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Pathogenic and Molecular Variability of *Aspergillus niger* Isolates Causing Collar Rot Disease in Groundnut

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ABSTRACT

Pathogenic variability studies of 26 isolates of collar rot pathogen (Aspergillus niger) of groundnut collected from different districts of Telangana and Andhra Pradesh states were conducted under greenhouse conditions by using two groundnut cultivars TMV-2 and JL-24. The mean seedling mortality ranged from 29.7 per cent (MBNRAn-1 (Palkapally)) to 94.4 per cent (ATPAn-1 (Jogannapet)). All the isolates of A. niger showed differential reaction with regard to seedling mortality on JL-24 and TMV-2 cultivars. Similarly genetic relatedness among eight virulent isolates (two isolates from each district) of A. niger from groundnut was assessed by using RAPD analysis. The similarity index values among the A. niger isolates varied from 0.571 (between WGL An-2 and CHT An-3) to 0.229 (between MBNR An-3 and WGL An-2).

Key words: *Groundnut, Collar rot, Aspergillus niger, Pathogenic variability, Molecular variability*

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important food legume grown in Asia and Sub Saharan Africa. It is also an important source of oil for majority of human population worldwide and is a rich source of protein for human and animal consumption. Approximately, groundnut kernels contain 48-50% of edible oil and 26-28 % protein, along with rich dietary fibre, minerals and vitamins[11]. Major groundnut growing states of India include Andhra Pradesh, Telangana, Gujarat, Karnataka and Tamil Nadu. Of these, Andhra Pradesh, Telangana and Gujarat contribute to more than half the crop area in the country [3]. Groundnut cultivation in India as a rainfed crop is often subjected to significant yield losses annually due to biotic and abiotic stresses are the major limiting factors for attaining high productivity in India. Of various biotic stresses, soilborne diseases account for reduced pod yields.

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Among soilborne diseases collar rot (*Aspergillus niger* Van Tieghem), causing major havoc in all crop growing areas. Collar rot disease is commonly manifested as a pre- and post-emergence damping-off of the affected seedlings. Occasionally, collar rot can continue up to crop harvesting stage resulting in damage to the seed[4]. The fungus also produces heat canker symptoms [2]. Though, the annual yield losses due to collar rot alone are approximately 5 %, the disease has a potential to damage the crop with 40% losses[1]. For successful implementation of management practices against soilborne diseases, knowledge on pathogen distribution and their diversity in major crop growing areas is essential. This will enable to devise location specific management practices to curb these soil borne diseases hitherto difficult to manage especially under high disease pressure. Therefore, the present study was taken up to identify the pathogenic and molecular variability among the isolates of *A. niger* collected from different areas of Telangana and Andhra Pradesh states.

MATERIAL AND METHODS

To identify the most effective and virulent isolate of *A. niger,* the susceptible cultivars TMV-2 and JL 24 were inoculated separately with each fungal isolate by soil inoculation technique[12] under greenhouse conditions. The test pathogen was mass multiplied on sorghum grains [5]. Sorghum grains were pre-soaked in water overnight. Later, the excess water was removed and the soaked grains were transferred into 1000 ml flasks @ 400 g and autoclaved at 15 lb psi (121.6^{o} C) for 20 min. The flasks were allowed to cool at room temperature and inoculated with 5 mm discs of actively growing 3-4 day old culture of *A. niger*. Seven discs per flask were added and the flasks were later incubated for 10 days at 28 ± 2^{o} C.

Soil inoculation technique was followed for inoculation of the test isolates. To each pot (5" diameter with sterilized soil), 40 g of inoculum multiplied on sorghum grains was added and mixed with the soil to a depth of 5 cm and covered with the soil. The pots were inoculated with *A. niger* pathogen 3 days prior to sowing and sprinkled with water. Later, five groundnut seeds were sown in each pot. Three replications were maintained for each isolate. Per cent seedling mortality was calculated at 30 DAS. The experiment was conducted in Randomized Complete Block Design (RCBD).

The molecular variability of *A. niger* were studied using Random Amplified Polymorphic DNA (RAPD). Two virulent isolates from each district were selected for each pathogen to study the molecular variability. A total of 13 primers were used. Standard protocols were used for the isolation of DNA and RAPD analysis[8].

Isolation of genomic DNA

Mycelium (100±150 mg) of respective isolates (*A. niger*) were crushed using liquid nitrogen into a fine homogenate. Lysis buffer (200 mM Tris pH 8; 500 mM NaCl; 100 mM EDTA pH 8 and .0.5% SDS;) was added (800 µl) to the tube containing homogenate and mixed thoroughly. Then the samples were incubated in a water bath (Gallen Ramp, England) at 65° C for 30 minutes and centrifuged at 12000 rpm for 10 minutes in a microcentifuge (Eppendorf Centrifuge 5415 D). The supernatant was transferred into fresh tube and to that, equal volume of extraction buffer (phenol: chloroform: isoamyl alcohol, 25:24:1, v:v:v) was added and stirred gently on a vortex mixture until an emulsion had formed. The contents were later centrifuged at 12000 rpm for 10 min in a micro-centrifuge at room temperature. An aliquot of the upper aqueous layer was collected, mixed with an equal volume of extraction buffer, and recentrifuged. The supernatant was transferred into new tubes, mixed with an equal volume of chloroform and isoamyl alcohol (24:1), and recentrifuged. The upper aqueous layer was collected into eppendorf and to this 3µl of RNase A (10 mg ml^{-1}) was added and the mixture was incubated at 37 °C for 30 min. Finally 2/3rd volume of ice cold isopropanol was added to the eppendorf tubes. The contents were later mixed by tilting the tubes gently and the tubes were kept at -20 ° C for

30 minutes to allow the DNA to precipitate. Later, the contents were spinned at 12000 rpm for 10 minutes in a Centrifuge and the DNA pellet was washed with 70 % ice cold ethanol two times to remove the salts and other impurities and the tubes were air dried for 10±15 min, suspended in 40 µl of TE buffer (10 mm Tris±HCl (pH 8±0) 1 mm EDTA). The DNA concentration was estimated by electrophoresis in a 0±8% agarose gel, staining with ethidium bromide (10 lg ml^{-1}) for 30 min and the staining intensity was compared with Lambda DNA markers under UV illumination.

Measurement of DNA Concentration

The quality and quantity of DNA were analyzed by running 1 µL of each sample mixed with 1 µL of 10x loading dye (Bromophenol blue) in 1% agarose gel. The DNA from all isolates produced clear sharp bands in one per cent agarose gel indicating the good quality of DNA. The DNA was quantified by comparing with the 1 kb size marker (Genei, Bangalore) and by spectrophotometer (Nanodrop ND1000).

RAPD Profiles through Polymerized Chain Reaction (Pcr)

Primer sequences (5'-3') for *Aspergillus niger*

S. No	Primers	Primer Sequence (5´- 3´)
1	OPA 2	TGCCGAGCTG
2	OPA 4	AATCGGGCTG
3	OPA10	GTGATCGCAG
4	OPA 20	GTTGCGATCC
5	R108	GTATTGCCCT
6	R151	GCTGTAGTCT
7	UBC90	GGGGGTTAGG
8	GLB-12	CCTTGACGCA
9	GLB-15	GGAGGGTGTT
10	GLK-08	GAACACTGGG
11	GLL-04	GACTGCACAC
12	GLL-05	ACGCAGGCAC
13	GLL-12	GGGCGGTACT

Thirteen oligonucleotide primers (Macrogen Inc. Oligo, Seoul) were screened for generating polymorphism among the isolates under the study. The experiment was repeated thrice and results were reproducible. The Oligonucleotide primer sequences used in RAPD technique are given below:

PCR amplifications were carried out in 0.2 mL eppendorf tubes with 25 µL reaction mixture which consists of 2.5 µL of 10x Taq buffer, 2 µL of 25 mM MgCl2, 1 µL of primer (5 picomolar / µL), 1 µl of 5 mM dNTP mix, 0.5µL of Taq polymerase enzyme (conc. 5 U µL-1) and 15.5 µL of sterile PCR water (Genei, Bangalore) and 2.5 µL (40-50 ng) of DNA sample. Amplification was carried out by 5 min of initial denaturation at 94°C followed by 40 cycles of denaturation of 94°C for 1 min; annealing at 37°C for 1 min; extension at 72°C for 2 min with final elongation at 72°C for 5 min. Amplified PCR products were subjected to 1.5 per cent agarose gel electrophoresis with 1.0 x TBE as running buffer. The banding patterns were visualized under UV trans-illuminator with ethidium bromide (10 mg mL^{-1}) staining. The DNA banding profiles were documented in the gel documentation system (Alpha Innotech) and compared with 1 kb DNA ladder (Genei, Bangalore).

Scoring and Data Analysis

Each amplified band was considered as RAPD marker and recorded for all samples. Data was entered using a matrix in which all observed bands or characters were listed. The data matrix thus generated (0, 1 for absence and presence of the bands) was used to calculate Jaccard's similarity coefficient for each pair wise comparison. The coefficients were calculated In Silico[7]. The similarity coefficients were subjected to Unweighted Pair-Group Method on Arithmetic Average (UPGMA) cluster analysis to group the isolates based on their overall similarities. A similarity matrix was generated from the binary data using DICE similarity co-efficient in NTSYs pc package[14].

RESULTS AND DISCUSSION

Pathogenicity of 26 isolates of *A. niger* collected from different locations were tested on susceptible groundnut cultivars JL 24 and TMV-2 under greenhouse conditions and the data presented in the Table 1. All the isolates were found more or less effective in inducing seedling mortality in both the susceptible cultivars. The mean seedling mortality ranged from 29.7 per cent (MBNRAn-1 (Palkapally)) to 94.4 per cent (ATPAn-1 (Jogannapet)). The isolates ATPAn-1 (94.4%), ATPAn-6 (76.4%), ATPAn-3 and CHTAn-3 (70.8%) did not show any significant difference in the seedling mortality. Among the isolates collected from Anantapur isolate ATPAn-4 showed a seedling mortality of 42.2 per cent while isolate ATPAn-1 recorded seedling mortality as high as 94.4 per cent. Similarly, for isolates from Mahaboobnagar, the seedling mortalities varied from 29.7 per cent (MBNRAn-1) to 65.6 per cent (MBNRAn-9). While with Chittoor isolates, the seedling mortality varied from 32.8 per cent (CHTAn-5) to 70.8 per cent (CHTAn-3). *Aspergillus niger* isolates from Warangal recorded seedling mortality of 41.7 per cent (WGLAn-1) to 59.7 per cent (WGLAn-2). Further it was also also observed that isolate ATPAn-1, the seedling mortality was 88.9 per cent in the cultivar TMV-2, whereas, 100 per cent mortality was observed on JL-24. In TMV-2, the seedling mortality ranged from 33.3 per cent (MBNRAn-1, 4 and %) to 88.9 per cent (ATPAn-1) and on JL-24, it varied from 22.2 per cent (MBNRAn-2) to 100 per cent (ATPAn-1). The average per cent seedling mortality of Anantapur isolates was 61.2 per cent on JL-24 and 65.9 per cent on TMV-2. Similarly with Mahaboobnagar isolates the seedling mortality of 49.1 per cent was recorded on JL-24 and 47 per cent on TMV-2 seedling mortality. For Chittoor isolates, the seedling mortality was 38.4 per cent on JL-24 and 70.9 per cent on TMV-2. The per cent seedling mortality of Warangal isolates was 42.8 per cent on JL-24 and 60.6 per cent on TMV-2. The isolate ATPAn-1 was highly virulent among all the *A. niger* isolates showing an average of 94.4 per cent mortality in both cultivars JL-24 and TMV-2 and the isolate which showed highest per cent mortality was used for further studies (Figure 1).

The present studies indicate the pathogenic variability of different isolates of *A. niger* collected from different regions. All the isolates of *A. niger* showed differential reaction with regard to seedling mortality on JL-24 and TMV-2 cultivars. Isolates ATP An-1, ATP An-3, ATP An-6, MBNR An-3, 5, 6 and 9 were highly virulent on JL-24. While the rest of the isolates were more virulent on TMV-2. Isolates from the same district have shown differential reaction on different cultivars this may be due to the characteristics of that particular cultivar or due to the agro ecological conditions of that locality from which the isolate was collected and also due to the variation in the characteristics of the isolate [15]. Similar type of variations with the *A. niger* on groundnut cultivars was also observed by the earlier workers where the

isolates (AN1 to AN5) showed marked differences in the per cent germination, per cent seed rot and per cent collar rot on groundnut cultivar AK12-24. The isolate AN1 showed lowest seed germination and highest collar rot incidence considered to be more virulent[9].

Molecular variability among isolates of *Aspergillus niger*

Genetic relatedness among eight virulent isolates (two highly virulent isolates (based on seedling mortality values) from each district) of *A. niger* from groundnut was assessed by using RAPD analysis. Thirteen primers viz., OPA 2, OPA 4, OPA 10, OPA 20, R108, R151, UBC90, GLB-12, GLB-15, GLK-08, GLL-04, GLL-05 and GLL-12 were screened of which 10 primers (OPA 2,OPA 4,OPA10, OPA 20, R108, R151, UBC90, GLB-12, GLB-15 and GLK-08) generated reproducible polymorphism among the eight tested isolates. A total of 653 reproducible bands were generated with 10 primers (Plate 1 and 2).

The data presented in the Table 2 indicated that similarity index values among the *A. niger* isolates varied from 0.571 (between WGLAn-2 and CHTAn-3) to 0.229 (between MBNRAn-3 and WGLAn-2) (Table 2). The isolates collected from Mahaboobnagar were highly variable and they share only 23 % similarity followed by Warangal 39.5 %, Chittoor 50% and Anantapur 55.7 %. Genetic similarity between the *A. niger* isolates was estimated based on UPGMA cluster analysis (Figure 2) and indicated that all the isolates were grouped into two major clusters with single isolates i.e., MBNR An-1 and MBNRAn-3. The first cluster was further subdivided into four isolates which was again subdivided into two sub cluster of two each *i.e.* ATPAn-1 and ATPAn-6; CHTAn-3 and WGLAn-2. The isolates present in second cluster (CHTAn-4 and WGLAn-3) were more diverse when compared to first one. Highest similarity was observed between isolates collected from Chittoor (CHTAn-3) and Warangal (WGL An-2). Remaining isolates showed considerable genetic variation with each other.

These findings have clearly shown that RAPD can genetically differentiate the isolates at inter specific level. Correlation was not observed between the similarity index and the place of collection of isolates and clustering also was not observed according to location except ATPAn-1, ATPAn-6. In each group or sub group isolates from different locations were present indicating high genetic diversity. Even though the isolates CHTAn-3 and WGLAn-2 were isolated from different geographical regions but they showed highest genetic similarity which may be due to the migration of isolates from one place to another. Similar type of variations with the *A. niger* isolates also observed by several researchers[13&6]. Similarly [10] studied the molecular variability of 17 mutants and a wild type strain of *A. niger* using 12 random primers. All the mutants showed a varying degree of genetic divergence based on their amplification profile and maximum number of amplifications in wild type *A. niger* strain were produced by primer K-16. All the mutants were genetically diverse with wild type strain. The maximum similarity (78.63%) with wild type strain was found in G-80-A. On the other hand, the most closely related mutants were UV-180-A and UV-180-B with the genetic similarity of 61.60 %. From our results study of genetic variation through RAPD primers is useful in estimating the diversity among the isolates, between and within same species collected from the same host. The present findings indicated that genetic differences between species of the same genus maintain genetic diversity within this population isolated from same host.

Table 1: Evaluation of pathogenic variability of *Aspergillus niger* isolates in groundnut under greenhouse conditions

***Aspergillus niger* isolate (*Identity number)**	**Per cent Seedling mortality**		
	TMV2	**JL24**	**Mean**
ATPAn-1	88.9 (78.2)	100.0 (90)	94.4 (**84.1)
ATPAn-2	55.6 (48.2)	44.4 (41.7)	50.0 (45)
ATPAn-3	66.7 (60.0)	75.0 (65)	70.8 (62.5)
ATPAn-4	55.6 (48.2)	28.9 (32.3)	42.2 (40.3)
ATPAn-5	66.7 (60.0)	43.9 (41.3)	55.3 (50.6)
ATPAn-6	61.1 (51.5)	91.7 (80)	76.4 (65.7)
ATPAn-7	66.7 (60.0)	44.4 (41.7)	55.6 (50.9)
MBNRAn-1	33.3 (30.0)	26.1 (30.6)	29.7 (30.3)
MBNRAn-2	52.8 (46.7)	22.2 (23.5)	37.5 (35.1)
MBNRAn- 3	55.6 (48.2)	61.1 (51.5)	58.3 (49.9)
MBNRAn- 4	33.3 (30.0)	33.3 (29.9)	33.3 (29.9)
MBNRAn- 5	33.3 (30.0)	72.2 (63.2)	52.8 (46.6)
MBNRAn- 6	38.9 (33.2)	77.8 (66.5)	58.3 (49.9)
MBNRAn- 7	66.7 (60)	23.3 (23.8)	45.0 (41.9)
MBNRAn- 8	55.6 (48.2)	48.3 (448.8)	51.9 (48.5)
MBNRAn-9	53.3 (46.9)	77.8 (71.7)	65.6 (59.3)
CHTAn-1	77.8 (66.5)	31.1 (28.7)	54.4 (47.6)
CHTAn-2	66.7 (59.9)	38.9 (38.5)	52.8 (49.2)
CHTAn-3	83.3 (74.9)	58.3 (55)	70.8 (64.9)
CHTAn-4	83.3 (74.9)	41.7 (40)	62.5 (57.4)
CHTAn-5	43.3 (41.1)	22.2 (23.5)	32.8 (32.3)
WGLAn-1	50.0 (44.9)	33.3 (30)	41.7 (37.5)
WGLAn-2	75.0 (69.9)	44.4 (41)	59.7 (55.8)
WGLAn-3	55.5 (48.2)	52.8 (46.7)	54.2 (47.5)
WGLAn-4	52.8 (46.5)	50.0 (45)	51.4 (45.7)
WGLAn-5	70.0 (61.9)	33.3 (30)	51.7 (45.9)
	S.Em±		CD at 5%
Isolates	11.93		33.46
Cultivars	3.3		9.282
Isolates*Cultivars	16.7		47.33

***** *Aspergillus niger* isolates are collected from Anantapur (ATP); Chittoor (CHT); Mahaboobnagar (MBNR); and WGL (Warangal) districts of Andhra Pradesh and Telangana

** Values in the parenthesis are angular transformed values and are means of three replications

Table 2: Similarity index values based on the genetic distance between isolates of *Aspergillus niger*

	ATPAn-1	**ATPAn- 6**	**MBNRAn-1**	**MBNRAn-3**	**CHTAn-3**	**CHTAn-4**	**WGLAn-2**	**WGLAn-3**
ATPAn-1	1.000							
ATPAn- 6	0.557	1.000						
MBNRAn-1	0.317	0.306	1.000					
MBNRAn-3	0.402	0.450	0.232	1.000				
CHTAn-3	0.420	0.430	0.380	0.296	1.000			
CHTAn-4	0.456	0.441	0.469	0.267	0.500	1.000		
WGLAn-2	0.500	0.434	0.403	0.229	0.571	0.460	1.000	
WGLAn-3	0.480	0.333	0.269	0.272	0.372	0.500	0.395	1.000

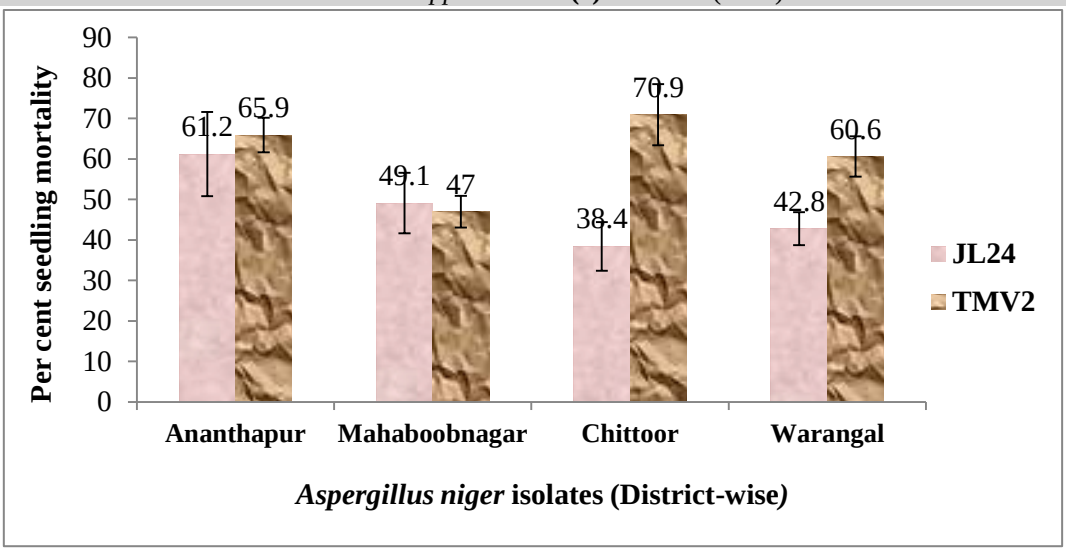

Fig. 1: Pathogenicity of isolates of *Aspergillus niger* in inducing seedling mortality in groundnut under greenhouse conditions

JL24 & TMV2 are the susceptible cultivars used, Seedling mortality was assessed at 30 days after sowing

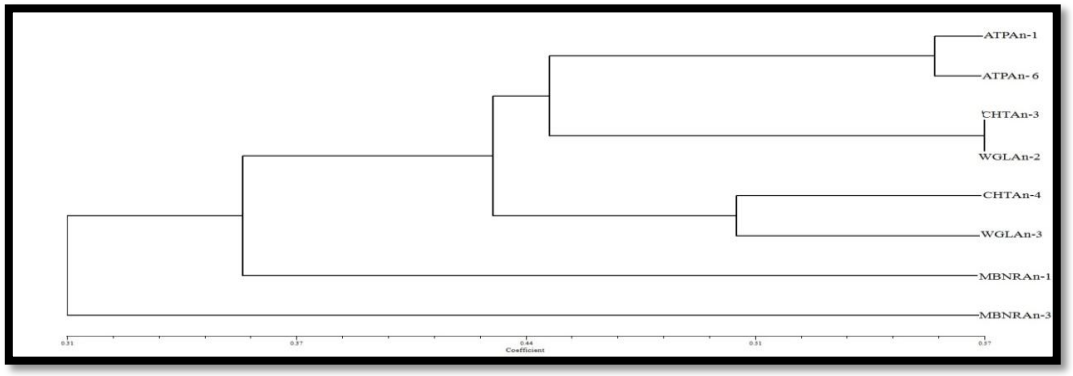

Fig. 2: Grouping of *Aspergillus niger* isolates based on genetic similarities using RAPD markers

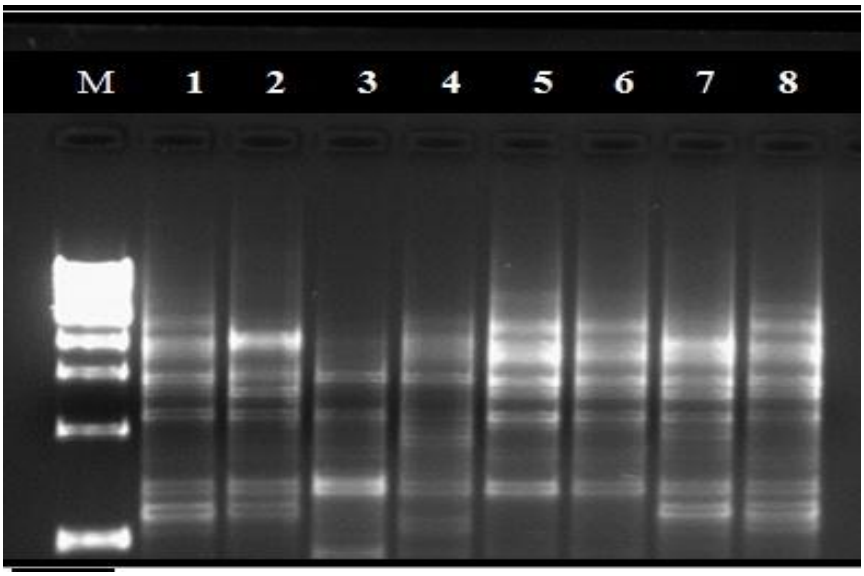

Plate 1: RAPD banding profile of *Aspergillus niger* isolates with primer OPA 2

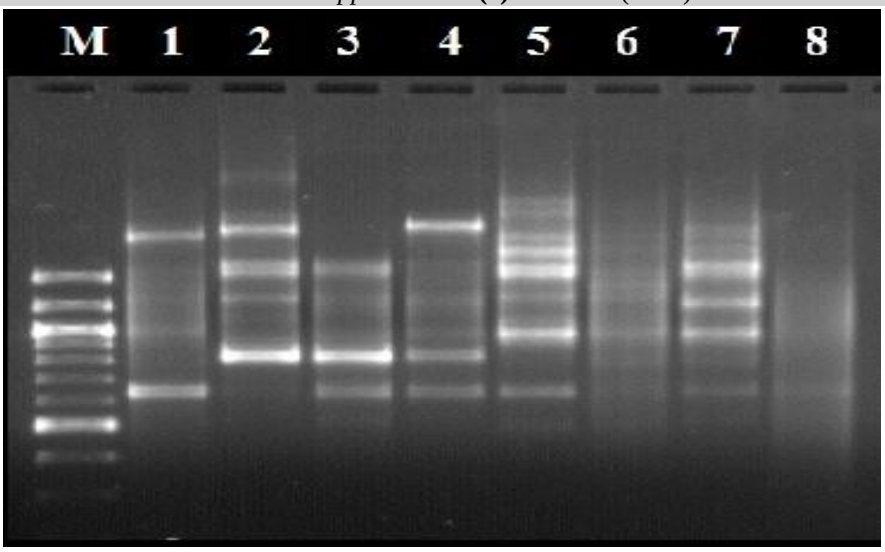

Plate 2: RAPD banding profile of *Aspergillus niger* isolates with primer OPA 2

Left-right Lane1: 9, (M) 1kb DNA marker
Lane 1-8: Randomly amplified DNA of fungal strains
Details of *A. niger* isolates

1) ATPAn-1 2) ATPAn- 6 3) MBNRAn-1 4) MBNRAn-3
5) CHTAn-3 6) CHTAn-4 7) WGLAn-2 8) WGLAn-3

CONCLUSIONS

Pathogenicity studies with 26 isolates of *A. niger* was tested on two susceptible groundnut cultivars JL-24 and TMV-2 under greenhouse conditions. The mean seedling mortality was highest (94.4%) with the isolate ATPAn-1 (Jogannapet) whereas, the lowest mean seedling mortality (29.7%) observed with the isolate MBNRAn-1 (Palkapally). Variation in relation to seedling mortality was observed between the isolates collected from different localities and also between isolate and cultivars. Molecular variability of eight virulent isolates of *A. niger* with RAPD marker indicated that considerable genetic variation was observed among the isolates. The similarity index values among the *A. niger* isolates varied from 0.571 (between WGL An-2 and CHT An-3) to 0.229 (between MBNR An-3 and WGL An-2). The isolates MBNR An-1 and MBNRAn-3 were grouped into different clusters which clearly indicate the highest level of genetic similarity between the isolates collected from same district.

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