

RESEARCH ARTICLE

# Genetic variability in isolates of *Exserohilum turcicum* causing turcicum leaf blight of maize

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## ABSTRACT

Turcicum leaf blight caused by *Exserohilum turcicum* is an economically important disease of maize. The genetic variability was studied of seven isolates of *E. turcicum* from maize and one isolate of *E. turcicum* from sorghum using RAPD markers. All the isolates exhibited considerable variation in their genetic level. Out of 60 polymers used for amplification, 20 were able to amplify the DNA of all the seven isolates of maize and one sorghum isolate. The number of amplified products were highest for the primer OPH7, followed by OPD11, OPD13 and OPA9. Least number of products were generated by OPA6 followed by OPC16 and OPH15. Co-efficient values of each isolate derived from RAPD study were presented in Table 1. Among the 7 maize isolates of test pathogen, highest similarity co-efficient 0.85 was shown between isolates of Guntur and Warangal followed by 0.70 between isolates of Karimnagar and Guntur, and between isolates of Karimnagar and Warangal. Lowest similarity co-efficient 0.51 was seen between Khammam and Karimnagar isolates, followed by 0.53 between Khammam and Almora isolates.

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## INTRODUCTION

*Exserohilum turcicum* (Pass.) Leonard and Suggs is a common fungal pathogen causing leaf blight in several members of Poaceae causing considerable economic damage (Abadi *et al.*, 1996). In maize it is one of the important foliar diseases causing severe reduction in grain and fodder yield to the tune of 16-98 per cent (Kachapur and Hegde, 1988). The disease is wide spread during humid and warm weather with heavy dew conditions (Indira *et al.*, 2002).

Variability in plant pathogen population can be studied through virulence and molecular analysis. Virulence analysis have limitation such as being less informative, requirement of genetically defined host differentials, time consuming and being laborious. The use of molecular markers involves the

detection of DNA sequence polymorphism and it helps to analyse a large number of genetic loci from the genome of an organism with high levels of efficiency and sensitivity (Weikert-Oleveria *et al.*, 2002; Wagara, 2004). RAPD markers are useful in measuring genetic relatedness and for detecting variation within and among populations of *E. turcicum* thus helping one to understand the ecology and biology of fungus (Abadi *et al.*, 1996; Jones and Dunkle, 1993). This technique is also effectively applied for differentiating various races of pathogenic fungi e.g., *Cochliobolus carbonum* (Jones and Dunkle, 1993), *Fusarium* (Sivaramakrishnan *et al.*, 2002), *Macrophomina* (Raj Kumar *et al.*, 2007; Das *et al.*, 2008). In this paper, the genetic variability was studied of seven isolates of *E. turcicum* from maize and one isolate of *E. turcicum* from

sorghum using RAPD markers.

## MATERIAL AND METHODS

### Fungal isolates :

Six isolates of *E. turicum* (Et) from six districts of Andhra Pradesh viz., Karimnagar, Guntur, Warangal, Khammam, Mahaboobnagar and RangaReddy during *Kharif* 2011-12. One sample was also collected from Almora district of Uttarakhand where the disease appears in high intensity. Further to ascertain the variability of *E. turicum* from sorghum to maize, one reference isolate was collected from sorghum from RangaReddy district. The pure cultures of the isolates were maintained on Potato dextrose agar (PDA) slants at 4°C at Department of Plant pathology, Acharya N.G. Ranga Agriculture University, Rajendranagar, Andhra Pradesh, India. The details of isolates are given in Table A.

### DNA Isolation :

Ten day old culture of all the isolates of the pathogen was used for DNA isolation. The mycelial mat was taken out from PDB and blot dried after thorough washing with sterile distilled water and 500 mg of mycelium was taken with respect to each of the isolate for isolation of DNA. The mat was grinded into fine powder in a small pestle and mortar by adding liquid nitrogen. Immediately the powder was poured in an eppendorf tube containing 1 ml of CTAB buffer and mixed thoroughly. Then the samples were incubated in a water bath at 65°C for 30 minutes and cooled to room temperature. One ml of chloroform and isoamyl alcohol (Chloroform : isoamyl alcohol = 24:1) was added to each tube, mixed and centrifuged at 3,500

rpm for 20 minutes in high speed refrigerated centrifuge (Eppendorf, USA). The supernatant was collected carefully into another eppendorf tube by 200 µl micropipette and re-extracted with 1.0 ml of chloroform: isoamyl alcohol (24: 1).

Finally the aqueous layer was collected in another eppendorf tube and 2/3 volume of isopropanol was added to it and the content was mixed by tilting the tubes gently and kept at -20°C for 30 minutes to allow the DNA to precipitate. After that a spin at full speed (10,000 rpm) was given for 3 minutes in a centrifuge and the DNA pellet was washed with 70 per cent ethyl alcohol 3 times to remove the salts and other metabolites and air dried. The DNA pellet was suspended in 200 µl of T<sub>10</sub>E<sub>1</sub> and stored at -20°C for further use. The quality and quantity of DNA was checked using gel electrophoresis and UV spectrophotometer.

### PCR Amplification of DNA :

Preliminary primer screening was carried out with 60 primers (Ten-Base oligonucleotide) and 20 primers from OPA, OPC, OPD and OPH series (Operon Technologies, Inc., USA) were employed for molecular variation analysis. The RAPD primer amplifications were carried out in a 20 µl of reaction mixture containing 3 µl of 50 ng of fungal genomic DNA, 2 µl of 10X PCR buffer, 0.6 µl MgCl<sub>2</sub>, 1 µl of 2.5 mM dNTPs, 1 µl of RAPD Primer and 0.2 µl of Taq polymerase (Invitrogen life technologies, USA). Master mix was prepared by mixing all the components except template DNA in a separate eppendorf tube. Then 17 µl of master mix was added to another tube containing 3 µl of template DNA and a spin was given. The amplification process was accomplished in a 0.2 ml of thin

**Table A : Details of the places surveyed for collection of turicum leaf blight samples**

Sr. No.	Isolates	District	Sr. No.	Isolates	District
1.	Et <sub>1</sub>	Karimnagar	5.	Et <sub>5</sub>	Khammam
2.	Et <sub>2</sub>	Almora (Uttarakhand)	6.	Et <sub>6</sub>	Mahaboobnagar
3.	Et <sub>3</sub>	Guntur	7.	Et <sub>7</sub>	Rangareddy
4.	Et <sub>4</sub>	Warangal	8.	Et <sub>8</sub> (Sorghum)	Rangareddy

**Table B : List of primers used in RAPD study**

Sr. No.	Primers name	Sequence	Sr. No.	Primers name	Sequence
1.	OPA-02	TGCCGAGCTG	11.	OPD-1	ACCGCGAAGG
2.	OPA-03	AGTCAGCCAC	12.	OPD-5	TGAGCGGACA
3.	OPA-05	AGGGGTCTTG	13.	OPD-7	TTGGCACGGG
4.	OPA-06	GGTCCTGAC	14.	OPD-11	AGCGCCATTG
5.	OPA-07	GAAACGGGTG	15.	OPD-15	CATCCGTGCT
6.	OPA-09	GGGTAACGCC	16.	OPD-16	AGGGCGTAAG
7.	OPA-10	GTGATCGCAG	17.	OPH-7	CTGCATCGTG
8.	OPA-17	GACCGCTTGT	18.	OPH-15	AATGGCGCAG
9.	OPA-18	AGGTGACCGT	19.	OPH-16	TCTCAGCTGG
10.	OPC-18	TGAGTGGGTG	20.	OPH-18	GAATCGGCCA

walled 96 well plates in a thermocycler. The samples were incubated at 94°C for initial denaturation. Then samples were subjected to 35 repeats of following cycle: 92°C for 0.45 min (denaturation), 36°C for 1 min (primer annealing), 72°C for 1 min (primer extension) and at the end of 35<sup>th</sup> cycle 72°C for 10 min for final extension. The samples were incubated at 4°C. The PCR amplicons were separated in electrophoresis unit (Bio-Rad, USA) in 1.5 per cent agarose gel in 1X Tris acetate EDTA (TAE) and 10mg/ml Ethidium bromide. The loaded samples were electrophoresed at 90 volts for 30 minutes and gels were visualized under UV-light documentation unit (Gene flash, Syngene Bioscience, USA). The primers that gave reproducible and scorable amplifications were used in the analysis of genetic variability of the isolates. DNA amplification with respect to all the fungal isolates was observed with the primers given in Table B. The RAPD procedure was repeated twice for each of the primer to test the reproducibility of DNA bands (Table B).

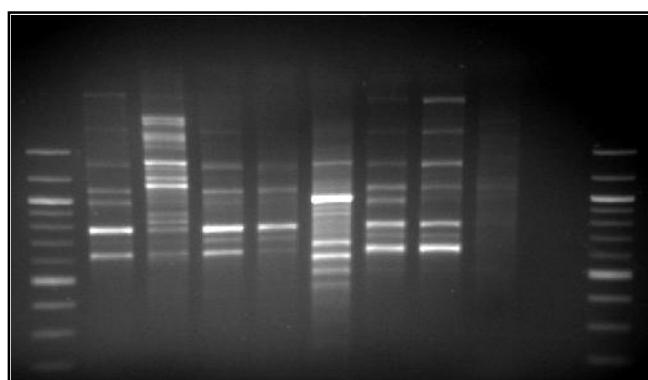
#### Data analysis :

The data generated from each primer gel run was recorded in a matrix identifying the presence (1) or absence (0) of each RAPD band of a particular molecular weight. A similarity matrix was generated from the binary data using DICE similarity co-efficient in SIMQUAL programme of NTSYS-pe package (Rohlf, 1993).

## RESULTS AND DISCUSSION

Polymorphism was observed among the seven *Exserohilum turcicum* isolates of maize and sorghum. RAPD analysis of seven *Exserohilum turcicum* isolates of maize and one *Exserohilum turcicum* isolate of sorghum was done with ten base pair oligonucleotide operon polymers. Out of 60 polymers used for amplification, 20 were able to amplify the DNA of all the seven isolates of maize and one sorghum isolate. Polymorphism of the *Exserohilum turcicum* isolates of maize and sorghum with primer OPA-9 is shown in Fig. 1. The numbers of amplified products were highest for the primer OPH7, followed by OPD11, OPD13 and OPA9. Least number of products were generated by OPA6 followed by OPC16 and

OPH15. Information on banding pattern with all primers were used to determine the similarity co-efficient and genetic distance between isolates and further to construct dendrogram.



M: Marker (100bp DNA ladder); B: Blank; 1 to 8 : Seven *Exserohilum turcicum* isolates from maize and one from sorghum; 1:Karimnagar; 2:Almora; 3:Guntur; 4:Warangal; 5: Khammam; 6: Mahaboobnagar; 7: RangaReddy; 8: Sorghum

**Fig. 1 : Random Amplified Polymorphic DNA (RAPD) banding profile of *Exserohilum turcicum* isolates of maize and sorghum with primer OPA-9**

Co-efficient values of each isolate derived from RAPD study are presented in the Table 1. Among the 7 maize isolates of test pathogen the highest similarity co-efficient 0.85 was shown between isolates of Guntur and Warangal followed by 0.70 between isolates of Karimnagar and Guntur, and between isolates of Karimnagar and Warangal. Lowest similarity co-efficient 0.51 was seen between Khammam and Karimnagar isolates, followed by 0.53 between Khammam and Almora isolates.

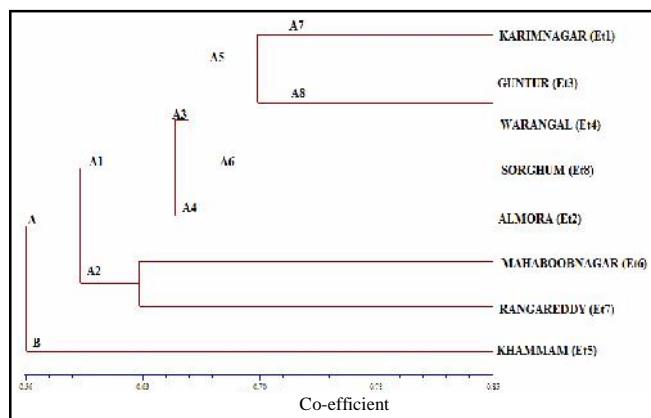
However, sorghum isolates has shown highest similarity co-efficient with Warangal and lowest similarity co-efficient with Khammam.

Data differentiated the test isolates into 2 major clusters A and B (Fig. 2). Cluster A was divided into sub-clusters A<sub>1</sub> and A<sub>2</sub>. A<sub>1</sub> was further classified in to A<sub>3</sub> and A<sub>4</sub>; A<sub>3</sub> was differentiated into A<sub>5</sub> and A<sub>6</sub>. A<sub>5</sub> was further divided into A<sub>7</sub> and A<sub>8</sub>, cluster A<sub>2</sub> included Mahaboobnagar and RangaReddy

**Table 1 : Similarity co-efficient values of *Exserohilum turcicum* isolates of maize and sorghum based on RAPD analysis**

Isolates	Et <sub>1</sub>	Et <sub>2</sub>	Et <sub>3</sub>	Et <sub>4</sub>	Et <sub>5</sub>	Et <sub>6</sub>	Et <sub>7</sub>	Et <sub>8</sub>
Et <sub>1</sub>	1.00							
Et <sub>2</sub>	0.58	1.00						
Et <sub>3</sub>	0.70	0.69	1.00					
Et <sub>4</sub>	0.70	0.68	0.85	1.00				
Et <sub>5</sub>	0.51	0.53	0.58	0.59	1.00			
Et <sub>6</sub>	0.57	0.50	0.57	0.57	0.58	1.00		
Et <sub>7</sub>	0.60	0.60	0.65	0.60	0.54	0.62	1.00	
Et <sub>8</sub>	0.62	0.64	0.66	0.69	0.51	0.56	0.63	1.00

isolates, A<sub>4</sub> included Almora isolate, A<sub>6</sub> included sorghum isolate, A<sub>7</sub> included Karimnagar isolate, A<sub>8</sub> included Guntur and Warangal isolates; cluster B included Khammam isolate.



**Fig. 2 :** Grouping of *Exserohilum turcicum* isolates of maize and sorghum based on RAPD banding pattern

In the present study, the RAPD analysis revealed genetic diversity among isolates of *E. turcicum* which was notably higher according to banding pattern observed. Out of 60 primers initially evaluated for amplification, 20 primers generating reproducible banding patterns were selected. Among these primers, three primers OPH7, OPD11 and OPD-13 have generated more polymorphic bands.

The Random Amplified Polymorphic DNA is a PCR based technique which reveals polymorphism with in completely unknown samples without the need of probe hybridization or DNA sequencing. RAPDs have also been used in race, pathotype and population studies (Crowhurst *et al.*, 1991 and Manalis *et al.*, 1994). This feature combined with their easy identification and makes it a valid type of marker potentiality useful in many areas of genetic research such as gene mapping and strain differentiation of *Trichoderma* spp. (Zimad *et al.*, 1994). In present study, detection of genetic variability of *E. turcicum* isolates was done by PCR using 60 random primers. The results revealed that the isolates were polymorphic and confirmed the genetic variability. Among the isolates, maximum genetic variation was high with minimum similarity co-efficient of 0.50 between Almora and RangaReddy district. These findings have clearly shown that RAPD can genetically differentiate the isolates at inter specific level. A total of 26 isolates representing 15 species of *Drechslera*, five *Bipolaris* and four *Exserohilum* and two formae speciales of *Drechslera teres* (Sacc.) Shoemaker were compared by RAPD analysis by Bakonyi *et al.* (1995). *Drechslera* formed a large, heterogeneous group, while species of *Bipolaris* and *Exserohilum* were more closely related. Considering their cultural characteristics and specificity to their major hosts, they confirmed that species of *Drechslera*, *Bipolaris* and

*Exserohilum* are genetically distinct. On the other hand, studies done by Oliveira (1995) with *B. sorokiniana* isolates identified genotypic variation among the samples.

According to the Burdon and Silk (1997), the plant pathogenic fungi most commonly rely on mutation and recombination as the main source of genetically based variation. Within a species, gene flow between populations supplement these processes as propagules spread from one epidemiological area to another. Gene flow, along with other evolutionary forces can result in the spread of single gene or DNA sequences, and even establishment of whole populations in different regions. Considering that *E. turcicum* reproduces asexually, the source of the high genetic diversity reported in this study can be attributed to mutation and migration. This variation driven by forces of selection and genetic drift further amplifying the genetic variation as is also reported by Zeigler *et al.* (1995). The cultivation of different maize varieties may have led to a higher selection pressure due to the host pathogen interaction and environmental constraints resulting in increased diversity in the population's structure of the pathogen.

### Conclusion :

Variability among the isolates may be attributed to long term influence of weather conditions of particular location and ability of the pathogen to adapt to the varieties developed in a specific situation. Highly virulent isolates exhibited higher infection types on different differentials, whereas less virulent isolates were unable to produce more infection as compared to virulent isolates. Thus, it clearly indicated the existence of different strains or virulence within *E. turcicum*.

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