Pak. J. Bot., 44(4): 1267-1270, 2012.

MOLECULAR POLYMORPHISM AND PHYLOGENETIC RELATIONSHIP OF SOME *ALTERNARIA ALTERNATA* ISOLATES

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Abstract

Random amplified polymorphic DNA technique (RAPD) was used to determine the finger print of 10 isolates of *Alternaria alternata* collected from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of Punjab, Lahore. Conventional methods often do not distinguish between the isolates of the same species. Therefore DNA based method was applied to find if the isolates differ significantly from each other. The RAPDs method was choosen because of its simplicity and ability to differentiate the isolates. This work was concentrated on *Alternaria alternata* because they are common pathogens of different plants and are particularly difficult to distinguish with conventional methods.DNA was isolated from the fungal hyphae of the isolates which were formerly purified by the single spore method. Five out of ten isolates were able to produce clear DNA bands. Those isolates were Aa1, Aa2, Aa3, Aa4, and Aa5. A total of 22 bands ranging in size from 250-1200 bp were generated by the primer GL-A-01. Nineteen of these 22 bands (86.36%) were monomorphic. Only three bands with sizes of 250, 600 and 1200 bp produced by primer GL-A-01, were polymorphic. Cluster analysis of similarity between band pairs showed that the fungal isolates were clustered in two distinctive groups. RAPD was found to be an important tool to identify genetic dissimilarities between different isolates of *Alternaria alternata*.

Introduction

Alternaria alternata (Fr.) Keissler has been isolated from a wide range of foods including fresh fruits and vegetables, nuts and cereals (Andersen *et al*., 2005). *Alternaria alternata* is a common saprobe found on many plants and other substrata worldwide; including pine needles (Lu *et al*., 2000; Grunden *et al*., 2001; Tokumasu & Aoiki, 2002).This species is also an opportunistic pathogen affecting many cultivated plants in the field and during post harvest storage of fruit and vegetables. The taxon is the principal causative agent of black mould of ripe tomatoes (Pearson & Hall, 1975; Davis *et al*., 1997), brown spot on citrus (Kohmoto *et al*., 1979), brown necrotic lesions on foliage and black pit disease of potatoes (Droby *et al*., 1984), and late blight on California pistachios (Aradhya *et al*., 2001). On the other hand, strains of *A. alternata* include some of the most destructive plant pathogens that affect a wide range of host plants, causing leaf spots, blights, blossom rots and fruit rots. More than 380 hosts, including lilacs, have been recorded in the USDA Systematic Botany and Mycology Fungus-Host Distribution Database (http://nt.ars-grin.gov) (Mmbaga *et al*., 2011). *Alternaria alternata* is also one of the most important allergenic molds in the USA (*Pharmacia Diagnostics*, 1992).

Detection of *Alternaria* fungus by PCR-based assays was reported in previous studies (Johnson *et al*., 2000; Konstantinova *et al*., 2002; Zur *et al*., 2002).The genetic variation of saprobic and pathogenic *A. alternata* isolates has previously been assessed based on the analyses of RAPD, RFLPs, DNA hybridization, AFLP and DNA sequences (Tanabe *et al*., 1990; Adachi *et al*., 1993; Morris *et al*., 2000; Aradhya *et al*., 2001; Peever *et al*., 2002).

The aim of the present study was to investigate the usefulness of RAPD for assessing intraspecific genetic variation and to address the questions of whether or not there are significant genetic differences within the *Alternaria alternata* population isolated from different sources, and there is relationship between fungal genotypes. This information will be used as the basis for further study of population genetic structure.

Material and Method

Culture Procurement: Ten isolates of *Alternaria alternata* were collected from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of Punjab, Lahore. The accession numbers are 0100, 0188, 0189, 0328, 0435, 0492, 0568, 0589 and 0597. These isolates were sub cultured on 2% Malt Extract (ME) agar and preserved at 4^{o}C for future use. For DNA isolation cultures were inoculated on 2% ME broth. Flasks were incubated at 24 ± 2^{o}C for four days. Mycelium was harvested by filtration on Whatmann No.1 filter paper.

Solutions: An extraction buffer consisting of 2% CTAB (w/v), Tris HCl pH 8.0 (0.5 M); EDTA pH 8.0 (0.5 M); NaCl (5.0 M) and β-mercaptoethanol 2.5 % (V/v) was prepared. Phenol: Chloroform: Isoamylalcohol (25:24:1v/v/v), Ethanol (70%, 100%) and TE buffer (Tris HCl, 10 mM, EDTA, 1 mM, pH 8.0) are the additional solutions required.

DNA isolation protocol

- Freshly harvested fungal sample (1 g) was ground in liquid nitrogen using a mortar and pestle. The grinded material was quickly transferred to 3 ml of freshly prepared prewarmed (65°C) extraction buffer and shaken vigorously by in- version to form slurry. The tubes were incubated at 65°C in hot air oven or water bath for 60-90 min with intermittent shaking and swirling for every 30 min.

- An equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed properly by inversion for 30 min and centrifuged at 13,000 rpm for 15 min at RT (room emperature) to separate the phases.

- The supernatant was carefully decanted and transferred to a new tube and was precipitated with equal volumes of cold lsopropanol, and gently mixed to produce fibrous DNA and incubated at –20°C for a minimum of 30 min.
- The samples were centrifuged at 13000 rpm for 15 min. The pellet was washed with 70% ethanol, air dried and resuspended in 3 ml of TE buffer and 5 µl of RNase was added and incubated O/N at 37°C (An overnight RNAse treatment helped achieving in proper genomic DNA).
- The dissolved DNA was extracted with equal volumes of phenol: chloroform: lsoamylalcohol (25:24:1, v/v/v) at 8000 rpm for 15 min.
- The aqueous layer was transferred to a fresh 15 ml tube and re extracted with equal volume of chloroform and lsoamylalcohol (24:1) by centrifuging at 12,000 rpm for 15 min.
- The supernatant was transferred to a fresh tube and equal volumes of absolute alcohol and 1/10 volume of sodium acetate were added and incubated at-20^{o}C for 30 min followed by centrifugation at 12,000 rpm for 15 min. The pellet was air dried and resuspended in TE buffer. All the centrifugation steps were carried out at RT to avoid precipitation with CTAB, DNA degradation and to obtain good quality DNA.

Amount and purity of DNA: The yield of DNA per gram of fungal tissue extracted was measured using a UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples on 0.8% agarose gel based on the intensities of band when compared with the Lambda DNA marker (used to determine the concentration). The nucleic acid concentration was calculated following Sambrook *et al*., (1989). Formula used for the quantity estimation of DNA is:

DNA (µg/ml) = absorbance at 260nm x dilution factor x 50

RAPD Analysis: Strains were genetically fingerprinted using a modified protocol described by William *et al*., (1990). All fingerprinting reactions were set up at final volume of 25µl containing: 50m M, KCl; 10m M, Tris-HCl (p H 8.3); 1.5 m M, MgCl2; 0.01 % (w/v), gelatin; 2 m M each dNTP (Fermentas Inc. 7520 Connelley Drive Maryland 21076, USA); 50pmole, each primer (BioBasic, USA); 1.5 U, Taq DNA polymerase (Fermentas, USA); 150ng, DNA template.

PCR profile was programmed at 94^{o}C for 4 minutes for initial denaturation followed by primer annealing at 32^{o}C for 1 minute and primer extension at 72^{o}C for 2 minutes for 40 cycles.

Analysis of amplified products: PCR products were analyzed on 1% agrose gel stained by ethidium bromide. After washing the gel, the photograph was taken with UV transilluminator. DNA bands on gel were scored as present (1) or absent (0) for all isolates studied. Data was analyzed using Minitab software and cluster analysis of variables obtained in the form of Dandogram.

Results

Alternaria alternata cultures were obtained from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of Punjab, Lahore. These isolates were identified by conventional methods and preserved as culture collection. An important question was whether the isolates of the same species are genetically identical or not. Conventional methods often do not distinguish between the isolates of the same species. Therefore DNA based method was applied to find if the isolates differ significantly from each other. The RAPDs method was chosen because of its simplicity and ability to differentiate the isolates. This work was concentrated on *Alternaria alternata* because they are common pathogens of different plants and are particularly difficult to distinguish with conventional methods.

Pure cultures were obtained after 7 days of inoculation on broth media, at room temperature. From ten *Alternaria alternata* isolates DNA was extracted by CTAB method which was found the best method for the extraction of DNA from *Alternaria alternata* isolates. The quality of extracted DNA was observed on 0.7% agrose gel (Fig. 1) in the form of visible bright bands under Ultra Violet transilluminator (Fig. 2). DNA was amplified by using random decamer GL-A-01. Three cycling conditions were tested for the amplification (Fig. 3). At Tm 32^{o}C the decamer amplified total 22 bands in fungal strains. No band was produced at Tm 29^{o}C and 30^{o}C. Five DNA samples gave positive results. The samples in which DNA quantity was found to be satisfactory were subjected to RAPD analysis. Average seven bands were obtained per sample.

Easily resolved DNA bands were considered present and scored. The bands were counted by starting from top of the lines to their bottom and correlation coefficient distance was obtained. A total of 22 bands ranging in size from 250-1200 bp were generated by the primer GL-A-01. Nineteen of these 22 bands (86.36%) were monomorphic. Only three bands with sizes of 250, 600 and 1200 bp produced by primer GL-A-01, were polymorphic. Thus, all were distinguished by the presence of DNA fragments sizes range from 300-1200bp. The gene tree produced by primer GL-A-01 by Minitab software is represented in Fig. 4.

Dendrogram obtained by primer GL-A-01 formed one cluster in which Aa1 was grouped with Aa2 and were 86.51% similar. Aa1 and Aa2 were 100% similar to Aa3. Aa3 and Aa4 was formed one subcluster and are 58% similar. Aa4 was nearer to Aa5 genetically. Aa5 show 45.55% similarity towards Aa1, Aa2, Aa3 and Aa4. Aa3 showed differences in genetic makeup as compared to other isolates of Aa.

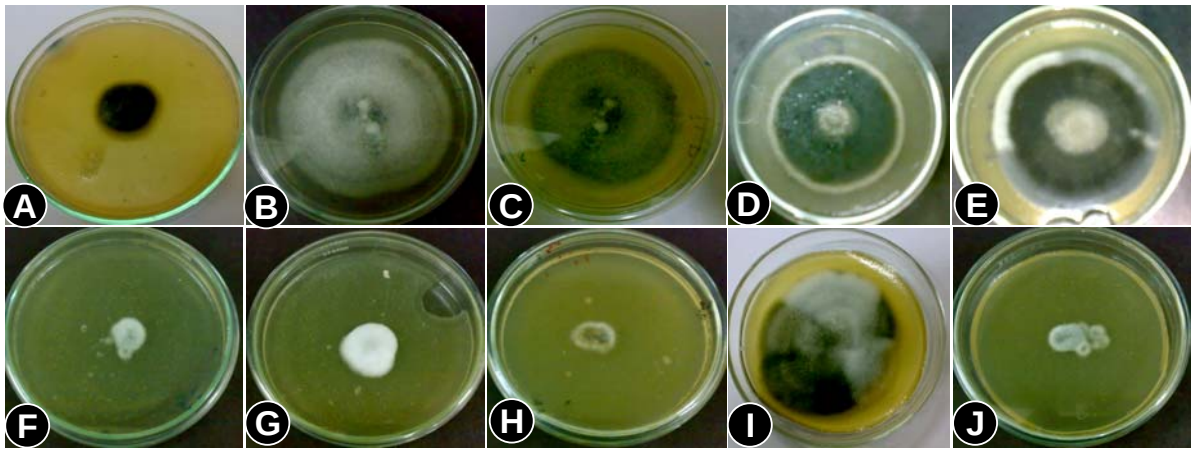

Fig. 1. Pure cultures on solid media, A: 589; B: 100; C: 189; D: 479; E: 492; F: 435; G: 568; H: 597; I: 188; J: 328.

Discussion

The genomic diversity in organism can be determined by adopting different techniques viz. RFLP, AFLP and RAPD (William *et al*., 1990). However RAPD gained more attention because of it's more economical than other techniques and can be successfully applied to find out the genomic polymorphism in different groups of organism including fungi (Welsch & McClelland, 1990). From ten *Alternaria alternata* isolates DNA was extracted by CTAB method and the quality of extracted DNA was observed on 0.7% agrose gel (Fig. 1) in the form of visible bright bands under Ultra Violet transilluminator (Fig. 2).

In RAPD analysis DNA bands of different size were amplified, from 400bp-1200bp (Fig. 2). Aa1, Aa2 and Aa3 represented the same banding pattern therefore do not show significant genomic variability (Fig. 3), however in case of Aa4 and Aa5 genomic variability was find out in terms of presence of some additional amplified DNA bands. Morris *et al*., (2000) distinguished 69 isolates of *A. alternata* isolated from tomato fruit with characteristic sunken black lesions in California using RAPD markers. Similarly, 26 genotypes were found in 65 *A. alternata* isolates sampled from citrus brown spot lesions of six countries using RAPD markers (Peever *et al*., 2002).

In case of *Alternaria* isolates Aa4 a bright band of Size 1200bp was amplified unlike other isolates while in case of *Alternaria* strain Aa5 a characteristics unique DNA band of size 600bp was amplified unlike in other isolates. So overall in isolates Aa4 and Aa5 more genomic variability was found as compared to *Alternaria* isolates Aa1, Aa2 and Aa3 (Fig. 3). Our results are in agreement with those of Welsch & McClelland (1990) who also reported the use of RAPD analysis to find out the polymorphism of genomic DNA.

This study describes the use of RAPD analysis to ascertain the genomic diversity in different *Alternaria* isolates obtained from First Fungal Culture Bank of Pakistan, Institute of Mycology and Plant Pathology, University of the Punjab, Lahore. This would further be useful in developing system for the management of *Alternaria* diseases in crops.

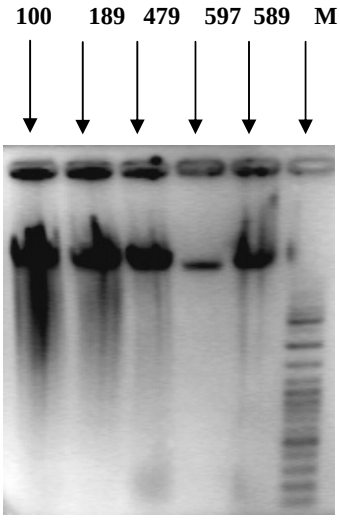

Fig. 2. Extracted DNA of *Alternaria alternata* isolates.

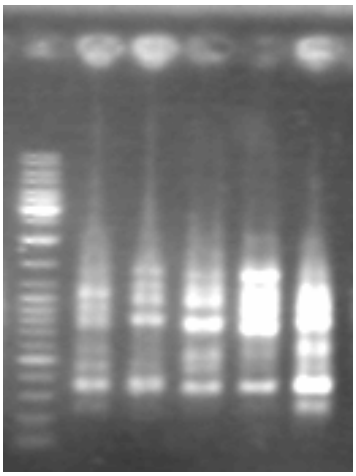

Fig. 3. RAPD reaction of five *Alternaria* isolates with primer GL-A-01, 1Kb DNA ladder was used as molecular weight size marker.

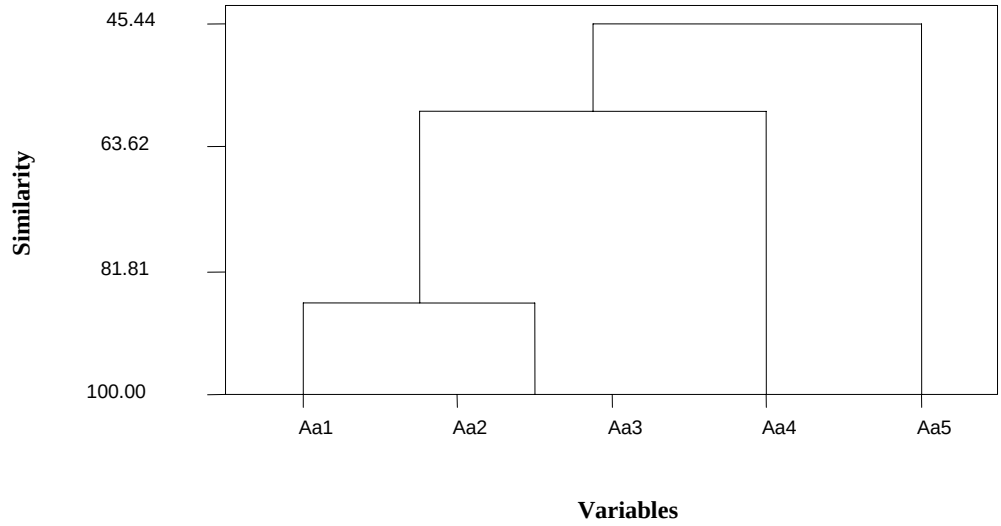

Fig. 4. Dendrogram obtained by primer GL-A-01, Aa stands for Alternaria alternata showing cluster analysis of variables.

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(Received for publication 18 May 2011)