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Investigation of Genetic Diversity in Fusarium Wilt of Egg Plant Caused by *Fusarium oxysporum* f.Sp. *melangene* (Schlecht) Mutuo and Ishigami in Marathwada Region of Maharashtra, India

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ABSTRACT

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The experiments described in this chapter were conducted during the Kharif-2018-19. The RAPD marker can be used to find out diversity among different isolates of *Fusarium oxysporum* f.sp. *melongenae*. The highest genetic similarity to the extent of 0.58 (i.e. 58%) was recorded between FOM-2 and FOM-7. This indicates that FOM-2 and FOM-7 isolates of *Fusarium oxysporum* f.sp. *melongenae* were showing maximum similarity based on selected RAPD primers. Whereas, FOM-1 and FOM-5 were found least genetic similar showing similarity 0.25 (25%). It found that the isolates collected from different places as mentioned in description were found to be different from each other. It states that the isolates show diversity among each other. The genetic variability in the FOM isolates may be due to differences in geographical and environmental conditions.

Introduction

Vegetables play an important role in human nutrition and health by providing minerals, micronutrients, vitamins, antioxidants and dietary fiber. Vegetables cultivation is significant part of the national agriculture economy, especially in developing world. Brinjal (*Solanum melongenae*) also known as

eggplant is a common and popular vegetables crop grown in the subtropics and tropics. It belong to family 'Solanaceae' it is one of the widely used vegetables crops by most of the people and is popular in many countries viz. central south and south east Asia, some parts of Africa and central America (Harish *et al.*, 2011) It is grown in 691,000 hectares with production of eight production of eight to nine

million tonnes (equivalent to one quarter of global production), which makes India the second largest producer of brinjal in the world. In India, brinjal is cultivated in 729 hectares with a total production of about 12616 million tonnes. The major brinjal producing states in India are Andhra Pradesh, Maharashtra, Karnataka, Orissa, Madhya Pradesh and West Bengal (IKISAN 2018).

Fusarium oxysporum* f.sp. *melongenae

Brinjal is susceptible to many diseases like *verticillium* wilt (*Verticillium dahliae*), *Fusarium* wilt (*Fusarium oxysporum* f. sp. *melongenae*) and bacterial wilt (*Ralstonia solanacearum*) (Kalloo and Berg, 1993) and (Sihachakr *et al.*, 1994). *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *melongenae*, is a major constraint in brinjal production in India. The disease is widely distributed in tropical, subtropical and some warm temperate regions of the world. The pathogen is difficult to control since it is soil-borne and has a wide host-range, including several hundred species representing 44 families of plants. Infection is through root-to-root transmission, movement of soil and dissemination by farm implements, and insect transmission. A combination of high temperature and poor drainage favor development of the disease which causes 75 to 81% yield loss during summer in India (Das and Chattopadhyay, 1953; Rai *et al.*, 1975; Rao *et al.*, 1976).

Fusarium wilt in brinjal is being managed by application of bactericides, copper fungicides and by crop rotation, with no adequate control. Once the disease develops and wilt symptoms appear in the field, application of bactericides and copper fungicides has no effect on the bacterium. Crop rotation is not a viable control method, as; the bacteria can persist indefinitely in infested fields (Jaworski and Morton, 1964; Sonoda, 1978). In the

absence of effective chemicals and bactericides for managing this disease, emphasis is laid on developing brinjal (brinjal) cultivars with resistance to *Fusarium oxysporum*. Though resistance to *Fusarium* wilt has been studied in several crops, especially tomato, there is little published work on *Fusarium* wilt resistance in brinjal (Chaudhary and Sharma, 2000; Zakir Hussain *et al.*, 2005; Mondal *et al.*, 2013).

Molecular markers

The recent development of random amplified polymorphic DNA (RAPD) has allowed a rapid generation of reliable reproducible DNA fragment that showed of great use in identifying pathogenic variation in several fungal plant pathogens (Jimenez-Gasco *et al.*, 2001).

The main objectives of this work were to determine if RAPDs could be of useful for the characterization of pathotypes and races of *Fusarium oxysporum* f. sp. *melongenae* isolates infecting Brinjal. The present study is with an objective to investigate genetic diversity in fusarium wilt of egg plant caused by *Fusarium oxysporum* f.Sp. *melangene* (Schlecht) Mutuo and Ishigami in Marathwada region of Maharashtra.

Materials and Methods

The present study “Investigation of Genetic Diversity of *Fusarium oxysporum* f. sp. *melongenae* Causing Wilt Disease In Brinjal By RAPD Marker” was carried out at the Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani during the year 2018-2019. The details of field and laboratory procedure followed during this research work are described in this chapter.

Collection of diseased samples

Eight wilt affected Eggplant samples were collected from different regions of Maharashtra (Fig. 1).

These diseased samples were preserved at 4°C in refrigerator and used for further studies.

Sterilization of root sample

The infected root samples were surface sterilized with 0.1% Mercuric chloride (HgCl₂) for 30 sec. and washed 3-4 times with sterilized double distilled water to remove traces of HgCl₂. The surface sterilized root samples were then placed on PDA medium containing streptomycin (0.12 gm/lit) and incubated at 26°C for 5-6 days.

Purification of the *Fusarium oxysporum* f. sp. *melongenae* isolates

All the isolates of the pathogen were purified by hyphal tip method (Dohroo and Shanna, 1992). Sufficient care was taken to maintain the purity of the isolates throughout the study.

Morphological characterization

To study morphology of macroconidia, microconidia and chlamyospore 10-15 day old culture of each isolate grown on PDA medium was stained with 0.1% Lactophenol cotton blue on slide and observed under compound microscope.

Genetic variability

Random amplified polymorphic DNA (RAPD) marker was used to detect the variations among the isolates of *Fusarium oxysporum* f. sp. *melongenae*. Standardized protocol was used for the isolation of DNA and RAPD analysis (Williams *et al.*, 1990).

DNA isolation

The standardized protocol of Cenis (1992) for DNA extraction was used with some modifications and yielded sufficient quantity of DNA which was amenable to PCR amplification.

Extraction of genomic DNA

One week old mycelial mat was filtered through Whatmann No.1 filter paper and air dried.

Mycelium (0.5gm) was transferred to a sterile mortar pestle and ground with glass wool.

1 ml extraction buffer was added to the mortar and the content transferred to the 1.5ml centrifuge tube.

200µl of 3M Sodium acetate (pH- 5.2) was added and tubes were placed at -20°C for 30 minutes.

Tubes were centrifuged at 13000 rpm for 5 minutes and supernatant was transferred to another tube.

Equal volume of Isopropanol was added. The DNA in each tube was precipitated by incubating the mixture at room temperature for 5 minutes.

The precipitated DNA was pelleted by centrifugation in a microfuge tube at 13000 rpm for 10 minutes.

The pellet was rinsed with 70% ethanol and air dried.

The pellet was re-suspended in 50µl of TE buffer.

Purification of DNA

RNase treatments

To degrade RNA present in DNA sample 1µl of RNase A (10 mg/ml) was added in DNA sample solution and incubated it at 37°C for 1 hour in water bath.

Dilution of DNA samples

A part of DNA sample was diluted with appropriate quantity of sterilized double distilled water to yield a working concentration of 30 ng/ μ l and stored at 4°C until PCR amplification.

Agarose gel electrophoresis

The gel electrophoresis unit was, cleaned properly before use 0.8% Agarose gel was prepared by dissolving 0.8 g of Agarose in 100 ml 1X TAE buffer and heated in microwave oven and 5 μ l Ethidium bromide (10 mg/ml) was added to it after cooling down to 45°C. The gel was then poured in mini casting tray in which comb was inserted previously and kept for 1 hour. After solidification the comb was removed. 5 μ l of DNA was mixed with 2 μ l 1X gel loading dye and loaded on the gel. The electrophoresis was carried out at 100 V for 1.5 hours using 1X TAE buffer.

DNA fingerprinting profile

The RAPD-PCR protocol was used with some modifications to produce DNA fingerprinting profile of 8 fungal isolates of *Fusarium oxysporum* f. sp. *melongenae* (Table 1 and Fig. 2–8).

The PCR amplification reaction was optimized by varying concentration of PCR components. Amplification reaction was carried out in 25 μ l reaction mixtures containing 30 ng of fungal genomic DNA, 1X PCR buffer, 1.5 mM MgCl₂, 0.25 mM dNTPs, 10 pmol primers and 1.50 U of *Taq* DNA polymerase. PCR amplification was performed in master cycler gradient, Eppendorf PCR thermocycler, and the program consisted of an initial denaturing at 94 °C for 4 min, followed by 39 cycles comprising denaturation at 94 °C, 1 min,

annealing at 37 °C and extension of 2 min. at 72 °C. The final extension was set at 72 °C for 10 min. PCR amplified product was separated by electrophoresis on 1.5 % agarose gel in 1X TAE buffer, stained with Ethidium bromide and visualized under gel documentation system.

RAPD Analysis

Five random primers viz., OPA-16, OPA-06, OPA-12, OPA-04, OPA-10, were screened which showed clear banding pattern, which are used for genetic diversity analysis of 8 FOM isolates. The PCR reactions were set in a 25 μ l reaction volume.

PCR reaction

Master mix was prepared with the above mentioned reagents and divided into 8 equal parts (each of 24 μ l) into 8 different PCR tubes. 1 μ l of 8 different genomic DNA samples of fungal isolates was added to master mix by changing tips to avoid contamination that leads to final quantity of 25 μ l. PCR tubes were then placed in thermal cycler for amplification of the genomic DNA as per the standardized protocol by Williams *et al.*, (1990) with some modifications according to selected primers.

Resolution of amplified product

The amplified products were resolved on 1.5% Agarose gel at 60 volts for appropriate hours. The gel was stained with Ethidium bromide (5 μ l/100ml). After electrophoresis, the gel was carefully removed from the casting tray and photograph was taken on a Gel documentation system (Alphaimager).

Data scoring and analysis

Amplified products in the gel images were scored for presence (1) or absence (0) missing

and doubtful case were scored as 9. Homology of bands based on the distance of migration of amplified DNA fragments according to their molecular weights in the gel was determined. Molecular weights of the bands were estimated using 100 bp DNA ladder as standard. Data analysis was performed using NTSYS-PC (Numerical Taxonomy System) Version 2.02 software (Rohlf, 1990).

Results and Discussion

The results of present study entitled Investigation of genetic diversity in fusarium wilt of egg plant caused by *Fusarium oxysporum* f.sp. *melongene* (Schlecht) Mutuo and Ishigami in Marathwada region of Maharashtra. studied 8 isolates belonging to different species of *Fusarium oxysporum* f. sp. *melongenae* were subjected to amplification by RAPD. The data obtained from RAPD marker clearly distinguished the isolates of fungus used for study of genetic relationships. The marker method differentiated clusters within species. The results obtained in the study are presented under following headings.

Isolation and Identification of the *Fusarium oxysporum* f. sp. *melongenae*

Fusarium oxysporum f. sp. *melongenae* were isolated from the roots of wilted plants of Brinjal. Eight FOM isolates of *Fusarium oxysporum* f. sp. *melongenae* were obtained. Growth of fungus was observed 3-4 days after incubation at 27°C in all FOM isolates. The isolates were fluffy in its growth while the colour was dull white, pinkish white and pink depending upon the isolates (Fig. no.2).

On the basis of macroconidia, microconidia and chlymadosores the isolates were identified as *Fusarium oxysporum* f. sp. *melongenae* on the basis of morphological characteristics feature mentioned in the

monograph by Booth (1971). The isolates were purified and mass multiplied for further studies.

DNA extraction

Genomic DNA of 8 *Fusarium oxysporum* f.sp. *melongenae* isolates was extracted from their mycelial mat grown on Potato dextrose broth by using protocol of Cenis (1992) with some modification and yielded 350-400ng/μl quantity of DNA which was amenable to PCR amplification.

Genetic diversity analysis of *Fusarium oxysporum* f.sp. *melongenae* using RAPD Marker

The DNA fingerprint was generated through RAPD primer (OPA-04, OPA-10, OPA-12, OPA-16, and OPA-06) as shown in figure 4 to 8. Based on the fingerprint data polymorphism percentage for each RAPD primer was calculated for eight isolates of *Fusarium oxysporum* f. sp. *melongenae*.

Percent Polymorphism (%) =

$$\frac{\text{No. of polymorphic bands}}{\text{Total no. of bands}} \times 100$$

Eight isolates of *Fusarium oxysporum* f. sp. *melongenae* were differentiated on the basis of their RAPD pattern. In all 47 bands were generated from 8 isolates using 5 arbitrary primers tested with an average of 9.4 bands per primer. The no. of amplified bands ranged from 5-13.

The primer OPA-10 produced lowest number of bands (05) whereas primer OPA-04 produced highest (13) bands. The most informative primer was OPA-04 with 13 polymorphic bands. In present study, the similarity coefficient value was ranged between 0.25 to 0.58 across eight isolates

indicated existence of genetic variation among selected eight isolates of *Fusarium oxysporum* f.sp. *melongenae*. The highest genetic similarity to the extent of 0.58 (i.e. 58 %) was recorded between FOM-2 and FOM-7. This indicates that FOM-2 and FOM-7 isolates of *Fusarium oxysporum* f.sp. *melongenae* were showing maximum similarity based on selected RAPD primers.

Whereas, FOM-1 and FOM-5 were found least genetic similar showing similarity 0.25 (25%). The highest genetic distance was found in FOM-1 and FOM-5, they were 0.75 (75%) diverse from each other. Least genetic distance was found in FOM-2 and FOM-7 which is 0.42 (42%). Whereas FOM-2 and FOM-7 were found lowest genetic distance showing 0.42 (42%) (Table 2–7).

Table.1 Isolates of *Fusarium oxysporum*. f. sp. *melongenae* collected from different regions of Marathwada

Sr.No	Isolate Codes	District	Tashil
FOM-1	FOM A3	Aurangabad	Soegaon
FOM-2	FOM B5	Beed	Ambajogai
FOM-3	FOM H7	Hingoli	Sengaon
FOM-4	FOM J10	Jalna	Badnapur
FOM-5	FOM L14	Latur	Chakur
FOM-6	FOM N16	Nanded	Kinwat
FOM-7	FOM O20	Osmanabad	Tuljapur
FOM-8	FOM P22	Parbhani	Gangakhed

Table.2 List of primers used for RAPD analysis

Sr. No.	Name of Primer	Sequence	Base pair
1	OPA-16	5'AGCCAGCGAA3'	10
2	OPA-06	5'GGTCCCTGAC3'	10
3	OPA-12	5'ACGGTACCAGT3'	10
4	OPA-04	5'CACCCCCTTG3'	10
5	OPA-10	5'GTGATCGCAG3'	10

Table.3 Components of RAPD-PCR

Sr. No.	PCR Components	Volume for 1 tube	Final Conc.
1	PCR Buffer	2.5µl	10X
2	MgCl ₂	2.0µl	25mM
3	dNTPs	1.25µl	10mM
4	Primer	1.0µl	30pmol
5	<i>Taq</i> DNA Polymerase	0.33µl	1U
6	Template DNA	1.0 µl	30ng
7	Nuclease Free Water	17.05 µl	---
Total Volume		25µl	

Table.4 Temperature profile used for DNA amplification by RAPD Marker

Sr. No.	Steps	Temperature (°C)	Duration	Cycle
1	Initial Denaturation	94	5 min	40 Cycles
2	Denaturation	94	1 min	
3	Annealing	36	1 min	
4	Primer Extension	72	2 min	
5	Final Extension	72	10	
Hold-4°C				

Table.5 Percent polymorphism of RAPD primers for eight isolates of *Fusarium oxysporum* f.sp. *melongenae*

Primer Name	No. of Bands	No. of Polymorphic Bands	No. of Monomorphic Bands	% Polymorphpism
OPA-16	11	8	3	72.72
OPA-10	5	4	1	80.00
OPA-12	12	11	1	91.66
OPA-06	6	6	0	100.00
OPA-04	13	13	0	100.00
Total	47	42	5	--
Average	9.4	8.4	1	88.87%

Average percentage of polymorphism by RAPD marker: 88.87%

Average number of bands amplified per primer: 9.4

Average number of polymorphic bands per primer: 8.4

Average number of Monomorphic bands: 1

Table.6 Jaccard' Similarity Matrix of eight isolates of *Fusarium oxysporum* f.sp. *melongenae* based on RAPD data

Isolates	FOM-A2	FOM-B5	FOM-H7	FOM-J10	FOM-L14	FOM-N16	FOM-O20	FOM-P22
FOM-A2	1.00							
FOM-B5	0.37	1.00						
FOM-H7	0.40	0.57	1.00					
FOM-J10	0.38	0.40	0.35	1.00				
FOM-L14	0.25	0.36	0.34	0.50	1.00			
FOM-16	0.39	0.50	0.31	0.56	0.51	1.00		
FOM-20	0.32	0.58	0.45	0.51	0.55	0.53	1.00	
FOM-P22	0.31	0.51	0.39	0.42	0.48	0.56	0.55	1.00

Table.7 Genetic distances between of eight isolates of *Fusarium oxysporum* f.sp. *melongenae* based on similarity index

Isolates	FOM-1	FOM-2	FOM-3	FOM-4	FOM-5	FOM-6	FOM-7	FOM-8
FOM-A2	0.00							
FOM-B5	0.63	0.00						
FOM-H7	0.60	0.43	0.00					
FOM-J10	0.62	0.60	0.75	0.00				
FOM-L14	0.75	0.64	0.66	0.50	0.00			
FOM-16	0.61	0.50	0.69	0.44	0.49	0.00		
FOM-20	0.68	0.42	0.55	0.49	0.45	0.47	0.00	
FOM-P22	0.69	0.49	0.61	0.58	0.52	0.44	0.45	0.00

Fig.1 Wilted plants of Brinjal



Fig.2 Pure cultures of *Fusarium oysporum* f.sp. *melongenae* collected from various regions of Marathwada

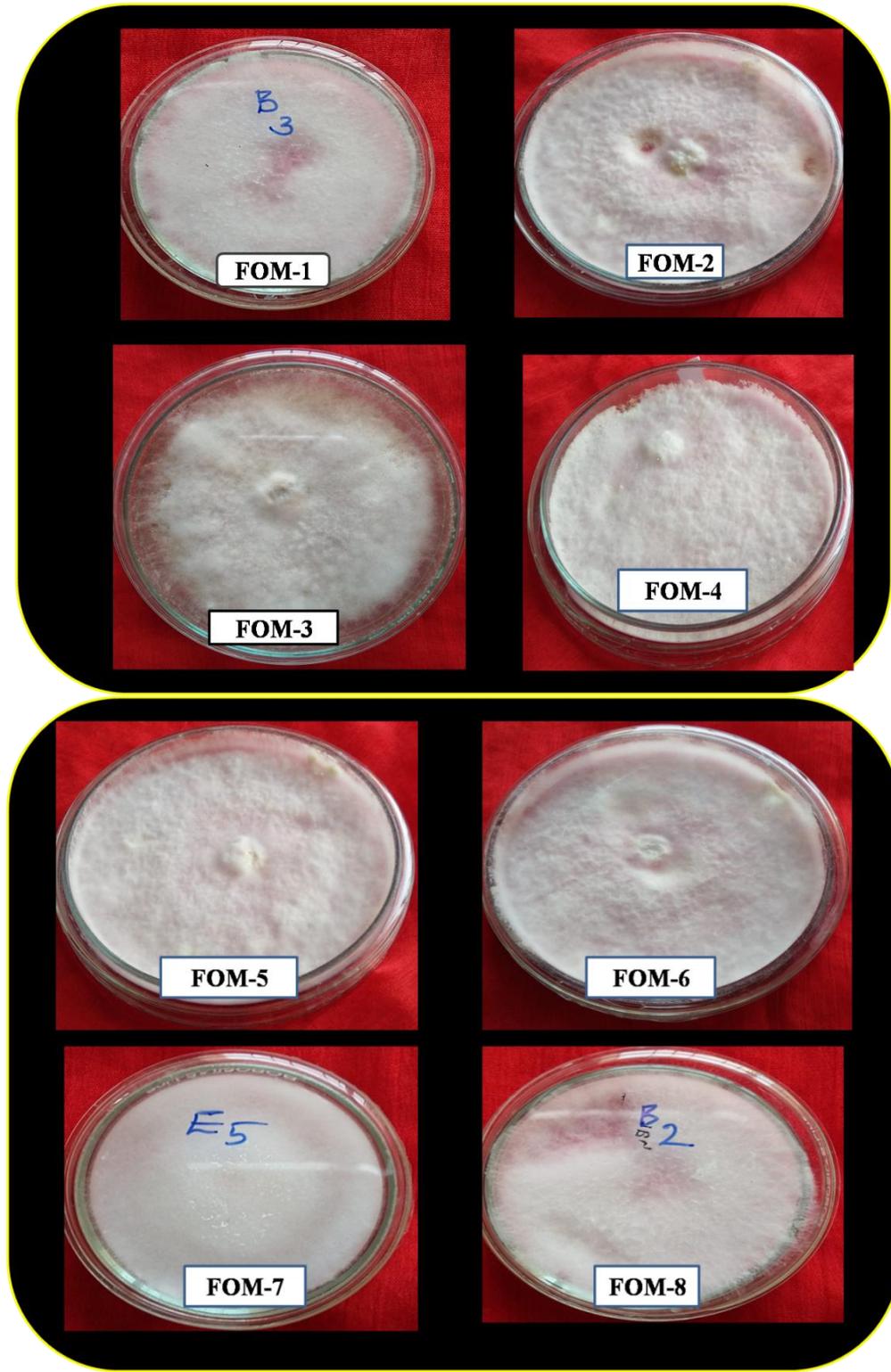


Fig.3 DNA was isolated from eight *Fusarium oxysporum* f.sp. *melongenae*

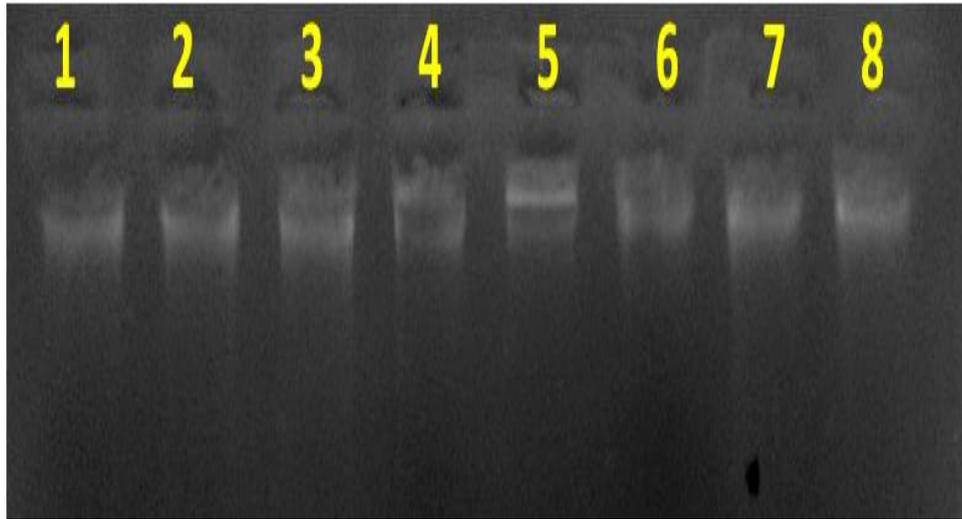


Fig.4 RAPD profile of FOM isolates generated by using primer OPA-16.

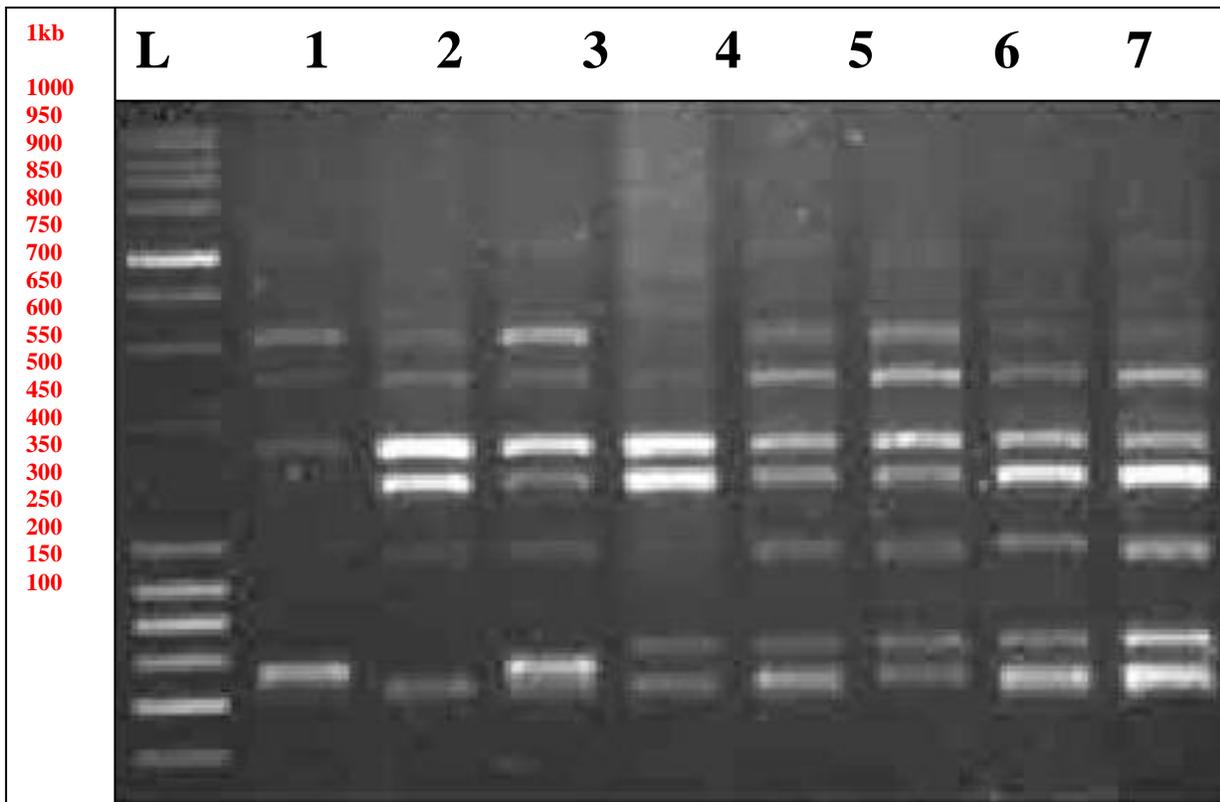


Fig.5 RAPD profile of FOM isolates generated by using primer OPA-10.
L-1kb DNA ladder; lane 1-8 FOM isolates described in table 1

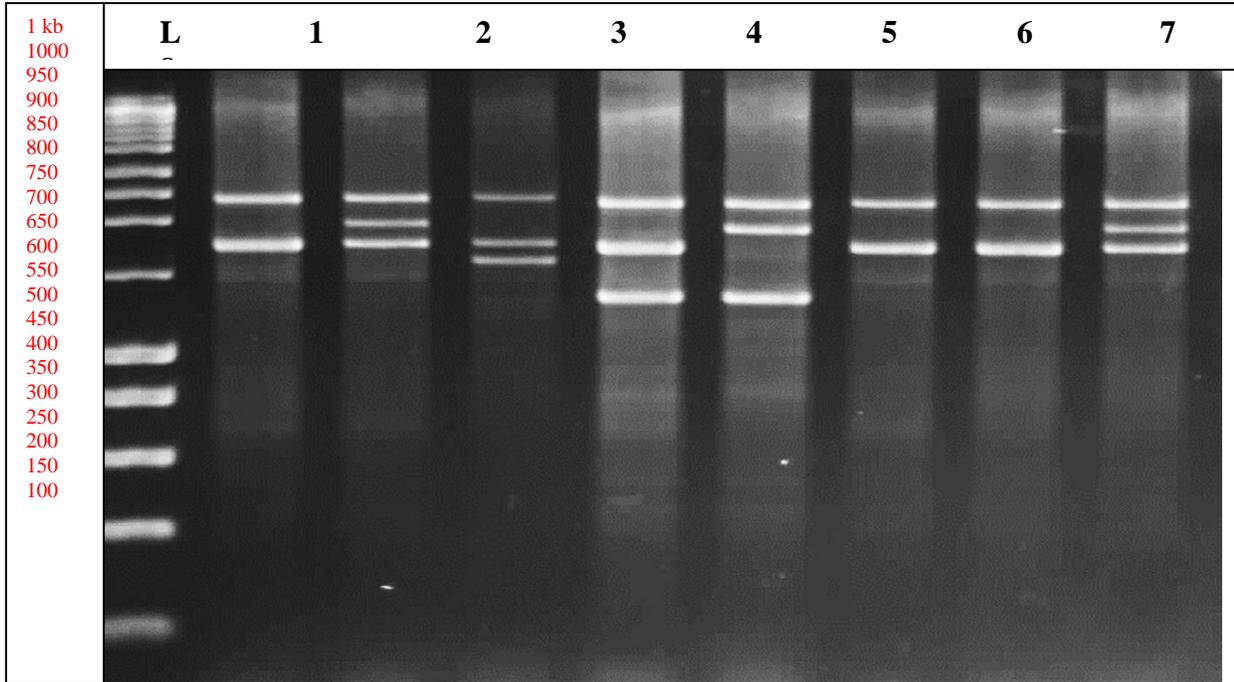


Fig.6 RAPD profile of FOM isolates generated by using primer OPA-12.
L- 1kb DNA ladder lane 1-8 FOM isolates described in table 1

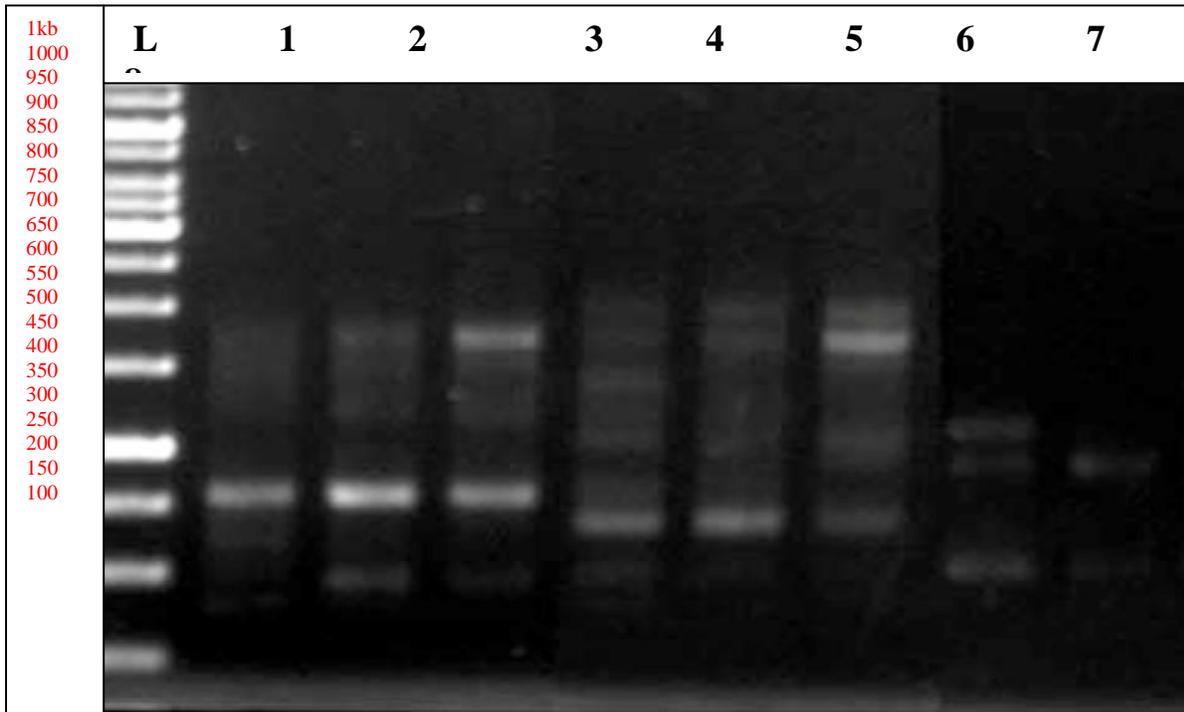


Fig.7 RAPD profile of FOM isolates generated by using primer OPA-06.
L-1kb DNA ladder; lane 1-8 FOM isolates described in table 1

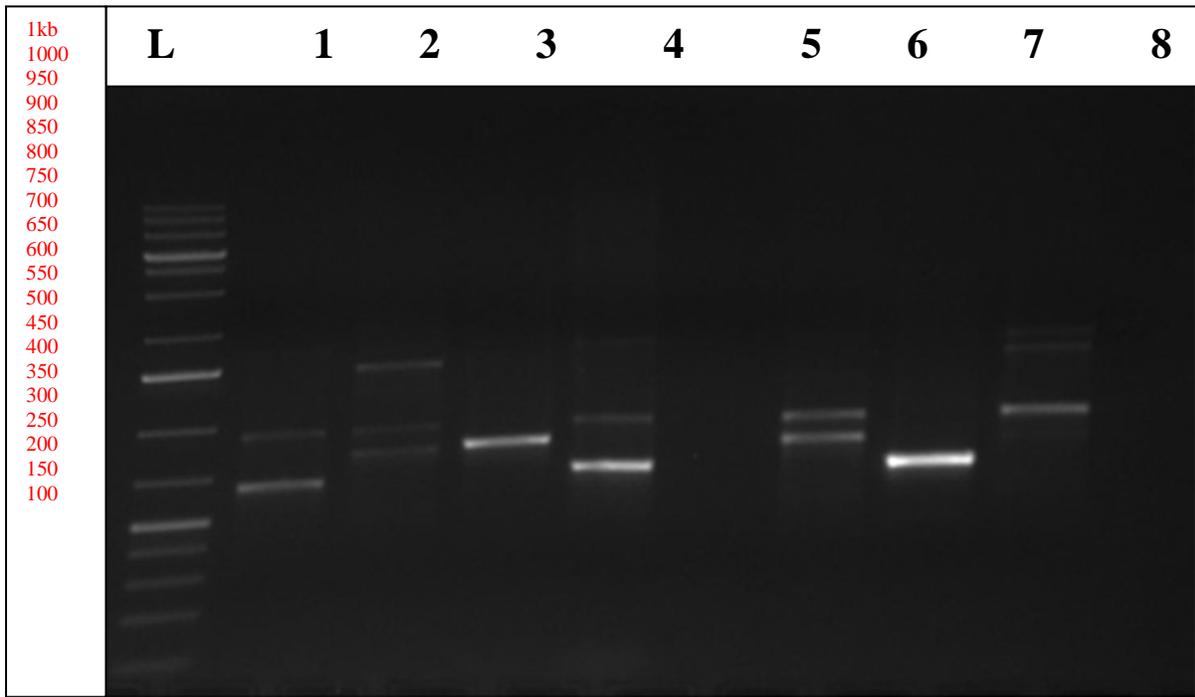


Fig. 8 RAPD profile of FOM isolates generated by using primer OPA-04
L-1kb DNA ladder; lane 1-8 FOM isolates described in table 1

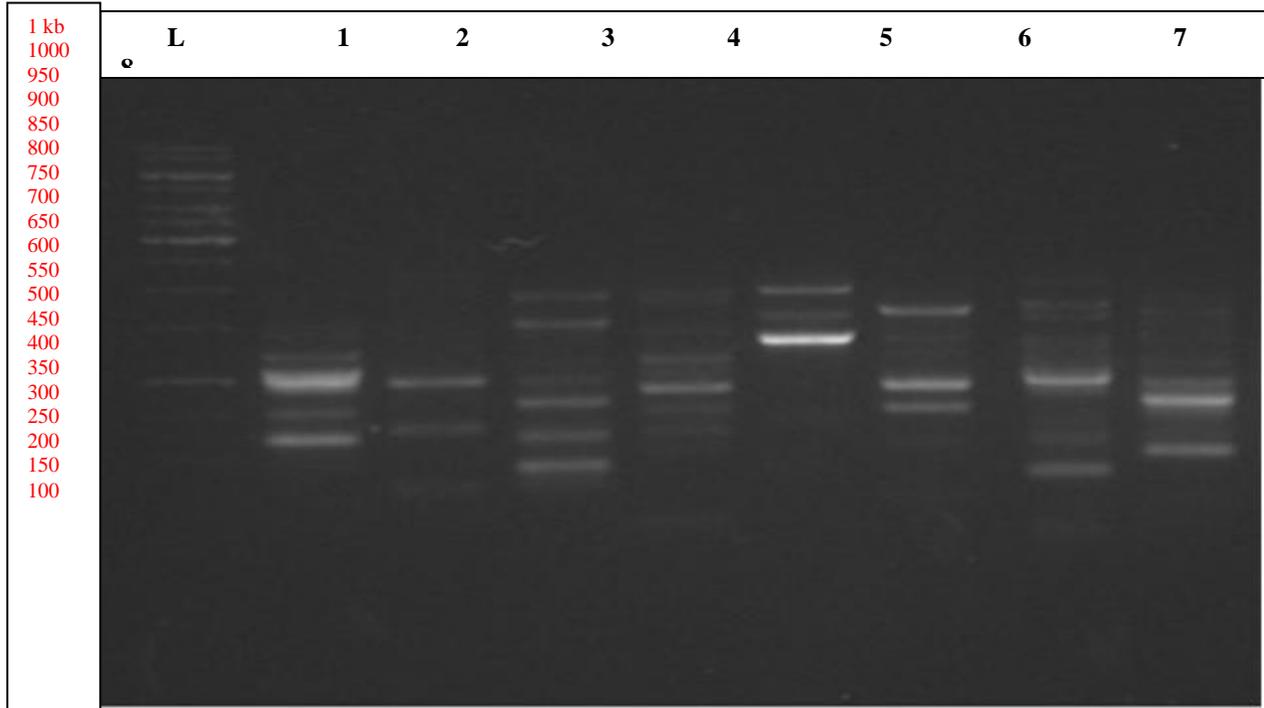
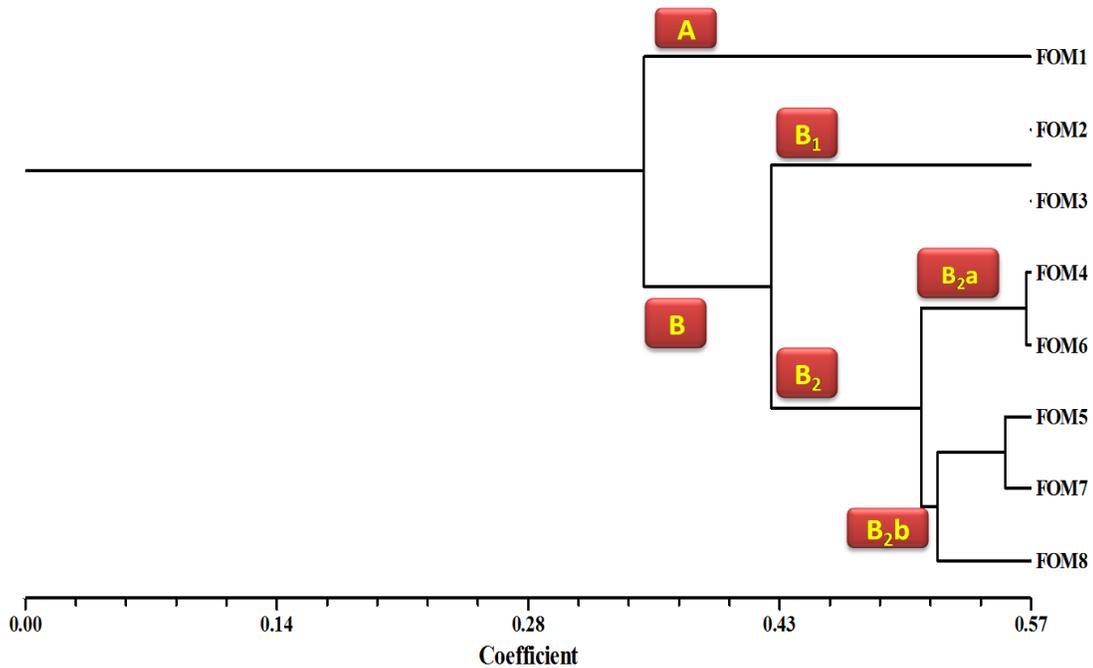


Fig.9 Dendrogram generated using NTSYS analysis demonstrating the relationship among eight FOM isolates



Genetic diversity analysis among eight FOM isolates based on dendrogram generated using RAPD Marker

Dendrogram was constructed on the basis of their variants from the entire data set of makers assisted identification and analysis of the 8 isolates of *Fusarium oxysporum* f. sp. *melongenae*.

Cluster based on RAPD marker analysis revealed the genetic distance ranged from 0.0 to 0.57, which was used to generate the dendrogram by Jaccards coefficient of similarity & UPGMA cluster analysis.

According to the cluster analysis, all the 8 FOM isolates were found distributed into 2 major cluster A and B at 35% similarity value. Cluster A contain only one isolates viz. FOM-1 at 35% similarity. Of the entire cluster formed the largest one is cluster B having seven FOM isolates viz. FOM-2, FOM-3, FOM-4, FOM-6, FOM-5, FOM-7, and FOM-8. Cluster B is subdivided into

cluster B₁ and B₂ at 42% similarity. Sub cluster B₁ contain 2 isolates viz. FOM-2 and FOM-3 whereas sub cluster B₂ contain 5 isolates viz. FOM-4, FOM-6, FOM-5, FOM-7, and FOM-8.

Cluster B₂ is further divided into B_{2a} and B_{2b}. Cluster B_{2a} comprises isolates FOM-4 and FOM-6 at 56% similarity. In cluster B_{2b} 3 FOM isolates are included viz. FOM-5 FOM-7 & FOM-8 at 51% similarity. Thus, in present studies molecular variability was observed among the isolates of *Fusarium oxysporum* f. sp. *melongenae* may be attributed to their geographic distribution in the Marathwada region of Maharashtra state, long term influence of weather parameters at a particular location and ability of the pathogen to adopt the chilli varieties grown.

Prasad *et al.*, (2004) have reported 30-100 per cent genetic similarity among the isolates of *F. oxysporum*. The high polymorphism among the isolates may be due to the nucleotide alterations, insertions and deletions

at initiation sites, recombination or gene flow, which may result in polymorphic DNA (Williams *et al.*, 1990; Burdon and Silk, 1997; Kang *et al.*, 2002). Previous studies by Kang *et al.*, (2002) have indicated that the URP region in fungi is conserved and any change in the region may enable us to detect the variations at the interspecific and intra-specific levels. Some monomorphic bands are shared by all the isolates specific to particular species or specific to all the isolates of *F. oxysporum* and *F. solani*, which support the presence of certain gene/region in those isolates, which were responsible for their specific amplification to be separated into two different species. The clustering of the isolates in the UPGMA analyses was associated with the geographic localities from which the isolates were obtained but some isolates demonstrated variation to a particular locality. The observed deviations could be due to migration of conidia of the isolates through air/planting materials from one location to another. DNA banding pattern (DNA fingerprints) very specific to species *F. oxysporum* and/or *F. solani* obtained in the present study would be highly useful for rapid identification and differentiation of *Fusarium* isolates belonging to different species.

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