Specific PCR-based marker for detection of pathogenic groups of *Fusarium oxysporum f. sp. cucumerinum* in India

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Abstract For the detection of *Fusarium oxysporum f. sp. cucumerinum* pathogenic groups, a specific PCR-based marker was developed. Specific random amplified polymorphic DNA (RAPD) markers which identified in four pathogenic groups I, II, III, and IV were cloned into pGem-Teasy vector. Cloned fragments were sequenced, and used for developing sequence characterized amplified regions (SCAR) primers for detection of pathogenic groups. *F. oxysporum f. sp. cucumerinum* isolates belonging to four pathogenic groups in India, cucumber nonpathogenic *F. oxysporum*, *F. oxysporum f. sp. moniliforme* and *melonis, Fusarium udum*, and isolate of *Alternaria* sp. were tested using developed specific primers. A single 1.320 kb, 770 bp, 1.119 kb, and 771 bp fragment were amplified from pathogenic group I, II, III, and IV isolates, respectively. Results showed the PCR based marker, which used in this research work, could detect up to 1 ng of fungal genomic DNA. The specific SCAR primers and PCR technique developed in this research easily detect and differentiate isolates of each *F. oxysporum f. sp. cucumerinum* pathogenic groups.

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1. Introduction

*Fusarium oxysporum f. sp. cucumerinum* (Owen) Snyder and Hansen (*Foc*) causal agent of Fusarium wilt of cucumber (*Cucumis sativus* L.), is one of the most important fungal pathogen of cucumber and can significantly reduce the production of cucumber in several areas. In India it is the most important disease affecting the high quality cucumber cultivars. Resistance to *Foc* is usually race specific and as a consequence correct identification of the local race is necessary for the choice of resistant cultivars. Disease diagnosis and pathogen identification by traditional methods, which involve isolating of the pathogen and characterizing it by inoculation and pathogenicity tests, are time consuming. The problems further are sometimes
complicated by the occurrence of saprophytic strains of *F. oxysporum* on diseased cucumber plants, which are morphologically similar to *Foc* but are nonpathogenic. Precise characterization is possible by using PCR based techniques and sequence characterized amplified regions (SCARs) which have been useful for the detection of many of plant pathogens [9,10,12,15]. By increasing the specificity, the results are less sensitive to changes in reaction conditions and are more reproducible [8]. The SCAR markers can be used to generate specific PCR primers based on informative RAPD markers that could discriminate isolates of *Foc* from nonpathogenic *F. oxysporum* from cucumber, other *F. oxysporum* formae speciales, and *Fusarium* spp. and identify the isolates of the pathogen. The aim of this study was to analyze a collection of pathogenic and nonpathogenic strains of *F. oxysporum* recovered from cucumber fields by RAPD markers, and to use this information to develop a PCR-based procedure that allows the identification of the prevalent pathogenic groups in India.

2. Materials and methods

2.1. Fungal isolates

Thirty (out of fifty) pathogenic and nonpathogenic strains of *F. oxysporum* collected from different parts of country are listed in Table 1. All the fifty strains of *F. oxysporum* either pathogenic or nonpathogenic on cucumber were recovered from cucumber fields in different parts of India and have been previously described [14]. Pathogenic isolates were grouped into four groups, according to the reactions on different cucumber cultivars characterized in the Division of Plant Pathology, Indian Agricultural Research Institute (IARI), India [14].

2.2. Extraction of DNA

Genomic DNA of the fungal isolates was extracted by using cetrimide tetradeyl trimethyl ammonium bromide (CTAB) method [13] with little modification. Ground mycelia was suspended in an extraction buffer [100 mM Tris–HCl (pH 8.0), 1.4 M NaCl, 50 mM EDTA (pH 8.0) 2% CTAB, and 2% β-mercaptoethanol fresh]. The mixture was emulsified with an equal volume of phenol/chloroform/isoamyl alcohol (25: 24:1), and centrifuged at 13,000 rpm at room temperature for 20 min. The aqueous phase was treated with RNAase and extracted with chloroform/isoamyl alcohol (24:1) followed by centrifugation at 10,000 rpm for 10 min. The last step was repeated once again to make a pure DNA. DNA was precipitated by adding cold isopropanol and 0.1 volume of 3 M sodium acetate and then kept in –20 °C for 3–4 h. The supernatant was poured off and the pellet was washed twice with 70% ethanol and dried at either room temperature at 37 °C. The DNA pellet was resuspended in 100–200 μl sterile distilled water and was analyzed on 0.70% agarose gels in 1× Tris–acetate–EDTA (TAE) buffer to estimate the concentration and quality of DNA. For PCR reactions, samples were diluted 25–50 ng/μl in TE buffer (1.0 M Tris–HCl, pH approx. 8.0, containing 0.1 M EDTA).

2.3. Use of RAPD primers and fragments

*Fusarium oxysporum* f. sp. *cucumerinum* pathogenic groups were characterized and cloned as follows: pathogenic group I, 1.320 kb (OPB-15), pathogenic group II, 0.770 kb (OPA-18), pathogenic group III, 1.119 kb (OPC-5), and pathogenic group IV, 0.771 kb (OPB-15). The amplifications were performed in a total volume of 25 μl containing 2.5 mM MgCl2, 200 μM each dNTPs, 0.5 μM each primer, 50 ng genomic DNA, and 1 U of DNA Taq polymerase (Fermentase) and 1× Taq buffer. The amplification conditions were programmed for 4 min at 94 °C, followed by 35 cycles of 1 min 94 °C, 1 min at 35 °C, and 2 min at 72 °C and a final incubation at 72 °C for 7 min. Negative controls, in which DNA template solution was replaced by water, were performed in all experiments to test for contamination. RAPD products were separated in 1.4% agarose gels in 1× TAE buffer pre-stained with ethidium bromide, and visualized under UV light. The 1 kb DNA ladder marker was used for electrophoresis. The RAPD markers of *F. oxysporum* f. sp. *cucumerinum* pathogenic groups were excised

### Table 1 Isolates of *F. oxysporum* collected from different places of India.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Isolate code</th>
<th>Geographical origin</th>
<th>Pathogenic group</th>
<th>Isolate No.</th>
<th>Isolate code</th>
<th>Geographical origin</th>
<th>Pathogenic group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CRK</td>
<td>Rajasthan</td>
<td>IV</td>
<td>16</td>
<td>CRD-4</td>
<td>Delhi</td>
<td>II</td>
</tr>
<tr>
<td>2</td>
<td>CRT-1</td>
<td>Rajasthan</td>
<td>II</td>
<td>17</td>
<td>CRD-5</td>
<td>Delhi</td>
<td>IV</td>
</tr>
<tr>
<td>3</td>
<td>CRD</td>
<td>Delhi</td>
<td>III</td>
<td>18</td>
<td>CFD</td>
<td>Delhi (market)</td>
<td>III</td>
</tr>
<tr>
<td>4</td>
<td>CRG</td>
<td>Haryana</td>
<td>IV</td>
<td>19</td>
<td>CRD-6</td>
<td>Delhi</td>
<td>np</td>
</tr>
<tr>
<td>5</td>
<td>CRB-1</td>
<td>Uttar Pradesh</td>
<td>IV</td>
<td>20</td>
<td>CRD-7</td>
<td>Delhi</td>
<td>III</td>
</tr>
<tr>
<td>6</td>
<td>CRB-2</td>
<td>Rajasthan</td>
<td>I</td>
<td>21</td>
<td>CSWB-1</td>
<td>West Bengal</td>
<td>II</td>
</tr>
<tr>
<td>7</td>
<td>CRA</td>
<td>Uttar Pradesh</td>
<td>I</td>
<td>22</td>
<td>CSWB-2</td>
<td>West Bengal</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>CRU</td>
<td>Uttar Pradesh</td>
<td>II</td>
<td>23</td>
<td>CSWB-3</td>
<td>West Bengal</td>
<td>I</td>
</tr>
<tr>
<td>9</td>
<td>CRT-2</td>
<td>Rajasthan</td>
<td>II</td>
<td>24</td>
<td>CSWB-4</td>
<td>West Bengal</td>
<td>II</td>
</tr>
<tr>
<td>10</td>
<td>CRS</td>
<td>Rajasthan</td>
<td>III</td>
<td>25</td>
<td>CSWB-5</td>
<td>West Bengal</td>
<td>np</td>
</tr>
<tr>
<td>11</td>
<td>CRD-1</td>
<td>Delhi</td>
<td>II</td>
<td>26</td>
<td>CSWB-6</td>
<td>West Bengal</td>
<td>II</td>
</tr>
<tr>
<td>12</td>
<td>CRD-2</td>
<td>Delhi</td>
<td>I</td>
<td>27</td>
<td>CCWB-1</td>
<td>West Bengal</td>
<td>II</td>
</tr>
<tr>
<td>13</td>
<td>CRD-3</td>
<td>Delhi</td>
<td>np</td>
<td>28</td>
<td>CCWB-2</td>
<td>West Bengal</td>
<td>I</td>
</tr>
<tr>
<td>14</td>
<td>CSD-1</td>
<td>Delhi</td>
<td>IV</td>
<td>29</td>
<td>CCWB-3</td>
<td>West Bengal</td>
<td>I</td>
</tr>
<tr>
<td>15</td>
<td>CSD-2</td>
<td>Delhi</td>
<td>III</td>
<td>30</td>
<td>CRWB-1</td>
<td>West Bengal</td>
<td>np</td>
</tr>
</tbody>
</table>

* C, cucumber; R, root; S, stem; 3rd and 4th alphabetic in isolate code, origin of sample; np, nonpathogenic.
from the agarose gel, and DNA was purified using the QIA-quick gel extraction kits (Qiagen). The purified RAPD products were cloned into the vector pGem-T easy (Promega, Madison, WI, USA) according to the manufacturer’s instruction and used to transform competent Escherichia coli cells strain DH 5α. For each cloned RAPD marker, plasmid DNA was released by the alkali lysis method [4] and to ensure that DNA inserts carried by selected bacterial clones corresponded to the putative correct RAPD marker, colony PCR using same primer and at same condition was done and the size of the DNA inserts were checked by EcoRI restriction digestion.

2.4. Sequencing of cloned RAPD markers and design of specific SCAR primers

Cloned RAPD inserts were sequenced at the DNA sequencing facility at Delhi University, South Campus, New Delhi, India, using T7 and SP6 sequencing primers. A search for sequence similarities was performed with BLAST programs of NCBI network service [1]. The DNA sequences were submitted to GenBank. For each cloned RAPD amplification product we designed a PCR primer pair so as to correspond to sequences between 18 and 21 nucleotides that were identified at both ends of the inserts to be used as SCAR primers. The multiple sequence alignment and pairwise alignment were made using BioEdit version 5.09 [7]. These primers included full nucleotide sequence of the Operon primer that amplified the RAPD marker originally. The designed primers, sequences for the specific primer are shown in Table 2, which were synthesized by Biochem Company,. Amplification of genomic DNA (1 ng/μl) with SCAR primers was done in a similar condition except annealing temperature, which were specific to each primer and are shown in Table 2, and finally amplification products were reserved electrophoretically in a 1.4% agarose gel.

3. Results

3.1. Cloning of RAPD markers

The following specific RAPD markers for each pathogenic group of *F. oxysporum* f. sp. *cucumerinum* were identified: pathogenic group I, 1.320 kb (OPB-15), pathogenic group II, 0.770 kb (OPA-18), pathogenic group III, 1.119 kb (OPC-5), and pathogenic group IV, 0.771 kb (OPB-15). These inserts were distinguished because of slight differences in molecular size, and distinct patterns of inserts digested with EcoRI endonuclease (Fig. 1). Colony PCR with original primers which were synthesized, could easily amplify them again (Fig. 1). The above results allowed selection of bacterial clones carrying inserts corresponding to each pathogenic groups of *F. oxysporum* f. sp. *cucumerinum* (Table 2).

3.2. Sequencing of cloned RAPD markers and design of SCAR primers specific for *F. oxysporum* f. sp. *cucumerinum* pathogenic groups I, II, III, and IV

The complete sequences of insert DNAs (clones 4-1, 5, 8, and 11-6) were obtained and compared with the GenBank database for the identification of sequence similarity to published gene sequences [1]. The insert from clone 8 showed 83% nucleotide similarity with Gibberella zeae PH-1 chromosome 1 hypothetical protein (FG01028.1) partial mRNA and 80% with Fusarium fujikuroi partial mRNA for putative chaperone protein, clone gsr10. For the insert from clones 4-1, 5, and 11-6 no significant similarity found with already presented sequences in GenBank. The sequences were deposited in the GenBank nucleotide sequence database (Accession No., Table 2). From the sequences of the clones, we designed four specific primer pairs for the identification of *F. oxysporum* f. sp. *cucumerinum* pathogenic groups I, II, III, and IV. Primer designation and sequence, target pathogenic group, and PCR products under optimized amplification conditions are presented in Table 2.

3.3. Identification of *F. oxysporum* f. sp. *cucumerinum* pathogenic groups by using SCAR primers

Primers derived from sequences of inserts in clones 4-1, 5, 8, and 11-6 amplified only a single PCR product from genomic DNA of isolates of each of pathogenic group II, IV, III, and I, respectively (Fig. 2). None of these products were amplified when DNA of isolates of *F. oxysporum* f. sp. *cucumerinum* groups other than the target pathogenic group, and of non-pathogenic isolates of *F. oxysporum*, *F. solani*, *F. oxysporum* f. sp. *Ciceris* (not showed), *F. moniliforme* (not showed), *F. oxysporum* f. sp. *melonis*, *F. udum*, and *Alternaria brassicicola* were used as template in specific PCR assays (Fig. 3). No cross-reactions or amplification of additional fragments were observed for isolates of other *F. oxysporum* formae speciales, other *Fusarium* spp., non-pathogenic *F. oxysporum* and also other genus like *Alternaria brassicicola*. Furthermore, the specific PCR assays correctly identified isolates of *F. oxysporum* f. sp. *cucumerinum* and assigned them to the correct pathogenic group. Nevertheless, these PCR assays proved problematic for some of tested isolates. Finally we tested the PCR assays,

![Figure 1](image-url) Colony PCR (A) with original primers (OPA-18 amplified 770 bp line 1–4) and (OPC-5 amplified 1.119 kb line 5–13) patterns of 1.119 kb insert digested with EcoRI endonuclease. (B) Numbers on the left are the molecular weight of the 1 kb DNA ladder (lane M).
all SCAR primer pairs developed were able to detect 1 ng of fungal DNA using the amplification conditions described herein (Fig. 4).

### 4. Discussion

#### 4.1. Application of SCAR markers

Collection, identification and characterization of *F. oxysporum* f. sp. *cucumerinum* isolates is very useful to understand the variability in pathogenic studies. This research work showed that SCAR primers developed from RAPD markers can be applied for specific detection of *F. oxysporum* f. sp. *cucumerinum* pathogenic groups I, II, III, and IV, which are represented in this study. These specific primers can be used to separate *F. oxysporum* f. sp. *cucumerinum* from other formae specialis of this species, other *Fusarium* spp., non-pathogenic *F. oxysporum* and other genera. The SCAR primers developed in this study can amplify a single fragment from all isolates of forma specialis and pathogenic groups collected from different states. Similarly, the genetic diversity among isolates of *F. oxysporum* from cucumber characterized by the RAPD markers previously has been reported [16]. The insert from clone 8 showed 83% nucleotide similarity with *Gibberella zeae* PH-1 chromosome 1 hypothetical protein (FG01028.1) partial mRNA and 80% with *Fusarium fujikuroi* partial mRNA for putative chaperone protein, clone gsr10. For the insert from clones 4-1, 5, and 11-6 no significant similarity found with already presented sequences in GenBank.

The SCAR primers and PCR assays are free of problems associated with RAPD assay that reduce its applicability for diagnosis of those races and enhance the possibility of fast, extensive, and reliable discrimination of the pathogen and races.

#### 4.2. Detection of isolates with specific SCAR primers

The correlation of molecular markers to pathogenic races in *F. oxysporum* by use of RAPD assays have been shown in different studies [3,6,11,16]. However, the number of works that subsequently have changed race-associated RAPD markers into SCARs and have designed specific primers

<table>
<thead>
<tr>
<th>Bacterial clone, primer</th>
<th>Isolate</th>
<th>Insert</th>
<th>Accession number</th>
<th>Sequence (5’–3’)</th>
<th>Product, pathogenic group</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-1/OPA-18</td>
<td>CRT-1</td>
<td>770 bp</td>
<td>ER935968</td>
<td>F: AGGTGACCGTGCTAGCAAGAGGTAAGCAAG</td>
<td>II</td>
<td>Annealing temp. 54 °C, 25 cycles</td>
</tr>
<tr>
<td>5/OPB-15</td>
<td>CRB-1</td>
<td>771 bp</td>
<td>ER935969</td>
<td>F: GGAGGGTGGTTAGAGATGC</td>
<td>IV</td>
<td>Annealing temp. 61 °C, 35 cycles</td>
</tr>
<tr>
<td>8/OPC-5</td>
<td>CRS</td>
<td>1119 bp</td>
<td>ER935970</td>
<td>F: GATGACGCCAATGTGACT</td>
<td>III</td>
<td>Annealing temp. 62 °C, 25 cycles</td>
</tr>
<tr>
<td>11-6/OPB-15</td>
<td>CSWB-3</td>
<td>1320 bp</td>
<td>ER935971</td>
<td>F: GGAGGTTAATACATCAAT</td>
<td>I</td>
<td>Annealing temp. 55 °C, 25 cycles</td>
</tr>
</tbody>
</table>

F, forward; R, reverse. Underlined bases indicate the sequences of Operon RAPD primer used.

**Table 2** GenBank accession number of sequence characterized amplified regions (SCARs), characterized of recombinant DNA bacterial plasmids, SCAR primers, and amplification conditions for specific polymerase chain reaction assays developed for identification of *F. oxysporum* f. sp. *cucumerinum* pathogenic groups I, II, III, and IV.

**Figure 2** Agarose gels showing amplification products from polymerase chain reaction (PCR) using genomic DNA from isolates of *Foc*, and sequence-characterized amplified region (SCAR) primers designed for pathogenic groups A (pathogenic group I), B (pathogenic group II), C (pathogenic group III), and D (pathogenic group IV). Numbers on the left and right are the molecular weight of the 1 kb DNA ladder (lane M).
for race detection is more limited [2,5,9]. The technique is very useful and efficient; it can separate the different pathogenic groups (races) within a same forma speciales from other *Fusarium* spp. In addition to that, these results practically can be used for diagnosis, controlling import and export of plant materials, epidemiology studies, and management strategies of *Fusarium* wilt of cucumber. Thus, the SCAR primers specific for *Fusarium oxysporum* f. sp. *cucumerinum* provide a useful tool for simply differentiate nonpathogenic *Fusarium* isolates as well as from *Fusarium* wilt-affected cucumber. Also, the race-specific SCAR primers provide an alternative and useful technique for the study of race structure in *Fusarium oxysporum* f. sp. *cucumerinum* population in comparison with the use of traditional methods such as pathogenicity tests which are time consumable. The SCAR primer pairs developed could detect 1 ng of fungal genomic DNA using the amplification conditions described before. This amount of DNA can be obtained easily from different host material that carries the target pathogen. This method could be envisioned for the detection of races of *Fusarium oxysporum* f. sp. *cucumerinum* in infested soil or infected plants [2], thus helping in understanding the pathogenic variability.

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**References**

