

Analysis of Genetic Diversity Amongst *Fusarium* spp. Associated with Root Rot of Apple

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The Genus *Fusarium* is associated with crop diseases of horticultural and agricultural commodities and is considered to be a destructive pathogen. Genetic diversity among ten different isolates of *Fusarium* species isolated from apple rhizosphere from different locations of Himachal Pradesh was analysed using Random Amplified Polymorphic DNA (RAPD-PCR). Molecular characterization using ITS sequencing revealed that out of 10 isolates of *Fusarium*, three belongs to *F. oxysporum*, two belongs to *F. solani*, one belongs to *F. equiseti* and four were *Fusarium* species. Seven random primers were used for their genetic characterization using RAPD analysis. The dendrogram obtained from the UPGMA study characterized ten isolates into one main cluster and nine independent branches on the basis of similarity value 0.65.

Key words: *Fusarium*, Apple, RAPD, ITS, TEF.

Fusarium is one of the devastating phytopathogenic fungi belongs to Division *Ascomycota*, Class: *Sordariomycetes*, Order *Hypocreales*, Family: *Nectriaceae*. This filamentous fungi, can smite any crop since having a broad host range including rice, wheat, horticultural crops, ornamentals and in almost all agricultural commodities (Supyani and Widadi, 2015). Species of *Fusarium* serving as pathogens to many diseases in crops such as vascular wilt, root rot, corm rot, damping-off, yellows, and others. Mostly *Fusarium* species are regarded as soilborne fungi because of their abundance in soil and their frequent association with plant roots, as parasites and saprophytes. However, many have active or passive means of dispersal in the

atmosphere and are common in colonizers of aerial plant parts, where they may result in diseases of substantial economic importance. *Fusarium* rot on apples by *Fusarium* species (Riad and Zeiden, 2015), Sugarcane wilt by *Fusarium sacchari* (Lin *et al.*, 2014), Pokkah Boeng in sugarcane by *Fusarium moniliforme* (Vishwakarma *et al.*, 2013), Bakanae in rice by *Fusarium fujikuroi* (Jain *et al.*, 2014), Oil palm wilt by *Fusarium oxysporum* f.sp. *elaidis* (Rusli *et al.*, 2015), Panama disease by *Fusarium oxysporum* f.sp. *cubensis* (Zhang *et al.*, 2013), are some important diseases caused by *Fusarium* spp.

Fusarium taxonomy is very complex and over the last 100 years has persistently been rationalized. The traditional diagnostic method based only on morphological characteristics observed on selective media under specific incubation conditions for detection and identification of *Fusarium* species. Considerable expertise is required for morphological identification while differentiating closely related

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species of *Fusarium* as their morphological features may overlap. Molecular biology has brought many powerful tools to fungal taxonomists including method for identification of isolates, means to illuminate the relationships among fungal species. To define fungal populations at species, intraspecific, race and strain levels, RAPD assays have been extensively used (Miller, 1996; Ingle *et al.*, 2009) and for detecting genetic variability RAPD-PCR technique is also used (Edwards *et al.*, 2002; Sabir, 2006).

The present study was aimed to estimate genetic relatedness among the *Fusarium* isolates, isolated from apple rhizosphere from different locations of Himachal Pradesh using RAPD markers.

MATERIAL AND METHOD

DNA isolation

Thirteen isolates of *Fusarium* species collected from different locations of Shimla, Kullu and Mandi were cultured on potato dextrose agar (PDA) plates containing 1 µg/ml streptomycin, incubated at 27°C for 3-5 days. The mycelia grown were harvested and total DNA was extracted using protocol described by Sambrook *et al.*, 1989. Crushing of harvested mycelia was done in 1-2 ml of extraction buffer (100mM Tris HCl, 50mM EDTA, 500mM NaCl, 0.01% β-mercaptoethanol). 130 µl of 10% sodium dodecyl sulphate (SDS) was added to the mixture per ml of extraction buffer and incubated at 65°C for 15 minutes. The sample was centrifuged at 8,000 rpm for 10 min. To the supernatant, equal volume of phenol/chloroform (1:1) was added, mixed thoroughly and centrifuged at 10,000 rpm for 10 min. Aqueous phase containing nucleic acid was collected and 2.5 volume of absolute ethanol was added to precipitate the nucleic acid. Sample was centrifuged at 12,000 rpm for 20 min to pellet down the precipitates. The pellet was then washed with 70% ethanol, air dried and resuspended in 50 µl of 10X TE buffer (10mM Tris HCl, 50mM EDTA). Bands corresponding to genomic DNA were observed by performing electrophoresis in 1% agarose gel.

Molecular characterization of fungal isolates by PCR

The ITS region and Transcription

elongation of the isolates was amplified with primers ITS-Fu-F (5'-CAACTCCCAAACCCCTGTGA-3'), ITS-Fu-R (5'-GCGACGATTACCAGTAACGA-3'), TEF-Fu3F (5'-GGTATCGACAAGCGAACCAT-3'), TEF-Fu3R (5'-TAGTAGCGGGAGTCTCGAA-3'), ITS-Fu1F (5'-ACAACCTCATAACCCTGTGAACAT-3'), ITS-Fu1R (5'-CAGAAGTTGGGTGTTTTACGG-3') (Arif *et al.*, 2012). PCR was conducted in 20 µl mixture containing 1 µl DNA (~50ng), 1.5mM dNTP mix, 10 pmole each of upstream and downstream primers and 0.045 units of Taq polymerase (Banglore Genei) with 1X buffer. Cycling conditions for PCR were: initial denaturation at 95°C for 3 min, 35 cycles at 95°C for 1 min, 46°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 10 min. The PCR product was analyzed by agarose gel electrophoresis.

PCR Purification of Amplified DNA Product

PCR amplified DNA products were purified from gel using gel purification kit (DNA gel/PCR purification miniprep kit, XcelGen) as per the instructions provided in the instruction manual.

Sequencing and Sequence analysis

The eluted DNA was sent to Xcelris™ lab Pvt Ltd (Ahmedabad) for sequencing. Sequence data obtained was analysed using BLAST software. Sequences obtained were submitted to European Nucleotide Archive and accession numbers were obtained.

Diversity Analyses of Isolated Fungal Strains Using Random Amplified Polymorphic DNA (RAPD)

Isolated DNA (Sambrook *et al.*, 1989) from *Fusarium* species was used to study the genetic variation using RAPD. The standard RAPD reaction was set up by using standard RAPD primers. Seven RAPD primers [TC-01 (5'-GGAGTACTGG-3'), TC-03 (5'-GAGTCTCAGG-3'), OPA-01 (5'-CAGGCCCTTC-3'), OPA-14 (5'-TCTGTGCTGG-3'), OPA-17 (5'-GACCGCTTGT-3'), OPG-5 (5'-CTGAGACGGA-3'), and OPC-13 (5'-AAGCCTCGTC-3')] were used in this study. PCR was conducted in 25 µl reaction mixture containing 1 µl DNA (~50ng), 2mM dNTP mix, 2 µl of random primers and 0.045 units of Taq polymerase (Banglore Genei) with 1X buffer. Cycling conditions for PCR were: initial denaturation at 94°C for 5 min, 40 cycles at 94°C for 30 sec, 36°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 7 min. The PCR product was analyzed by agarose gel

electrophoresis.

RAPD product scoring and data analysis

Data was compiled as binary 0-1 matrix, (1) represented the presence of a band and (0) the absence of a band at a particular position. All RAPD bands were considered in statistical analysis. Dendrogram was produced from the distance matrix by Unweighted Pair-Grouped Method by Arithmetic average, contained in the software package NTsys 2.2 version.

RESULTS AND DISCUSSION

DNA isolation

To study the diversity among the *Fusarium* species, samples were collected from apple rhizosphere in orchards of Himachal Pradesh, India.

All the strains have been purified by growing the single spores on PDA plate and DNA of thirteen isolates was extracted manually using phenol/chloroform method in a good quantity.

Molecular Characterization

Isolated *Fusarium* strains were confirmed by molecular characteristics i.e. by PCR amplification of ITS region and transcription elongation factor - alpha, recommended as a

universal DNA barcode marker for fungi (Schoch *et al.*, 2012). For a single-locus identification in *Fusarium*, TEF markers has become a choice (Geiser *et al.*, 2004). Three primers sets: ITS FuF-FuR, ITS Fu1F-Fu1R corresponding to ITS region and TEF Fu3F-Fu3R corresponding to transcription elongation factor - alpha region showed amplification at 400 bp which confirmed that ten of the thirteen isolates under study were *Fusarium* (Table 1). Arif *et al.*, (2012) designed the primers for ITS, TEF for rapid detection of genus *Fusarium* and *F. solani*. The primers had shown accurate identification and discrimination of genus *Fusarium* and *F. solani*, which have number of applications in screening of infected plants, disease diagnosing and breeding programs. Purified PCR product of primers ITS FuF-FuR and TEF Fu3F-Fu3R of ten fungal isolates were sent to Xcelris™ lab Pvt. Ltd. for sequencing. Sequence data obtained was analysed using BLAST software and found that out of ten *Fusarium* isolates three isolates (isolate 4, isolate 5 and isolate 12) belongs to *F. oxysporum*, two isolates (isolate 2 and isolate 13) belongs to *F. solani*, one isolate (isolate 9) to be *F. equiseti* and other four belongs to strains of *Fusarium* species. The sequences were assigned accession numbers by submitting to European

Table 1. PCR amplification of fungal isolates with ITS and TEF primers

Primer name	Name of isolates												
	1	2	3	4	5	6	7	8	9	10	11	12	13
ITS FuF-FuR	+	+	-	+	+	-	+	+	+	+	-	+	+
ITS Fu1F-Fu1R	+	+	-	+	+	-	+	+	+	+	-	+	+
TEF Fu3F-Fu3R	+	+	-	+	+	-	+	+	+	+	-	+	+

(+ indicates amplification, - indicates non amplification)

Table 2. Sequence of primers, number and size of fragments of *Fusarium* species amplified by RAPD primers

Sr No	Primers	Sequences 5'-3'	Amplified fragments	Polymorphic fragments	Maximum band size (bp)	Minimum band size (bp)
1	OPA 1	GGAGTACTGG	28	7	1750	450
2	OPA 14	GAGTCTCAGG	20	5	1000	250
3	OPA 17	CAGGCCCTTC	15	6	910	400
4	TC 1	TCTGTGCTGG	21	5	1700	400
5	TC 3	GACCGCTTGT	17	3	1250	480
6	OPG 5	CTGAGACGGA	43	6	1820	460
7	OPC 13	AAGCCTCGTC	41	6	2960	400

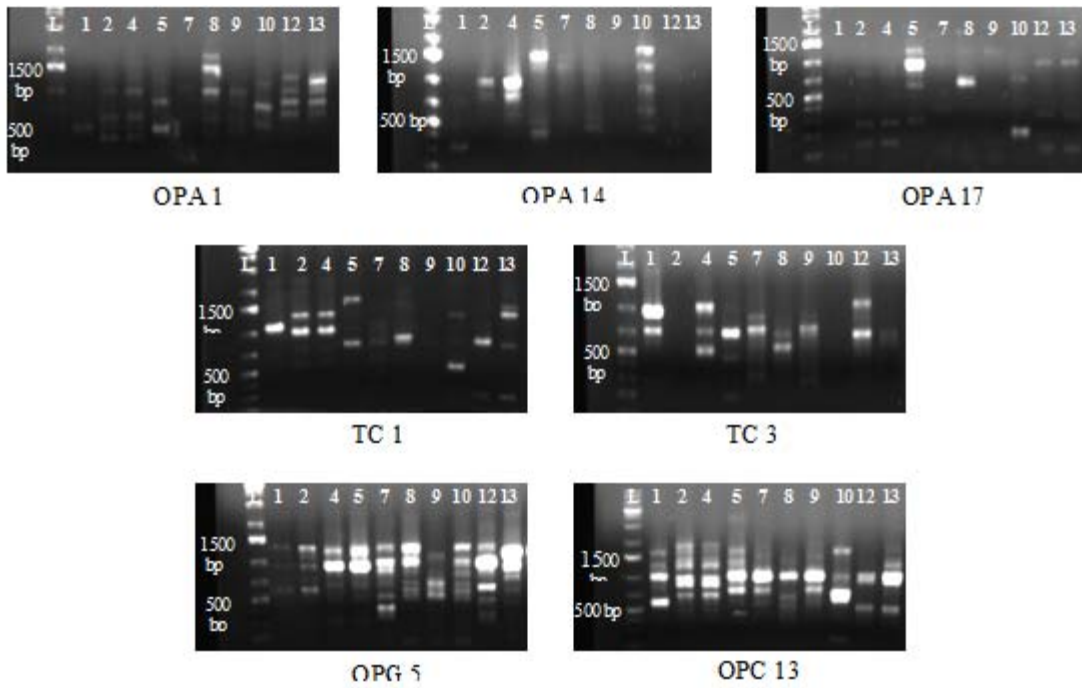


Fig. 1. RAPD banding patterns of ten *Fusarium* isolates using seven selected random primers on 1% agarose gel. lane L: ladder 1kb plus, lane 1-13 ten isolates of *Fusarium* species, respectively

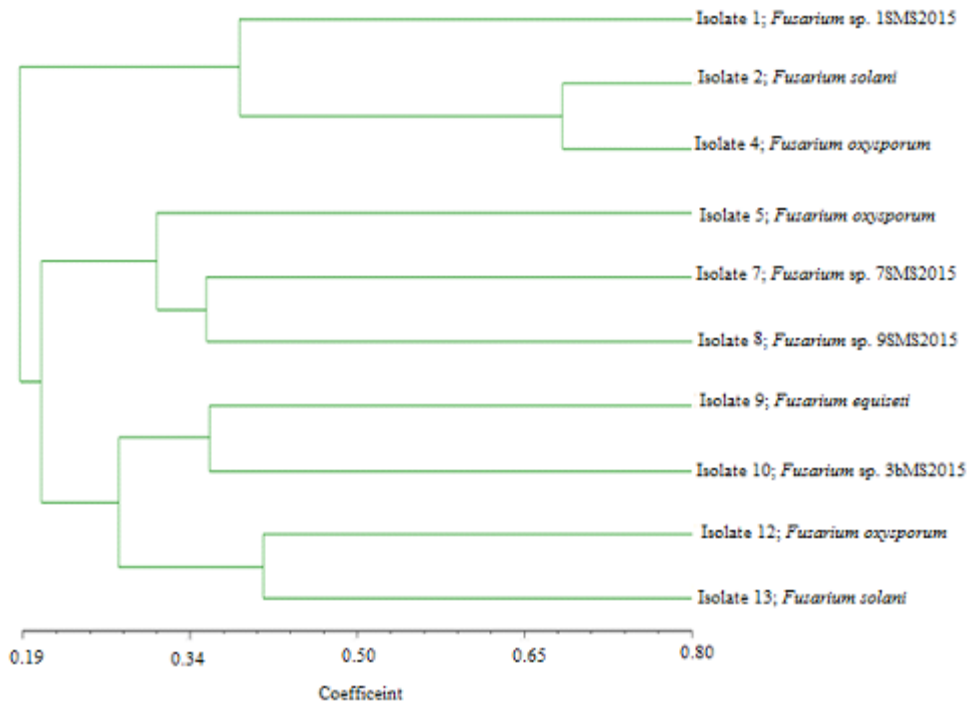


Fig. 2. Dendrogram of *Fusarium* species derived from RAPD fingerprints generated by using seven different primers produced from the distance matrix by Unweighted Pair-Grouped Method by Arithmetic average, contained in the software package NTsys 2.2 version

Nucleotide Archive the entries permanently available from the ENA browser at <http://www.ebi.ac.uk/ena/data/view/LN886527-LN886536>.

RAPD Analysis

Isolated DNA from ten isolates of *Fusarium* species was used to study the genetic variation using RAPD. RAPD, a method widely used to characterize isolates, indicated a high level of genetic variability among isolates of Fungi (Bahmani *et al.*, 2012). The standard RAPD reaction was set up by using standard RAPD primers. Seven random primers *viz*: OPA-01, OPA-14, OPA-17, TC-01, TC-03, OPG-5 and OPC-13, which generated reproducible RAPD patterns were used for a comparative analysis of ten selected fungal isolates (Figure 1). For RAPD, a total of 28, 20, 15, 21, 17, 43 and 41 bands were scored by using specific primers OPA-01, OPA-14, OPA-17, TC-01, TC-03, OPG-5 and OPC-13 respectively (Table 2). Out of total amplified fragments obtained from each primer 7, 5, 6, 5, 3, 6 and 6 polymorphic fragments were shown by OPA-01, OPA-14, OPA-17, TC-01, TC-03, OPG-5 and OPC-13 respectively. The number and size of amplified products resulted from random primers were varied between 15 – 43 and 250 – 2960 bp respectively. The dendrogram obtained from the UPGMA study characterized ten isolates into one main cluster and nine independent branches on the basis of similarity and on similarity value 0.65 (Figure 2). The independent branches consist of isolate 1, 5, 7, 8, 9, 10, 12 and 13. However, isolate 2 and 4 were found to be similar with each other on the similarity value nearly 0.70. Al-Tuwaijri, (2015) in Saudi Arabia characterized eight isolates of *F. oxysporum f. sp. cucumerinum*, the causal agent of *Fusarium* wilt of cucumber. Their isolates were subdivided into two sub clusters, one of them included isolate Fus 6 only and second include the rest of seven *Fusarium* isolates with different similarity values. Bonde *et al.*, (2014) studied genetic variations in ten isolates of *F. equiseti* isolated from different fruits and vegetables by RAPD. Hierarchical clustering distinguished the isolates into three groups on the basis of similarity and 0.02-0.15 was the range of similarity coefficient for all isolates. Hafizi *et al.*, (2013) in Malaysia studied morphological and molecular characterization of *F. solani* and *F. oxysporum* associated with crown disease of oil

palm. Cluster analysis revealed that the isolates in both *Fusarium* species were divided into two main clusters with the percentage of similarity from 87%-100% for *F. solani* and 89%-100% for *F. oxysporum* and isolates were highly variable. Gupta, (2012) reported PCR-RAPD profiling of *Fusarium* species; *F. oxysporum f. sp. psidii* and *F. solani* causing Guava wilt disease in India. Cluster analysis clearly separated the isolates of *F. oxysporum* and *F. solani* into three and two clusters respectively, showing a higher degree of similarity. Gupta *et al.*, (2009) studied genetic polymorphism in isolates of *F. solani* collected from different places in India on the basis of Carboxylesterases isozyme pattern and DNA polymorphism. Results revealed polymorphism within the isolates and established DNA fingerprints useful for genetic characterization.

Our results suggest the existence of significant genetic variation among *Fusarium* species isolated from apple rhizosphere. *Fusarium* species contribute to many diseases such as root rot, vascular wilt and fruit rot in apple. So to combat these problems, the present study of genetic diversity of *Fusarium* species will be useful.

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