Int.J.Curr.Microbiol.App.Sci (2020) *9*(5): 818-828

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 9 Number 5 (2020)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2020.905.091

Morphological Characterization and Genetic Diversity of *Fusarium* spp. Infecting Bitter Gourd

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Keywords

Bitter gourd, Fusarium spp., Pathogenicity, PCR amplification, Genetic diversity.

Article Info

Accepted:
05 April 2020
Available Online:
10 May 2020

A B S T R A C T

Bitter gourd (*Momordica charantia*L.) is an important cucurbitaceous plant in which the deleterious diseases namely vascular wilt, damping off occurs commonly and causes 30 to 50 percent losses in crop. The present investigation was aimed to study the morphological and molecular characterization of disease causing pathogen to understand the etiology of the disease.Survey was conducted at different locations of Coimbatore, Erode and Dharmapuri and samples were collected for the isolation of the pathogen. The pathogen was isolated from the brownish discoloured vascular tissue of stem portion which was typically wilted. Pathogenicity assay was proved and the virulence of the fusarium isolates were identified under artificial inoculation studies. The morphological studies of *Fusarium* isolates were studied using the microscopic observation to study the micro and macro conidial variation .The molecular confirmation of pathogen was done by PCR amplification using ITS 1 and ITS 4primer at~560 bp. The different species of *Fusarium solani*, *Fusarium equiseti*, *Fusarium falciforme*, *Fusarium chlamydosporum* and *Fusarium incarnatum*were identified in these molecular studies. Among the species, *Fusarium solani* is a major pathogen associated with bitter gourd wilt disease. The genetic variability studies among different *Fusarium*spp. was carried out using RAPD marker. The variability studies revealed that virulence isolates *viz, F. solani* VP, *F. solani* EL, *F. solani* TP, *F. falciforme* IJ and *F. chlamydosporum* TN were grouped into cluster II which are more virulent and the same results were shown in pathogenicity. The wilt pathogen of bitter gourd isolated from the palee hybrid was shown more virulence when compared to the other isolates.

Introduction

Bitter gourd (*Momordica charantia* L.) is one of the important cucurbitaceous vegetable grown in India. Among the cucurbits, it is prized vegetable which is having high nutritive value especially ascorbic acid and iron (Behera2004). For culinary preparations immature fruits and tender vine tips are used. It is a most common vegetable cultivated throughout India during warm season (Satkar *et al.,* 2013). Bitter gourd has been used in various herbal medicine systems. Phytochemical compounds like dietary fiber, minerals, vitamins, flavonoids and antioxidants involved in health promoting and

disease prevention. It is also used for reduction of blood sugar levels in the treatment of type-2 diabete (Singh *et al.,* 2013). The crop is cultivated with an area of 99,000 ha in India with an annual production of 11,98,000 MT and the productivity of 12.18MT/ha (Indiastat 2018-19). Wilt disease caused by *Fusarium* spp. the main constraint with bitter gourd cultivation in India. (Tamilselvi2014). It is the most devastating soil borne disease and one of the major yields limiting constraint which cause profound economic losses ranging from 30 to 50 per cent under dry warm conditions (Tamilselvi and Pugalendhi 2015). The knowledge on host pathogen interaction and predominating of causative agents are not explained. Hence considering this idea in mind, the present investigation was carried out to study the morphological and molecular variability of the pathogen and to the etiology of the disease.

Materials and Methods

Survey and collection of plant materials

The survey was conducted in around Coimbatore, Erode and Dharmapuri districts of Tamil Nadu. Thediseased plants showing typical disease symptom were collected from ten different fields. Thetotal numbers of plants wilted were recorded in sq. meter of area and the Percent Disease Incidence (PDI) was calculated for each field location as methodology explained by Muhammad *et al.,* (2019).

Symptomatology

A symptom of fusarium wilt includes damping-off, seedling disease or wilt during any stage of plant development. Symptoms on mature plants typically appear as a dull grey green appearance of the leaves followed by yellowing of the crown foliage, wilting during the day and eventual death. Brown stripes will develop on stems and branches of infected plants (Tamilselvi *et al.,* 2016). Vascular discoloration is visible inside the stem and stem collarturn dark brown (Fig.1).

Isolation and purification of pathogen

The wilt infected vascular tissue was taken from the infected parts of the plant, sterilized in a 0.5% sodium hypochlorite solution, rinsed with sterilized water for three times and placed on Potato Dextrose Agar (PDA) medium. The medium was supplemented with $0.5gL^{-1}$ of streptomycin sulphate to avoid the bacterial contamination. After that the Petri dishes were incubated at 25±1°C for 7 days (Muhammad *et al.,* 2019). The colony produced from diseased sample were re-isolated using a single spore where the fungal colonies emerged from diseased samples were transferred to different Petri dishes containing fresh PDA medium for pure culture.

Pathogenicity

In order to confirm the pathogenic nature of isolated fungal pathogen, the pathogenicity test was conducted in earthen pots.

Inoculum preparation and inoculation

Sand maize medium was used for mass multiplication of the fungal isolate in the laboratory. The medium was prepared with ratio of 19:1 sterilized in an autoclave at 15 lbs psi for 30 minutes. Sand maize medium was inoculated with pure culture of *Fusarium* isolates in aseptic conditions and incubated in an incubator at 28 + 2^0C for 15 days (Ashwathi *et al.,* 2017).

Preparation of planting material

Bitter gourd seeds CO1 were sown in the earthen pots containing sterilized potting soil.

Sand maize medium was prepared and mixed with proportion of 100mg inoculum/kg of potting mixture and sowing was taken.

Morphological and microscopic identification of pathogen

The fungal pure culture was transferred to new Potato Dextrose Agar (PDA) to facilitate for the growth of mycelia and sporulation of conidia for the identification of pathogen through Labomed camera model LX400 microscope with image analyser pixel pro program at 40x magnification using sterile water (Karthick *et al.,* 2019). The pathogens were identified based on the microscopic analysis, microconidia and macroconidia characteristics such as colour, shape and size, and also the presence chlamydospore were studied and tabulated.

Molecular identification of the isolated fungi

The pure fungal isolates were taken for the DNA isolation using CTAB method described by Chowdhury *et al.,* (2019). The isolated DNA were amplified through polymerase Chain Reaction (PCR) technique using universal primers, ITS1 (5'-TCC GTA GCT GAA CCT GCC G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') developed by White *et al.,* (1990) and Hot Start Green Master Mix (Promega, USA). PCR was performed in a 50µl reaction mixture containing 25µl of Hot Start Green Master Mix (2X), 2.0µl of each forward and reverse primer, 2.0µl of genomic DNA and rest of the PCR water. The performing PCR program was as follows: pre heat at 95°C for 2 min, followed by 40 cycles of denaturation step at 95°C for 1min, primer annealing at 58°C for 1min, primer extension at 72°C for 1min with a final extension at 72°C for 5 min, hold at 10°C for overnight. The amplicons were separated by 1% agarose (V3125, Promega, USA) gel electrophoresis (Karthick *et al.,* 2019a). The quality and quantity of isolated DNA were checked by NanoDrop Spectrophotometer (ND2000, Thermo Scientific, USA). Finally, The PCR products were purified and used for sequencing analysis in Barcode Bioscience Pvt ltd, Bangalore(India). The sequenced data were analysed using similarities of nucleotide sequences between isolates through the BLAST procedure (http://blast.ncbi.nlm.nih.gov).

Molecular variability studies

RAPD-PCR analysis of *fusarium* spp.

Genotypic characterization of the *Fusarium* spp. isolateswas done by using a PCR-based fingerprinting of randomly amplified polymorphic DNA (RAPD) markers method described by Bentley *et al.,* (1995). PCR amplification was performed using an Eppendorf nexus gradient master cycler and a 20µl total volume containing 2.0 units of Taq polymerase (Bangalore Genei Pvt Ltd, India), 2µl of 10X buffer, 1.5µl of 2.5 mM $MgCl_2$, 1µl of 2.5 mM dNTP, 2µl of 10µM primer, 4µl of genomic DNA and sterile distilled water. The PCR was performed, using Eppendorf – Master Cycler nexus gradient S (Eppendorf, A G, Hamburg, Germany), with an initial denaturation step for 5 min at 94°C, followed by 35 cycles of 1 min for denaturation at 94°C, 1 min for annealing at 36°C and 1 min for extension at 72°C, with a final extension for 5 min at 72°C. Following amplification, 20µl of each PCR product was separated by electrophoresis in 1.0% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer.To visualize amplified DNA, fragments gels were stained with an ethidium bromide (0.1 mg l^{-1}) and then photographed under transmission ultraviolet light, using an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA, USA).

The whole RAPD analyses experiment was repeated at least three times for all primers and isolates and only the RAPD bands which appeared consistently were evaluated for polymorphism. Using the software DARwin 6, the dendrogram constructed. The RAPD primers are given below table.1.

Results and Discussion

Isolation and purification of pathogen

In this study, the different fusarium wilt disease infected fields were surveyed which showing typical symptoms. The wilt pathogens were isolated and listed in the Table.2. The results revealed that the disease incidence was maximum at Madhampatti (59.3%) followed by Papampatti (55.4%) villages of Coimbatore district. The least disease incidence (15.6%) was observed at TNAU vegetable garden followed by the Injampalayam (20.3%) Erode district.

Pathogenicity test

The assay on pathogenicity, plants were shown different period of wilting incidence after the inoculation of pathogen. After inoculation of pathogen, plants initially had shown yellowing of foliage, pale green appearance of leaves, day wilting followed by death. Inside the stem, vascular browning of tissues was found (Fig.2).

The present findings were similar with the Ashwathi *et al.,* (2017) studies that the fusarium wilt of coriander symptom expression was observed under the sand maize inoculation of pathogen which shown reddish brown lesions at collar portion, gridling and toppling down of seedlings on 15^{th} day. The Koch's postulates were proved. This study revealed that the isolate *Fusarium solani* was more virulent followed by *Fusarium equiseti*(Table.3).

Morphological and molecular identification of pathogen

In morphological identification of pathogen, *Fusarium solani,* macro conidia have slightly curved and relatively thick with a slightly hook cell were similar with the information described by Leslie and Summerell (2006). The hypha of the *Fusarium* spp. was hyaline, septate, smooth and branched. The microconidia were oval or falcate and macroconidia were fusiform or falcate ('canoe-shaped') having 3-5 septation with large in numbers (Table.4). The morphological variations were clearly recorded that *Fusarium solani* macroconidia was fusiform with moderately curved. In case of *F. chlamydosporum* was shown that slightly curved with fusiform macroconidia which is varied from *F. incarnatum* and shown fusiform to falcate. The macroconida of *F. equiseti* was shown fusiform to falcate. The different species of Fusarium were recorded in bottle gourd crop and the species like *F. equiseti*, *F. moniliforme* and *F. solani* which is responsible for wilt disease (Shah *et al.,* 2014). The *F. equiseti* and *F. oxysporum* are the causal agents which is mainly responsible for causing fusarium wilt in bitter gourd reported by Chowdhury *et al.,* (2019).

Molecular identification of the isolated fungi

To identify the Fusarium species associated with bitter gourd wilt disease, the ITS 1 and 4 region PCR based amplification and sequencing was carried out for the amplicon of ~560bp. The results were coincided with work of Chowdhury *et al.,* (2019) who has been confirmed *Fusarium* spp. infecting bitter gourd in Bangladesh. On 1% agarose gel electrophoresis, the genomic DNA isolated from the fungal isolates showed higher molecular weight and bright band, 1kb DNA ladder was used as a marker.

Table.1 RAPD primers used for genetic variability analysis

S.No	CODE	PRIMER
1.	M1	OPF 01 – 5'CCCAAGGTCC 3'
2.	M2	OPE 02 – 5' GGTGCGGGAA 3'
3.	M3	OPE 03 – 5' CCAGATGCAC 3'
4.	M4	OPA 04 – 5' AATCGGGCTG 3'
5.	M5	OPA 05 – 5'AGTCAGCCAC 3'
6.	M6	OPF 06 – 5' GGGAATTCGG 3'
7.	M7	OPA 07 – 5' GAAACGGGTG 3'
8.	M8	OPF 08 – 5' GGGATATCGG 3'
9.	M9	OPA 09 – 5' GGGTAACGC 3'
10.	M10	OPA 11 – 5' CAATCGCCGTS 3'

Table.2 Survey of Fusarium wilt in bittergourd in Coimbatore, Erode and Dharmapuri districts for assessing disease incidence

S.NO	LOCATION/DIST	LATITUDE/ LONGITUDE	SOIL TYPE	STAGE	CODE	VARIETY	DISEASES INCIDENCE (%)
1.	Vettaikaranputhur (Pollachi)	10.5534°N / 76.8889°E	Red calcareous	Vegetative	VP	Palee	32.8^{e} (35.59)
2.	Papampatti (CBE)	10.9434°N / 77.1126°E	Red calcareous	Harvesting (IInd Picking)	PA	Palee	55.4^{b} (46.01)
3.	Theetharahalli (Dharmapuri)	12.3251°N / 78.0469°E	Black calcareous	Harvesting (Ist Picking)	DPI	Palee	45.5^{d} (41.53)
4.	Thelungupalayam (CBE)	10.9945°N / 76.9248°E	Black soil	Harvesting (IIst Picking)	TP	Palee	51.7^{c} (44.33)
5.	Madhampatti (CBE)	10.9728°N / 76.8576°E	Red loamy	Harvesting (IIIrd Picking)	MA	Palee	59.3^{a} (47.78)
6.	Vadugapatti (Erode)	11.1281°N / 77.7406°E	Deep loamy soil	Harvesting (Ist Picking)	VPT	Palee	26.3^{f} (32.51)
7.	Archalur (Erode)	11.1176°N / 77.6881°E	Red loamy	Harvesting (Ist Picking)	AR	Palee	24.5^{g} (31.34)
8.	Elumathur (Erode)	11.1868°N / 77.7738°E	Deep loamy soil	Harvesting (IInd Picking)	EL	Palee	31.8^{e} (35.10)
9.	Injampalayam (Erode)	11.2043°N / 77.8196°E	Black calcareous	Harvesting (Ist Picking)	IJ	Palee	20.3^{h} (28.97)
10.	TNAU (CBE)	11.0123°N / 76.9355°E	Loamy	vegetative	TN	Co 1	15.6^{i} (26.05)

*Values are mean of three replicates
Values in parentheses are arcsine transformed values
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Table.3 *In vitro* pathogenicity assay of *Fusarium* spp. in bittergourd

S.NO	ISOLATE CODE	ISOLATES	WILTING (DAI)
1.	VP	*Fusarium solani*	13^{a}
2.	PA	*Fusarium equiseti*	23^{cd}
3.	DPI	*Fusarium falciforme*	26^{def}
4.	TP	*Fusarium solani*	18^{b}
5.	MA	*Fusarium incarnatum*	29^{f}
6.	VPT	*Fusarium solani*	22^{c}
7.	AR	*Fusarium chlamydosporum*	28^{ef}
8.	EL	*Fusarium solani*	15^{ab}
9.	IJ	*Fusarium falciforme*	25^{cde}
10.	TN	*Fusarium chlamydosporum*	24^{cd}

*Values are mean of three replicates
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT

Table.4 Morphometric analysis of Fusarium spp. under light microscope at 40xmagnification

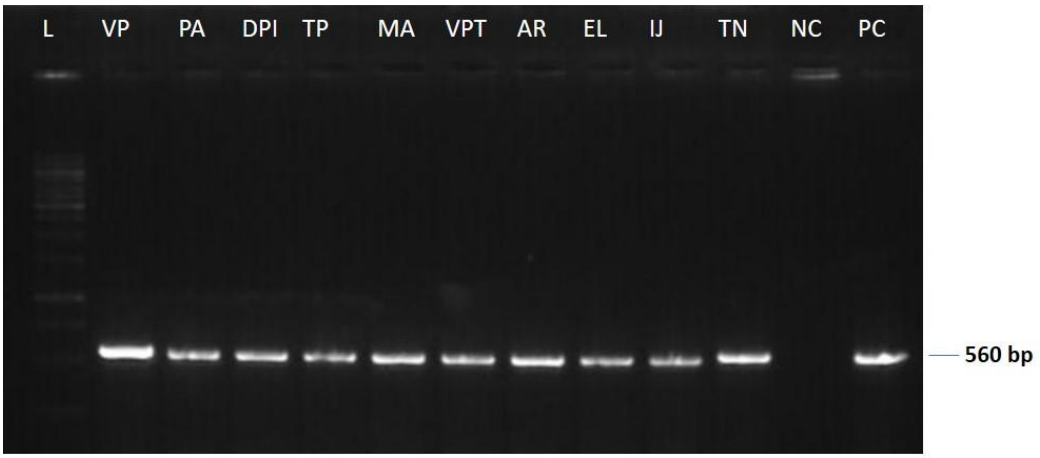
S.NO	ISOLATES CODE	ISOLATES	MORPHOLOGICAL CHARACTERS				
			MICROCONIDA		MACROCONIDIA		CHLAMYDOSPORE
			SHAPE	SIZE (µm)	SHAPE	SIZE (µm)	
1.	VP	*Fusarium solani*	Oval	8-16 × 3-5	Fusiform, often moderately curved	28 – 42× 4-6	Terminal and intercalary
2.	PA	*Fusarium equiseti*	Oval and elongated	8-16 ×2.5-5	Fusiform to falcate	20 -32 × 4-5	Terminal and intercalary
3.	DPI	*Fusarium falciforme*	Oval	11-13 × 4-4.6	Falcate	22-26.5 × 5-6	Terminal and intercalary
4.	TP	*Fusarium solani*	Oval	7-15 × 4.5 -5	Fusiform, often moderately curved	30 -41 × 4-5.5	Terminal and intercalary
5.	MA	*Fusarium incarnatum*	Oval and elongated	9-10.5 ×3-4.2	Fusiform to falcate	28 -33.5 × 4-4.7	Not found
6.	VPT	*Fusarium solani*	Oval	8-16 × 3-5	Fusiform, often moderately curved	30 -41 × 4-6	Terminal and intercalary
7.	AR	*Fusarium chlamydosporum*	Oval to elongated	6-26 × 2-4	Fusiform, slightly curved	30-38 ×3-4.5	Terminal and intercalary
8.	EL	*Fusarium solani*	Oval	7.5-15 × 3.5-5	Fusiform, often moderately curved	29-40 ×4-6	Terminal and intercalary
9.	IJ	*Fusarium falciforme*	Oval	12-13.5 × 3-4.6	Falcate	22.2-26.3 × 5.4-6.1	Terminal and intercalary
10.	TN	*Fusarium chlamydosporum*	Oval to elongated	7-25.5 × 3-4	Fusiform, slightly curved	31-36.5 × 3-4.4	Terminal and intercalary

Table.5 The sequencing results of 10 isolates of pathogen

S.No	Code	Pathogen	Accession no
1.	VP	*Fusarium solani*	MN999964
2.	PA	*Fusarium equiseti*	MN999973
3.	DPI	*Fusarium falciforme*	MN999971
4.	TP	*Fusarium solani*	MN999965
5.	MA	*Fusarium incarnatum*	MN999974
6.	VPT	*Fusarium solani*	MN999966
7.	AR	*Fusarium chlamydosporum*	MN999969
8.	EL	*Fusarium solani*	MN999967
9.	IJ	*Fusarium falciforme*	MN999972
10.	TN	*Fusarium chlamydosporum*	MN999970

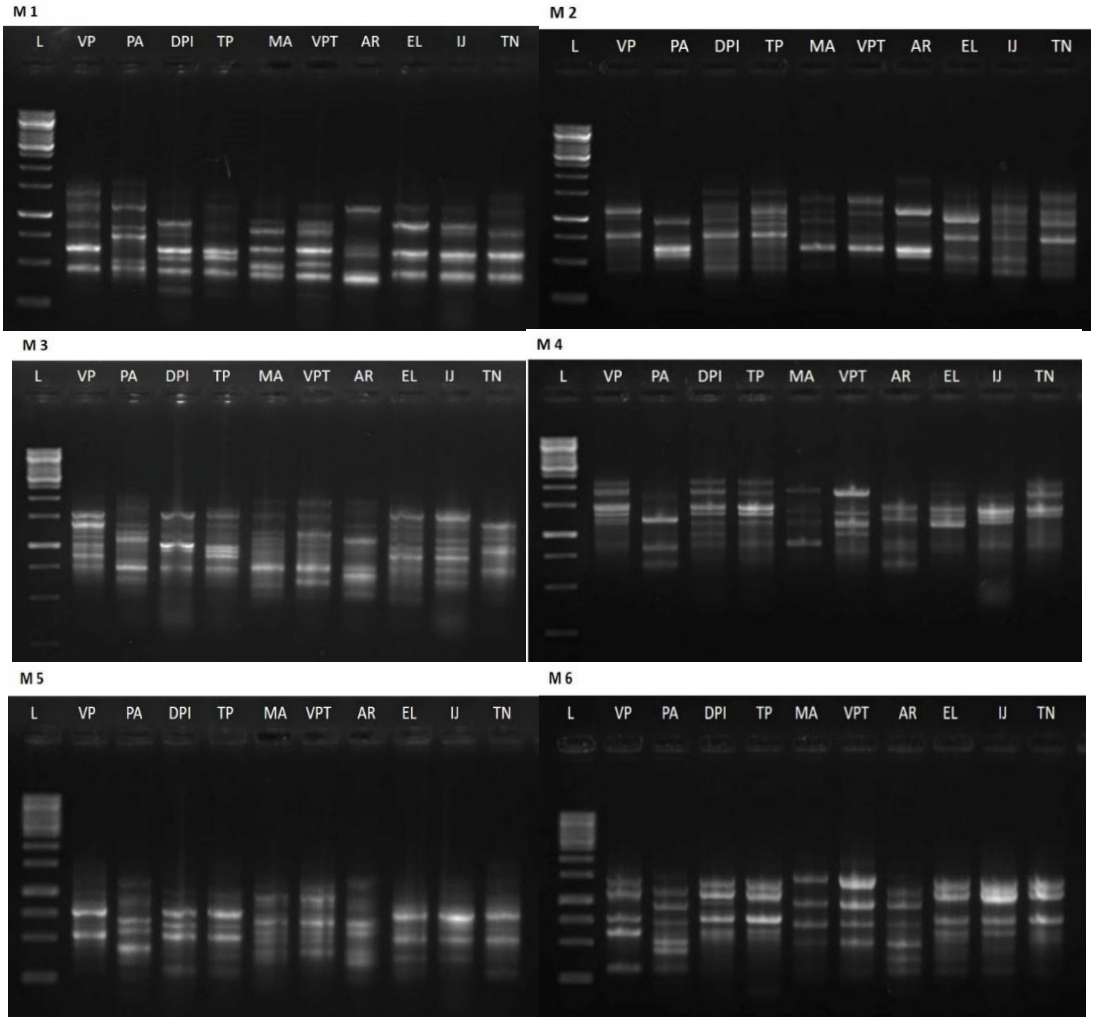
A) Yellowing of crown leaves B) Complete wilting and death
C) Dark brown vascular discoloration
Fig.1 Survey and collection of wilt infected plant samples from farmer's field

Yellowing of leaves B) Wilting of leaves C) Drying and wilting of leaves compared to control
Fig.2 Pathogenicity assay under glass house condition



L – 1 Kb ladder NC – Negative control
PC – Positive control (*Fusarium oxysporum f. sp. cubense*)

Fig.3 PCR amplification and gel electrophoresis of ITS region of *Fusarium* spp. infecting bitter gourd



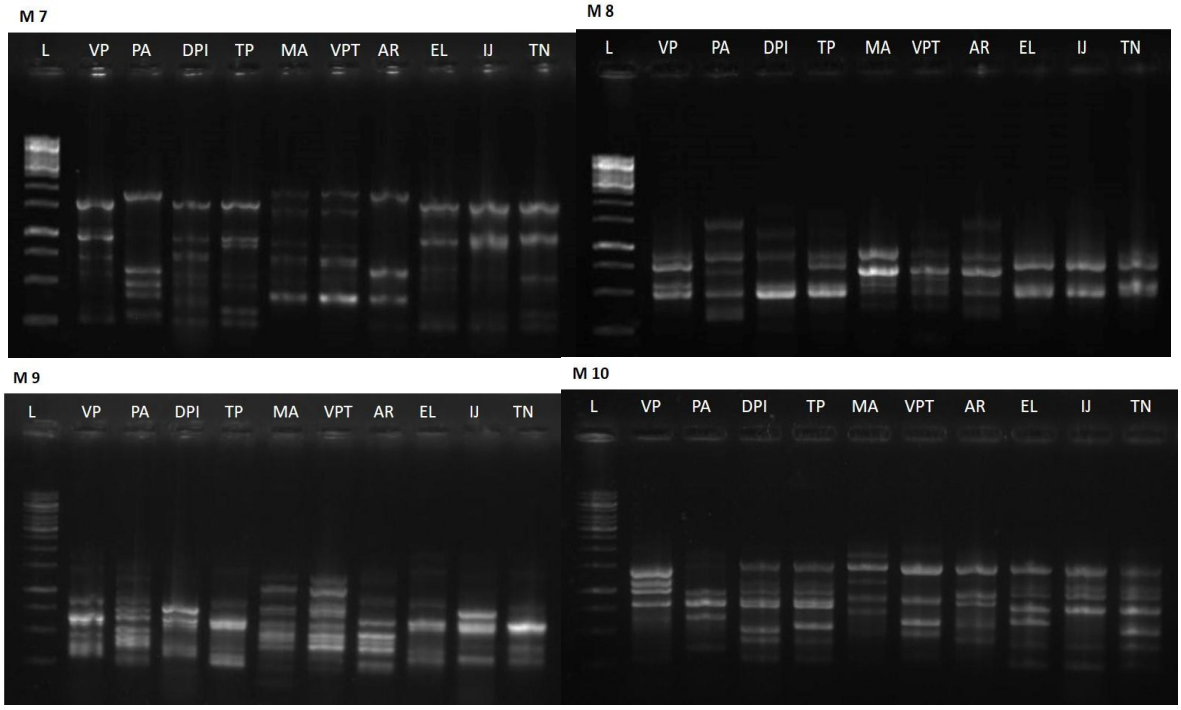


Fig.4 RAPD marker analysis of genetic variability of *Fusarium* spp. infecting bitter gourd

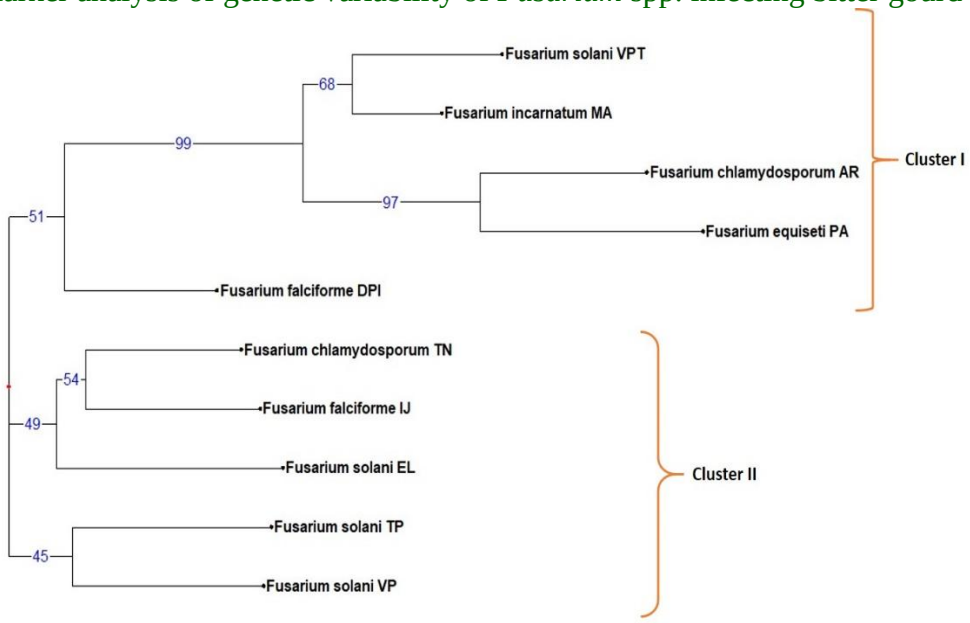


Fig.5 Phylogenetic tree analysis by RAPD marker

The universal primers, ITS-1 5' TCCGTAGCTGAACCTGCCG 3' and ITS-4 5' TCCTCCGCTTATTGATATGC 3', were used to amplify a region of fungal genome named the 18S of ribosomal DNA gene of different *Fusarium* spp. isolates. The PCR amplified fragments of the isolates yielded band of around 560bp (Fig.3). The result was coincided with the findings of Sreegayathri *et al.*, (2018) who has been confirmed the *F.Solani* amplified at ~560bp. The sequenced results of the different isolates were shown more than 97% similarity with respective species of the *Fusarium*. The sequenced result

and accession number for individual isolates were given in Table.5.

Molecular variability of *fusarium* spp.

Gupta *et al.,* (2012) utilized RAPD analysis in order to study the genetic diversity and fingerprinting of fusarium spp. infecting guava. The results revealed that *Fusarium oxysporumf.sp. psidii* divided into three clusters, were as *Fusarium solani* were formed two clusters based on number banding pattern. The genetic variations among the Fusarium spp. were observed which indicates RAPD is a potential marker for genetic characterization (Fig.4).

The result of RAPD analysis revealed that there are two major clusters were formed (Fig.5). In custer1, two sub clusters were formed. In sub cluster I*Fusarium solani* VPT, *Fusarium incarnatum* MA occupied a separate branch. In sub cluster II, *Fusarium chlamydosporum* AR, *Fusarium equiseti* PA occupied separate branch. The *Fusarium falciforme* DPI occupied separate clusters that are well separated from another *Fusarium* spp. in major cluster I.

In major cluster II, there are two subclusters were formed, which showed more virulence in pathogenicity assay. The *Fusarium chlamydosporum* TN, *Fusarium falciforme* IJ and *Fusarium solani* EL grouped under subcluster. In sub cluster II *Fusariumsolani* TP, *Fusarium solani* VP were clustered together. The results revealed that variability in the Fusarium spp. according geographical location at molecular level. The variability of pathogen reflected between and within the species.

Acknowledgments

The authors are thankful to the Director, CPPS, Head of the Department and Professor of Department of Plant Pathology, TNAU, Coimbatore for providing support, guidance and financial assistance. The authors would like to acknowledge DST-FIST and UGC-SAP-DRSI for providing facilities at Department of Plant Pathology.

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How to cite this article:

Saran Kumar. M., S. K. Manoranjitham, V. Sendhilvel and Rajasree. V. 2020. Morphological Characterization and Genetic Diversity of *Fusarium* spp. Infecting Bitter Gourd. *Int.J.Curr.Microbiol.App.Sci.* 9(05): 818-828. doi: https://doi.org/10.20546/ijcmas.2020.905.091