Morphological and molecular characterization of Seed-borne *Alternaria* species

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Morphological and molecular characterization of Seed-borne *Alternaria* species

SULOCHANA RATHOD
Government College of Arts and Science, Aurangabad- 431004, Maharashtra

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Alternaria species showed their incidence on seeds along with the other seed-borne fungi. *Alternaria* isolates were obtained from various seeds of cereals, pulses and oil seeds collected from different store houses. For all isolates, morphological characteristics of the colony and sporulation apparatus were determined. For molecular characterization ten isolates of seed borne *Alternaria* species were selected. Molecular characteristics of these isolates were determined using random amplified polymorphism DNA (RAPD) analysis and polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of nuclear intergenic space. Based on morphological characteristics, the isolates were grouped as identical or very similar. The studies would be helpful to understand and classify different groups of *Alternaria* occurring on crop seeds. To know the genetic or molecular variation in different species of *Alternaria* isolated from different hosts the molecular analysis was carried out by using AFLP technique. For which 10 species of *Alternaria* were screened against 120 different markers. Finally based on polymorphic bands and dimorphic bands twelve markers were selected and genetic similarity was understood based on dendrogram.

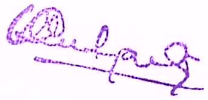
Key words. AFLP technique, Dendrogram, restriction fragments, markers

INTRODUCTION

In agriculture, seeds of many crops are known to carry various types of pathogenic and non-pathogenic fungi which are commonly known as seed mycoflora or seed-borne fungi. Depending upon the presence of fungi either on the seed coat or in the seed it is further called external seed-borne fungi and internal seed-borne fungi. Different types of seed abnormalities occur due to dominant fungi like *Aspergillus, Curvularia, Drechslera, Fusarium, Penicillium, Rhizoctonia, Verticilastum,* and *Alternaria.*

Seed is the basic and most vital input in crop production. About 90 % of world food crops are produced by using seeds. The seeds are also found to be responsible for transmission of diseases. More than 3000 diseases are known to be transmitted through seeds. This transmission takes place either in the field or in storage conditions. It is accepted that over all losses due to diseases can vary from 10 – 25 % annually throughout the world. In India even it will take conservative estimates of around 15 % losses we are losing an average 30 metric tones of food grains, 4 metric tonnes of oilseed, 36 metric tonnes of cane, 23 metric tones of fruit and vegetables. The incidence of seed-borne pathogens mainly depends on climatic / physical conditions under which the seed crops are grown in the field. Several research works have reported on seedborne fungi of several solanaceous and other crops in different countries (Al-Askar *et al.* 2013; Balogun *et al.* 2005) but little information is available on seedborne fungi of pepper seeds in different pepper growing communities in Ghana. Seed-borne fungi are either pathogenic or saprophytic. Pathogenic seed-borne fungi infect seeds in the field and reduce seed vigour, weaken the plant at its initial growth and cause disease epidemic in the field. Saprophytic seed-borne fungi affect seeds during storage and cause seed discoloration, reduce seed weight and reduce seed germination (Al-Askar *et al.* 2013) Similarly, storage conditions also enhance to develop various types of seed damages due to associated seed-borne pathogens such seeds show great loss in

*Correspondence : rathod sulochana@gmail.com



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Govt. College of Arts & Science
Aurangabad

their chemical content. The literature cited on seed pathology clearly reveal that the seed-borne fungi have found to be composed of great diversity which mainly include species of *Alternaria, Aspergillus, Curvularia, Dreshclera, Helminthosporium, Fusarium, Penicillium, Rhizopus, Rhizoctonia, Trichoderma, Vertisilatum* etc. Species of *Alternaria* cause range of diseases with great economic importance on large variety of commercially cultivated tropical crop plants which include cereals, legumes, oil seeds and large number of post harvest crops. The disease severity was more common during humid weather and symptoms were most pronounced on nutrient deficient leaves and seeds (Nagrale, 2007; Nagrale *et al.* 2012). *Alternaria* is a genus of ascomycetous fungi. *Alternaria* species are known as major plant pathogens. There are 299 species in the genus *Alternaria* (Kirk *et al.*2008). *Alternaria* species causing early blight disease to the plants are known to cause widespread damage in tropical crops. It shows diversity at morphological, physiological, pathological and molecular level. Identification of early blight pathogen is generally based on conidial morphology, but for conidial production, the pathogen needs specific media, temperature, relative humidity and light (Loganathan *et.al* .2014) Considering the diversity of conidium shapes and sizes among *Alternaria* spp. in general, there have been efforts in developing generic groupings of species based upon similar characteristics. Most species of *Alternaria* exhibit considerable plasticity in morphology depending on cultural conditions such as temperature, light and humidity. Besides the morphological characteristics, polymerase chain reaction (PCR)-based assays have also been employed for identification of *Alternaria* pathogens (Pryor and Gilbertson, 2000). A PCR assay reported by Konstantinova et al. (2002) can detect *Alternaria* at genus level. Therefore, it was decided to study in detail the morphological and molecular characters of the fungal pathogen.

MATERIALS AND METHODS

Studies of seed-borne fungi

Collection of seed samples

For the collection of seed samples, the method described by Neergaard (1973) has been adopted. Accordingly, random samples of different varieties of seeds were collected from fields, storehouses, marketplaces, and seed companies. A composite sample of each variety was prepared by mixing the individual samples, preserved in cloth bags in laboratory conditions at room temperature during the studies.

Detection of seed mycoflora

The seed mycoflora was isolated by using the standard moist blotter method (SBM) and Agar plate methods (APM) as recommended by International Seed Testing Association (ISTA 1966; Neergaard 1973; Agarwal 1976).

Identification of seed-borne fungi

The fungi occurring on every seed in the plates were identified preliminary based on sporulation characters like sexual or asexual spores with the help of a stereoscopic binocular microscope. The identification and further confirmation of seed-borne fungi were made by preparing slides of the fungal growth and observing them under the compound microscope. The identification was made with the help of manuals. Pure cultures of these fungi were prepared and maintained on potato dextrose agar (PDA) slants.

DNA Fingerprinting of Alternaria species

DNA extraction

Each *Alternaria* species were grown separately in a glucose nitrate medium contained in 250 ml Erlenmeyer flasks and grown at 25 °C for 7 days in an orbital shaker (120 rpm). Mycelium was harvested by filtration through No. 3 Whatman filter paper and immediately frozen in liquid nitrogen. The frozen mycelium was pulverized, freeze-dried and ground to a fine powder using a sterile pestle and mortar. The mycelia powder was stored at -20°C until needed. DNA was isolated from a ground fine powder of mycelium using DNeasy Plant mini kit (Quigen, USA) following the manufacture's protocol.

Amplified fragment length polymorphism (AFLP)

The amplified fragment length polymorphism (AFLP) analyses were carried out as described in Vos. *et al.* (1957) AFLP technology is DNA fingerprinting technique that combines classical hybridization-based fingerprinting and PCR-based finger-

Table 1: Growth of *Alternaria* species on different liquid media

	Different Liquid Media			
Alternaria species	RB	CZ	GN	AF
A.alternaria	0.213 +++	0.053 +++	0.250 +++	0.197 +++
A.citri	0.193 +++	0.186 +++	0.190 +++	0.115 +++
A.crassa	0.075 ++	0.052 ++	1.180 +++	0.048 ++
A.dianthicola	0.067 ++	0.121 +++	0.102 +++	1.050 +++
A.macrospora	0.173 +++	0.054 ++	0.941 +++	0.70 ++
A.tenuissima	0.123 +++	0.068 +++	0.151 +++	0.181 +++

Table 2: Growth of *Alternaria* species on different solid media

	Different Solid Media											
	RBA			CZA			GNA			AFA		
Alternaria species	Colour	Morphology	Dia (mm)	Colour	Morphology	Dia (mm)	Colour	Morphology	Dia (mm)	Colour	Morphology	Dia (mm)
A. alternata	Black	Smooth	5.4	Black	Smooth	4.5	Black	Smooth	5.4	Black	Smooth	5.4
A. citri	Black	Verruculos	5.4	Black	Verruculos	5.2	Black	Verruculos	5.6	Black	Verruculos	5.3
A. crassa	Brown	Smooth	4.9	Brown	Smooth	4.6	Brown	Smooth	5.0	Brown	Smooth	4.5
A. dianthicola	Brown	Smooth	4.6	Brown	Smooth	5.6	Brown	Smooth	5.5	Brown	Smooth	5.4
A.microspora	Brown	Smooth	5.7	Brown	Smooth	4.5	Brown	Smooth	5.8	Brown	Smooth	4.7
A.tenuissima	Grey	Smooth	5.5	Grey	Smooth	4.4	Grey	Smooth	5.5	Grey	Smooth	5.7

Table 3: Details of polymorphism revealed by different AFLP primer combinations on *Alternaria* isolated

Primer Combination		Total Band	Total Monomorphic Band	Total Polymorphic Band	% Polymorphism Per P.C	PIC Value
M-CTA	E-ACC	42	30	12	28.57%	0.8743
M-CAT	E-AAG	38	25	13	34.21%	0.854
M-CTC	E-ACA	50	27	23	46.00%	0.9276
M-CAA	E-AAC	48	31	17	35.41%	0.9034
M-CAG	E-AAG	31	21	10	32.25%	0.8544
M-CAT	E-ACA	50	26	24	48.00%	0.9468
Total		259	160	99	224.44%	5.3405
Average		43.17	26.67	16.50	38.22%	0.89

Thus the sequence of the adapter and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotide Introduction 43 extending into the restriction fragments are added to the 3'ends of the PCR primers such that only a subset of the restriction fragments is recognized. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. To generate the fingerprint the subsets of amplified fragments are then analyzed by denaturing polyacrylamide gel electrophoresis. When the DNA fingerprints of related samples are compared, common Bands, as well as different bands will be observed. These differences, referred to as DNA polymorphism, are observed in an otherwise identical fingerprint. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence including mutations abolishing or creating a restriction site, and insertions, deletions, or inversions between two restriction

printing strategies. It is based on the selective amplification of a subset of genomic restriction fragments using PCR. DNA is digested with restriction endonucleases, and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generated template DNA for amplification.

Table 4: Genetic similarity matrix of different species of *Alternaria*, based on PCR markers using Jaccord's similarity coefficient

Al.ten	*A.cat*	*A.ten*	*A ten*	*A.por*	*A.dia*	*A.cra*	*A.cit*	*A.alt*	*A.mac*
1.00									
0.725	1.00								
0.879	0.648	1.00							
0.824	0.637	0.879	1.00						
0.648	0.681	0.729	0.714	1.00					
0.571	0.626	0.626	0.593	0.659	1.00				
0.505	0.582	0.560	0.571	0.615	0.538	1.00			
0.582	0.527	0.549	0.626	0.538	0.549	0.373	1.00		
0.736	0.637	0.703	0670	0.604	0.615	0.593	0.670	1.00	
0.703	0.582	0.692	0.681	0.549	0.538	0.516	0.571	0.813	1.00

Alen = Alternaria tenuissima. Acar = A. carthami. Apor=A.porri, Adia = A dianthicola, Acra = A. crassa, Acit = A. citri, Aalt = alternata, Amac = A. macrospora

sites. The DNA polymorphisms identified using AFLP are typically inherited in Mendelian fashion.

AFLP protocol in brief

• A 250ng aliquot of genomic DNA was digested with the restriction enzymes EcoPJ and MseI at 37°C incubation for 2hrs, followed by 70°C for 15 min to inactivate the enzyme.
• The DNA fragments were ligated to adaptors at 20°C for 2 hrs.
• The pre selective amplification cycles were carried out with E-A, MC for big genome and E-0, M-C for small genome
• Selective amplification cycles using two additional selective Introduction 44 nucleotides.
• The amplification products thus obtained by using three selective nucleotides for both EcoPJ and MoseI were run on a 6% denaturing polyacrylamide gel with TBE buffer (pH- 8.3) along with a size standard 30-300 bp AFLP ruler. (Invitrogen life technology, California).
• The gels were stained with sliver stain kit (Pirece, USA)
• Gels were scanned with the Densitometer scanner GS-800 densitometer scanner (Bio-Rad, USA).
• The gels were scored manually for the presence and absence of the band. The AFLP bands were named with the three selective nucleotides of EcoRI and the three selective nucleotides of MseI. 4).

Data recording and estimation of Genetic similarity –DNA fingerprints generated by the PCR assay of different isolates were compared and grouped according to their banding pattern. The banding pattern of each isolate was coded in binary form. 1 representing the presence and 0 representing the absence of a particular band in a lane. Bands that were monomorphic across the entire set of genotypes were omitted from the analysis. Data of all the isolates were used to estimate the similarity based on the number of shared amplified bands. The similarity was calculated with the SIMQUAL function of software NTSYS-pc, which computes a variety of similarity coefficients.

The similarity matrix values based on Jacquard's coefficient of similarity were calculated as, a/n-d Where, a, d, and n represent several matches, unmatched and total number of bands respectively. The polymorphism information content (PIC) was calculated for each primer combination using the formula, PIC=2Pi(1-Pi), Where, pI is the frequency of the Ith AFLP band . The marker index was calculated for each primer combination as, MI = PIC x nβ, Where, PIC is the mean PIC value. n = Number of bands. β = Proportion of the polymorphic bands. Percent polymorphism = (No. of polymorphic bands / Total No. of bands) x 100. A dendrogram was generated with the SAHN (Sequential, Agglomerative, Hierarchical Nested) clustering method, a program (Rohlf 1998) of NTSYS-PC (Numerical Taxonomic System, version 2.11, Exeter Software, Setauket, NY) following the 'unweighted pair- group method using arithmetic averages' (UPGMA).

RESULTS

The growth of different species of *Alternaria* experiments was carried out with different species of *Alternaria* with five different media, both liquid and solid.

Growth on different liquid media

To compare the response of different media for growth and sporulation of *Alternaria* species four

different media like GN; RB, CZ, AF are used. The flasks were incubated for six days at room temperature and the results are summarized in Table 1.

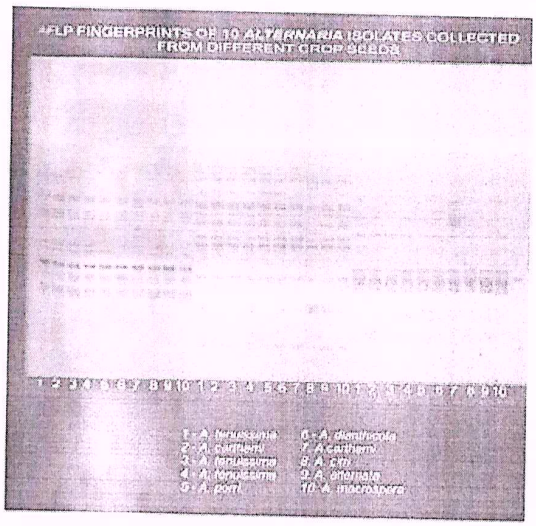

Fig. 1 : AFLP Finger printings of 10 *Alternaria* isolates

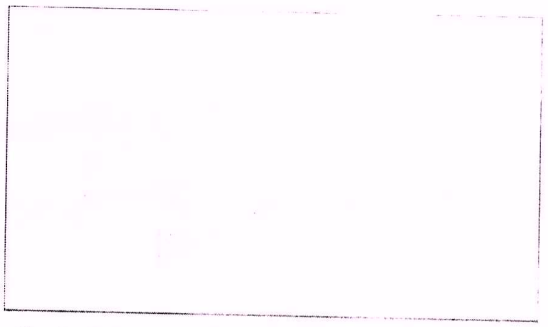

Fig. 2 : Dendrogram showing diversity of *Alternaria* species

It is observed from the results that all media favored the growth of the *Alternaria* species. However, glucose nitrate (GN) medium proved highly stimulatory for the growth and sporulation for all the species whereas alfalfa leaves media also proved stimulatory to all the species of *Alternaria* except *A. crassa*. It is interesting to note that *A. citri* and *A. alternata* grew maximum in all the media.

Growth on different solid media

In order to study growth and morphology of different species of *Alternaria* they were grown on four different solid medium. It is clear from the data summarized in Table 2 that all the species of *Alternaria* showed maximum growth on GNA medium (Glucose Nitrate Agar Medium) followed by Martin's Rose Bengal Agar (RBA) alfa alfa leaves agar (AFA) and Czapek Dox Agar medium (CZA). The morphology of the colony shows variation in its texture as well as change in the colony color is also appeared

DNA Finger printing of Alternaria species

Molecular characterization of ten *Alternaria* species through DNA fingerprinting was done by AFLP technique. It was carried out by using AFLP small genome kit (In nitrogen USA). AFLP analysis with 06 primer pair combination produced total of 259 reproducible fragment fig. 142 out of 259 bands 99 bands were observed polymorphic in nature with an average of 38.22 % polymorphism (Table 3 and Fig. 1). Six different primer combinations amplified 31 to 50 fragments with an average of 43.17 fragments per PC.

The number of polymorphic fragments for each PC varied from 10 to 24 with an average of 16.50 polymorphic fragments per PC. The percentage of polymorphism per primer combination shows that the lowest polymorphism is in PC m – CTA / E – AAC (28.57 %) whereas the highest polymorphism is in PC M – cat / E – ACA (48.00 %). Similarly, PIC values ranged from 0.834 to 0.9468. Based on 06 AFLP primers combination the data was generated and presented in the dendrogram.

Similarly, genetic similarity matrix of *Alternaria* species based on PCR marker was generated by using software NTSYS – PC with Jaccard's coefficient of similarity. The similarity matrix of these 10 genotypes is presented in table no.4 The similarity coefficient ranged from 373 to 879. Isolate *A. carthami* isolated from safflower has the lowest similarity coefficient with isolate N o08, *A. citri* isolated from citrus, followed by isolate No. 10 *A. macrospora* isolated from cotton whereas the highest similarity was observed in A. tenuissima isolated from chicko and *A. tenuissima* isolated from wheat. Clustering was done by using symmetry matrix of similarity coefficient by using UPGMA using SHAN module of NTSYS – PC. The 'r' (Cophenetic correlation) value was 0.85 which indicated good fit for phylogenetic tree. The dendrogram thus obtained is presented in Table 4. Ten isolates were grouped into 07 major cluster at 0.54 %, similarity isolate *A. tenuissima* isolated from chicko, wheat and safflower, form a major cluster followed by isolate *A. alternata* and

DISCUSSION

Ten dominant species of *Alternaria* were studied for their molecular characterization by AFLP technique. All the species showed great diversity at species level whereas *A. tenuissima* isolated from chickoo, safflower and wheat showed more genetic similarity which means that genetic diversity has not affected by the host but it may be totally due to genetic constitution of the species.

Seed-borne fungi are one of the major biotic constraints in seed production worldwide. They are responsible for both pre- and post-emergence death of seeds reducing germination, seedling health, and plant morphology (Pecchia *et al.* 2019) The seeds are incubated in the solid media and liquid media allowing the development of all *Alternaria* species without the utilization of selective media that require specific knowledge It also allows for the development of all the seed-borne *Alternaria* species present on/in the seeds and promotes the development of the target fungus biomass before DNA extraction and amplification by PCR. This step is usually very useful in cases of low infection levels (loos *et al.* 2009)

Morphological variation and the growth pattern of different *Alternaria* species studied in detailed on solid as well as liquid medium. When the fungi were grown on different nutritional medium, it changes its morphological behavior as well as mycelia growth. However colour of sporulation do not affect the content of the medium. This variation in morphology and growth pattern clearly indicates that *Alternaria* species isolates from different group of seed have its own choice of food or chemical content where it grows luxuriantly. DNA fingerprinting markers such as RAPDs using polymerase chain reactions (PCR) have been widely used for detecting genetic diversity among the microorganisms (Sharma *et al.*2013). The genetic diversity within *Alternaria* species have also been widely studied using the RAPD and DNA sequencing (Khan *et al.* 2017; Sofi *et al.* 2013).

In order to know the genetic diversity amongst different species of *Alternaria* isolated from different seeds, AFLP technique is important. Several authors have reported the use of AFLP for molecular cahracterization of different species of *Alternaria* (Bock *et al.* 2002; Dini-Andreote *et al.* 2009). It is observed that irrespective of host crop seed similar species occurring on different category of seed shows maximum genetic similarity, whereas different species occurring on the same type of seeds shows maximum genetic diversity. Such types of genetic studies amongst different species of same fungi and different strains of same species will be helpful to know the pathogenicity of fungi, which in turn, will be helpful for the management of disease caused by pathogens.

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