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Identification of Molecular and Biochemical Variations in *Alternaria* spp

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Abstract
A total of eight species of Alternaria denoted by nine isolates were collected from diverse crops and variation among them was examined by protein profiling and RAPD-PCR techniques. Most of the isolates amplified similar bands of DNA which were used as measures of the degree of resemblance among isolates. The phylogenetically linked out group genus Stemphylium could not be clearly separated using RAPD analysis. No correlation was found between RAPD cluster analysis and pathogenic abilities of the isolates tested.

Keywords: Alternaria, RAPD-PCR, Primer, Protein, SDS-PAGE.

Introduction
Alternaria is a ubiquitous saprobic, endophytic and pathogenic fungal genus. The species of *Alternaria* are found to be associated with diverse substrates comprising plants, seeds, agricultural products, human being and animals. Alternaria was firstly defined by Nees (1816) based on *Alternaria tenuis* as the only species. The characteristics of the genus include production of dark-coloured phaeodictyospore in the chains, and a beak of tapered apical cell. The prime temperature range for their existence and growth is 25 to 30 °C. *Alternaria* species usually have ability to sporulate in most of the cultures. Potato dextrose agar and oat meal agar media are found to be the most suitable media. Incubation under alternate 12 h light/dark circle is found to be most suitable for sporulation.
Identification of variability amongst *Alternaria* species on the basis of molecular approaches and biochemical studies are important. Such studies may explain a true consequence of different *Alternaria* species and provide a base to know variability of the Alternaria either inter or intra species. On the basis of above background the present study was undertaken with the objective to analyze variation among Alternaria collected from different hosts at molecular level.

Materials and methods
Isolation and purification of *Alternaria* spp.
Isolations of *Alternaria* species were made from cumin, sesame, tomato brinjal, onion, cauliflower, chilli, datura and potato, exhibiting blight symptoms (Dhingra and Sinclair, 1985) [2]. Small bits of 2-3 mm length were washed thoroughly in sterilized water to remove the inert matter. Surface disinfested with 0.1% Mercuric Chloride (1000 ppm) for 20-30 seconds and subsequently rinsed with 2-3 changes of sterilized water to ensure freeness from traces of mercuric chloride. Pre-sterilized blotting paper sheet in laminar flow was used to remove excess moisture. Surface disinfested pieces were placed on potato dextrose agar (PDA) in Petri dishes for isolation. Later; Petri dishes were incubated at 28±2 °C in BOD incubator. The cultures were purified by single spore method (Toussoun and Nelson, 1976) [3] and maintained for investigations. The isolates were transferred at one month intervals. The cultures slants of purified isolates were and stored in refrigerator (4 °C). Seven days old freshly developed cultures of *Alternaria* spp. on PDA were used for further analysis.

Biochemical analysis
Identification on biochemical variations of *Alternaria* spp was done using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).The mycelial mats were ground in pre-chilled sterilized pestle and mortar into fine powder with liquid nitrogen and transferred to

centrifuge tubes, containing extraction buffer (Tris-HCl 0.05 M pH 7.4, glycerol 5%, sodium dodecyl sulphate (SDS) 0.5% and mercaptoehanol 0.1%). The tubes were allowed to stand for one hour on ice. The samples were then centrifuged at 10,000 rpm for 45 min, at 4ºC the clear supernatant was collected. Seven volumes cold acetone was added and kept at -20°C for overnight to these supernatant. The proteins were precipitated by centrifugation at 10000 rpm for 20 min at 4°C, the pellet was washed two times with cold acetone and air dried. The pellet was dissolved in sample buffer for SDS-PAGE and samples were loaded after denaturation. The gel was stained in coomassie blue solution.

Polyacrylamide gel was prepared from the stock solutions by mixing them in following proportion. Gel was poured immediately after addition of TEMED and APS for polymerization. 50 µl of crude protein sample was mixed with 2X sample buffer in 1:1 ratio. Sample was boiled in water bath for 5min. and centrifuged at 10,000 rpm for 10 min and loaded on gel. Molecular weight of marker in the range of 14-100 kDa (Genei) was 10µl/well) used as standard.

Gel was pre-run for 15min at 80V. Samples were loaded and electrophoresed at 80 volts till the sample is in stacking gel and voltage raised up to 120 volts for separating gel. Allowed the gel to run till the dye reached 0.5cm (7 – 8 hrs.) from the lower edge of the gel. After the electrophoresis, the gel was stained in staining solution containing Coomassive brilliant blue (R 250) for overnight. The gel was destained in destaining solution (3%Nacl).

The SDS-PAGE Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All gels were repeated at least twice and only reproducible bands were considered for analysis. The similarity coefficient obtained were transformed into Olsen-Jaccard's pattern i.e. those strains having the same banding pattern were given 1 and those not having that band were designated 0 and this 0-1 pattern was utilized to obtain the Euclidian distance matrix among the strains. UPGMA dendrogram was prepared based on the similarity co-efficient value with the help of NTSYS pc version 2.02i software.

Molecular analysis

DNA isolation and purification

Isolates of *Alternaria* spp (Table 1) in different hosts potato, tomato, chilli, onion, brinjal, cauliflower, datura, cumin and sesame were cultured on potato dextrose broth (PDB) medium. The plates were incubated at 28°C for seven days. The mycelium from pure fungal colonies was used for DNA isolation.

Fresh fungal mycelium (200-300 mg) was weighed and ground to fine powder in presence of liquid nitrogen by using pre-chilled pestle and mortar. Samples were transferred to a sterilized 1.5 ml micro centrifuge tube with the help of sterilized scalpel and 800 µL of extraction buffer (0.1 M Tris-HCl, pH 8, 10 mM EDTA pH 8, 2.5 M NaCl, 3.5% CTAB, 150 µL of 20 mg/mL proteinase K) was added. The mixture was vortex at high speed on a homogenizer for 5 min. The samples were placed in a water bath at 65ºC for 30 min. The samples were then centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was collected and equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was mixed. The samples were again centrifuged at 10,000 rpm for 10 min at room temperature. Supernatant was again collected and equal volume of chloroform-isoamyl alcohol (24:1) was mixed. Samples were again centrifuged under the conditions mentioned above. Supernatant was collected and equal volume of ice-cold isopropanol was added. Samples were incubated at −20ºC for 1-2 hrs. The samples were centrifuged for 10 min at 10,000 rpm to pellet the DNA. Supernatant was decanted and DNA pellet was washed with 800 µL of 70% ethanol. DNA pellet was air-dried and dissolved in 200 µL TE buffer (10 mMTris-HCl pH 8, 1 mM EDTA). 5 µL RNaseA (20 mg/mL) was added to DNA samples, mixed and incubated at 37ºC for 1 hrs. DNA was recovered and air-dried as described above. DNA was reconstituted in TE buffer for further use for PCR amplification.

Quality of the extracted DNA was obtained by means of electrophoresis on 0.8% agarose gels, followed by staining with ethidium bromide. The purity of the DNA was estimated from the A260/A280 ratio, whereas the yield was obtained by measuring absorbance at 260 nm with a spectrophotometer. A variety of PCR-based assays were done to check the suitability of extracted DNA for downstream analysis.

PCR amplification

PCR amplification of fungal DNA was carried out using different eight RAPD markers. Primers based on reported primer sequences were got synthesized from Operon Technologies, USA. Table 4 shows the Tm, sequence details of RAPD primers. PCR amplification was performed in 20 µL reaction mixture containing: 1U *Taq* DNA polymerase (Genei) along with 10X PCR buffer (Genei), 200 µM each deoxynucleoside triphosphate (dNTPs), 20 pM primers and 50 ng genomic DNA. The reaction conditions were as follows: initial denaturation step at 94°C for 5 min, 45 amplification cycle of denaturation at 94°C for 1 min, annealing at 37°C for 1 min and primer extension at 72°C for 2 min; followed by a final extension at 72°C for 10 min. PCR amplifications were carried out using thermal cycler. PCR products were analyzed on 1.2% (w/v) agarose gels (Sigma,) by horizontal gel electrophoresis. Amplicons were visualized by UV excitation after staining with ethidium bromide (0.5 mg/L) under gel documentation system.

Band scoring and data analysis

Amplified products were used for band scoring as 1 for presence and 0 indicates absence. Binary matrix data were used for phylogenetic tree construction using UPGMA cluster analysis through NTSYS-pc.

Results

Biochemical analysis

Banding patterns of soluble proteins have shown higher variability among all the species of *Alternaria* in different crops. A total of 80 protein bands were observed with molecular weight between 3.0 – 29.0 KD *Alternaria* Nevertheless, no protocol is perfect, but phosphate buffer extraction method was found more effective to resolve low abundant proteins of species of *Alternaria* on SDS-PAGE. All the proteins are not present in equal abundance. Highest (17) bands were found in *Alternaria burnsii* in cumin, *Alternaria alternata* from chilli and *Alternaria solani* an isolates of tomato which exhibited higher variation in proteins, whereas minimum bands were found in *Alternaria brassicae* isolated from cauliflower (1 band) and *Alternaria tenuissima* of datura (2 bands) which did not exhibit more proteins as compared to other species. The results obtained with electrophoresis supported the use of these macromolecular criteria as an aid in differentiating the species of *Alternaria* encountered on various vegetable, spice and oil yielding crops. The precise and reliable identification of plant pathogenic Alternaria

species is of major concern for executing effective disease management strategies.

Molecular analysis

Isolates of *Alternaria* spp in different crops like tomato, chilli, cumin cauliflower, brinjal, potato, datura, sesame and onion, were used for molecular characterization and diversity analysis. A total of 20 RAPD primers were used for amplification of mentioned fungal template DNA samples. Out of 20 RAPD markers only 8 were found to be suitable for amplification (Table 2). Primers OPH-02 and OPH-04 amplified the highest 6 bands with all samples. Primer OPH-08 amplified only 2 bands and all were found to be monomorphic. Two primers OPH-06 and OPH-09 amplified a total of 5 bands with 2 polymorphic (40%) each. Among all primers only 3 were amplified polymorphic banding pattern (OPH-02, OPH-06 and OPH-09).

Cluster analysis

A dendrogram was generated based on Jaccard's similarity coefficient using UPGMA cluster analysis. All the fungal isolates were grouped into two clusters i.e. one major and one minor. The major cluster consisted of 8 isolates while minor cluster contained only one isolate. The major cluster was further divided into two sub clusters. Major subgroup again contained 7 isolates while minor sub cluster had single isolate. Isolates 1, 2 showed 100% similarity with each other and grouped together. Isolates 3, 4 and 8, were 9 also grouped together with 100% similarity with each other (Table 3).

Discussion

The precise identification of *Alternaria* species is very crucial for implementing effective disease management strategies. As identification of *Alternaria* species based on morphological characters has limitations, the species were organized into species groups (Simmon and Roberts, 1993) [4]. In this study, it has have shown that electrophoresis of soluble proteins can be used to separate nine species of *Alternaria* encountered. Isolates in different species of *Alternaria* had distinctive and reproducible SDS–dissociated protein patterns, whereas isolates within three species, namely *A. burnsii* (cumin), *A. alternata* (chilli) and *A. solani* (tomato), produced largely homogenous banding patterns. Earlier studies on molecular approaches using different gene sequence phylogenies failed to distinguish *Alternaria* species within the species-groups due to lack of sufficient genetic information Electrophoretic patterns of soluble proteins were shown to be more valuable as diagnostic aids in distinguishing fungal isolates whose variability in morphology is indeterminate and overlapping Pryor and Bigelow, 2003 [5]; Peever *et al.,* 2004 [6]; Andrew *et al.,* 2009 [7]. Thus, polyacrylamide gel electrophoresis of soluble mycelial protein patterns obtained in this study can be used as a reproducible and sensitive fingerprint for nine species of *Alternaria* encountered on vegetable, fruit and oil seed crops for rapid identification.

Isolates of *Alternaria* spp in different crops Potato, tomato, chilli, onion, Brinjal, cauliflower, Datura, Cumin and sesame were used for molecular characterization and diversity analysis. RAPD-PCR has been used in fungi to study the genetic variation at level of genus, species and/or subspecies. In present study a total of 20 RAPD primers were used for amplification of mentioned fungal template DNA samples. Out of 20 RAPD markers only 8 were found to be suitable for amplification. Primers OPH-02 and OPH-04 amplified the highest 6 bands with all samples. Primer OPH-08 amplified only 2 bands and all were found to be monomorphic. Two primers OPH-06 and OPH-09 amplified a total of 5 bands with 2 polymorphic (40%) each (Table 2). Among all primers only 3 were amplified polymorphic banding pattern (OPH-02, OPH-06 and OPH-09). RAPD markers have been used to study intraspecific and interspecific variation in the *Alternaria* genus Weir *et al.,* 1998 [8]; Roberts *et al.,* 2000 [9]. Sharma and Tiwari (1998) [10] also found RAPD analysis more easy, efficient, fast and reproducible in the detection of intra-specific variation in three *Alternaria* species *i.e. A. brassicae, A. brassicicola* and *A. raphani* pathogenic to crucifers. A dendrogram generated based on Jaccard's similarity coefficient using UPGMA cluster analysis. All fungal isolates were grouped into two clusters i.e. one major and one minor. The major cluster consisted of eight isolates while minor cluster was contained only one isolate. The major cluster further divided into two sub clusters. Major subgroup again contained seven isolates while minor sub cluster having single isolate. Isolates 1, 2 showed 100% similarity with each other and grouped together. Isolates 3, 4 and 8,9 also grouped together with 100% similarity with each other. *Alternaria* isolates from Cruciferous hosts were divided into 3 groups and banding pattern was not related to host or geographical distribution.Morris *et al.* (2000) [11] analyzed 60 isolates of *A. alternata* from tomato using 29 RAPD primers, also showed genetic diversity among the isolates studied.

Table 1: Details of RAPD markers used for diversity analysis among *Alternaria* spp.

S.	Isolate	Host Plant
1.	*A. brunsii*	Cumin
2.	*A. sesami*	Sesame
3.	*A. solani*	Tomato
4.	*A. melongenae*	Brinjal
5.	*A. porri*	Onion
6.	*A. brassicae*	Cauliflower
7.	*A. alternata*	Chilli
8.	*A. tenuissima*	Datura
9.	*A. tenuissima*	Potato

Table 2: Details of RAPD markers used for diversity analysis among *Alternaria* spp.

Marker	Sequences 5'-3'	GC%	Ta	TB	MB	PB	PP
OPH-02	TCGGACGTGA	60	37	6	5	1	16.7
OPH-03	AGACGTCCAC	60	37	4	4	0	0
OPH-04	GGAAGTCGCC	70	37	6	6	0	0
OPH-05	AGTCGTCCCC	70	37	3	3	0	0
OPH-06	ACGCATCGCA	60	37	5	3	2	40.0
OPH-07	CTGCATCGTG	60	37	5	5	0	0
OPH-08	GAAACACCCC	60	36	2	2	0	0
OPH-09	TGTAGCTGGG	60	37	5	3	2	40.0

Ta-Annealing temperature, TB-Total bands, MB-Monomorphic bands,
PB- Polymorphic bands, PP-Percentage polymorphism

Table 3: Jaccard's similarity coefficients among *Alternaria* spp.

Isolates	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8	I-9
I-1	1.000								
I-2	1.000	1.000							
I-3	0.906	0.906	1.000						
I-4	0.906	0.906	1.000	1.000					
I-5	0.767	0.767	0.750	0.750	1.000				
I-6	0.867	0.867	0.844	0.844	0.821	1.000			
I-7	0.875	0.875	0.969	0.969	0.774	0.871	1.000		
I-8	0.935	0.935	0.969	0.969	0.774	0.871	0.938	1.000	
I-9	0.935	0.935	0.969	0.969	0.774	0.871	0.938	1.000	1.000

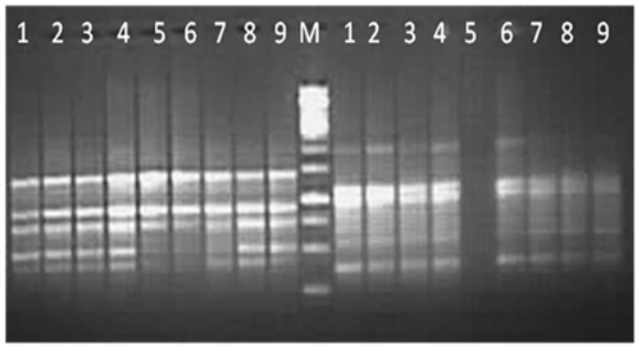

Fig 1: Electrophoretic banding pattern of RAPD markers OPH6 and OPH7. Lane 1-9 as mentioned in table 1. M-1 kb DNA ladder

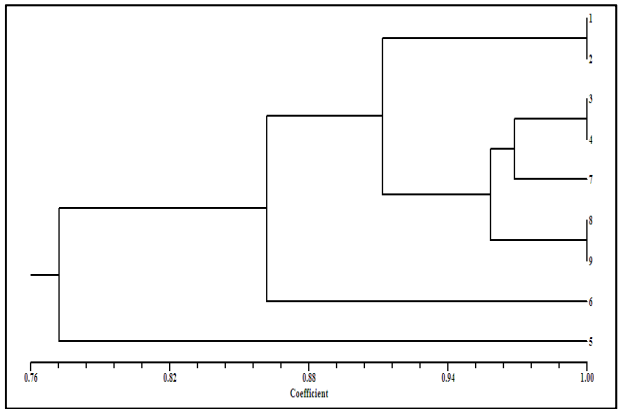

Fig 2: Dendrogram showing relationship among nine isolates of Alternaria species using RAPD marker based on UPGMA cluster analysis. 1. *A. burnsii* 2. *A. sesami* 3. *A. solani* 4. *A. melongenae* 5. *A. porri* 6. *A. brassicae* 7. *A. alternata* 8. *A. tenuissima* (Datura) and 9. *A. tenuissima* (Potato)

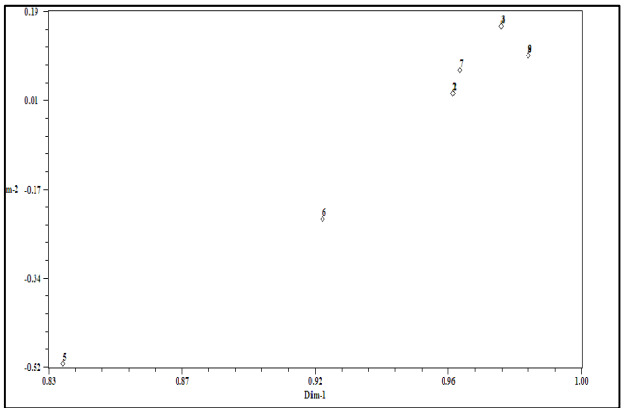

Fig 3: Two dimensional principal component analysis among nine isolates of *Alternaria* species based on UPGMA

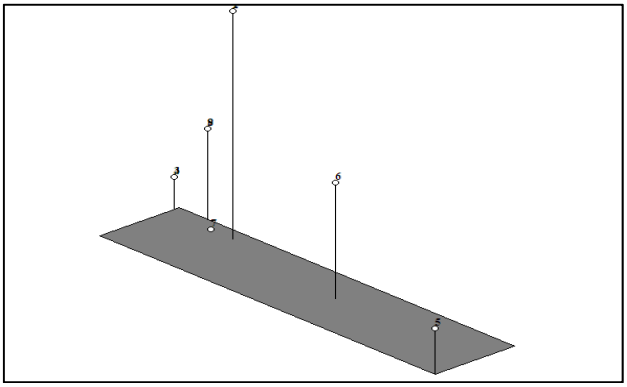

Fig 4: Three dimensional principal component analysis among nine isolates of *Alternaria* species based on UPGMA

Conclusion

Molecular approaches and biochemical studies generated good information in respect of variability. For precise and reliable identification of plant pathogens it is essential to study different *Alternaria* species according to their host range. By using this technique are can effectively consider different *Alternaria* spp. encountered on various vegetable, fruits and oil yielding crops. In present study used RAPD markers were able to differentiate *Alternaria* isolates in terms of diversity present among them. Isolates separated into two groups showed the diversity at genetic level

References

1. Nees von, Esenbeck CG. Das system der pilze und schwamme. Wurzburg, Germany, 1816.
2. Dhingra OD, Sinclair JB. Basic Plant Pathology Methods. CRC Press, Irc, 1985.
3. Toussoun TA, Nelson PE. A Pictorial Guide to the Identification of *Fusarium* species, Second Edition. Pennsylvania State University Press, University Park, 1976, 43.
4. Simmons EG, Roberts RG. *Alternaria* themes and variations (73). Mycotaxon. 1993; 48:109-140.
5. Pryor BM, Bigelow DM. Molecular characterization of *Embellisia* and *Nimbya*species and their relationship to *Alternaria, Ulocladium*, and *Stemphylium.* Mycologia 2003; 95:1141-1154.
6. Peever TL, Su G, Carpenter-Boggs L. Molecular systematics of citrus-associated *Alternaria* species Mycologia. 2004; 96(1):119-134.
7. Andrew M, Peever TL, Pryor BM. An expanded multilocus phylogeny does not resolve species within the small-spored *Alternaria* species complex. Mycologia. 2009; 101:95-109.
8. Weir TL, Huff DR, Christ BJ, Romaine CP. RAPD-PCR analysis of genetic variation among isolates of *Alternaria*

solani and *Alternaria alternata* from potato and tomato. Mycologia. 1998; 90:813-821
9. Roberts RG, Reymond ST, Anderson B. RAPD fragment pattern analysis and morphological segregation of small-spored *Alternaria* species and species group. Mycological Research. 2000; 104:151-160.
10. Sharma TR, Tiwari JP. RAPD analysis of three *Alternaria* species pathogenic to crucifers. Mycological Research. 1998; 102:807-814.
11. Morris PF, Connolly MS, Clair DA. Genetic diversity of *Alternaria alternata* isolated from tomato in California assessed using RAPDs. Mycology Research. 2000; 104:286-292.