Full Length Research Paper

Molecular diversity in Indian isolates of *Fusarium oxysporum* f.sp. *lentis* inciting wilt disease in lentil (*Lens culinaris* Medik)

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Accepted 15 June, 2011

For integrated management of wilt disease, foolproof knowledge about the genetic diversity among the prevalent isolates is very important. One hundred (100) isolates of *Fusarium oxysporum* f. sp. *lentis*, causing agent of vascular wilt in lentil were collected from different agro-climatic regions of India. Finally, fifteen distinct *F. oxysporum* f. sp. *lentis* isolates were selected for molecular characterization by three molecular markers. Twenty randomly amplified polymorphic DNA (RAPD) primers produced a total of 105 reproducible bands, out of which 81 (77.14%) were polymorphic and 24 (22.85%) were monomorphic. Nine simple sequence repeats (SSR) primer pairs amplified 21 alleles with 2.33 alleles per primer. Considerable length variations (561 to 668 bp) in rDNA regions were found and restriction digestion of amplified rDNA region produced forty eight different DNA bands. Three molecular markers revealed varying degree of genetic diversity in the selected isolates ranging from 54% in case of RAPD to up to 35% with ITS markers. Based on the coefficient of similarity, the isolates grouped into two major clusters in the dendrogram. Isolates from North Indian regions grouped in same cluster, whereas isolates from north east regions and eastern region fell in another cluster. This information will be helpful for pathologists and plant breeders to design effective resistance breeding programs in lentil taking into account the diversity in wilt pathogen.

**Key words:** Indian isolates, *Fusarium oxysporum* f.sp. *lentis*, vascular wilt, molecular diversity.

INTRODUCTION

Lentil (*Lens culinaris* Medikus sub sp. *culinaris*) was among the first crops domesticated and has become an important food legume crop in the farming and food systems of many countries globally (Sarker and Erskine, 2006). Among the diseases, *Fusarium* wilt caused by pathogenic fungi *Fusarium oxysporum* f.sp. *lentis* is the most important biological constraints to productivity of lentil worldwide (Bhalla et al., 1992). No physiological races of this pathogen have been reported so far. Although, *F. oxysporum* f.sp. *lentis* isolates exhibit great variability in morphology and aggressiveness (Abbas, 1995; Belabid et al., 2004), *Fusarium* vascular wilt disease can be managed by the use of resistance cultivars (Jalali and Chand, 1992) and for the development of resistant cultivars, knowledge about the existing variability within the pathogen is a pre requisite.

Accurate and rapid identification of pathogens is necessary for appropriate management of plant diseases. Identification of *Fusarium* spp. by morphological characters like size, shape of conidia and pigmentation are highly variable as all these characters are influenced by nutritional composition of the medium and cultural conditions. However, DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity and also phylogeny relationships of *Fusarium* spp. numerous workers have already worked on molecular variation in *Fusarium* spp. (O'Donnell, 2000).

RAPD analysis has been applied widely in the detection and genetic characterization of phytopathogenic fungi, including race differentiation in several formae

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**Abbreviation:** Fol, *Fusarium oxysporum* f.sp. *lentis*; PIC, polymorphic information content; rDNA, ribosomal DNA; SSR, simple sequence repeat; ITS, internal transcribed spacer.
speciales of *F. oxysporum*, such as *F. oxysporum* f.sp. *cubense* (Bentley et al., 1995). RAPDs applied to the carnation wilt pathogen *F. oxysporum* f. sp. *dianthi* and they were able to identify specific band patterns that were subsequently used as probes to distinguish between races of the pathogen (Manulis et al., 1994).

Simple sequence repeat (SSR) markers amplify small fragments of DNA in which motifs of 1 - 6 bases occur in tandem repeats. SSRs provides a powerful tool for taxonomic and population genetic studies (Britz et al., 2002). Alleles vary according to the number of repeat units present but other mutations have also been shown to be responsible for allele length variation in SSR analysis (Burgess et al., 2001; Slippers et al., 2004b). Microsatellites have been used to study polymorphism of several plant pathogenic fungi including *S. sclerotiorum* (Sirjusingh and Kohn, 2001) and *R. solani* (Mwang’Ombe et al., 2007).

Regions of ribosomal DNA (rDNA) also have been used in diversity and phylogenetic studies of several *Fusarium* spp. (Alves-Santos et al., 2002). These regions are highly conserved and can easily be investigated using PCR amplification. Out of various regions of rDNA, the internal transcribed spacer (ITS) and intergenic spacer (IGS) of the nuclear rDNA repeat units have been reported to be evolved fast and may vary among species within a genus or among populations and hence can be used for phylogenetic studies at these taxonomic levels (O’Donnell, 2000). Molecular phylogenetic analyses have helped to clarify ambiguities in traditional classification systems of *Fusarium* species by ITS marker (LoBuglio et al., 1993).

This study was undertaken to assess the genetic variation among vascular wilt pathogen of lentil in India. The use of RAPD, SSR and ITS-RFLP markers along with morphological and pathogenic data for characterization of the *Fol* isolates could greatly enhance the understanding of the variability within this important fungus.

**MATERIALS AND METHODS**

**Fungal Isolates and pathogenicity test**

One hundred (100) representative of different morphological and cultural group of *Fol* isolates were collected from wilt infested lentil plants from India during 2006 – 2007 (Figure 1). These *Fol* isolates were multiplied on sand- maize meal medium in the glass house

![Figure 1. Map of India showing areas of collection of isolates of *F. oxysporum* f. sp. *lentis* from different regions.](image-url)
A total of 9 SSR primers were synthesized from Operon Technologies, USA (Table 2b) and used for the amplification of individual microsatellite loci (Bogale et al., 2005). PCR amplification was performed in 25 µl total volume containing 2.5 µl of 10X Taq Buffer, 0.2 mM of dNTPs mix (100 mM of each dNTPs), 1 µl of each forward and reversed primer (25 ng each primer), 0.6 U of Taq polymerase with 25 ng of template DNA. PCR conditions for SSR were as follows; the PCR programme had one initial denaturation step at 94°C for 4 min followed by 35 cycles of 94°C for 30 s, annealing for 30 s (appropriate annealing temperature were used for each primers set, Table 2a). The thermal cycles were terminated by a final extension of 10 min at 72°C. Amplified products were resolved in 2.0% agarose gel @ 60 V cm⁻¹ using 1X TBE buffer.

DNA region amplification and PCR-RFLP analysis

An internal transcribed spacer (ITS) region of rDNA was amplified using the primers ITS-1(5`TCCGTAGGTGAACCTGCGG3`) and ITS-4 (5`TCTTCCGCTTTATTGATATG3`) according to White et al. (1990). Amplification was performed in total volume of 50 µl containing 0.7 U Taq DNA polymerase, 0.2 mM each dNTPs, 1 µl of each ITS-1 and ITS-4 (25 pmol) primers and approximately 50 ng template genomic DNA with the following condition; an initial denaturation for 1 min at 94°C followed by 30 s denaturation at 94°C, 30 s annealing at 55°C, 30 s elongation at 72°C repeated 31 times with the final elongation step of 5 min at 72°C. The PCR products were precipitated by adding 0.1 volume of 3 M sodium acetate and 2 volume of absolute ethanol to remove excess dNTPs and primers and resuspended in sterilized distilled water. Five restriction enzymes; EcoRI, EcoRV, SmaI, PstI and HindIII were used to digest the amplified products according to manufacturers instruction. The restricted fragments were electrophoresed on 2.5% agarose gel buffer. Stained with ethidium bromide (0.5 µg/ml) and photographed under UV light using gel documentation system (Alpha Digi Doc).

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**Table 2a.** RAPD primers used in this study and % polymorphism detected by 20 selected primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Monomorphic band</th>
<th>Polymorphic band</th>
<th>Total number of band</th>
<th>% Polymorphism Size range of band (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₁</td>
<td>5’TGCGTGCTTG 3’</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>85.71 300 - 2635</td>
</tr>
<tr>
<td>K₂</td>
<td>5’ACTTCCGCAC 3’</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>75.00 300 - 1264</td>
</tr>
<tr>
<td>K₃</td>
<td>5’GGCTCAGTG 3’</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>80.00 700 - 2400</td>
</tr>
<tr>
<td>K₄</td>
<td>5’CAAACGTGGG 3’</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>66.67 300 - 1600</td>
</tr>
<tr>
<td>K₅</td>
<td>5’CGAGGTCAGCGGTATCG 3’</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>50.00 300 - 1000</td>
</tr>
<tr>
<td>K₆</td>
<td>5’CACCGCCAAAATGGCCAC 3’</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>80.00 700 - 2400</td>
</tr>
<tr>
<td>K₇</td>
<td>5’GTCTCTAGTCCCCCAATCCC 3’</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>60.00 550 - 2300</td>
</tr>
<tr>
<td>P₁</td>
<td>5’CGTGGATGC 3’</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>80.00 500 - 2350</td>
</tr>
<tr>
<td>P₂</td>
<td>5’TACGGCTGGC 3’</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>73.33 300 - 1750</td>
</tr>
<tr>
<td>P₃</td>
<td>5’GCGGCATTGT 3’</td>
<td>1</td>
<td>6</td>
<td>7</td>
<td>85.71 450 - 2200</td>
</tr>
<tr>
<td>P₈</td>
<td>5’CGGGCCTTCTC 3’</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>66.66 430 - 1200</td>
</tr>
<tr>
<td>P₁₇</td>
<td>5’TACGGCTGGC 3’</td>
<td>1</td>
<td>4</td>
<td>6</td>
<td>75.00 300 - 2150</td>
</tr>
<tr>
<td>P₁₉</td>
<td>5’GCGGCATTGT 3’</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>75.00 300 - 1400</td>
</tr>
<tr>
<td>P₂₁</td>
<td>5’CCAGACAAGC 3’</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>75.00 300 - 1400</td>
</tr>
<tr>
<td>OPD 11</td>
<td>5’AGCGCCATTG 3’</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>75.00 300 - 1400</td>
</tr>
<tr>
<td>OPD 16</td>
<td>5’AGGGCGTAAG 3’</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>83.33 550 - 2350</td>
</tr>
<tr>
<td>OPA 11</td>
<td>5’CAATCGCCGT 3’</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>100.00 700 - 2700</td>
</tr>
<tr>
<td>OPF 01</td>
<td>5’ACGGATCCTG 3’</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>83.33 430 - 1000</td>
</tr>
<tr>
<td>OPF 05</td>
<td>5’CCGAAATTCCC 3’</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>80.00 450 - 1500</td>
</tr>
<tr>
<td>OPI 07</td>
<td>5’CAGCGACAAG3</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>50.00 300 - 1100</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>24</td>
<td>81</td>
<td>105</td>
<td>Average % polymorphism 65.82</td>
</tr>
</tbody>
</table>

(RAPD analysis

By using 20 RAPD primers, a total of 105 reproducible bands were amplified, out of which 81 (77.41%) were polymorphic and 24 (22.85%) were monomorphic. On an average of 4.37 bands per primer were amplified and size of bands were varied from 0.3 to 3.0 kb. Primers K₁, P₃, OPF and OPA-11 were the most informative (Figure 2a). A dendrogram based on UPGMA analysis indicated that 15 isolates formed two major clusters (Figure 3a). Major cluster ‘A’ comprised of eight isolates from central north region and one isolates (Fol 140) from north region. Maximum similarity of 72% was found in between Fol 95 and Fol 183, both from central north region. In the cluster A, isolate Fol 100 was most dissimilar from other seven isolates. The second cluster ‘B’ comprised of seven isolates from north and north east regions. Isolates Fol 141 and Fol 167 showed maximum similarity (67%) in the cluster B. Genetic similarity coefficient among the 15 isolates of Fol varied from 0.28 - 0.72 with RAPD

**RESULTS**

Fifteen distinct Fol isolates were selected for molecular characterization. All pathogenic isolates were pigmented with greenish black mycelia, while one pathogenic isolate Fol 167 was non-pigmented, with white mycelia. Two moderately virulent isolates (Fol 262 and Fol 265) were pigmented with greenish black mycelia, while all other moderate isolates were white in colour (Table 1). Isolates Fol 83, Fol 141, Fol 156 and Fol 167 were more virulent caused > 50% wilt incidence and remaining ten isolates were moderately pathogenic caused 30 - 50% wilt.
Table 2b. Allele amplification of 15 *F. oxysporum* f. sp. *lentis* with nine SSR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Repeat motif</th>
<th>Primer sequence (5'-3')</th>
<th>Tm (°C)</th>
<th>Number of alleles</th>
<th>Amplified alleles size (bp)</th>
<th>PIC value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB 2</td>
<td>(GT)$<em>{11}$(GA)$</em>{6}$</td>
<td>TGCTGTGTATGGATGGATGG CATGGTGGATGCT</td>
<td>57</td>
<td>2</td>
<td>250, 225</td>
<td>0.780</td>
</tr>
<tr>
<td>MB 5</td>
<td>(TG)$_{9}$</td>
<td>ACTTGGAGAAATGGGCTTC GGATGGAGTTTAATATAATCTGG</td>
<td>54</td>
<td>2</td>
<td>150,100</td>
<td>0.633</td>
</tr>
<tr>
<td>MB 9</td>
<td>(CA)$_{9}$</td>
<td>TGGCTGGGATACTGTGTAATTG TTAGCTTCAGCCCTTTGG</td>
<td>51</td>
<td>2</td>
<td>175, 150</td>
<td>0.818</td>
</tr>
<tr>
<td>MB 10</td>
<td>(AAC)$_{6}$</td>
<td>TATCGAGTCCGGCTTCCAGAAC TTGCAATTACCTCCGATCCAC</td>
<td>48</td>
<td>1</td>
<td>300</td>
<td>0.966</td>
</tr>
<tr>
<td>MB 11</td>
<td>(GGC)$_{7}$</td>
<td>GTGGACGAACACCTGCATC AGATCCCTCACCCACTCCACC</td>
<td>68</td>
<td>5</td>
<td>500, 400, 300, 225, 150</td>
<td>0.918</td>
</tr>
<tr>
<td>MB 13</td>
<td>(CTTGGAAGTTAGCGG)$_{14}$</td>
<td>GGGAGATGAGCTCGATGAAG CTAAGCCTGACTACACCCCTCG</td>
<td>68</td>
<td>5</td>
<td>500, 350, 300, 250, 150</td>
<td>0.935</td>
</tr>
<tr>
<td>MB 14</td>
<td>(CCA)$_{5}$</td>
<td>CGTCTCTGAAACCACCTTCATC TTCTCCGATCCATCCG</td>
<td>57</td>
<td>1</td>
<td>700</td>
<td>0.923</td>
</tr>
<tr>
<td>MB 17</td>
<td>(CA)$_{21}$</td>
<td>ACTGATTCACGGATCTTGG GCTGGCGTGGACTTGTATTCGG</td>
<td>57</td>
<td>1</td>
<td>325</td>
<td>0.876</td>
</tr>
<tr>
<td>MB 18</td>
<td>(CAACA)$_{6}$</td>
<td>GGTAGGAATGACGAAAGCTGAC TGAGCCTCTAGCACTACCAAC</td>
<td>57</td>
<td>2</td>
<td>300, 275</td>
<td>0.802</td>
</tr>
<tr>
<td>Mean PIC value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.850</td>
</tr>
</tbody>
</table>

markers.

**SSR analysis**

The SSR markers developed by Bogale et al. (2005) for species of *F. oxysporum* complex were tried against Indian isolates of *Fol* and all primers showed good polymorphism. Nine SSR primers were amplified a total of 21 alleles. Maximum of 5 alleles were amplified by primer pairs of MB 11 and MB 13 followed by 2 alleles from MB 2, MB 5, MB 9 and MB 18 (Figure 2b). Size of the amplified alleles varied between 100 and 850 bp. On an average, 2.33 alleles per locus were amplified by the *Fol* population. The mean number of PIC value of SSR primer pairs in this study was 0.80 (Table 2b). The SSR primers clustered all 15 *Fol*
isolates into two main clusters (Figure 3b). In the cluster A₁, the maximum similarity (73%) was found in between two isolates from central north region of India, Fol83 and Fol100. The second group B₁ subdivided into two sub groups. In the first sub group, three isolates Fol140, Fol235 and Fol265 separated in one cluster with 58% similarity. The second sub group consisted of virulent isolates, Fol141, Fol156 and Fol242 with 55% similarity. Genetic similarity coefficient among the 15 isolates of Fol varied from 0.26 - 0.73 with SSR markers. The highly
pathogenic isolates Fol 161, Fol 167 and Fol 156 were grouped in one clad in cluster A₁ and B₁.

**rDNA region amplification and PCR-RFLP analysis**

ITS-1 and ITS-4 primers amplified a single band ranging from 561 to 668 bp (Figure 2c). The amplified ITS region was restricted with the five different hexa cutter restriction enzyme and only two restriction enzymes, EcoRI and HindIII were cleaved in ITS region (Figure 2d). These two enzymes revealed the extensive polymorphism in ITS regions in three isolates Fol 140, Fol 167 and Fol 208. With the enzyme EcoRI, all isolates showed three bands, which ranged 668 - 242 bp. Restriction enzymes HindIII could also detect variation in the restriction sites in another isolates Fol 208, Fol 235, Fol 242, Fol 248 and Fol 262, as these isolates were restricted and others were not. The dendrogram constructed from ITS-RFLP data also grouped all 15 isolates in two major clusters with 65.49% of average similarity (Figure 3c).

In cluster A₂, almost all isolates from north central region were clustered and others were in cluster B₂. The dendrogram also supported the restriction digestion data of the isolates Fol 140, Fol 167 and Fol 208, which showed the different banding patterns by PCR-RFLP patterns and were separated from all other isolates.

**DISCUSSION**

The prevalence of different Fol isolates in lentil growing regions makes it essential to identify region specific pathogen to devise strategies for conferring resistance against them in the respective agro-climatic regions. RAPD markers grouped the Fol isolates into two major groups based on their geographical location as earlier reported by Belabid et al. (2004). Highly pathogenic isolates, Fol 83 and Fol 115, clustered in one cluster, while the remaining three isolates, Fol 141, Fol 156 and Fol 167, grouped in second subcluster. Similar studies on other plant pathogenic fungi have emphasized the importance of molecular approaches to characterize genetic diversity within and between isolates (Sivaramakrishnan et al., 2002; Bentley et al., 1995). The pathogen can be divided in two genetic subpopulations and can be characterized by a low polymorphism in same subgroups subpopulations. Similar results were also obtained with *F. oxysporum* f. sp. *erythroxyl* and by *F. oxysporum* f. sp. *lentis* (Belabid et al., 2004). By SSR
markers, different allele sizes were recorded in all the loci in the genome of \textit{Fol}, the smallest allele size was detected as 100 bp and the largest one was 850 bp. The polymorphic character of SSRs produces highly discriminating fingerprints that often allow characterization of fungi at a strain level (Migheli et al., 1998; Barre’s et al., 2006). The variable numbers of alleles per loci is an indication of high level of polymorphism and was reported by Mwang’Ombe et al. (2007) in Kenyan \textit{R. solani} isolates. The mean number of PIC value of SSR markers in this study was 0.80. Primer MB-10 gave maximum PIC value with 0.97, whereas MB-5 gave minimum PIC value of 0.63. This PIC value indicates that the Indian isolates had a high degree of biodiversity. Polymorphism has been observed in other fungi as a direct record of genetic evolution (Sanders, 2002; Scharald and Craven, 2003). SSR markers placed all the \textit{Fol} isolates in two major groups which were separated from 27% of genetic similarity. Earlier, Dubey and Singh (2008) reported the clustering of 64 \textit{F. oxysporum} f. sp. \textit{ciceris} isolates into three categories at 25% genetic similarity and into two major categories at 30% genetic similarity with ISSR and RAPD markers. Thus, they were found suitable for the study of genetic diversity in the pathogen. MB 13, MB 11 and MB 05 were given the maximum allelic variation in \textit{Fol} isolates. Earlier, MB 05, MB 14 and MB 17 were also given the good allelic variation in \textit{F. oxysporum} f. sp. \textit{ciceris} isolates from Indian isolates (Dubey and Singh, 2008).

ITS-RFLP analysis has been used extensively to distinguish many \textit{Fusarium} species and remains an important tool for species identification in ECM fungal communities (Joshi et al., 2006). Isolates from central north region grouped in first cluster \textit{A}₀, except four isolates \textit{Fol 137}, \textit{Fol 208}, \textit{Fol 248} and \textit{Fol 252} which did not group in first cluster. All isolates from north east regions grouped in the second major cluster \textit{B}. The dendrogram constructed from ITS-RFLP data also grouped all 15 isolates in two major clusters with 65.49% of average similarity.

Considerable variability was also found in some isolates belonging to the same agro climatic regions like two isolates (\textit{Fol 137} and \textit{Fol 208}) from north India. All the three molecular techniques used in this study separated the isolates into two major clusters. Similar results were also reported in \textit{F. oxysporum} f.sp. \textit{phaseoli} by Woo et al. (1996) and in \textit{F. oxysporum} f. sp. \textit{ciceris} by Jimenez-Gasco et al. (2001).

The three methods provided similar resolution, although, there were differences in the distribution of isolates and the ratio of genetic similarity in the dendrogram. Similar findings have also been reported in the Ethiopian \textit{F. oxysporum} isolates by AFLP, SSR and ITS sequence analysis by Bogale et al. (2006). Genetic variation in \textit{Fol} detected by molecular marker indicates the ability of a pathogen to adapt to different life cycle condition according to different climatic regions, cultural practices and crop rotation. This has far reaching consequences on breeding programs. Resistance genes against all major pathogenic races should be pyramided according to their agro climatic regions for effective management of the wilt disease.

REFERENCES


