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Molecular characterization of *Fusarium oxysporum* f. sp. *melongenae* (Schlecht) mutuo and Ishigami in Marathwada region of Maharashtra by using ITS-RFLP Markers

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Abstract
ITS-RFLP (Internal Transcribed Spacer-Restriction Fragment Length Polymorphism) marker were used to study the genetic variability among *Fusarium oxysporum* f. sp. *Melongenae* isolates from different regions of Marathwada collected during the summer 2018-2019. A total 2 restriction enzymes were used for the digestion of ITS region of the Fom isolates Study of ITS-RFLP analysis for genetic diversity shows the genetic relationship between the 8 Fom isolates. The major cluster I consisted of seven isolates *viz.* Fom-1 (Soegaon), Fom-2 (Ambajogai), Fom-4 (Badnapur), Fom-5 (Chakur), Fom-6 (Kinwat), Fom-7 (Tuljapur) and Fom-8 (Gangakhed) while second major cluster II comprises single isolates Fom-3 (Sengaon) shown 33% genetic similarity with cluster I and genetic diversity 67% with all isolates.

Keywords: Molecular characterization, *Fusarium oxysporum* f. sp. *melongenae* (Schlecht), ITS-RFLP Markers

Introduction
Brinjal (*Solanum melongena* L.) is one of the most common, and highly productive vegetables grown globally and cultivated widely in India. Brinjal belongs to the family *Solanaceae* and is the most important and widely consumed vegetable in India. It is grown in 691,000 hectares with 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).
Brinjal, *Solanum melongena*, also known as aubergine or eggplant, is an important vegetable crop of central, south and south-east Asia and several African countries (Kalloo, 1988). It is a good source of minerals and vitamins, in total its nutritional value is comparable to other vegetables, and has several medicinal properties (Khan, 1979) [22]. The area between India and Indochina is considered to be the centre of brinjal diversity (Vavilov, 1992) [41]. The National Bureau of Plant Genetic Resources (NBPGR), New Delhi has the major responsibility of collecting, characterizing and conserving the brinjal diversity in the country, and presently maintains over 2500 accessions.

Fusarium wilt of brinjal
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) [20]. *Fusarium* wilt, caused by *Fusarium oxysporum*, 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 favour 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) [13, 34, 35].

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 (Sonoda, 1978) [40]. In the absence of effective chemicals and bactericides for managing this disease, emphasis is laid on developing 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) [8, 43, 28].

ITS marker

Molecular markers are versatile and highly informative tools for fungal pathogen identification and diagnosis (Majer *et al*., 1996) [26]. They can be used to evaluate levels of genetic diversity and phenotypic relationships within and between species and to identify particular races and pathotypes (Brown, 1996). Nuclear RDNAS, and particularly the ITS regions, are a good target for phylogenetic analysis in fungi (White *et al*., 1990; Bruns *et al*., 1991; Chillali *et al*., 1998) [42, 5, 10] due to presence of variable region suitable for PCR amplification, restriction analysis and sequencing procedure (Pryor and Gilbertson, 2000) [33]. Internal transcribed spacers (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan and Daniels, 1995) [6]. In addition to standard ITS-Fu-F and ITS-Fu-R primers used by most laboratories, several taxons specific primers have been described that allow selective amplification of fungal sequence (Gardes and Bruns, 1993) [18]. In the broader context, taxon selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. Taxon selective ITS amplification has already been used for the detection of fungal pathogens such as *Fusarium* (O'Donnell, 1992) [32] and *Verticillium* species (Nazar *et al*., 1991) [30]. Therefore we focused on ITS region of the ribosomal genes for the analysis of genetic variability among *Fusarium oxysporum* f. sp. *Melongenae* isolates.

The present investigation was undertaken to assess genetic variability among *Fusarium oxysporum* f. sp. *Melongenae* isolates causing wilt in brinjal with following objective, Isolation of *Fusarium oxysporum* f. sp. *Melongenae* from various region of Marathwada. And Analysis of genetic diversity by using ITS-RFLP marker.

Materials and Methods

The present study entitled "Molecular Characterization of *Fusarium oxysporum* f. sp. *Melongenae* (Schlecht) Mutuo and Ishigami in Marathwada region of Maharashtra by using ITS-RFLP 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.

Experimental materials

The wilt affected brinjal plant root samples were collected in sterile bags from different brinjal growing regions of Marathwada (Table No.1) and preserved at 4 °C in refrigerator for further studies.

Table 1: List of *Fusarium oxysporum* f. sp. *Melongenae* isolates 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

Methods

Isolation of *Fusarium oxysporum* f. sp. *Melongenae*

The infected root samples were surface sterilized with 0.1% Mercuric chloride ($HgCl_2$) for 30 sec. and washed 3-4 times with sterilized double distilled water to remove traces of $HgCl_2$. The surface sterilized root samples were then placed on PDA medium containing streptomycin (0.12 gm/lit) and incubated at 26±2 °C for 5-6 days.

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

All the isolates of the *Fusarium oxysporum* f. sp. *melongenae* pathogen were purified by hyphal tip method (Dohroo and Sharma, 1992). Sufficient care was taken to maintain the purity of the isolates throughout the study.

Morphological characterization

Observation on cultural characteristics of *Fusarium oxysporum* f. sp. *melongenae* viz. colony colour, growth, pigmentation and sporulation were recorded a week after inoculation. To study morphology of macroconidia, microconidia and chlamydospore 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.

Identification and maintenance of FOM isolates

The fungal growth showing different colouration on PDA media depending on the isolates were identified on the basis of morphological characteristics i.e. microconidia, macroconidia and chlamydospores mentioned by Booth (1971) [4]. Microphotographs of the *Fusarium oxysporum* f. sp. *melongenae* isolates were taken to describe spore morphology. All the isolates were then maintained on PDA medium at 4 °C and subculture every three months.

Genetic variability

Internal Transcribed Spacers (ITS) and RFLP analysis was used to detect the variations among the isolates of *Fusarium oxysporum* f. sp. *melongenae*. Standard protocols were used for the isolation of DNA (Cenis, 1992) and ITS analysis (Das, 2015).

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, which is as follows:

- 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.

- One ml of extraction buffer was added to the mortar and the content transferred to the 1.5ml centrifuge tube.
- 200 µl 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 centrifuge tube at 13000 rpm for 10 minutes. The pellet was rinsed with 70% ethanol and air dried.
- The pellet was re-suspended in 50µl TE buffer.

ITS amplification of FOM isolates

ITS amplification of genomic DNA was carried out by polymerase chain reaction using ITS primer. The variability among fungal isolates was studied by using ITS-RFLP and subjected to reveal diversity, based on cleaved polymorphic sequences.

Table 2: Nucleotide sequence and annealing temperature of ITS marker.

Sr. No.	Primer ID	Sequence	Base pair (bp)	Annealing Temperature (°C)
1.	ITS-Fu-F	5'CAACTCCCAAACCCCTGTGA3'	20	54.5
2.	ITS-Fu-R	5'GCGACGATTACCAGTAACGA3'	20	53.7

Table 3: Components of PCR for ITS amplification.

Sr. No.	Components	Stock conc.	Volume/Reaction (µl)
1.	Nuclease free water	-	13.82
2.	PCR buffer	10X	2.5
3.	$MgCl_2$	25mM	2.0
4.	Primer forward	10pM	1.3
5.	Primer reverse	10pM	1.3
6.	DNTPs	10mM	1.25
7.	Taq DNA polymerase	3U/µl	0.33
8.	Genomic DNA	25ng	2.5
Total volume			25 µl

Table 4: Temperature profile used for amplification of RDNA its region of *Fusarium oxysporum* f. sp. *Melongenae* isolates.

Step no.	Steps	Temperature	Cycle	Time
1.	Initial Denaturation	94 °C	1	4 min
2.	Denaturation	94 °C	35	1 min
3.	Annealing	56 °C		1 min
4.	Extension	72 °C		1.5 min
5.	Final extension	72 °C	1	6 min
6.	Hold	4 °C	1	∞

Resolution of amplified product

The amplified products were resolved on 1.2% Agarose gel at 60 volts for 1 hour. The gel was stained with Ethidium bromide (5µl/100ml). After electrophoresis, the gel was carefully removed from the casting tray and photographed in a Gel Documentation System (Alpha-Innotech, USA).

Restriction digestion of rDNA ITS region

PCR amplified rDNA product of *Fusarium oxysporum* f. sp. *melongenae* isolates was subjected for restriction digestion with unique restriction enzyme *viz. Eco*RI and *Hae*III. The details of restriction enzymes used is as follows (Table no. 5).

Table 5: List of restriction enzymes and their sources.

Sr. No.	Restriction enzymes	Recognition Sequence	Source
1.	*Eco*RI	GAATTC	*E. coli*
2.	*Hae*III	GGCC	*Haemophilus aegyptius*

Restriction enzyme analysis was performed in 50µl reaction mixture according to the manufacturer's instructions (Table No. 6).

Table 6: Components of restriction digestion.

Sr. No.	Reaction components	Volume/reaction (µl)
1.	10X Restriction assay buffer	5
2.	100X with BSA	0.5
3.	Restriction enzymes (5U/µl)	0.5
4.	Amplified PCR product	10
5.	Nuclease free water	34
Total volume		50µl

Restriction digestion was carried out at 37 °C for 4 hours in water bath. The digested product was separated on 4% Agarose gel and visualized in Gel Documentation System.

Analysis of genetic diversity of FOM isolates from RFLP data

The restriction digestion analyses were performed in 50µl purified PCR product of FOM isolates. Two restriction enzymes *viz. Eco*RI and *Hae*III were used to digest the amplified products. The restricted fragments were analyzed on 4% Agarose gel in 1X TAE buffer at 60V for 1 hour. The molecular size of each fragments were estimated using 100bp ladder (Genei, Banglore).

Data scoring and analysis

The amplified products generated from RFLP were resolved on Agarose gel. The amplicons which distinguishes *Fusarium oxysporum* f. sp. *melongenae* isolates were observed and scored. The images were scored for presence (1), absence (0) and missing and doubtful case scored as 9. Band size was determined by using software Alpha Ease FC 4.0 with reference to 100bp DNA ladder respectively for ITS and ITS-RFLP products. The data were entered into binary matrix and subsequently analyzed using NTSYS pc (Numerical Taxonomy system), version 2.02*i* (Rohlf, 1998) [36]. Coefficients of similarity were calculated by using Jaccard's similarity coefficient by SIMQUAL programme. Dendrogram was constructed by agglomerative technique using the Un-weighted Pair Group Method with Arithmetic Mean (UPGMA) method based on Jaccard's Similarity Coefficient.

Results

The results of present study entitled "Molecular Characterization of *Fusarium oxysporum* f. sp. *Melongenae*

Causing Wilt in Brinjal By using ITS-RFLP Marker" is described below. In this study 8 isolates collected from different regions of Marathwada were subjected to amplification by ITS-RFLP marker. The data obtained from RFLP 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 FOM isolates

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 ºC in all FOM isolates. The culture was fluffy in its growth while the colour was dull white, pinkish white and pink depending upon the isolates (Plate no.1). On the basis of macroconidia, microconidia and chlymadospores the isolates were identified as *Fusarium oxysporum* f. sp. *Melongenae* on the basis of morphological characteristics feature mentioned in the monograph by Booth (1971) [4]. The isolates were purified and mass multiplied for further studies.

Fig 1: Typical symptoms of Fusarium wilt in brinjal

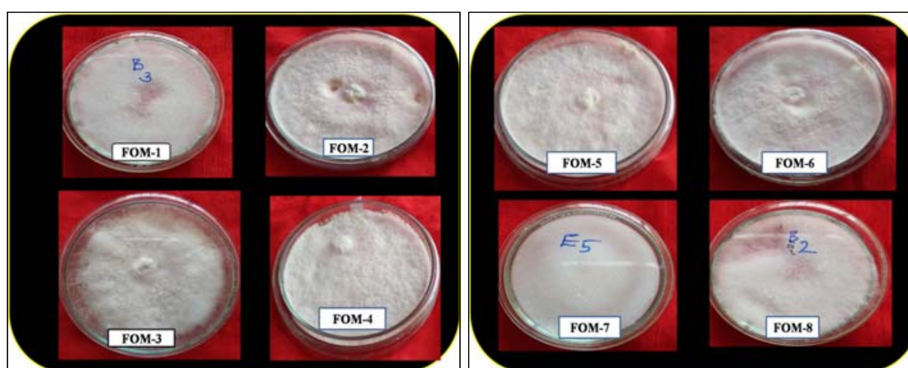

Fig 2: Pure cultures of *Fusarium oxysporum* f. sp. *melongenae* collected from various regions of Marathwada

DNA extraction

Genomic DNA of 8 FOM 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.

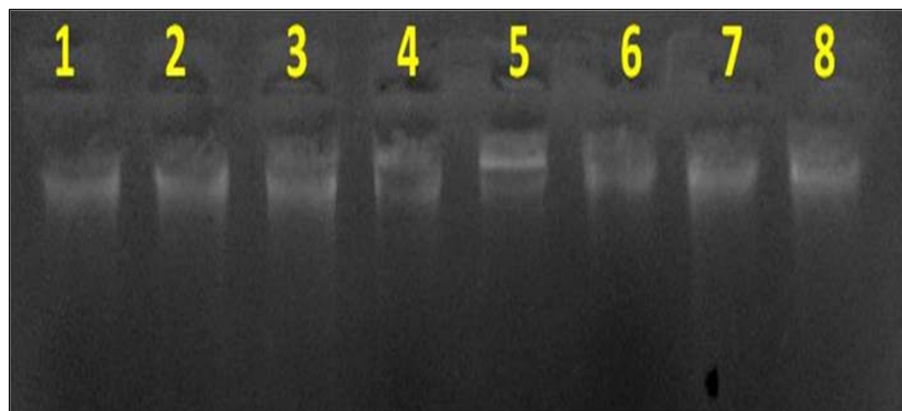

Fig 3: Plate No.2 Genomic DNA of eight *Fusarium oxysporum* f. sp. *Melongenae* isolates.

RDNA ITS amplification of FOM isolates

Internal Transcribed Spacer region of rDNA provides excessive template for amplification during polymerase chain reaction. Because of its high variability at genus, species, and strain level it provides more information related with divergence among isolates. The ITS primer pair ITS-Fu- F

and ITS-Fu-R was used in PCR to amplify the rDNA ITS regions of 8 *Fusarium oxysporum* f. sp. *melongenae* isolates.

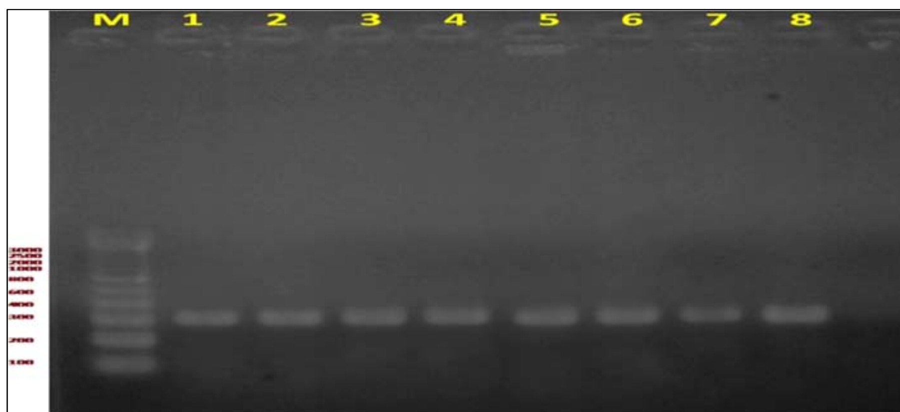

Fig 4: Plate No.3 amplification of ribosomal DNA internal transcribed spacer region from F sp. *Melongenae* isolates by primers ITS-Fu-F and ITS Fu-R: M, 100bp DNA ladder 1-8.

Analysis of genetic diversity among FOM isolates

ITS-PCR products of 302bp generated by primers ITS-Fu-F and ITS-Fu-R were digested using two restriction enzymes viz., *Eco*RI and *Hae*III to assess the genetic variability among the FOM isolates, isolated from different regions of Marathwada. These two enzymes enabled to digest PCR product of ITS region amplified by primer ITS-Fu-F and ITS-Fu-R (Plate no. 4. And 5). The digested PCR product was resolved on 4% Agarose gel in 1X TAE buffer, stained with Ethidium bromide and visualized in Gel Documentation System.

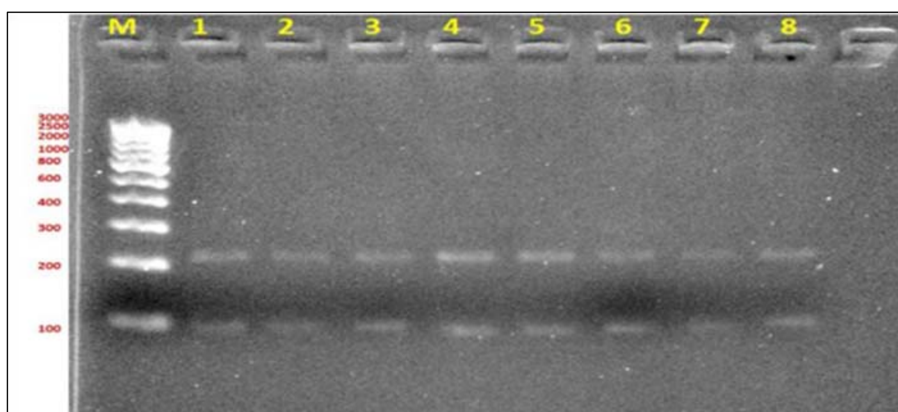

Fig 5: Plate No.4 ITS-RFLP profile of RDNA of FOM isolates 1-8 with *Eco*RI restriction endonuclease. Lanes L: 100bp DNA ladder, 1-8 FOM isolates as described in table.

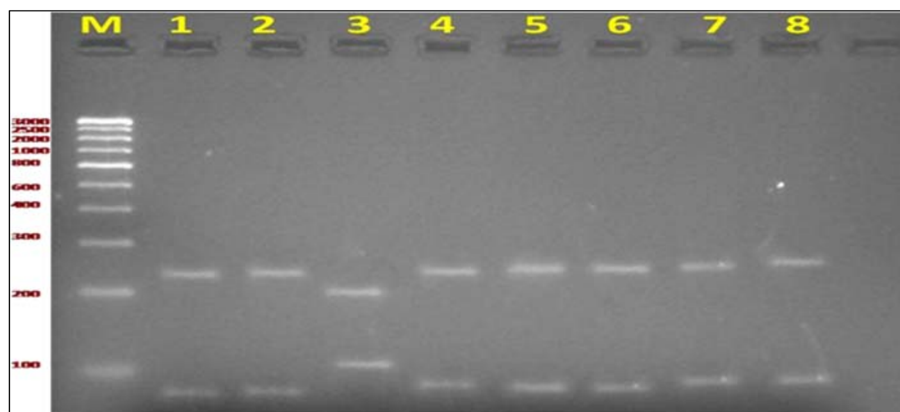

Fig 6: Plate No.5 ITS-RFLP profile of RDNA of FOM isolates 1-8 with *Hae*III restriction endonuclease. Lanes L: 100bp DNA ladder, 1-8 FOM isolates as described in table

Table 8: Restriction fragment size (in base pairs) of 8 FOM isolates

Sr. No	Enzymes Isolates	*Eco* RI	*Hae* III
1	FOM-1	220,82	230,72
2	FOM-2	220,82	230,72
3	FOM-3	220,82	200,102
4	FOM-4	220,82	230,72
5	FOM-5	220,82	230,72
6	FOM-6	220,82	230,72
7	FOM-7	220,82	230,72
8	FOM-8	220,82	230,72

In plate no 5 all isolates shows same type of banding pattern. The ITS region is fragmented into 2 bands one has length 220bp and another one has 82bp.

In ITS fragment digestion with enzyme *Hae* III, generated a banding pattern. In which all 8 FOM isolates shows different banding pattern. In which two types of band generated, one band has length 230bp and another one has 72bp. Third isolate showed different banding pattern with 200bp and 102bp.

Table 9: Jaccard' similarity matrix of eight *Fusarium oxysporum* f. sp. *Melongenae* isolates based on ITS-RFLP data.

Isolates	FOM-1	FOM-2	FOM-3	FOM-4	FOM-5	FOM-6	FOM-7	FOM-8
FOM-1	1.00							
FOM-2	1.00	1.00						
FOM-3	0.33	0.33	1.00					
FOM-4	1.00	1.00	0.33	1.00				
FOM-5	1.00	1.00	0.33	1.00	1.00			
FOM-6	1.00	1.00	0.33	1.00	1.00	1.00		
FOM-7	1.00	1.00	0.33	1.00	1.00	1.00	1.00	
FOM-8	1.00	1.00	0.33	1.00	1.00	1.00	1.00	1.00

All isolates are similar to each other except third isolates showed least similarity with all isolates. Only third isolates show less similarity with all isolates. It is dissimilar to all isolates showing 33% similarity with all isolates. Others are genetically similar to each other.

Table 10: Genetic distance between eight isolates *Fusarium oxysporum* f. sp. *melongena* based on similarity index.

Isolates	FOM-1	FOM-2	FOM-3	FOM-4	FOM-5	FOM-6	FOM-7	FOM-8
FOM-1	0.00							
FOM-2	0.00	0.00						
FOM-3	0.67	0.67	0.00					
FOM-4	0.00	0.00	0.67	0.00				
FOM-5	0.00	0.00	0.67	0.00	0.00			
FOM-6	0.00	0.00	0.67	0.00	0.00	0.00		
FOM-7	0.00	0.00	0.67	0.00	0.00	0.00	0.00	
FOM-8	0.00	0.00	0.67	0.00	0.00	0.00	0.00	0.00

All isolates are similar but isolate third shows 0.67 (67%) genetic distance with all isolates. It shows genetic difference with all isolates. Isolates FOM-1 (Soegaon), FOM-2 (Ambajogai), FOM-4 (Badnapur), FOM-5 (Chakur), FOM-6 (Kinwat), FOM-7 (Tuljapur) and FOM-8 (Gangakhed) have 0% genetic variation.

Genetic diversity analysis of eight FOM isolates based on ITS-RFLP data

Genetic similarity matrix (Jaccard's coefficient) based on ITS-RFLP banding pattern was used for cluster analysis and generated dendrogram among eight FOM isolates. The eight isolates were grouped into two main clusters (Figure 1). The major cluster I consisted of seven isolates *viz*., FOM-1 (Soegaon), FOM-2 (Ambajogai), FOM-4 (Badnapur), FOM-5 (Chakur), FOM-6 (Kinwat), FOM-7 (Tuljapur) and FOM-8 (Gangakhed) while second major cluster II comprises single isolates FOM-3 (Sengaon) shown 33% genetic similarity with cluster I and genetic diversity 67% with all isolates.

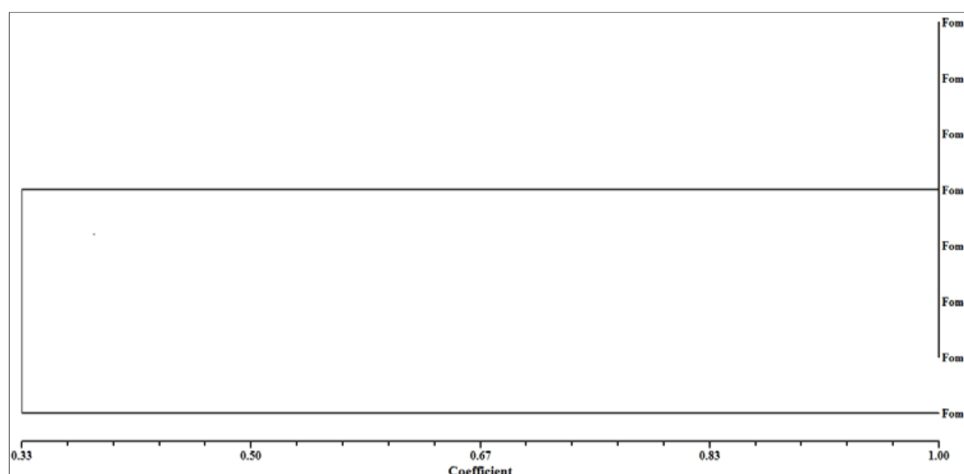

Fig 7: Dendrogram generated using NTSYS analysis demonstrating the relationship among 8 FOM isolates based on ITS-RFLP data

Discussion

The experiments described in this chapter were conducted during the summer 2018-2019. The present investigation entitled "Molecular Characterization of *Fusarium oxysporum* f. sp. *Melongenae* (Schlecht) Mutuo and Ishigami in Marathwada region of Maharashtra by using ITS-RFLP Marker" was carried out at Vilasrao Deshmukh Collage of Agricultural Biotechnology, Latur (M.S.).

Study of ITS-RFLP analysis for genetic diversity shows the genetic relationship between the 8 FOM isolates.

The major cluster I consisted of seven isolates *viz*., FOM-1 (Soegaon), FOM-2 (Ambajogai), FOM-4 (Badnapur), FOM-5 (Chakur), FOM-6 (Kinwat), FOM-7 (Tuljapur) and FOM-8 (Gangakhed) while second major cluster II comprises single isolates FOM-3 (Sengaon) shown 33% genetic similarity with cluster I and genetic diversity 67% with all isolates.

The genetic variability among the FOM isolates may be due to the differences in geographical and environmental conditions.

The present result was also in conformity with the other workers (Larena *et al*., 1999; Arif *et al*., 2013; Datta and Lal, 2013) [24, 3, 14]. The work of Zhang *et al*. (2012) support present findings, which amplified a product of 315 bp size specifically in F. *oxysporum* isolates. Larena *et al*. (1999) [24] used ITS5/ITS4 and ITS1F/ITS4 primer pair for identification of F. *oxysporum* f. sp. *lycopersici* and F. *oxysporum* f. sp. *Radicis-lycopersici* and the result were similar to those with the ITS1/ITS4 primer pair. Molecular systematic studies among *Fusarium* spp. and their identification, based on nuclear ribosomal DNA have been previously analyzed by many workers (Abd-El-Salam *et al*., 2003; Korabecna, 2007; Nilsson *et al*., 2008; Leong *et al*., 2009; Chehri *et al*., 2011; Datta *et al*., 2011; Schoch *et al*., 2012; Naqvi *et al*., 2013; Kaur *et al*., 2015) [23, 31, 25, 9, 38, 29, 21].

The cluster of ribosomal DNA consists of tandem repeats of three coding (18S, 5.8S and 28S) and two noncoding ITS and Intergenic spacer (IGS). The 18S, 5.8S and 28S RNA genes are highly conserved and the sequence variations in these genes reflect major evolutionary events and hence these can therefore be used to reconstruct higher order phylogenies. The three RNA-encoding regions are separated by internal transcribed spacer (ITS) regions that are less conserved and are useful to study lower order phylogeny (Bruns *et al*., 1991; Samuels and Seifert, 1995) [5, 37]. Internal transcribed spacer (ITS) regions in fungi have been found to evolve faster and therefore may contain sufficient nucleotide sequence variations to infer relationships between the species. Sequence variation in rRNA genes allows the use of these genes as targets for differential amplification. In a broader context, taxon-selective amplification of the ITS region is likely to become a common approach in molecular identification of the pathogens. Therefore, these ITS primer combinations, which targeted and amplified a particular gene/region in the internal transcribed spacer (ITS) regions, could further be utilized for easy and early identification of these two species viz., F. *oxysporum* and F. *solani* from the infected plant tissues.

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