Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Mesapogu et al., J Plant Pathol Microbiol 2011, 2:2
DOI: 10.4172/2157-7471.1000107

Research Article Open Access

Rapid detection and quantification of *Fusarium udum* in soil and plant samples using real-time PCR

Sukumar Mesapogu[1]*, Bandamaravuri Kishore Babu[1], Achala Bakshi[1], Somasani S. Reddy[1], Sangeeta Saxena[2], Alok K. Srivastava[1] and Dilip K. Arora[1]

[1]*National Bureau of Agriculturally Important Microorganisms (NBAIM), Post Box 06 Maunath Bhanjan-275 101, Uttar Pradesh, India*
[2]*Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow-226 025, India*

Abstract

Real-time PCR based detection assay was developed for *Fusarium udum,* causing vascular wilt of pigeonpea. The Histone-3 gene of *F. udum* was targeted to design species-specific primers and probe. A comparative study was undertaken to develop a reliable and reproducible procedure for detection and quantification of *F. udum* from diverse samples. The sensitivity and specificity of oligonucleotides were evaluated through dot blot hybridization, standard and real-time PCR assays. The probe HFUSP showed high degree of sensitivity for DNA obtained from pure cultures of *F. udum* to that of environmental DNA samples. The qPCR assay specifically differentiated the *F. udum* from closely related species of *Fusarium*, other test microbes and environmental samples. The single melting curve at 84.17 and a monomorphic band of 200 bp indicates the specificity and authenticity of the PCR assays. Thus, real-time PCR assay can be used as a rapid and effective procedure that can detect minute amounts of *F. udum* from complex environments. Therefore, the real-time PCR assay demonstrated in the present study can be successfully used for detection and monitoring of *F. udum* for early infection and disease epidemiology of vascular wilt of pigeonpea.

Keywords: *Fusarium udum*; Histone-3 gene; SYBR green; Dot blot hybridization

Introduction

Fusarium wilt caused by *Fusarium udum* Butler (1906), alternate teleomorph, (*Gibberella* indica) [37] is an important biotic constraint in pigeonpea production in Indian subcontinent. In India alone, the loss due to this disease was estimated to be US $71 million per year and the percentage of disease incidence varies from 5.3 to 22.6% [17]. Identification and detection of pathogenic *Fusarium* sp. was traditionally based on either symptom on the host or culture dependent isolation of the pathogen from affected host tissue [1]. These classical approaches are becoming increasingly problematic because more than one forma specialis may occur on a given host, along with non-pathogenic, common soil and rhizosphere inhabitants [10]. In addition, the morphological identification is not feasible to quantify pathogen load in different plant tissues during the growing season or in commodities after harvest. Moreover, isolation and culture dependent enumeration may introduce a bias in favor of faster-growing species [38].

PCR-based methods have been reported for the detection of soil-borne *Fusarium* species [7,9,10,23,30]. However, with advent of real-time PCR (=RT-PCR), plant pathologists possess the unprecedented ability to accurately quantify a specific pathogen within a host plant [15]. Since last one decade efforts have been made in detection and quantification of various plant pathogens [29,24,2]. For instance, RT-PCR with SYBR green chemistry for quantification of *F. solani* f.sp. *phaseoli* in both sterile and non-sterile soil [11] and RT-PCR based on TaqMan probe chemistry to quantify the *F. culmorum* in infected plant tissue [36].

Nuclear rRNA, including the small and large subunits and the internal transcribed spacer (ITS) region, proved as an ideal target for the detection of different isolates/species of *Fusarium* such as *F. oxysporum* f. sp. *vasinfectum*, *F. oxysporum* f.sp. *ciceri* and *F. sporotrichioides* [26,16,20,12]. Besides ITS region, several researchers applied other conserved genes such as translational elongation factor 1- α (TEF1-α) [13,4,28] Cellobiohydrolase-C [14], Histone-3 [35] and Topoisomerase-II [39] for development of species-specific PCR primers. In recent studies, primer set were developed targeting to *esyn*-1 gene encoding multifunctional enzyme enniatin synthetase to identify enniatin-producing *Fusarium* species [21]. Similarly, toxin producing genes were also targeted to design species specific ologonucleotides for identification, detection and quantification of different pathogenic *Fusarium* species [33,19].

Though Yadav et al. [39] recently developed Topoisomerase-II gene based real-time PCR for identification and detection of *F. udum*, those assays were not validate with *F. acutatum*, another important wilt causing pigeonpea pathogen, which is almost undistinguishable morphologically with *F. udum* [22]. Keeping in view the present study was aimed to develop a rapid and sensitive RT-PCR assay using species-specific oligonucleotides complementary to the H3 gene for detecting *F. udum* and to evaluate these assays on diverse samples such as, artificially infected seedlings, plant materials and agricultural field samples. The specificity and suitability of these species-specific oligonucleotides were validated by using dot-blot hybridization, conventional and real-time PCR.

Materials and Methods

Fungal isolates and culture conditions

Different pathogenic isolates of *F. udum* including four reference cultures from different geographical origin infective on three different alternate hosts viz, *Cicer arietinum* (241426), *Crotalaria verrucosa*

***Corresponding author:** Sukumar Mesapogu, 1National Bureau of Agriculturally Important Microorganisms (NBAIM), Post Box 06 Maunath Bhanjan-275 101, Uttar Pradesh, India, Tel: +91 547 2530080; Fax: +91 547 2530358; E-mail: sukumarmm@yahoo.co.in

Received April 20, 2011; **Accepted** July 04, 2011; **Published** July 12, 2011

Citation: Mesapogu S, Babu BK, Bakshi A, Reddy SS, Saxena S (2011) Rapid detection and quantification of *Fusarium udum* in soil and plant samples using real-time PCR. J Plant Pathol Microbiol 2:107. doi:10.4172/2157-7471.1000107

(170431), *Cajanus indicus* (193652) and *Cajanus cajan* (1708) were obtained from the Centre for Agriculture and Biosciences International (CABI), U.K and National Agriculturally Important Microorganism Culture Collection (NAIMCC), India (Table 1). These cultures were referred all throughout the paper for validation. In all validation experiments, different tester microbes obtained from, India were used (Table 2). All fungal cultures were grown on potato dextrose agar (PDA) plates at 25±2°C while bacterial cultures were maintained on nutrient agar (NA) plates.

Preparation of seed samples

Conidial suspensions were prepared by culturing *F. udum* (193652) on PDA plates at 25±2°C under dark for 10 days. The surface of the fully grown culture plate was flooded with 2 mL of sterile distilled water (SDW) and agitated for 2 min. The conidial suspension was serially diluted to adjust 5×10^6 conidia mL^{-1} using haemocytometer. Pigeonpea seeds were disinfected with 1% NaOCl, followed by rinsing twice with SDW and seeds were soaked for 1h in a conidial suspension at different dilutions. To enrich the fungal culture on seed surface, the healthy seeds were placed in a sterile test tube (10 seeds tube per tube), mixed with 0.5 mL of liquid culture of MDP medium (2% malt extract, 2% dextrose, 0.1% peptone) and incubated at 25°C for 48h. The tubes were vortexed briefly and seed free suspension (mycelia and conidia) was collected to microcentrifuge tube through millipore filtration (0.45 µm).

Pigeonpea seeds (susceptible cultivar T-20) were sown in different pots (15 cm diameter) filled with garden soil. The pots were watered daily and maintained at 22±2°C with a 14h photoperiod in a greenhouse. Three weeks after sowing, the plants were injected with 50 µL of conidial suspension (5×10^6 conidia mL^{-1}) at the base of the stem. After 40 days of inoculation, root and stem samples from the plants showing typical symptoms of the infection were harvested. Three replicates were maintained for each set of experiment.

Extraction of Genomic DNA

Total genomic DNA, from fungal cultures was isolated using the protocol described earlier Babu et al. (2007). DNA from bacteria and actinomycetes was extracted using Wizard Genomic DNA Purification Kit (Promega, USA). For isolation of genomic DNA from *F. udum* infested soils and pigeonpea plant materials (artificially and naturally collected samples) was carried by using MoBio soil DNA isolation kit (MoBio Inc., CA) and DNeasy plant DNA extraction kit (Qiagen Inc., Canada) respectively and the samples were stored at -20°C for further use.

PCR amplification and sequencing of Histone-3 gene

The Histone-3 (H3) gene was amplified using the primer pair H_3-1a (5'-ACTAAGCAGACCGCCCGCAGG-3') and H_3-1b (5'-GCGGGCGAGCTGGATGTCCTT-3') and PCR was carried out in Dyad Peltier thermal cycler (BioRad, USA) in a 25µL volume with conditions as described earlier [35]. PCR amplified products were resolved on a 1.2% agarose gel containing ethidium bromide (0.2 µg mL^{-1}) and visualized under UV light. The amplified products from four reference strains of *F. udum* were gel extracted, purified and subjected for direct sequencing on an ABI automated DNA Sequencer using ABI Big Dye termination cycle sequencing ready reaction kit, following the manufacturers protocol.

Sequence analysis and design of specific oligonucleotides

All the H3 gene sequences were compared with the available H3

Sl. No.	Accession No.	Biological origin	Geographical location
1	CABI 241426	*Cicer arietinum*	Hyderabad, Andhra Pradesh
2	CABI 170431	*Crotalaria verrucosa*	Hyderabad, Andhra Pradesh
3	CABI 193652	*Cajanus indicus*	Hyderabad, Andhra Pradesh
4	NAIMCC-F-1708	*Cajanus cajan* (Stem)	Kanpur, Uttar Pradesh
5	NAIMCC-F-1703	*Cajanus cajan* (Stem)	Fatehpur, Uttar Pradesh
6	NAIMCC-F-556	*Cajanus cajan* (Stem)	Dholpur, Rajasthan
7	NAIMCC-F-557	*Cajanus cajan* (Stem)	Baran, Rajasthan
8	NAIMCC-F-563	*Cajanus cajan* (Stem)	Rohtak, Haryana
9	NAIMCC-F-1688	*Cajanus cajan* (Stem)	Banda, Uttar Pradesh
10	NAIMCC-F-234	*Cajanus cajan* (Stem)	Andhra Pradesh
11	NAIMCC-F-1686	*Cajanus cajan* (Stem)	Banda, Uttar Pradesh
12	NAIMCC-F-567	*Cajanus cajan* (Stem)	Bhiwani, Central Haryana
13	NAIMCC-F-1713	*Cajanus cajan* (Stem)	Varansi, Uttar Pradesh
14	NAIMCC-F-1687	*Cajanus cajan* (Stem)	Banda, Uttar Pradesh
15	NAIMCC-F-564	*Cajanus cajan* (Stem)	Thazzar, Eastern Haryana
16	NAIMCC-F-1211	*Cajanus cajan* (Root)	Tandawa, Jharkhand
17	NAIMCC-F-559	*Cajanus cajan* (Stem)	Faridkat, Central Punjab
18	NAIMCC-F-1213	*Cajanus cajan* (Stem)	Sangbaria, Jharkhand
19	NAIMCC-F-1215	*Cajanus cajan* (Stem)	Maurshidabad, West Bengal
20	NAIMCC-F-571	*Cajanus cajan* (Stem)	Faridabad, Haryana

Table 1: Cultures of *F. udum* collected from different hosts and various geographical locations of India.

Sl. No.	Name of the microorganism	NBAIM accession no.
1	*Fusarium acutatum*	NAIMCC-F-00760, CABI 375327
2	*Fusarium oxysporum*	NAIMCC-F-00810
3	*Fusarium oxysporum ciceri*	NAIMCC-F-00858, CABI 241424
4	*Fusarium oxysporum ciceri*	NAIMCC-F-00857, CABI 259237
5	*Fusarium oxysporum* f.sp. *carthami*	NAIMCC-F-00833
6	*Fusarium oxysporum* f.sp. *carthami*	NAIMCC-F-00834
7	*Fusarium solani*	NAIMCC-F-01028
8	*Fusarium proliferatum*	NAIMCC-F-00964
9	*Fusarium culmorum*	NAIMCC-F-00772
10	*Fusarium culmorum*	NAIMCC-F-00773
11	*Fusarium graminearum*	NAIMCC-F-00778
12	*Fusarium moliniformae*	NAIMCC-F-00789
13	*Macrophomina phaseolina*	NAIMCC-F-01260
14	*Neurospora crassa*	NAIMCC-F-01390
15	*Alternaria alternata*	NAIMCC-F-00072
16	*Alternaria brassicicola*	NAIMCC-F-00096
17	*Trichoderma viride*	NAIMCC-F-01808
18	*Aspergillus niger*	NAIMCC-F-00290
19	*Aspergillus parasiticus*	NAIMCC-F-00331
20	*Chaetomium globosum*	NAIMCC-F-00494
21	*Bacillus megaterium*	NAIMCC-B-00067
22	*Pseudomonas fluorescens*	NAIMCC-B-00323
23	*Pseudomonas putida*	NAIMCC-B-00325
24	*Streptomyces*	NAIMCC-B-00475
25	*Sinorhizobium meliloti*	NAIMCC-B-00471
26	*Streptomyces aminophilus*	NAIMCC-B-00483
27	*Serratia marcescens*	NAIMCC-B-00459
28	*Rhizobium* sp.	NAIMCC-B-00442
29	*Gluconobacter* sp.	NAIMCC-B-00301
30	*Nocardia*	NAIMCC-B-00315
31	*Mesorhizobium ciceri*	NAIMCC-B-00313
32	*Burkholderia cepacia*	NAIMCC-B-00273
33	*Lactobacillus acidophilus*	NAIMCC-B-00304
34	*Klebsiella pneumoniae*	NAIMCC-B-00302
35	*E. coli*	NAIMCC-B-00283
36	*Bacillus thuringiensis*	NAIMCC-B-00147

Table 2: Different groups of microorganisms used for the testing of specific oligonucleotide primers and probe.

Name of the DNA sample	Dot blot hybridization	Conventional PCR	Real-time PCR	
			Mean of C_T value	Log of DNA concentration (ng/µL)
Conidial suspension*	–	+	19.75	4.2
Pure culture #	+	+	14.00	100
Assay for Artificially infected samples				
Seedling (ISD1-3)	–	+	22.50	0.8
Plant root (IPR1-3)	–	+	21.75	1.5
Plant stem (IPS1-3)	weak signal	+	19.25	6.5
Assay for Agricultural field samples				
Plant root (APR1-3)	–	+	27.50	0.002
Plant stem (APS1-3)	–	+	26.75	0.021
Rhizosphere soil (ARS1-3)	–	+	33.25	0.0015

*-5×10^6 conidia mL^{-1}, + presence, − absence, #- *F. udum* 193652

Table 3: Evaluation of species specific probe and primers under three different assays.

gene sequences in the GenBank database using BLASTn program. Multiple H3 gene sequences from *F. udum* (241426, 170431, 193652, 1708) and other H3 gene reference sequences retrieved from GenBank database were aligned using Gene Doc (version 2.6.002) [27]. The sequence alignment was visually checked for regions having similarity among the isolates of *F. udum* and variable in those of other species. The specific locations thus obtained were subjected for Primer3 online software [32] to design a pair of PCR primers and a single oligonucleotide probe. The forward (HFUSF) and reverse (HFUSR1) primers were evaluated *in silico* to yield a product of 200 bp size. The theoretical specificity of the three oligonucleotides was checked against sequences of closely related fungi in GenBank. Parameters such as the % G+C content and the absence of self complementarity in oligonucleotides and complementarity between primers were analyzed using the computer program Gene Runner (Hastings Software, USA). The oligonucleotides were custom synthesized by Bangalore Genei, India.

Validation of the primers in standard PCR

The designed species-specific primers HFUSF and HFUSR1 were used for PCR amplification of *F. udum* isolates, other representative test microbes, infected seedlings, plant materials and agricultural field samples (Table 1-3). PCR protocol was standardized with variable cycle numbers, annealing temperatures and different concentrations of target DNA. The amplifications were carried out (25µL) by mixing 2.5µL of 10X PCR buffer, 0.2mM, 2.5mM each dNTPs, 2µL of 5% glycerol, 0.5µL of 10 mg mL^{-1} BSA, 0.5µL of 5% DMSO, 5pmol of each primer, 1 U of *Taq* DNA polymerase and 35ng of template DNA. The PCR reaction performed for 40 cycles of denaturation at 95°C for 1 min, followed by annealing at 54°C for 10 s, extension at 72°C for 20s and the final extension step of 72°C for 10 min.

Dot blot hybridization assay

The oligonucleotide probe HFUSP (3'-CGCCACATCAACCA-CAGCTCAACACT-5') was labelled with digoxigenin-3-O-methylcarbonyl-amino-caproyl-5-(3-aminoallyl)-uridine-5-triphosphate (DIG-11-dUTP) using DIG DNA labeling kit (Roche Diagnostics, Germany), following manufacturer's instructions. Initially, the DIG labelled probe specificity was tested with the genomic DNA of different strains of *F. udum* (Table 1). Later the dot blot hybridization assay was carried out for genomic DNA from different test microbes and agricultural field samples (Tables 2 & 3) as unknown target and genomic DNA from four reference strains of *F. udum* were used as control. All target DNA samples irrespective of their concentrations (4-8µL) were blotted manually onto positively charged nylon membrane (Boehringer Mannheim). Pre-hybridization treatments for membrane such as denaturation, neutralization and cross-linking were carried out as described by [2]. The assay was optimized by adjusting the conditions like: hybridization temperature, concentration of the probe (0.5–2 pmol mL^{-1}) and salt concentration of the wash buffer were determined as described elsewhere [10]. Detection of the hybridized DIG labeled probes were carried out and then visualized with the colorimetric substrates NBT/BCIP. Dot blot hybridization assay was performed at least thrice under the optimized conditions.

Specific real-time PCR assay for *F. udum*

RT-PCR assay was performed with Step-One™ real-time PCR system (Applied Biosystems, USA) using SYBR green fluorescent molecules. All reactions were performed in 0.5mL thin-walled, optical grade PCR tubes with a reaction volume of 20µL. The reaction mixture consists of 10µL of 2X SYBR® Green I Master Mix, 900nM of each primer (HFUSF and HFUSR1), 2µL of DNA template and water to the final volume. The amplification conditions of the reaction were set at 95°C (10 min), 40 cycles at 94°C (15s), 54°C (10 s), and 72°C (20s), and fluorescence read at 72°C at the end of each cycle, followed by final melting curve analysis at 65-95°C with an increment of 0.1°C s^{-1}. Determination of cycle threshold (C_T) and data analysis were carried out with the help of Sequence Detection System Software v.1.2, provided by the Applied Biosystems, USA. The specificity of SYBR green assay was verified using different *F. udum* isolates obtained from various agro-climatic regions, along with other tester microbes and agricultural field samples (Tables 1-3). The DNA extractions were preformed as described above. Template DNA (5-60ng) added to the reaction mixture, *F. udum* genomic DNA used as positive control and DNA from other test microbes and agricultural field samples were used as unknown targets.

Sensitivity and standard curve analysis

Genomic DNA from *F. udum* (241426) with an estimated initial concentration of 100ng $µL^{-1}$ was serially diluted (1:10) with SDW. The results were analyzed by plotting the Log of template concentration against C_T values. The sensitivity or minimum detection limit of the assay was estimated so as to quantify and detect the lowest amount of target DNA, when the cycle threshold was being attained up to 40 cycles.

Detection of *F. udum* in seedlings, plant and agricultural samples

Artificially infected seed and plant samples were prepared and their DNA was extracted as mentioned above. *Fusarium* infected pigeonpea plant and rhizospheric soil samples were collected from long-term pigeonpea growing agricultural fields of Kusmaur (25°53'57"N, 83°29'01"E), Mau district, Uttar Pradesh, India. The infected rhizosphere soil and plant material DNA was extracted as mentioned above. The extracted DNA from each sample was used as unknown targets for identification and detection of *F. udum*. Genomic DNA of *F. udum* (193652) was used as positive control while healthy and uninfected (pigeonpea) plant DNA was used as negative control. The RT-PCR assay for standard graph was performed under the similar conditions as described above.

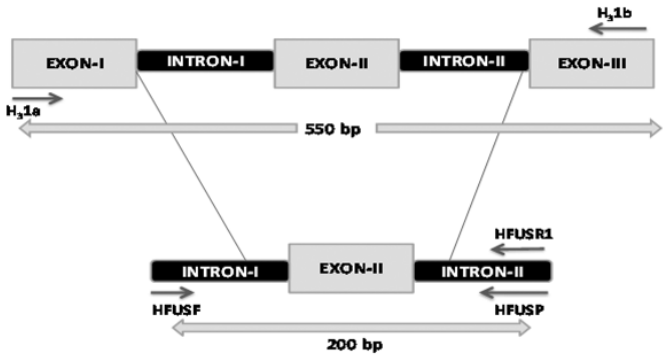

Figure 1: Schematic representation of partial Histone-3 gene: $H_3$1a and $H_3$1b represent the universal primers used for partial amplification of Histone-3 gene. HFUSF and HFUSR1 indicate the designed species-specific primers and probe for *F. udum*.

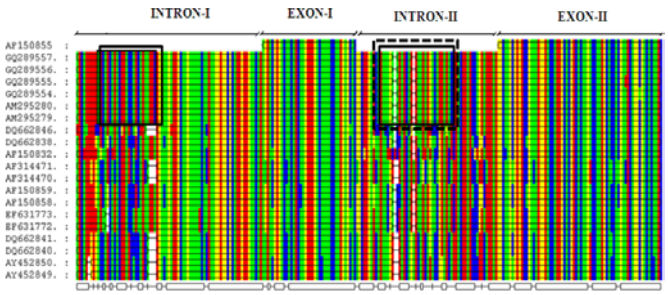

Figure 2: Development of specific oligonucleotide primers and probe: Alignment of partial H3 sequences from four isolates of *F. udum* (in this study) and other *Fusarium* reference sequences were taken from GenBank. Nucleotides are shown in colour bars (A-red, G-yellow, T-blue and C-green). The solid line rectangles indicate specific nucleotide regions used for the development of specific oligonucleotide primers and the dashed rectangles shows the specific region used for designing of the probe.

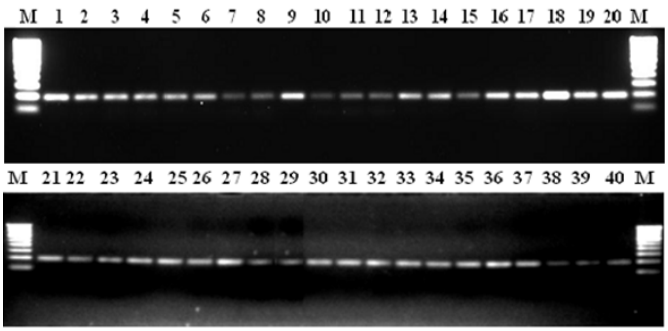

Figure 3: Species specific PCR Amplification of H3 gene cluster: Primers HFUSF and HFUSR1 were used for amplification of 200 bp fragment of H3 gene cluster of *F. udum* from different samples. (a) Lane 1 to 20 are *F. udum* isolates listed in the Table 1, (b) Lane 21-conidial suspension, Lane 22-pure culture, Lane 23,24 infected seedlings (ISD1-3), Lane 26–28 infected plant root (IPR1-3), Lane 29–31 Infected plant stem (IPS1-3), Lane 32–34 Agricultural plant root (APR1-3), Lane 35–37 Agricultural plant stem (APS1-3), Lane 37–40 Agricultural rhizosphere soils (ARS1-3) as listed in Table 3. M-1 kb molecular ladder.

Results

Histone-3 amplification and sequence analysis

Partial Histone-3 gene (nearly about 550 bp) was amplified from all the isolates of *F. udum* using H3 universal fungal primers. The partial sequence of H3 gene consists of three exons, which were separated by two intron regions (Figure 1). Multiple sequence alignment of H3 region of the four representative isolates with other related fungal species revealed that the two intron regions were conserved in *F. udum* isolates and variable among the other related fungi. The edited H3 gene sequences have been deposited in GenBank and accession numbers were obtained (GQ289554 – GQ289557). After editing and realignment of H3 gene sequence the two intron regions were selected

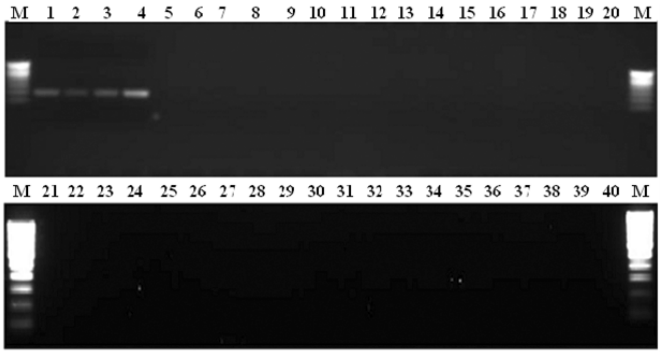

Figure 4: Validation of Species-specific primers (HFUSF and HFUSR1) : PCR amplification of *F. udum* strains (241426, 170431, 193652 and 1708) giving 200 bp in lane 1-4. Lane 5– 40 showing no amplified product with different test microbes listed in the Table 2. M-1 kb DNA ladder.

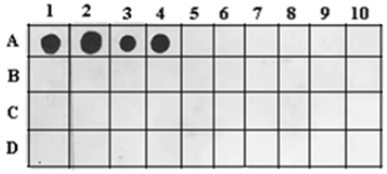

Figure 5: Dot blot hybridization: Hybridization of oligonucleotide probe HFUSP with genomic DNA from representative microbial groups. Lane A1 to A4 representing positive controls (241426, 170431, 193652 and 1708), A5 to D10 were test microbes listed in the Table 2.

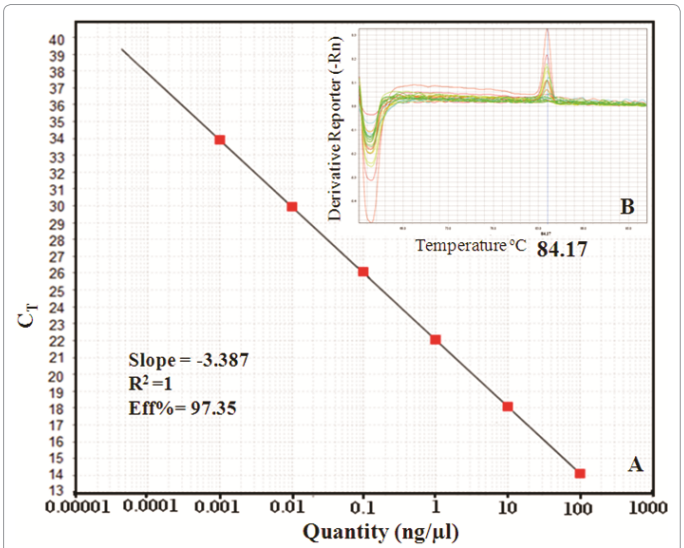

Figure 6: Standard curve Analysis: a. Standard curve of *F. udum* (CABI 241426) DNA concentration standards against the cycle threshold (CT). **b. Melting Curve Analysis:** Melting temperature (Tm) at 84.17 with a single peak indicates the specificity of the species specific the primers HFUSK and HFUSR1 to *F. udum*.

for development of species-specific oligonucleotides. The forward primer HFUSF (5'-ATCATCACTAACTTCATCACCAAT-3') was designed from intron-I region, while reverse primer HFUSR1 (3'-TGTCGAATGTTAGTAAGTGTTG-5') and one probe of 26mer HFUSP (3'-CGCCACATCAACCACAGCTCAACACT-5') were designed from intron-II (Figure 2).

Specificity of primers by standard PCR

In conventional PCR all the isolates of *F. udum*, artificially infected seedlings, infested plant and soil samples (artificially and naturally) from green house and agricultural field samples of pigeonpea yielded a single amplified product of 200bp (Figure 3). The primers were specific for *F. udum* as none of the other tester microbes exhibited any amplification (Figure 4, Table 2).

Conditions for specific hybridization of probe

Detectable hybridization signals were obtained with the oligonucleotide probe at 1.5pmol mL^{-1} concentration; and optimum hybridization temperature was 43°C for 15h. The variable parameters were fixed at optimum as mention: membrane washing twice, 5 min per wash, in 2X SSC containing 0.1% of SDS, then twice, 5 min per wash, in 0.1× SSC, 0.1% SDS, followed by 15 min per wash per two times, in 0.1× SSC containing 0.1% SDS (20±2°C). The dot blot assay carried out with 5µL of genomic DNA allowed the specific detection of all the *F. udum* strains. Probe HFUSP selectively hybridized with strains of *F. udum* but failed to do so with all other tester microbes (Figure 5; Table 2). In case of DNA samples obtained from artificially infected seedlings, plant material and naturally infected field samples the probe fails to produce detectable signals, exception with plant sample (IPS1) where a weak signal was obtained (Data not shown) (Table 3).

Sensitivity and standard curve analysis

Under optimized conditions the RT-PCR assay showed standard fluorescence amplification representing exponential growth of PCR products and a standard curve was obtained at least 6 orders of magnitude. The lowest detection limit of the assay was 1 pg, obtained at C_T value of 34.0. The standard curve revealed that the primer set used in the present study was quite accurate over a linear range and high correlations between C_T and DNA quantities were obtained with a slope of -3.387 and R^2 =1. Amplification efficiency of the target gene was 97% among all the samples used in this study (Figure 6a). The mean C_T values recorded for the DNA samples obtained from reference strain of *F. udum*, conidial suspension, artificially infected pigeonpea seedlings, plant materials (root and stem), and naturally obtained diseased pigeonpea plants and rhizospheric soil batches ranged from 14.00 to 33.25. While the mean limit of detection ranged from1.5 pg to 100ng (Table 3). The real time PCR SYBR green assay enabled specific detection of *F. udum* with a single melting curve at 84.17°C without cross detecting any of the representative tester microbes (Figure 6b).

Discussion

Molecular diagnostics for plant pathogens are important to detect fungal contaminants in crops, commodities and also act as valuable tools for plant quarantine purposes [5,38]. However, quantitative estimation of a pathogenic population is also essential to investigate the ecology and epidemiology of disease, which leads pathologists to monitor harmful microbial populations spread and distribution over time and space [24]. In particular, when plant diseases caused by any member of Fusaria species complex, it is very crucial to diagnose or quantify such an individual population to develop disease management strategies and breeding for resistance programs. Moreover, accurate and rapid tools are required to investigate and monitor the global spreading and disease aggressiveness. Early identification of pathogen is very crucial for framing disease management strategies and breeding programs. For example, selection and breeding for resistance in cereals are facilitated by the use of species specific markers to diagnose the various fungi implicated in *Fusarium* ear blight [30,34]. The situation for *F. udum* is equally complex as this species was associated with a wide range of diseases in diverse plant species under diverse agro-climatic regions [6].

In the present study, we targeted on rRNA, β-tubulin, TEF1-α and Histone-3 genes to design and develop species-specific probe and primers for *F. udum*. Sequencing of each gene, followed by multiple sequence alignment revealed that the rRNA and β-tubulin genes exhibited minimum variability to differentiate *Fusarium* sp. to that of *F. udum*. Further, the sequence alignment of TEF1-α have multiple variations within the isolates of *F.udum* (data not shown). Therefore, these genes were not considered as suitable targets to design species-specific primers and probes. However, partial sequences of Histone-3 gene showed that this gene was conserved among the isolates of *F. udum* and showed sufficient variability amongst the *Fusarium* sp. The Histone-3 gene was conserved among the isolates of *F. udum* and showed significant variability with *F. oxysporum* sp. *ciceri*. Moreover, in BLASTn analysis of the primers and probe showed 100% similarity with *G. indica,* an alternate teleomorph of *F. udum* [37] while no significant similarity was observed with its closely related *F. xylarioides* and *F. acutatum* sequences [22]. Thus, oligonucleotide primers (HFUSF and HFUSR1) and probe (HFUSP) can be used for specific amplification and detection of *F. udum* and its telomorph *G.indica.*

The genetic diversity analysis of *F. udum* revealed high degree of genetic diversity among populations [18]. Therefore, in present study, the real-time PCR assays have been validated with many target organisms along with tester microbes. Similarly, in our previous study also we have been included an array of target species to validated specificity of the real-time PCR assays [3]. PCR Optimization especially of the annealing temperature at 54°C for 10s allowed specific amplification of *F. udum* among all the isolates analyzed. Thus, the protocol developed in our study was tested with twenty different isolates of *F. udum* from various biological and geographical origins. Further, in dot bolt assay the probe HFUSP showed high specificity towards the target DNA and produced strong signals with pure cultures expect with agricultural field sample (IPS1), where weak signals were obtained. This might be because of the low concentrations or may be due to the short length of the probe (26 mer). On the other hand the probe did not show any false positive or non-specific hybridization with other representative microbial isolates (Table 2). Our results clearly demonstrated that *F. udum* could be differentiated from that of other *Fusarium* species, using these specific primers and probe. Recently, several researchers demonstrated this approach for the detection of *Fusarium* sp. in different hosts [25,28]. As evident from the RT-PCR studies, the sample IPS1 consists of 6.5ng $\mu L^{-1}$of targeted DNA (Table 3), while a weak signal was observed on dot blot analysis. The efficiency may be used as a rough estimate for how well real-time PCR works in a given sample type. When the results of the H3-targeted dot-blot hybridization was compared, a reasonable level of sensitivity was obtained using a standard conventional PCR, while real-time PCR had a superior sensitivity and also proved to be a more convenient and less expensive method for identification and quantification of *F. udum* populations. Thus, the developed RT-PCR assay can be considered as far more superior and novel tool for detecting *F. udum* especially from that of its closely related species

like *F. acutatum* infecting the same host and also for quantification of this pathogen in soil and plant tissue at picogram level of target DNA. The specific primers and probe generated in the present study could be further exploited in epidemiological studies of *F. udum*. In conclusion, the novel detection system developed in the present study will be a useful tool in monitoring early infection of *F. udum* in disease outbreaks and can be helpful for formers to administrate disease management practices.

Acknowledgements

We thank Indian Council of Agricultural Research, New Delhi, India for providing financial assistance under the Application of Microbes in Agriculture and Allied Sectors (AMAAS) project.

References

1. Baayen RP (2000) Diagnosis and detection of host-specific forms of *Fusarium oxysporum*. *EPPO Bulletin* 30: 489–491.
2. Babu KB, Saxena AK, Srivastava AK, Arora DK (2007) Identification and detection of *Macrophomina phaseolina* by using species-specific primers and probe. *Mycologia* 99: 797–803.
3. Babu BK, Mesapogu S, Sharma A, Reddy SS and Arora DK (2011) Quantitative Real-Time PCR assay for rapid detection of plant and human pathogenic *Macrophomina phaseolina* from field and environmental samples. Mycologia 103: 466-473.
4. Bogale M, Wingfield BD, Wingfield MJ, Steenkamp ET (2007) Species-specific primers for *Fusarium redolens* and a PCR-RFLP technique to distinguish among three clades of *Fusarium oxysporum*. *FEMS Microbiol Lett* 271: 27–32.
5. Boonham N, Glover R, Tomlinson J, Mumford R (2008) Exploiting generic platform technologies for the detection and identification of plant pathogens. *European Journal of Plant Pathology* 121: 355–363.
6. Booth C (1978). *Fusarium udum* Descriptions of Pathogenic Fungi and Bacteria 575. Commonwealth Mycological Institute Kew, U.K.
7. Brandfass C, Karlovsky P (2006). Simultaneous detection of *F. culmorum* and *F. graminearum* in plant material by duplex PCR with melting curve analysis. *BMC Microbiol* 6: 4.
8. Butler EJ (1906) The wilt disease of pigeonpea and pepper. Agricult J India 1:25–26.
9. Demeke T, Clear RM, Patrick SK, Gaba D (2005) Species-specific PCR-based assays for the detection of *Fusarium* spp. and a comparison with the whole seed agar plate method and trichothecene analysis. *Int J Food Microbiol* 103: 271–284.
10. Edel V, Steinberg C, Gautheron N, Alabouvette C (2000) Ribosomal DNA targeted oligonucleotide probe and PCR assay specific for *Fusarium oxysporum*. *Mycological Research* 104: 518–526.
11. Filion M, Arnaud St M, Jabaji-hare S.H (2003) Direct quantification of fungal DNA from soil substrate using real-time PCR. J Microbiol Meth 53: 67-76.
12. Gayatri G, Barve M, Giri A, Gupta V (2009) Identification of Indian pathogenic races of *Fusarium oxysporum f. sp. ciceris* with gene specific, ITS and random markers. *Mycologia* 101: 484–495.
13. Geiser DM, Jemenez-Gasco DM, Kang S, Makalowska I, Veeraraghavan N, et al. (2004) *Fusarium*-ID v.1.0: a DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology* 110: 473–479.
14. Hatsch D, Phalip V, Jeltsch JM (2002) Development of a bipartite method for *Fusarium* identification based on cellobiohydrolase-C: CAPS and Western blot analysis. *FEMS Microbiol Lett* 213: 245–249.
15. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real-time quantitative PCR. *Genome Res* 6: 986–994.
16. Jimenez-gasco MM, Jimenez-diaz RM (2003) Development of a specific polymerase chain reaction-based assay for the identification of *Fusarium oxysporum* f. sp. *ciceris* and its pathogenic races 0, IA, 5, and 6. *Phytopathology* 93: 200–209.
17. Kannaiyan J, Nene YL, Reddy MV, Ryan JG, Raju TN (1984) Prevalence of pigeonpea diseases and associated crop losses in Asia, Africa and Americans. *Tropical Pest Management* 30: 62–71.
18. Kiprop EK, Baudoin JP, Mwang'ombe AW, Kimani PM, Mergeai G, et al. (2002) Characterization of Kenyan Isolates of *Fusarium udum* from Pigeonpea [*Cajanus cajan* (L.) Millsp.] by Cultural Characteristics, Aggressiveness and AFLP Analysis. *Journal of Phytopathology* 150: 517–525.
19. Kristensen R, Berdal KG, Holst-Jensen A (2007). Simultaneous detection and identification of trichothecene- and moniliformin-producing *Fusarium* species based on multiplex SNP analysis. *J Appl Microbiol* 102: 1071–1081.
20. Kulik T, Fordoński G, Pszczółkowska A, Płodzień K, Łapiński M (2004) Development of PCR assay based on ITS2 rDNA polymorphism for the detection and differentiation of *F. sporotrichioides*. *FEMS Microbiol Lett* 239: 181–186.
21. Kulik T, Pszczólkowska A, Fordonski G, Olszewski J (2007) PCR approach based on the esyn1 gene for the detection of potential enniatin-producing *Fusarium* species, *Int J of Food Microbiol* 116: 319–324.
22. Kvas M., Marasas WFO, Wingfield BD, Wingfield MJ, Steenkamp ET (2009). Diversity and evolution of *Fusarium* species in the *Gibberella fujikuroi* complex. *Fungal Diversity* 34: 1–21.
23. Lievens B, Brouwer M, Vanachter AC, Levesque CA, Cammue BP, et al. (2003) Design and development of a DNA array for rapid detection and identification of multiple tomato vascular wilt pathogens. *FEMS Microbiol Lett* 223: 113–122.
24. López MM, Llop P, Olmos A, Marco-Noales E, Cambra M, et al. (2009) Are Molecular Tools Solving the Challenges Posed by Detection of Plant Pathogenic Bacteria and Viruses? *Curr Issues in Mol Biol* 11: 13–46.
25. López-Errasquín E, Vázquez **C**, Jiménez M and González-Jaén MT (2007) Real-Time RT-PCR assay to quantify the expression of fum1 and fum19 genes from the Fumonisin-producing *Fusarium verticillioides*. *J Microbiol Methods* 68: 312–317.
26. Moricca S, Ragazzi A, Kasuga T, Mitchelson KR (1998) Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant Pathology* 47: 486–494.
27. Nicholas KB, Nicholas HB Jr, David W. Deerfield II (1997) Gene Doc: Analysis and Visualization of Genetic Variation.
28. Nicolaisena M, Supronienb S, Nielsena LK, Lazzaroa I, Spliida NH, et al. (2009). Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *J of Microbiol Methods* 76: 234–240.
29. Okubara PA, Schroeder KL, Paulitz TC, (2005) Real-time polymerase chain reaction: applications to studies on soilborne pathogens. *Canadian Journal of Plant Pathology* 27: 300–313.
30. Ramsfield TD, Dobbie K, Dick MA, Ball RD (2007) A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and f. sp. *radicis-cucumerinum*. *Environ Microbiol* 9: 2145–2161.
31. Lievens B, Claes L, Vakalounakis DJ, Vanachter AC, Thomma BP (2007) A robust identification and detection assay to discriminate the cucumber pathogens *Fusarium oxysporum* f. sp. *cucumerinum* and f. sp. *radicis-cucumerinum*. *Environ Microbiol* 9: 2145–2161.
32. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods Mol Biol* 132: 365–386.
33. Sarlin T, Tapani Y, Marika J, Aldo R, Sari P, et al. (2006) Real-time PCR for Quantification of Toxigenic *Fusarium* spp. in Barley and Malt. *European Journal of Plant Pathology* 114: 371–380.
34. Schilling AG, Miller EM, Geiger HH (1996) Polymerase chain reaction-based assays for species-specific detection of *Fusarium culmorum, F. graminearum,* and *F. avenaceum*. *Phytopathology* 86: 515–522.
35. Steenkamp ET, Wingfield BD, Coutinho TA, Wingfield MJ, Marasas WF (1999) Differentiation of *Fusarium subglutinans* f. sp. *pini* by Histone Gene Sequence Data. *Appl Environ Microbiol* 65: 3401–3406.
36. Strausbaugh CA, Overturf K, Koehn AC (2005) Pathogenicity and real-time PCR detection of *Fusarium* spp. in wheat and barley roots. *Canadian Journal of Plant Pathology* 27: 430–438.
37. Upadhyay RS, Rai B (1992). Wilt of pigeonpea. In: *Plant Diseases of. International Importance: Diseases of Cereals and Pulses*. A. N. Singh, Mukhopadhyay J, Kumar J, Chaube HS, eds., Prentice-Hall, Englewood Cliffs, NJ, I: 389-414.

38. Waalwijk C, van der Heide R, de Vries I, van der Lee T, Schoen C, et al. (2004) Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology* 110: 481–494.

39. Yadav MK, Babu BK, Saxena AK, Singh BP, Singh K, et al. (2011) Real-time PCR assay based on Topoisomerase- II Gene for Detection of *Fusarium udum*. *Mycopathologia* 171: 373-381.