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Molecular variability among different isolates of *Fusarium solani* causing root rot in chickpea by SSR marker

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Abstract
The Genetic diversity of all the isolates of *Fusarium solani* was analysed by using Simple Sequence Repeat (SSR) markers. The SSR primers were tested for amplification of genomic DNA of *Fusarium solani* isolates. The SSR analysis total 6 primers were screened against six isolates of *Fusarium solani* i.e. Fs1, Fs2, Fs3, Fs4, Fs5 and Fs6. Out of 6 primers 3 primers amplified scorable banding pattern. In which 6 SSR primer produced 41 scorable bands with 80.48% level of polymorphism. In the dendrogram two major clusters were obtained on the basis of analysis Cluster A and Cluster B. Cluster A include Fs4 and Fs6. Cluster B include Fs1, Fs2, Fs3 and Fs5. The similarity matrix indicated that six isolates of *Fusarium solani* exhibited in between 0.880 to 0.323 percent similarity coefficients for SSR primer. The results show that SSR analysis were suitable for the evaluation of genetic polymorphisms among fungal populations.

Keywords: *Fusarium solani*, root rot, SSR, marker, chickpea isolates

Introduction

The genus *Cicer* belongs to the Leguminosae family and to the sub-family Papilionoideae. Most of the *Cicer* species are diploid (2n = 29 = 16), cool season pulse crop with a genome size of 740 Mbp, is widely grown in more than 50 countries representing all the continents (Upadhyaya *et al.* 2011) [9]. Production of chickpea in India during 2015-16 and 2016-17 are 7060 and 9120 thousand tonnes and percent share in total production 43.18 and 41.20 respectively (Directorate of Economics and Statistics). In India Chickpea has 8.60 million ha production area in 2015-16. The chickpea root rot caused by *Fusarium solani* is one of the major factors limiting production of this pulse crop. One of our main reason for lower productivity is several soil borne pathogen were reported to be involved in causing root rot complex of chickpea (Nene and Reddy, 1987) [6]. Fungal disease is the most destructive factor in chickpea production. Root rot caused by *Fusarium solani* is known as one of the major yield reducers. Incidence of wilt and black root rot disease ranged from 9.7%-13.8% and 6.6%-7.4% respectively. Black root rot disease was found in Andhra Pradesh and Karnataka states mainly (Ghosh *et al*., 2013) [5]. The disease occurs at any stage. Affected plants turn yellow and wilt. Rotting of tap roots and lateral roots scattered in the field. The root system of affected plant was rotten, most of the finer roots shed, and the remaining roots turn black, affected plants dry prematurely but may go on producing new roots if sufficient moisture is available. Excessive moisture and moderately high temperature (25-30 ^{0}C) encourage disease development (ICRISAT information bulletin 2012) [6].

The pathogen has ability to infect a large number of hosts. The high amount of variability exists. Hence the present investigation was aimed to analyse the molecular variability among the isolates of *Fusarium solani* causing root rot by SSR primers. Studies of variability is important to understand the diversity of this pathogen for the development of effective management strategies to identify and deploy host resistance. To understand the ecology and pathogenicity aspects of the *Fusarium solani*, it is essential to study about the phenotypic and genetic variability among isolates.

Molecular techniques allow the detection and identification of non-culturable microorganism and useful to determine the genetic differences between two species is important in trying to find out which genetic vulnerabilities and resistance a certain species of *Fusarium solani.* So detection of genetic variability based on molecular markers is reliable technique to assess

variability in the population of pathogen.

Materials and Methods

Isolation

The 6 isolates of *Fusarium solani* was obtain from different places from root rot infected chickpea. The isolates were maintained on Potato Dextrose Agar (PDA) media. The mycelium hypha was picked up from the fungal growth marked, with the help of sterile inoculating needle and transferred on fresh PDA media. The plates were incubated at 27 ± 2 °C for 7 days to obtain pure culture. The 8 isolates were named as FS1, FS2, FS3, FS4, FS5, FS6. Pure cultures of isolates were stored at 4 °C for storage and subsequent studies.

Table 1: List of different isolates of *Fusarium solani*

Sr. No.	Isolates	Location
1.	Fs1	Fs1804
2.	Fs2	Fs1984
3.	Fs3	Fs4352
4.	Fs4	Akola
5.	Fs5	Yavatmal
6.	Fs6	Amravati

DNA isolation

DNA was extracted following CTAB method of DNA extraction by Murray and Thompson (1980). For DNA isolation, Erlenmeyer flasks (250 ml) containing 100 ml of potato dextrose broth were inoculated with two 1-cm discs of actively growing cultures of *Fusarium solani* The cultures were placed on a rotary shaker (100 revs min-1) and incubated at 27 °C for 2-3 days. Mycelia were harvested by filtration through cheesecloth, blotted dry with sterile paper towels and used immediately for DNA extraction. Total genomic DNA was isolated from the fungus following the CTAB method. The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel (containing ethidium bromide @ 0.5 mg/ml) in a horizontal gel electrophoresis system. The gel was observed under UV light with the help of Bio-Rad Gel documentation system and DNA quality was confirmed.

PCR amplification and electrophoresis

The six SSR (Simple sequence repeats) primers of MB-series which indicated a higher degree of polymorphism, were selected for the diversity analysis studies. The identification of *Fusarium solani* isolates was done on molecular basis by using Internal transcript Spacer (ITS) forward (ITS 1) and reverse (ITS 4) (White et al. 1990). The sequence of ITS 1 and ITS 4 are given in the Table 3. The DNA sequences (5'-3') of all primers used for DNA amplification are given in table. Amplification reactions were carried out in volumes of 12.5 µl containing 2 µl (50 ng) template DNA, 1.25 µl Taq Reaction buffer (10 X), 0.75 mM dNTPs, 1 µl forward primer, 1 µl reverse primer, 1.25 µl of (25 mM) MgCl2, 0.5 µl of Taq polymerase (5 U/µl) and 4.75 µl sterile distilled water. Amplification was performed in a thermal cycler (Eppendorf, Germany) programmed for 40 cycles at a temperature regime of 94 °C for 1 min, 55 °C (Annealing temperature is varied from primer to given in table 2) for 45 seconds, 72 °C for 2 minute after an initial denaturation at 94 °C for 5 minutes. Following the cycling, the mixture was incubated at 72 °C for 10 minutes and then kept at 4 °C hold till electrophoresis.

Table 2: List of its primers with their sequence used for identification of *Fusarium solani*

Sr. No.	Oligo Name	Primer Sequence (5' - 3')
1.	ITS 1	TCCGTAGGTGAACCTGCGG
2.	ITS 4	TCCTCCGCTTATTGATATGC

Table 3: List of SSR primers used with their sequence

Sr. No.	Primer name	Primer sequence (5-3')	Annealing temperature (°C)
1	MB-2	F:TGCTGTGTATGGATGGATGG R:CATGGTCGATAGCTTGTCTCAG	56.0
2	MB-5	F:ACTTGGAGGAAATGGGCTTC R:GGATGGCGTTTAATAAATCTGG	54.0
3	MB-10	F:TATCGATCCGGCTTCCAGAAC R:TTGCAATTACCTCCGATACCAC	53.0
4	MB-13	F: GGAGGATGAGCTCGATGAAG R:CTAAGCCTGCTACACCCTCG	55.0
5	MB-17	F:ACTGATTCACCGATCCTTGG R:GCTGGCCTGACTTGTTATCG	57.0
6	MB-18	F:GGTAGGAAATGACGAAGCTGAC R:TGAGCACTCTAGCACTCCAAAC	57.0

Separation on polyacrylamide gel electrophoresis and silver staining

SSR-PCR amplified product was separated on 10% polyacrylamide gel (PAGE) assembly. 10% of PAGE (100 ml) was made by using urea, acrylamide, bisacrylamide and 10X TBE. To the gel 0.085% of 10% Ammonium per sulphate solution and 0.075% of TEMED was added before use. The glass plates, spacers, combs etc. washed and cleaned with ethanol. The polyacrylamide gel solution was poured between the glass plates and comb was placed onto the gel and allowed to solidify for one to one and half hours. 1X TBE buffer was used as tank buffer. After solidification of the gel, comb was removed and pre-running of the gel for 30-60min was followed. After pre-running of gel, the SSR-PCR product (7-10 ul) with 5 ul loading dye and each 5ul sterile water was loaded in a well followed by loading of 100bp DNA ladder (1 ul) with 5 ul loading dye in the first well. The gel was run on 70-80V and 250-300mA V till dye comes closer to bottom. After running of the DNA sample, gel was removed carefully and placed in a plastic tray, rinsed with distilled water and gel staining procedure was followed.

It was then transferred into fixing solution (30 ml Methanol, 1.5 ml Glacial acetic acid, and 270 ml distilled water) and kept for 5 min. giving gentle shake. Then transferred it into staining solution and kept for 2.5 min. After which it was rinsed again in distilled water for 5 min. Finally, it was transferred into developing solution for 10-20 min. The developer was removed and the gel was rinsed again with distilled water. The bands were observed under gel documentation unit and photographed

Data analysis

The gel image was captured and visualized under light in gel documentation system. Data were scored as the presence (1) or absence (0) of individual band for each isolate. This binary data was used to compute the similarity coefficient using the Pearson's similarity coefficient with the help of Numerical Taxonomy System Version 2.2 (NTSYSpc). The similarity matrix was used to construct the dendrogram by Unweighted pair group method of arithmetic average (UPGMA) using the SAHN (Sequential Agglomerative Hierarchal Nested) cluster analysis module (Rohlf, 2000) [8].

Results and Discussion

The investigation was carried out in the laboratory of the Department of Plant Pathology and Biotechnology Centre, Dr. PDKV, Akola. The six isolates of *Fusarium solani* i.e. Fs1, Fs2, Fs3, Fs4, Fs5, Fs6 were collected from different places.

Molecular characterization with SSR primers

The 6 SSR primer of MB Series were screened to evaluate the genetic variability among six isolates of *Fusarium solani*, the six primer produced reproducible and scorable bands with high degree of polymorphism. Genetic diversity was studied among eight isolates using 6 SSR primers of MB series. In which 6 SSR primer produced 41 scorable bands with 80.48% level of polymorphism. Out of 41 bands, 33 bands were polymorphic. The primer MB-10 amplified maximum of 12 bands within the size 1000 bp to 200 bp while Primer MB-2 and MB-17 amplified 7 and 6 bands within the size 950 bp to 175 bp and 700 bp to 105 bp respectively. Primer MB-2 showed 100% polymorphic bands. In the dendrogrm was found to have higher value of similarity coefficient 0.880 whereas found to have lower value of similarity coefficient 0.323. The 8 SSR markers was sufficient for study of genetic diversity in Fusarium complex allelic diversity at the nine loci ranged from 0.0003 to 0.895, (Bogale *et al.* 2005) [3]. Two major clustures were obtained on the basis of analysis. First group is named as clusture A, include Fs6 (Amravati), Fs4 (Akola). Second group is named as cluster B which include Fs5 (Yavatmal), Fs3 (New Delhi), Fs2 (New Delhi), Fs1 (New Delhi). Molecular diversity using SSR marker showed that the Fs3 (New Delhi) had found to be higher similarity index with Fs5 (Yavatmal) and Fs1 (New Delhi) was found to have a lower similarity index with Fs4 (Akola). Respectively, for the confirmation of fungus pathogen *Fusarium solani* ITS marker was done with using ITS1 and ITS4 primer which gives band size of 570bp and 620bp.

Table 4: Similarity coefficient for SSR analysis

	Fs1	Fs2	Fs3	Fs4	Fs5	Fs6
Fs1	1					
Fs2	0.667	1				
Fs3	0.469	0.759	1			
Fs4	0.323	0.364	0.400	1		
Fs5	0.394	0.667	0.880	0.414	1	
Fs6	0.344	0.382	0.419	0.407	0.387	1

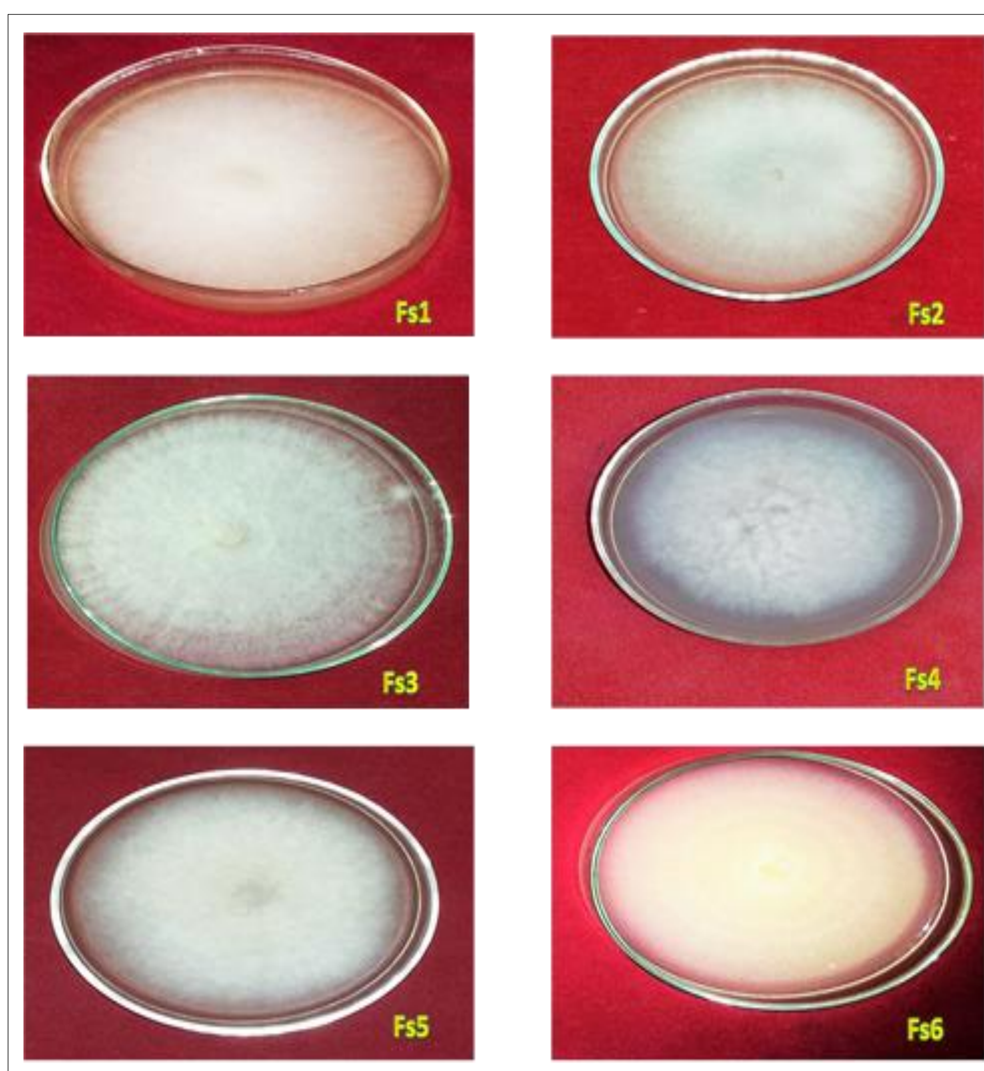

Plate 1: Growth of *Fusarium solani* isolates on PDA media

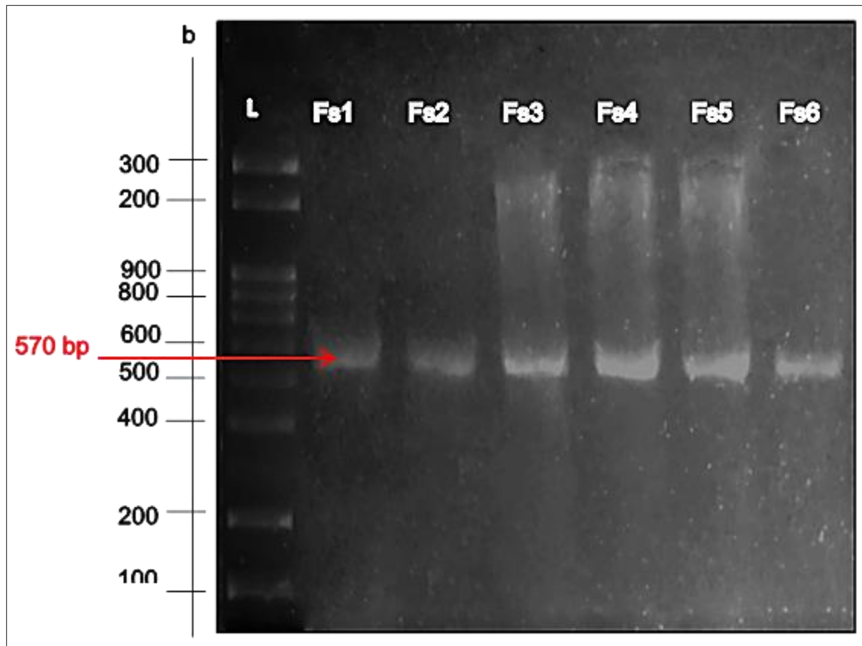

Plate 2: Its banding pattern of primer

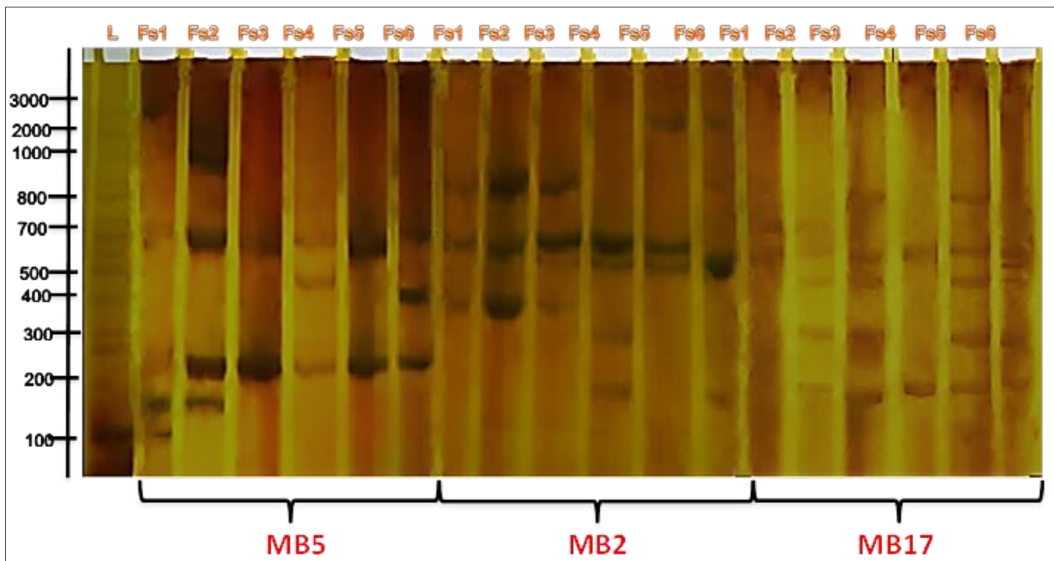

Plate 3: SSR banding pattern of primer MB5, MB2, MB-17

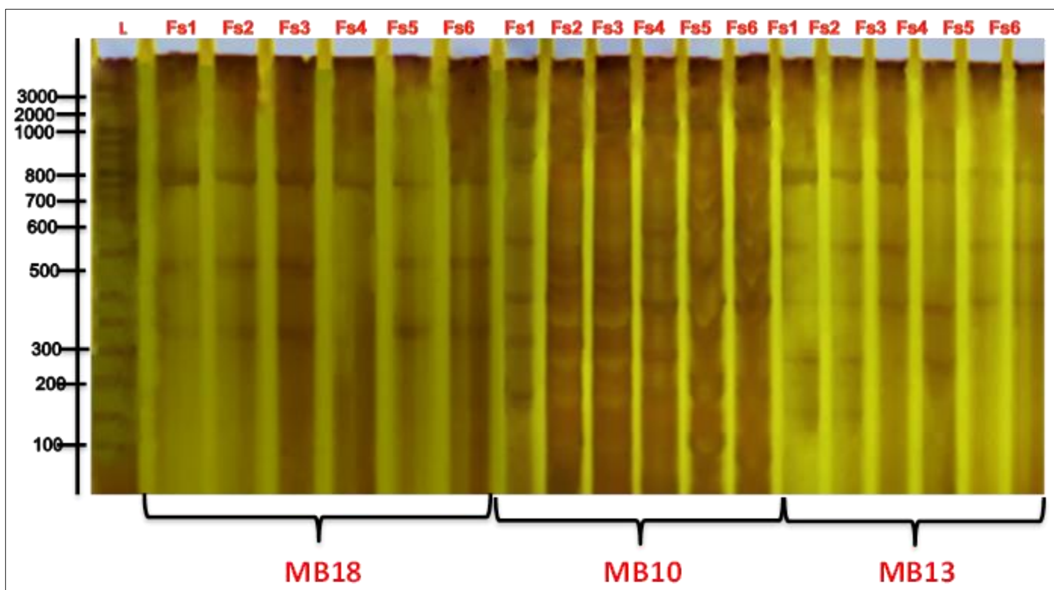

Plate 4: SSR banding pattern of primer MB10, MB13, MB-18

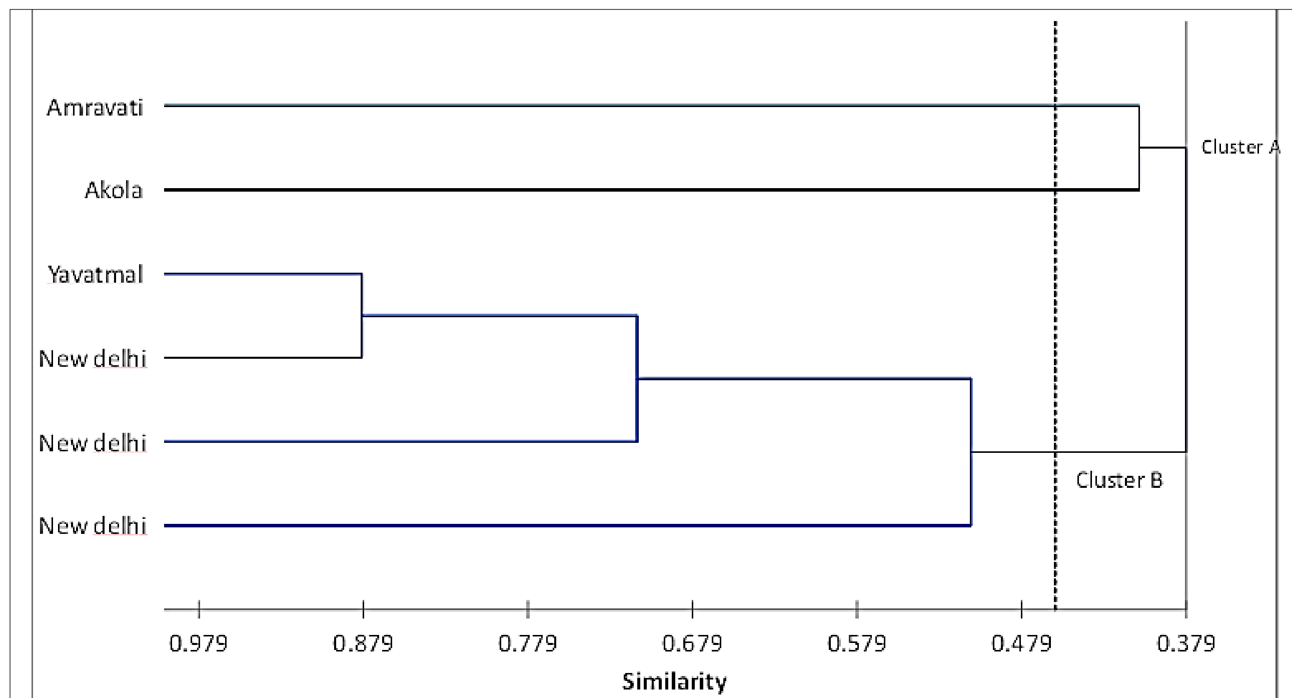

Fig 1: The SSR UPGMA dendrogram of six isolates of *Fusarium solani* based on Jaccard's similarity coefficient

Conclusions

The SSR analysis total six primers were screened against eight isolates of *Fusarium solani*. 6 SSR primer produced 41 scorable bands. Out of 41 bands, 33 bands were polymorphic. The similarity coefficient value ranged from 0.880 to 0.323 across the 6 isolates of *Fusarium solani* indicating high degree of polymorphism in respect to genetic similarity. The Dendrograms generated primer grouped into two major clusters, Cluster-A and Cluster-B. In SSR total % polymorphism among different isolates of *Fusarium solani* is 80.48% observed. Variability was observed among all the 6 isolates of *Fusarium solani* by using SSR molecular markers.

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