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Comparative Genetic Variation Among *Alternaria brassicae* Isolates Infecting Oilseed Brassica In India

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ABSTRACT

Alternaria blight caused by Alternaria brassicae is among the most devastating disease of brassica crop. The present study was carried out to know about the cultural and morphological variability among the different Alternaria brassicae isolates collected from geographically diverse regions of India. Rapid amplified polymorphic DNA (RAPD) markers was used to know the genetic relationships among the different isolates .Sixteen 10-mer primers of arbitrary nucleotide sequences were tested for amplification of Alternaria brassicae DNA by using PCR. Of these nine primers amplified the fungal genomic DNA from all the twenty isolates and produced reproducible RAPD profiles. The UPGMA analysis showed that the isolates collected from different regions can be classified into four major group. A close relationship was revealed between two isolates based on the cluster analysis data. Our results proved the existence of genetic variability among Alternaria brassicae isolates infecting oilseed brassica which is not based on their location and could be utilized for development of resistance against this pathogen.

Key words: *Alternaria brassicae, PCR, RAPD markers.*

INTRODUCTION

More than thirty diseases are better-known to occur on genus Brassica crops in India[16]. Alternaria blight disease caused by *Alternaria brassicae* (Berk.) Sacc. is the most catastrophic disease of Indian mustard that causes heavy yield loss of upto 47% specially in humid regions[1,8]. The pathogen affects the aerial parts of the host at all stages of growth however the special targets include the leaves and the pods that mainly influence the yield content[16]. Minute brown to black dots are seen as initial symptoms which further multiply and enlarge to form concentric rings. The whole leaf gets affected gradually and ultimately falls down[3]. Symptoms observed are generally similar on all infected host species but there may be variations in shape, size, colour, formation of concentric rings, yellow halo around the lesions which is because of different agro-ecological zones, host genotypes, nutritional status of soil and pathotypes involved[16].

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Traditionally plant diseases are controlled by applying fungicides, but it requires high input cost and also increases environmental pollution[11,12]. The variability study of pathogen population can be utilized to make resistance breeding approaches stronger. Recently, few reports have been presented on aggressiveness, diversity and distribution of *A. brassicae* isolates infecting Brassica in India[10]. Variation among pathogen populations usually observed with morphological, cultural, pathogenic and molecular specificity helps in the study of variation among the pathogen population[9]. For genotypic identification of pathogen PCR-based marker techniques were used extensively. DNA markers provides an opportunity for characterization of genotypes and measurement of genetic relationships. RAPD markers are very fast and simple to develop because of the arbitrary sequence of the primer [2]. RAPD markers are used successfully for developing the knowledge of phyletic relationship among and inside species of genus Brassica and its connected genera [6]. A fast assessment of genetic variability and study of intra-specific variability among different isolates of fungus can be easily done by using RAPD markers. Similar work has also been done by Jankar *et al.*[7], and Mehra amd Tiwari [13]. Genetic variation in populations of Alternaria species has been characterized by RAPD [17].There is little information gathered till date on genetic variation among geographically distinct isolates of *A. brassicae*. Keeping this in view RAPD-PCR markers was considered in the present study.

MATERIAL AND METHODS

Collection of *A. brassicae* isolates

For the study a total number of twenty *Alternaria brassicae* isolates were collected from different geographical locations of India where oilseed brassica is grown and maintained in the laboratory on Potato dextrose Agar (PDA) media at $\pm$ 4^{o}C at ICAR-Directorate of Rapeseed-Mustard Research, Bharatpur, Rajasthan, India (Table 1).

Cultural variability of different *A. brassicae* isolates

Twenty *A. brassicae* isolates were inoculated on V8 juice agar petri plate and incubated at ±25°C temperature in incubator. The cultural characteristics was documented 7 days post inoculation. Characters like colony color, appearance, were recorded by direct observation of culture plates.

Fungal genomic DNA extraction

The genomic DNA was extracted from five to seven days old fungal cultures grown in V- 8 juice broth medium. A total 0.2g of lyophilized mycelia was grounded in a pestle mortar into a very fine powder with the help of liquid nitrogen. DNA was extracted using the protocol followed by Cenis[4]. The concentration of DNA in RNAse treated samples was determined by measuring the absorbance at 260 nm using eppendorf basic spectrophotometer. For checking the quality of DNA and suitability for downstream application in RAPD analysis the samples was subjected on 0.8% agarose gel electrophoresis.

RAPD analysis

Reaction mixture (12 µl) contained 8.25 µl PCR grade water, 100ng of genomic DNA, 1.25 µl of 10X reaction buffers, 0.5µl of 10mM dNTPs mix, 0.5µl (10 pmol/µl) of random decamer oligonucleotide primer, and 0.5µl (5 U/µl) of Taq DNA polymerase. Ten-mer oligonucleotide primers (Integrated DNA technologies) were used in each polymerase chain reaction (PCR). Amplifications were performed in a veriti 96 well thermal cycler (Applied biosystems).Cycling conditions were as follows: 15 cycles , each consisting of a initial denaturation step of 5 min at 94°C,denaturation step of 1 min at 94°C,followed by an annealing step of 1 min at 35°C and an extension step of 2 min at 72°C.The next 30 cycles ,each consisting of denaturation step of 1 min at 94°C,followed by an annealing step of 1 min at 42°C, an extention step of 2 min at 72°C,and an final extention for 10 min at 72°C. The amplified products were electrophoresed in 1.5% (w/v) agarose and visualized by staining with ethidium bromide (0.5 µg/ml) at 90 V for 3.0

to 3.5 h and photographed using gel documentation system (AlphaImager TM 3400). All polymorphic bands were scored for the presence versus absence of amplification products and further the RAPD reaction results were analyzed using software Darwin. In order to analyze the relatedness among the species, a dendrogram based on unweighted pair group method with arithmetic average (UPGMA) and Nei and Li genetic distance matrix [14] value was obtained.

RESULTS AND DISCUSSIONS

Cultural variability of *A. brassicae* isolates

Isolates of *A. brassicae* showed variable cultural variability like colony color varied from white to light green, grayish black, and white to brownish grey. The growth appeared as cottony, flurry and feathery. Colony growth varied from slow to medium on the PDA medium while medium to fast on the V8 juice agar medium. Based on these characteristics, all *A. brassicae* isolates were grouped into four colony types. The colony was circular, profuse growth with concentric rings, cottony and olivaceous black with all types of margins (Table 3). Similar results of variability among different *A. brassicae* isolates were also reported earlier [15, 9].

Molecular variability of different isolates

Of the sixteen primers tested on twenty *A. brassicae* isolates template DNA 9 primers produced scoreable bands. On an average 25 bands per primer were generated resulting in a total of 211 scoreable polymorphic bands. The UPGMA (un-weighted pair group method with arithmetic mean) dendrogram was constructed using Jaccard's similarity coefficient of RAPD marker data of 9 polymorphic primers generated for twenty *A. brassicae* isolates employing the program Darwin 6. The dendrogram was prepared by using the similarity coefficients (Figure1)[5] to show phenetic representation of genetic relationship between the different isolates of *A.brasssicae* .The number of polymorphic bands produced was maximum (42 and 39) with the primers LC 99 and LC 94, while minimum (5) with primer LC 68 and LC 86. The dendrogram categorized twenty *A. brassicae* isolates into three major groups i.e. A , B and C. Group A, Group B and Group C were differentiated from each other on the basis of similarity coefficient. The dendrogram analysis based on RAPD basis revealed 4 groups. Group A was composed of three isolates namely, BAB-02, BAB-05, and BAB-19 showed 38-40% similarity and 58-60% dissimilarity. Group B was composed of six isolates namely BAB-03, BAB-09, BAB-42, BAB-43, BAB-44 and BAB-47 showed 20-27% similarity and 73-80% dissimilarity. Group C was composed of six isolates namely BAB-12, BAB-13, BAB-45, BAB-49, BAB-50 and BAB-52 showed 10-17% similarity and 83-90% dissimilarity. Group D comprised of five isolates namely BAB-18, BAB-53, BAB-54, BAB-55 and BAB-56 showed 5-10% similarity and 90-95% dissimilarity. Cluster analysis of pathogenic variability data revealed a close relationship between BAB-02 and BAB-05 representing from Jammu, (J & K) and Mau, (U.P) respectively.

DISCUSSION

By direct observation of cultures a remarkable variability was noticed on the basis of growth and appearance .The twenty *Alternaria brassicae* isolates were divided into four groups,the isolates in the same group shared similar growth and appearance pattern.Colony colour was variable like white,green,blackish with cottony, feathery and powdery appearance. Analysis of results revealed that RAPD primers could be used efficiently as it involves low cost and is less time consuming. The observation obtained in the present study could be utilized for further characterization of genetic diversity of *Alternaria brassicae*. The study revealed about the less association between the geographical distance and genetic variability as the isolates obtained from diversified regions showed similarity with each other. Similar results have also been reported by Goyal *et al*.[11,12] and thus strengthens our investigations. The dendogram analysis disclosed close relationship between three fungal isolates BAB 02, BAB 05, BAB, 19 that are collected from varied regions of

India. Therefore this helped in concluding that accessions belonging to different locations were genetically similar. Similar conclusions have been drawn by Sharma *et al*.[17].

Table 1: Alternaria brassicae isolates used under study, their accession numbers, source and place of isolation

Isolates No.	Accession Catalogue No.	Host	Plant part	Location	DNA yield µg/200mg of fungal mass
BAB-02	NAIMCC-F-02600	*B. napus*	Leaf	Jammu, J & K	189.4
BAB-03	NAIMCC-F-02601	*B. juncea*	Leaf	Mohanpur, WB	76.7
BAB-05	NAIMCC-F-02603	*B. juncea*	Leaf	Mau, UP	53.4
BAB-09	-	*B. juncea*	Leaf	Pantnagar, Uttarakhand	931.7
BAB-12	NAIMCC-F-02605	*B. juncea*	Leaf	Jagadhari, Haryana	370.7
BAB-13	-	*B. juncea*	Leaf	Wazirpur, Haryana	294.1
BAB-18	NAIMCC-F-02606	*B. juncea*	Leaf	Pantnagar, Uttarakhand	131.9
BAB-19	-	*B. juncea*	Seed	Bharatpur, Rajasthan	423.9
BAB-42	NAIMCC-F-02613	*B. juncea*	Leaf	Jhansi, UP	111.2
BAB-43	NAIMCC-F-02614	*B. juncea*	Leaf	Hazaribag, Jharkhand	479.8
BAB-44	NAIMCC-F-02615	*B. juncea*	Leaf	Bijnor, UP	96.6
BAB-45	NAIMCC-F-02616	*B. juncea*	Leaf	Jodhpur, Rajasthan	552.4
BAB-47	NAIMCC-F-02617	*B. juncea*	Leaf	Tonk, Rajasthan	833.7
BAB-49	NAIMCC-F-02619	*B. juncea*	Leaf	Jaipur, Rajasthan	118.7
BAB-50	NAIMCC-F-02620	*B. juncea*	Leaf	Gwalior, MP	69.5
BAB-52	-	*B. juncea*	Leaf	Pantnagar, Uttarakhand	323.8
BAB-53	-	*B. juncea sp. rugosa*	Leaf	Pantnagar, Uttarakhand	93.5
BAB-54	-	*B. juncea*	Leaf	Ambabai, Datia, MP	760.1
BAB-55	-	*B. juncea*	Leaf	Simardha, Jhansi	380.1
BAB-56	-	*B. juncea*	Leaf	Chirula, Datia, MP	95.7

Table 2: List of RAPD primers sequences used for the study

S.No	Name of Primer	Base Sequence (5'-3')
1	LC68	AATCGGGCTG
2	LC 78	GTGATCGCAG
3	LC80	CAGCACCCAC
4	LC86	GTTGCGATCC
5	LC90	GTGAGGCGTG
6	LC 94	GTCGCCGTCA
7	LC 96	TTGGCACGGG
8	LC 97	GTGTGCCCCA
9	LC99	AGCGCCATTG

Table 3: Cultural variability of *A. brassicae* isolates

Group	Culture ID	Cultural variability
1	BAB-03, BAB-12, BAB-13, BAB-18, BAB-47, BAB-54, BAB-53, BAB-49	white colonies with a cottony and flurry to feathery
2	BAB-50, BAB-55, BAB-56, BAB-05	white to blackish gray colonies
3	BAB-52, BAB-09, BAB-19, BAB-42, BAB-44, BAB-45	green colonies with powdery appearance
4	BAB-43	black colonies with powdery appearance

Table 4: Similarity coefficient for A. brassicae isolates using RAPD primers

	2	3	5	9	12	13	18	19	42	43	44	45	47	49	50	52	53	54	55	56
2	1																			
3	0	1																		
5	0.444	0	1																	
9	0	1	0	1																
12	0	1	0	1	1															
13	0	1	0	1	1	1														
18	0	1	0	1	1	1	1													
19	0.667	0	0.667	0	0	0	0	1												
42	0.333	0	0.75	0	0	0	0	0.5	1											
43	0.333	0	0.4	0	0	0	0	0.5	0.2	1										
44	0.556	0	0.8	0	0	0	0	0.833	0.6	0.6	1									
45	0.444	0	0.333	0	0	0	0	0.429	0.167	0.4	0.5	1								
47	0.556	0	0.5	0	0	0	0	0.833	0.333	0.6	0.667	0.286	1							
49	0.333	0	0.167	0	0	0	0	0.5	0.2	0.2	0.333	0.4	0.333	1						
50	0.667	0	0.25	0	0	0	0	0.5	0.125	0.286	0.375	0.429	0.375	0.5	1					
52	0.556	0	0.286	0	0	0	0	0.571	0.333	0.333	0.429	0.5	0.429	0.6	0.375	1				
53	0.444	0	0.6	0	0	0	0	0.667	0.4	0.4	0.5	0.143	0.8	0.167	0.25	0.286	1			
54	0.667	0	0.667	0	0	0	0	1	0.5	0.5	0.833	0.429	0.833	0.5	0.5	0.571	0.667	1		
55	0.556	0	0.5	0	0	0	0	0.571	0.333	0.6	0.667	0.5	0.429	0.333	0.571	0.429	0.286	0.571	1	
56	0.111	0	0	0	0	0	0	0.167	0	0.333	0.2	0.25	0.2	0.333	0.167	0.2	0	0.167	0.2	1
Total	**7.667**	**5**	**6.92**	**4**	**3**	**2**	**1**	**8.071**	**4.191**	**5.252**	**5.504**	**3.937**	**4.399**	**3.433**	**2.863**	**2.486**	**1.953**	**1.738**	**1.2**	**1**
simlar	**0.383**	**0.25**	**0.346**	**0.2**	**0.15**	**0.1**	**0.05**	**0.404**	**0.21**	**0.263**	**0.275**	**0.197**	**0.22**	**0.172**	**0.143**	**0.124**	**0.098**	**0.087**	**0.06**	**0.05**

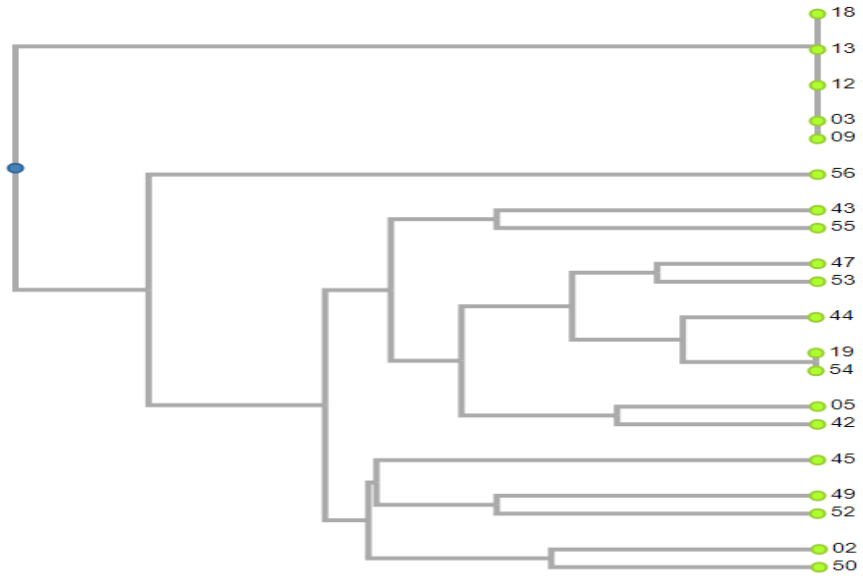

Fig. 1: Dendrogram showing relationships of 20 isolates of Alternaria brassicae estimated from band-sharing of 211 RAPD-PCR bands. The dendrogram was constructed using the Darwin 6 package

CONCLUSION

The presence of genetic diversity among the different *A. brassicae* isolates was detected using RAPD analysis as well as the cultural variability was noticed by direct observation .A significant variation in cultural and molecular variability was observed among *A. brassicae* isolates irrespective to geographical locations and *Brassica* spp.

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