Bulletin of Pure and Applied Sciences.
Vol.39 B (Botany), No.2.
July-December 2020: P.77-83
Original Research Article

Print version ISSN 0970 4612
Online version ISSN 2320 3196
DOI: 10.5958/2320-3196.2020.00012.9
Available online at www.bpasjournals.com

Molecular Markers for the Detection of Variability of Wilt Pathogen in Groundnut

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Received on 29.04.2020
Accepted on 28.09.2020

Keywords:
Tamil Nadu, *F. oxysporum, RAPD,* Microconidia, Groundnut wilt

Abstract
A survey was conducted in major groundnut growing areas of Tamil Nadu for the occurrence of *Fusarium* wilt of groundnut and wilt incidence was observed up to 66.00 per cent in the district. In pathogenicity tests, characteristic symptoms of wilt were observed under glass house condition. Of the five isolates tested for their cultural characters, I_1 showed a faster growth rate of 1.29 cm/day. The isolates varied in morphological features and all of them produced microconidia, macroconidia and chalamydospores. Using RAPD markers the genetic similarity among the five isolates of *F. oxysporum* infecting groundnut was studied and the similarity was observed between 27 to 55% indicating high variability among the isolates.

INTRODUCTION

Groundnut (*Arachishypogaea* L.) is an important oilseed crop of India and it is cultivated in about 6.1 M ha. and production is 9.36 Metric tonnes with a productivity of 1460 kg / ha. Among the groundnut producing states of India, Tamil Nadu occupies fourth place in area and ranks second in production (http: //www. dacnet. nic.in/2006). In spite of their important positions in national agricultural economy and the multiplicity of crops and crop growing situations, the countries are lagging far behind the requirement. The groundnut production is constrained by various factors and the major constraints include frequent drought stress, low input use and socio economic infrastructure and higher incidence of disease and pest attack. Though the groundnut is attacked by a number of diseases, the soil borne fungal disease, stem rot caused by *Fusarium* sp. is a potential threat to groundnut production and it causes yield losses. The geographical variability among *Fusarium* sp. population shows variability in cultural morphology, sclerotium formation, phenolic constituents and other degradative enzymes. Understanding the variability nature of the pathogen, developing and relay on single antagonism become challengeable and give way to explore and identify the suitable alternate antagonist against the disease. There are well known antifungal biocontrol agents that inhibit several plant pathogenic fungi (Errakhi *et al*., 2007). With the background of the above facts, the present study has been undertaken with the following objectives. 1. Survey the stem rot incidence of groundnut in major groundnut growing areas of the Tamil Nadu and 2. Variability of *Fusarium* sp.

MATERIALS AND METHODS

Collection of samples

A survey was conducted during 2011 to assess the occurrence of *Fusarium* wilt of groundnutat Madurai, Sivagangai and Theni districts of Tamil Nadu. The disease incidence was assessed by counting the number of affected plants out of total number of plants in each field. In each area three fields were assessed and the disease incidence was calculated. Diseased samples of plants were collected from these areas.

Isolation of pathogen

The pathogen causing wilt in groundnut was isolated from the samples by tissue segment method on potato dextrose agar (PDA) and the fungus was purified by single spore isolation and maintained on PDA. The causal organism was identified based on colony morphology, colour and conidial features.

Pathogenicity

Detached seedling technique

A five- mm culture disc of *F. oxysporum* was placed closer to the root region of either a pinpricked or non pinpricked healthy and cleaned groundnut seedling, kept in 150-mm-dia Petri dish over a layer of moistened cotton. An empty five- mm disc of PDA served as control. Three replications were maintained and the plates were incubated at room temperature ($28 \pm 2^{o}C$). The formation of lesion on stem was closely monitored and the lesion length was recorded at regular intervals up to16 days.

Pathogenicity in glasshouse

Multiplication of inoculum

The fungus was multiplied on sand-maize medium (Riker and Riker, 1936). The medium containing 1900 g of sand and 100 g of maize powder (19:1) was mixed, moistened with 400 ml of water kg^{-1} and filled in empty salaine bottles. The bottles were sterilized at 1.4 kg cm^{-2} pressure for two h for two alternate days. Each bottle was inoculated with two nine- mm culture disc of actively growing *F. oxysporum* and incubated at room temperature ($28 \pm 2^{o}C$) for 30 days to be used as inoculum.

Earthen pots of 25 cm- dia were filled with five kg of potting medium (red soil:sand:FYM @ 1:1:1). The pot mixture was sterilized in an autoclave at 1.4 kg cm^{-2} pressure for two h on two successive days and inoculated with 5 g inoculum of *F. oxysporum* multiplied on sand maize medium. Healthy groundnut seeds were sown in pots with proper control. The pots were maintained in glasshouse by uniform and judicious watering and plants were constantly observed for the development of symptoms. The pathogen was re isolated from plants showing symptoms of wilt.

Morphological features and growth of *F. oxysporum* isolates on PDA

Five isolates of *F. oxysporum* collected during the survey were grown on PDA to study their growth and variability in colony characters. From the seven-day-old culture plates, five- mm disc of the fungus was cut by a sterilized cork borer and placed at the center of each sterile Petri dish (90-mm-dia) containing 15 ml of sterilized and solidified PDA. The plates were incubated at room temperature ($28 \pm 2^{o}C$) for seven days. The mycelial growth colony morphology and color were recorded seven days after inoculation (DAI). One of the five isolates that were fast in growth was carried for further studies.

Variability studies using molecular markers

Extraction of DNA

Five isolates of *F. oxysporum* were used for DNA extraction. Isolates were grown in 100 ml of potato dextrose broth for four days at room temperature (28 ±) 2°C. Mycelial mat was filtered, dried and 2 g of the mat was ground to a fine powder using liquid nitrogen. Powdered mycelia were vortexed in pre- warmed lysis buffer (10mM Tris (pH: 8.5), 250 mMNaCl, 0.5 mM EDTA and 0.5 % SDS) and incubated at 65°C for 30 min followed by the addition of 1.7 M potassium acetate. The contents were gently mixed and incubated at -20 °C for 30 min. Samples were extracted with equal volumes of choloroform and the total nucleic acid was precipitated with equal volumes of chilled isoproponal. The pellet after centrifugation was dried and dissolved in Tris EDTA buffer (10 mMTris and 1 mM EDTA (pH: 8.0). The DNA was purified with phenol: choloroform (1:1 v/v) and further using choloroform: isoamylalcohol (24:1 v/v). Finally the DNA was precipitated with chilled ethanol after adding 1/10th volume of 3M sodium acetate. The DNA was dissolved in 50µl of TE buffer.

Polymerase chain reaction (PCR)

Three random oligo primers were used for PCR. The details of the primers are as follows.

Primer	Sequences
OPL05	5' – ACG CAG GCA C - 3'
OPL07	5' – AGG CGG CAA C - 3'
OPB15	5' – GGA GGG TGT T - 3'

Amplification reactions of RAPD were performed in 25µl reaction volume containing 50ng of template DNA, 1 X *Taq* polymerase (1unit),PCR buffer (10 Mm Tris (pH 8.0), 2.5 mM $MgCl_2$, 50 mMKCl, 0.01% gelatin) 20µl of primer , 150µM of each dNTPs. PCR was carried out in an Eppendorf master cycle gradient thermal cycler.

The amplification profile consisted of one cycle of initial denaturation at 94ºC for 5 min followed by 40 cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min, extension at 72ºC for 2 min with a final extension at 72ºC for 5 min. Amplified products were electrophoresed in 2 per cent agarose gel.

Agarose gel electrophoresis

Agarose of required quantity was weighed and melted in 1X Tris borate EDTA buffer (10 mMTris HCL (pH 8.3), 83mM boric acid, 0.5M EDTA) until clear solution was obtained. The solution was cooled to 50ºC and 2 µl of ethidium bromide was added from the stock and mixed well. Comb of appropriate size was placed at one end of the gel tray. The agarose was poured into the gel tray and allowed to set. The comb was removed after solidification and the gel was placed in electrophoresis chamber containing 1 X TBE buffer. The DNA samples were mixed with 6 X loading buffer at 5:1 and loaded into the well. Electrophoresis was carried out at 100 Vand the gel was documented using an Alpha Imager (Alpha Innotech Corporation, San Leandro, CA, USA).

Cluster analysis

The presence or absence of bands was converted into binary data (1 for presence and 0 for absence of each band) to determine the genetic relationship among the isolates. Similarity matrices were calculated with Jaggard's coefficient and the SIMQUAL program of NTSYS-pc (Exeter software, Setauket, NY, USA). Cluster analysis was completed within the SHAN program using the unweighed pair-group method with arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

Symptoms

Groundnut plants affected with wilt exhibited greyish green discolouration and flaccidity of leaves followed by yellowing of foliage and wilting. Vascular browning of internal tissues was also noticed. In the pathogenicity tests carried out *in vitro* as well as *in vivo*, plants inoculated *F. oxysporum* produced the same symptoms as observed in the field. The findings corroborate with that of Jofee (1973) who observed bleaching of foliage, drying of canopy with vascular browning of tap roots in wilt of groundnut caused by *F. oxysporum.* In cotton infection caused by *F. oxysporum* f. sp. *vasinfectum* lead to loss of leaf turgidity, leaf yellowing and withering. Wilting was either partial or complete. Tap roots were stunted with browning and blackening of vascular tissues (Prakasam *et al.,* 1993). In gingelly symptoms of Fusarium wilt include partial or total wilting of plants at flowering and podding, with a purple band extending from the base upwards. When the main stem or primary branches were split browning or blackening of internal tissue was noticed (Correll, 2005).

Morphological features of F. *oxysporum*

Morphological characters are important criteria in identifying *Fusarium* spp. In the present study, the isolates of *F. oxysporum* varied in growth and morphological features. Of the five isolates, $I_1$was fast in growth. The colony colour of the isolates varied from white to pinkish yellow. The isolates were dense, sparse and flat in nature. All the isolates produced macroconidia, microconidia and chlamydospores. Macroconidia were 3 to 5 septate while the microconidia were single celled and oval in shape. In cymbidium, *F. oxysporum* produced microconidia that were oval to cylindrical in shape. The fungus produced large number of fusoid, falcate macroconidia with 3 to 5 septa (Lee *et al*., 2002). The growth of *F.o.*f.sp. *pisidii* was cottony and pinkish in nature with abundant macroconidia and microconidia (Gupta *et al*., 2010).

Variability studies using molecular markers

Molecular tools have been used to characterize the diversity among pathogenic isolates of *F.oxysporum.* Molecular markers of RAPD have been used extensively as genetic markers in different fungal populations (Bentley *et al*., 1994 and Bridge *et al*., 1997). In the present study, the genetic similarity among the *F. Oxysporum* collected from five locations was between 27% to 55%. Forty isolates of *F.o.*f.sp.*vasinfectum* were characterized by RAPD and AFLP markers and cluster analysis showed two groups of isolates. Both techniques generated specific genomic patterns, which differentiated closely related races.

Unique fingerprint profiles generated by the RAPD and AFLP techniques can be exploited for race identification but additional pathogenicity testing on differential cultivars is needed to ascertain their precise determination (Abd-Elsalam *et al.,* 2004). High diversity among the 20 isolates of *F.o.*f.sp.*ciceri* was observed by RAPD technique. Though some of the isolates appeared similar in morphological and virulence patterns. RAPD finger printing could differentiate them. This indicates that molecular markers have high discrimination power than morphological traits and pathogenic reactions (Mandhare *et al.,* 2011). Malathi (2010) using RAPD markers observed that the similarity between isolates of *F.o.*f.sp.*cepae* was between 14-85 percent. Isolates collected from two distant areas had a genetic similarity of 85% revealing that isolates from different geographical area could share close genetic relationship.

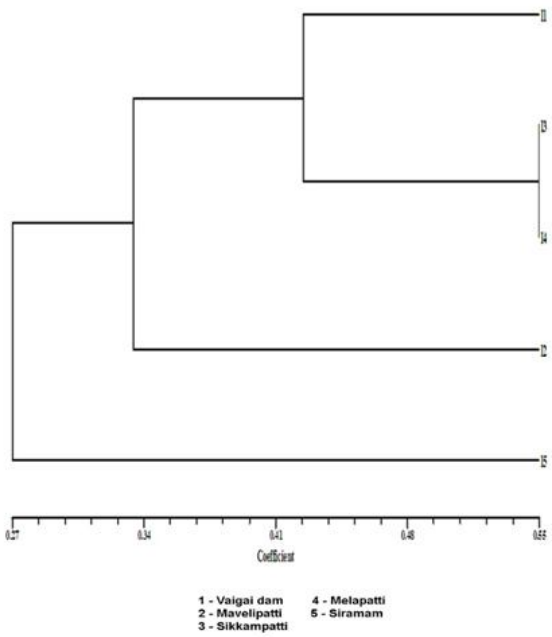

Figure 1: Dendrogram depicting the genetic relationship among the isolates of *F. oxysporum* based on DNA fingerprinting patterns generated by RAPD primers

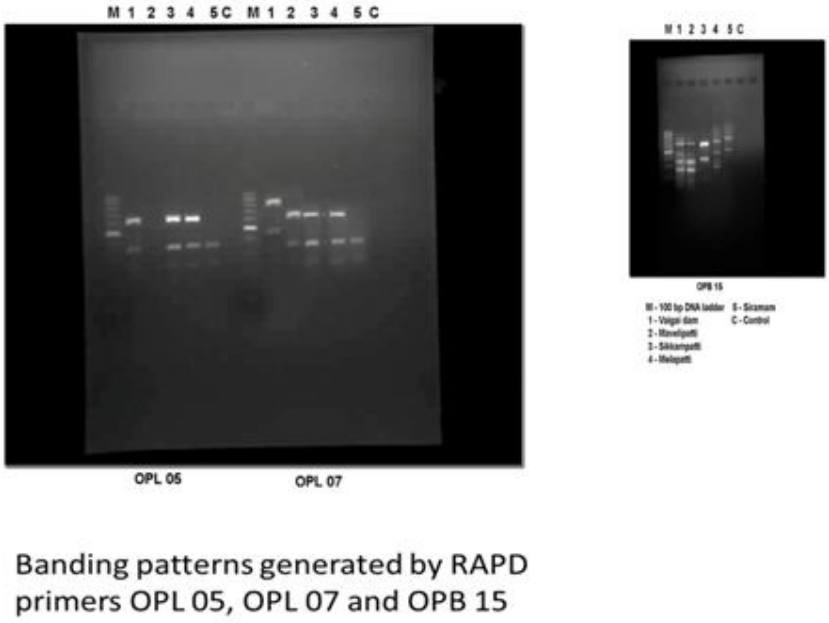

Figure 2: Molecular variability among the five isolates of Fusarium oxysporum

Table 1: Occurrence of Fusarium within groundnut growing areas

Sl. No.	Isolate code	Location	Variety	Age of the crop (days)	Disease Incidence (%)
1	I_1	Vaigai dam (Theni Dt.)	TMV 7	30	66.00
2	I_2	Mavelipatti (Madurai Dt.)	Local	65	44.00
3	I_3	Sikkampatti (Madurai Dt.)	VRI 2	70	20.00
4	I_4	Melapatty (Madurai Dt.)	Local	35	42.00
5	I_5	Siramam (Sivagangai Dt.)	Local	70	23.00

Table 2: Pathogenicity of *F. oxysporum* on groundnut *in vitro*

Days after incubation	Lesion length(mm)			
	Inoculated		Control	
	Pin prick	Without Pin prick	Pin prick	Without Pin prick
10	12.50	-	-	-
11	43.50	-	-	-
12	69.00	-	-	-
13	90.50	-	-	-
14	142.00	-	-	-
15	142.00	35.00	-	-
16	142.00	66.00	-	-

Table 3: Morphological characters and growth of different isolates of *F. oxysporum*on potato dextrose agar

Sl. No.	Isolate code	Colony type	Colour	Mycelial growth (cm) 7 DAI*	Mycelial growth rate (cm/day)
1	I_1	Sparse	Pinkish yellow	9.00	1.29
2	I_2	Dense	White	7.20	1.03
3	I_3	parse	White with yellow tinge	7.80	1.11
4	I_4	Dense	White	6.80	0.97
5	I_5	Flat	White	7.10	1.01
CD				0.19	-

*DAI - days after inoculation

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