, with PCR primers ITS-Fs8, ITS-Fs7 and ITS-Fs2 respectively, provided good evidence that they can be used to identify the presence of this pathogen. Additionally, the diagnostic 390 bp and 420 PCR fragments was not amplified from a range of other fungal pathogens, including the cotton pathogens, *R. solani* and *M. phaseolina* or from the taxonomically related *Fusarium* species when the same primers were used.

Sequences of the ribosomal DNA genes, including internal transcribed spacer regions ITS1 and ITS2 surrounding the 5.8S gene, have been used to develop specific primers for detection of several phytopathogenic fungi (O'Donnell *et al.* 2000). *F. solani*-specific primers were designed by using 18S rRNA gene sequences from *F. solani*. Ribosomal genes were chosen as targets for amplification, as they are highly conserved genes that exist as multiple copies in the fungal genome (Maleska and Clark-Walker 1993; Olsen and Woese 1993). This was reflected in the sensitivity of the system, which required 100 fg of DNA from *F. solani.* Li *et al.* (2003) Developed PCR assay on the specific detection of *F. solani* f. sp. *glycines* in plants and soil samples.

The time savings of the present approach are considerable. With the real-time PCR cycler used in these experiments, 40-cycle amplification was completed in only 35 to 45 min. The fact that no gel analysis is required for routine experiments reduce the time needed by at least 1 h and could facilitate the implementation of high-throughput screening.

In conclusion, the designed primers amplified all *F. solani* isolates tested and did not amplify DNA extracted from other *Fusarium* species other fungal genera. The time course for identification was reduced from 4 to 7 days with morphological methods to 7 and 10 h with single PCR assay. PCR detection has time saving advantages over traditional isolation methods for detection of *F. solani*.

The PCR assay tested on DNA from pure cultures explained in this research can also be used in DNA isolated from cotton infected roots using a protocol which allows to process a high number of samples and to decrease the time of analysis in comparison with traditional methods.

Future investigations will be performed in order to demonstrate the value of these PCR assays for the detection of *F. solani* in tissue samples of infected cottons.

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