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SCAR marker development for quick detection of *Fusarium oxysporum* f. sp. *lentis*

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Lentil (*Lens culineris* Medik.) commonly known as *Masur*, is an important pulse crop in India, which is mostly grown in northern plains, central and eastern parts of India. The major lentil producing states are Madhya Pradesh, Uttar Pradesh, Bihar, Uttarakhand and West Bengal. The major constraints for the low yield of lentil include lack of availability of good quality seed, and losses due to biotic and abiotic stresses. Among the biotic stresses, several fungal, bacterial and viral diseases affect the quality and yield of the crop such as wilt (*Fusarium oxysporum* f. sp. *lentis*), collar rot (*Sclerotium rolfsii*), root rot (*Rhizoctonia solani)* and rust (*Uromycis fabae*) (Gautam *et al.* 2013).

Among diseases, Fusarium wilt is the most widespread and important fungal disease causing maximum yield loss by reducing the crop stand in the field (Akhtar *et al.* 2012). The conventional detection methods are based on symptoms and fungus morphology. However, DNA-based techniques have increasingly become the tool of choice for understanding the genetic diversity (O'Donnell 2000).

The genetic diversity among the *F. oxysporum* f. sp. *lentis* (*Fol)* isolates have been reported to be high (Al-Husien *et al.* 2017, Mohammadi *et al*. 2011). The random amplified polymorphic DNA (RAPD) marker-based detection is a widely used method for various reasons including its easiness, adaptability, reasonability and the ability to reveal insignificant differences easily (Datta *et al.* 2011). Sequence characterized amplified region (SCAR) markers in fungal pathogens can be obtained from particular strain, gene, or from sequences of randomly developed decamers. Due to the soil and seed-borne nature of *Fol*, its initial diagnosis is very important to devise management strategies to minimize the crop losses and maximize the yield. There are no standard diagnostic markers available to detect *Fol* fungus from either soil or seed or any other plant part. In the present study, we aimed to develop the RAPD derived precise SCAR markers for the detection of *Fol* from infected lentil seed.

Current study was done in the Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi during 2018–20. The *Fol* infected seed samples were collected in the *rabi* season of year 2018–19 from major lentil growing areas of seven states of central India based on visual symptoms of the wilt disease. Single spore colonies of actively growing margin of *Fol* isolates were grown on potato dextrose agar (PDA) medium. The DNA extracted from freeze-dried fungal mycelium and powdered using liquid nitrogen following manufacturer's protocol of Nucleopore^{R}g DNA Fungal Bacterial Mini Kit (Genetix Biotech Asia Pvt Ltd). DNA samples were quantified in Spectrophotometer (Nano Drop 1000, Thermo Scientific Wilmington, USA) at 260 nm, diluted to 30-50 ng/µl, and stored in -20°C until further use.

A total of 6 RAPD primers were used for the diversity analysis of all the *Fol* isolates (Table 1). The primers that gave reproducible and scorable amplicons were used in the analysis of genetic variability existing in the selected pathogenic *Fol* isolates. DARwin 6.0.21 (dissimilarity analysis representing windows software) was used for subsequent dendrogram preparation using sequential agglomerative hierarchical non-overlapping clustering program (Rohlf 1998). Twenty-five RAPD primers (Bangalore Genei Pvt-Ltd) were employed for genetic diversity of the Fol. The PCR conditions were 94°C for 5.0 min (Initial denaturation), followed by 94°C for 1 min of final denaturation with 40 cycles, annealing temperature of 35°C for 1 min with an extension of 72°C for 2 min and 72°C for 5 min with final extension. A PCR mixture of 25 µl was prepared with 2.5 µl Taq buffer (Scientific TM 10X DreamTaq buffer), 2 µl of $MgCl_2$ from 2.5 mM, 1µl of 10 mM dNTPs, 2 µl of 10nM RAPD primer, 0.2 µl of Taq polymerase from 5U/µl (Thermo Scientific TM DreamTaq polymerase) and 2 µl template DNA at 50 ng for PCR amplification (Master cycler nexus gradient thermal cycler, Eppendorf, Germany). An agarose gel of 1.5% with lab safe dye was used to observe RAPD amplified bands

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Table 1 Properties of RAPD primers in isolates of *Fusarium oxysporum* f. sp. *lentis*

Primer	Sequence (5'-3')	Total number of bands	Monomorphic band	Polymorphism (%)	Size range of amplicons (bp)
OPD-3	TCGGCGATAG	7	1	85.71	100-1200
OPE-14	TTCCGAACCC	8	0	100	200-1517
OPN-12	ACCAGGGGCA	6	0	100	500-1200
OPI-18	TGCCCAGCCT	6	1	83.33	300-1200
OPS-30	GTGATCGCAG	10	0	100	200-1000
OPN-18	GGTGAGGTCA	9	0	100	200-1571
	Total	46	-	-	-

under a Trans-UV illuminator. Each RAPD test performed thrice to ensure reproducibility of results. The amplified fragment of RAPD specific to *Fol* isolates were visualized on agarose gel, eluted using a GeneiPure^{TM}Gel Extraction Kit (Genei, Bangalore) and quantified using spectrophotometer (Thermo Scientific™ NanoDrop 2000). The OPI-18 primer amplified the *Fol* DNA that generated different fragments. The excised monomorphic bands were ligated into pGEM-T vector as per the instructions provided by the manufacturer (Bangalore Genei, India) and subsequently recombinant plasmid transformation was done by using *E. coli* DH5a. The plasmid DNA of *E. coli* were cloned (GeNei™ Plasmid Preparation Teaching Kit) by inserting sequences from forward and reverses directions using OPI-18 primer. The obtained sequences (Genei, Bangalore) were processed for the homology analysis using NCBI BLASTN programme (http://blast.ncbi.nlm.nih.gov).

The eluted fragment was cloned, sequenced and NCBI blast searches were performed for confirmation of sequence identity (https://blast.ncbi.nlm.nih.gov/Blast.cgi), which showed 97.63% homology with *Fol*. Based on unique sequence of *Fol* specific fragment, three sets of SCAR primers (SCAR Fol 1 (F/R), SCAR Fol 2 (F/R) and SCAR Fol 3 (F/R) were developed using the Primer 3 software (http://bioinfo.ut.ee/primer3/) using the sequences obtained from RAPD cloned fragment. SCAR markers (SCAR Fol 1) were designed based on analysis of GC content and Tm using computer primer analysing programs (https://eu.idtdna.com/calc/analyzer), later primers were sent for the commercial synthesis (Sigma Aldrich, Bangalore, India). PCR conditions for SCAR markers amplification were standardized as: 94°C for 5.0 min followed by 40 cycles with final denaturation of 94°C for 1 min, annealing of 58.9°C for 1 min, elongation of 72°C for 2 min, and final extension at 72°C for 5 min. The PCR reaction volume 25 µl consisted of 1 µl (40-50 ng) of DNA template, 2.0 µl of Taq buffer (Thermo ScientificTM 10X Dream Taq buffer), 1.0 µl of 10 mmol/µl dNTPs (Bangalore Genei), 0.5 µl of each 10 mmol SCAR primers (SCAR Fol 1) (Sigma Aldrich, Bangalore), 0.3 µl of 5 U/µl Taq polymerase (Thermo ScientificTM DreamTaq polymerase) and 20.7 µl of nuclease-free water. The SCAR marker amplified products were visualized on lab safe dye stained 1.5% agarose gel under UV Trans illuminator (Genei, Bangalore) in which size of the amplicon was determined using 100-bp DNA ladder (Gene Ruler, Genei, Bangalore).

Specificity of SCAR marker was verified using four isolates of *Fol* DNA samples (*Fol*-101, *Fol*-102, *Fol*-103 and *Fol*-104) and four other fungal species, which are pathogenic to the crop plants viz., *Alternaria alternata, Ascochyta fabae* f. sp. *lentis*, *Stemphylium botryosum* and *Cercospora lentis* infecting lentil. The diversity analysis of 40 *Fol* isolates using 6 primers (Table 1) revealed the existence of genetically variable isolates. A total of 46 reproducible bands were amplified by the primers, out of which 44 were (94.92%) polymorphic and only four (5.08%) were monomorphic. The number of prominent DNA fragments varied from 6 to 10 with an average of 7.6 bands per primer and size ranging between 100 to1571 bp.

Genetic diversity of all the 40 *Fol* isolates was analysed by using 25 RAPD primers. Of which, 6 primers showed polymorphic bands in all the *Fol* isolates. Among them, primer OPI-18 (5'-AGTCAGCCAC-3') produced monomorphic band of 353 bp in all the isolates of *Fol* and this monomorphic band was absent in other fungal species viz., *A. alternata, A. fabae* f. sp. *lentis, S. botryosum* and *C. lentis.* This desirable amplified band (353 bp) from target RAPD primer was selected for SCAR marker development and detection of *Fol.* Among the three SCAR primers viz., (SCAR Fol 1 (F/R), SCAR Fol 2 (F/R) and SCAR Fol 3 (F/R) tested, SCAR Fol 1 F Primer 5'-TCTACCCTGCAGAAACACCA-3' and R Primer 5'-CCTTGAGGAAGAGGGTCACA-3' showed reliable and reproducible amplification (353bp) in representative isolates and infected lentil seed. Validation against representative isolates confirmed the specificity of these markers wherein the expected amplification was obtained in *Fol* isolates but not in other seed and soil borne fungi used as control in SCAR Fol 1(F/R) marker.

Fusarium wilt is one of the most widespread and important disease which causes maximum yield losses to the tune of 25 to 95% in the crop by reducing crop standing in the field (Stoilova *et al.* 2006). 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 (Datta *et al.* 2011).

Molecular marker-based detection is a highly reliable

method, which provides deeper insight in unravelling genetic characterization and identification of pathogen species. These methods provide quicker results for identification of *Fol* with ambiguity. Because of instant and high efficiency in comparison with traditional diagnostic techniques, SCAR markers derived from RAPD were widely used for detection of numerous plant pathogens or plant parts (Nithya *et al.* 2012). RAPD is being widely used DNA based molecular marker, easy to handle, does not require prior DNA sequence information of the species and shows high degree of polymorphism (Farahani *et al.* 2015). Therefore, SCAR marker derived from RAPD is the most reliable, reproducible and accurate one for rapid detection of Fol at an early stage of infection as well as seed based detection for quarantine purpose. The developed SCAR marker derived from RAPD primer is accurate which could be used for quick detection of Fol from genomic DNA of fungi, infected plants and seed. It will also facilitate rapid decision-making to reduce the risk of Fol epiphytotics and segregation of quarantine seed material during phytosanitary certification. Further, the present SCAR marker is technically simple and requires small quantity of DNA for the detection of the Fol isolates.

SUMMARY

The Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lentis* is recognized as a major seed and soil-borne disease of lentil worldwide. Rapid detection of Fusarium wilt is important for the management of disease at an early stage and thereby preventing the epiphytotic. Forty cultures of *Fol* were purified after routine isolation from infected seeds from different geographical locations. The amount of genetic diversity was evaluated using PCR amplification with a set of 6 RAPD primers of selective nucleotide primer pairs. The phylogenetic relationship of RAPD marker revealed diversity among the isolates. The RAPD monomorphic bands were cloned and sequenced. The final SCAR marker designed, provides the amplicon size 353bp with *Fol* isolates. The RAPD derived SCAR marker is the first marker with high sensitivity genomic DNA and species specific for the detection of *Fol.* This species-specific SCAR marker can be employed for the quick detection of fungus in seed during seed certification or seed health testing for long-term conservation of lentil.

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