

MORPHOLOGICAL AND GENETICAL DIVERSITY ANALYSIS OF *ALTERNARIA* ISOLATES FROM DIFFERENT HOST PLANT

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Received-01.12.2017, Revised-25.12.2017

Abstract: *Alternaria* is a plant pathogenic fungus with a wide host range and causes disease in different types of plant. In the present study, eleven *Alternaria* isolates were collected from infected samples of different host plant belonging to diverse families. Identification and study of diversity study was done based on the morphological features of the isolates i.e., colony characteristics size, septation and beak length of conidia. Genetical variability among the isolates was carried out by using three RAPD primers. All isolates were identified as belonging to the genus *Alternaria*. The diversity analysis showed that seven of the isolates were different from each. However, TD7 and TD2 resembled in their morphological as well as genetical characters, similarly TD17 and TD12 were also found similar. No significant relationship could be established between host plant infected and pathogen morphological or genetics. It was however apparent that *Alternaria* sp. may be morphologically similar but genetically dissimilar. The genus *Alternaria* was highly variable in their morphological and genetical characters. All the eleven isolates, including the two pairs of similar isolates, showed significant genetical variability.

Keywords: *Alternaria* sp., Conidia, Plant pathogen, RAPD

INTRODUCTION

Alternaria is a plant pathogenic fungus belonging to the Phylum Ascomycota. The genus has more than 300 species. It causes black spot disease in different types of plants including cereal and vegetables crops, ornamentals and fruits. *Alternaria* disease has been reported to cause huge economic loss throughout the world (Solel, 1991; Strandberg, 1992; Rotem, 1994; Shabana et al., 1995; Thomma, 2003; Akimitsu et al., 2003; Kosiak et al., 2004; Peever et al., 2004). The infection in plants can be easily identified by its characteristic symptoms of black spot with concentric rings and surrounded by a yellow colored halo. The genus is diverse in terms of its morphology, conidial structure and host range (Elliot, 1917; Wiltshire, 1947; Simmons, 1967) and its taxonomy and affinities are controversial (Simmons, 1992). Therefore, various molecular tools (e.g., rDNA sequencing, RAPD, RFLP) have been employed by different workers, to identify the organism and study its diversity (Kumar et al., 2008; Saikia et al., 2006; Morris et al., 2000; Roberts et al., 2000; Weir et al., 1998). Morphology of different species of *Alternaria* is different and it is not easy to differentiate the species from other closely related fungi (e.g., *Ulocladium* sp., *Embellisia* sp., *Chalastospora* sp., *Nimbya* sp.) (Pryor and Gilbertson, 2000; Woudenberg, 2013). Molecular tools, especially rDNA sequencing method is helpful in such a situation to differentiate from one another (Pryor & Gilbertson, 2000).

Alternaria is an opportunistic pathogen and the dark colored spores which are in chains, have vertical and

horizontal septations. Molecular analyses of plant pathogen populations are vital in comprehending epidemiology, host-pathogen relationship, and disease management methods. In this study, eleven *Alternaria* isolates were obtained from different hosts and their variability studied morphologically and their RAPD analysis.

MATERIAL AND METHOD

Isolation, characterization and identification of pathogen

Alternaria sp. infected leaf samples of plants including cereal and vegetable crops and ornamentals and fruits were collected from the Gangetic alluvial regions of Nadia District, West Bengal, India during 2009- 2013. The causal organism was isolated from the infected leaf pieces by surface sterilizing in 0.1% mercuric chloride solution followed by 3-4 washings with sterilized distilled water. The pieces were then transferred into potato carrot dextrose agar (PCA) plate and incubated at 28^oC. Pure cultures were made from single spore suspension of the pathogen and maintained at 4^oC - 5^oC, for further study. Identification of the cultures was done based on the morphological characteristics and spore size and shape.

Isolation of genomic DNA

Genomic DNA was extracted by slight modification of the CTAB protocol (Manicom *et al.*1987). 1 g fresh mycelium of each isolate was harvested, dried on sterile blotting paper and ground in liquid nitrogen to make a fine powder. DNA was extracted

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following the protocol of Raeder and Broda (1985), with slight modification. Presence of the genomic DNA was confirmed using 0.8% agarose gel electrophoresis. Quantification of the genomic DNA was done in a spectrophotometer (CECIL, CE7200, 7000 Series, England) at 260 nm and the purity of the genomic DNA measured by taking the absorbance ratio of 260:280.

RAPD analysis of isolates

Three 10-mer oligonucleotide primers (GeNei™) were tested on the different *Alternaria* isolates (Table 1). 50µL PCR reaction volumes containing 2µL (40 ng) of DNA, 5µL of 10 × buffer, 3 µL of 25 mM

MgCl₂ (GeNei™), 5µL of dNTP mixture (2.5 mM each, 5µL of primer (10 pico mole/ µl), 0.5µL of Taq polymerase (GeNei™) 3U/µl and nuclease free water (GeNei™), to complete the volume was taken. 1.6% agarose gel with 1 X TAE buffer was used, and visualized as bands by staining in 0.5µg/mL aqueous ethidium bromide with

Low Range DNA Ruler Plus MBD27 (GeNei™) as a standard marker. The RAPD bands were analyzed and phylogenetic tree draw by GenAIEx.

Table 1. RAPD Primers used

Primer	Primer Sequence (5'→ 3')	Temperature profile	Reference
1	CACAGCTGCC	95°C-7min; 95°C-15sec; 36°C-15sec; 72°C-15sec; 72°C-10min; cycle-40	Roberts <i>et al.</i> , (2000)
2	CCCGTTGCCT	95°C-7min; 95°C-15sec; 33°C-15sec; 72°C-15sec; 72°C-10min; cycle-40	Roberts <i>et al.</i> , (2000)
4	GTGATCGCAG	95°C-7min; 95°C-15sec; 38°C-15sec; 72°C-15sec; 72°C-10min; cycle-40	Cooke <i>et al.</i> , (1998)

RESULT AND DISCUSSION

The genus *Alternaria* is highly variable (Kusaba & Tsuge, 1995). Morphological and genetical diversity

of the eleven isolates studied was observed to be highly significant (Table 2).

Table 2. Host plant and isolate name of 11 *Alternaria* sp.

Name of the organism	Host plant	Host plant family	Isolate name
<i>Alternaria</i> sp.	<i>Capsicum frutescens</i> (fruit)	Solanaceae	TD2
<i>Alternaria</i> sp.	<i>Spinacia oleracea</i>	Amaranthaceae	TD7
<i>Alternaria</i> sp.	<i>Capsicum frutescens</i> (leaf)	Solanaceae	TD10
<i>Alternaria</i> sp.	<i>Raphanus sativus</i>	Brassicaceae	TD11
<i>Alternaria</i> sp.	<i>Brassica rapa</i>	Brassicaceae	TD12
<i>Alternaria</i> sp.	<i>Solanum lycopersicum</i>	Solanaceae	TD13
<i>Alternaria</i> sp.	<i>Triticum aestivum</i>	Poaceae	TD14
<i>Alternaria</i> sp.	<i>Brassica juncea</i>	Brassicaceae	TD17

<i>Alternaria</i> sp.	<i>Capsicum annuum</i>	Solanaceae	TD30
<i>Alternaria</i> sp.	<i>Brassica oleracea</i>	Brassicaceae	TD31
<i>Alternaria</i> sp.	<i>Musa paradisiaca</i>	Musaceae	TD32

The length of conidia ranged from 7.7-12.2 μm . Isolates TD10, TD14, TD7, TD2, TD13, TD32 produced large sized conidia compared to the others (Table 3). While most isolates showed three horizontal and one vertical septation, TD12 produced large conidia with four horizontal and two vertical septa. The length of the conidial beak also varied and ranged from 5.2 – 0.1 μm . On the basis of beak length, three groups could be identified. Long beaked (TD32), medium-sized beak (TD17 and TD13) and short beaked which included all the rest. All the conidia were dark colored the characteristic feature of typical *Alternaria* genus (Woudenberg et al., 2013). The conidial character is independent of host plant it infects and its morphology depends upon other factors (Misaghi et.al., 1978). The similarity and dissimilarity in length, breadth and beak sizes of

the 11 isolates did not reflect any relation with the host plant in our study also.

The colony colour of all the isolates was dark and varied from black, grey and their different shades with lighter coloured margins, which was typical of the *Alternaria* genus (Fig.2). Isolates TD2 and TD7 produced black colonies, colonies of TD10, TD12 and TD17 were greyish to lighter shades of black while TD11, TD14 and TD32 showed blackish gray colonies. Isolates TD13 and TD30 produced deep brown and grey ash colonies.

Isolates TD2 and TD7 produced colonies with irregular margins while in the rest the margins were smooth. The margin colour of the colonies also varied. It ranged from creamy white to dull white and grey. Mycelial growth of the *Alternaria* isolates was also different. It was either cottony or flat or raised (Table 4).

Table 3. Spore Characters of the *Alternaria* isolates

Isolates	Avg. Length (μm)	Avg. Breadth (μm)	Avg. Horizontal septa	Avg. Vertical septa	Avg. beak length (μm)
TD2	12.2	5.8	3	1	0.2
TD7	11.1	5.7	3	1	0.2
TD10	13.3	4.8	3	1	0.1
TD11	8.6	5.3	2	1	0.1
TD12	8.8	5.4	4	2	2.4
TD13	10.0	1.2	3	2	2.0
TD14	12.1	5.3	3	1	0.2

TD17	7.7	4.3	3	1	2.7
TD30	7.8	5.0	3	1	0.1
TD31	8.7	4.4	3	1	0.1
TD32	10.7	6.6	3	1	5.2

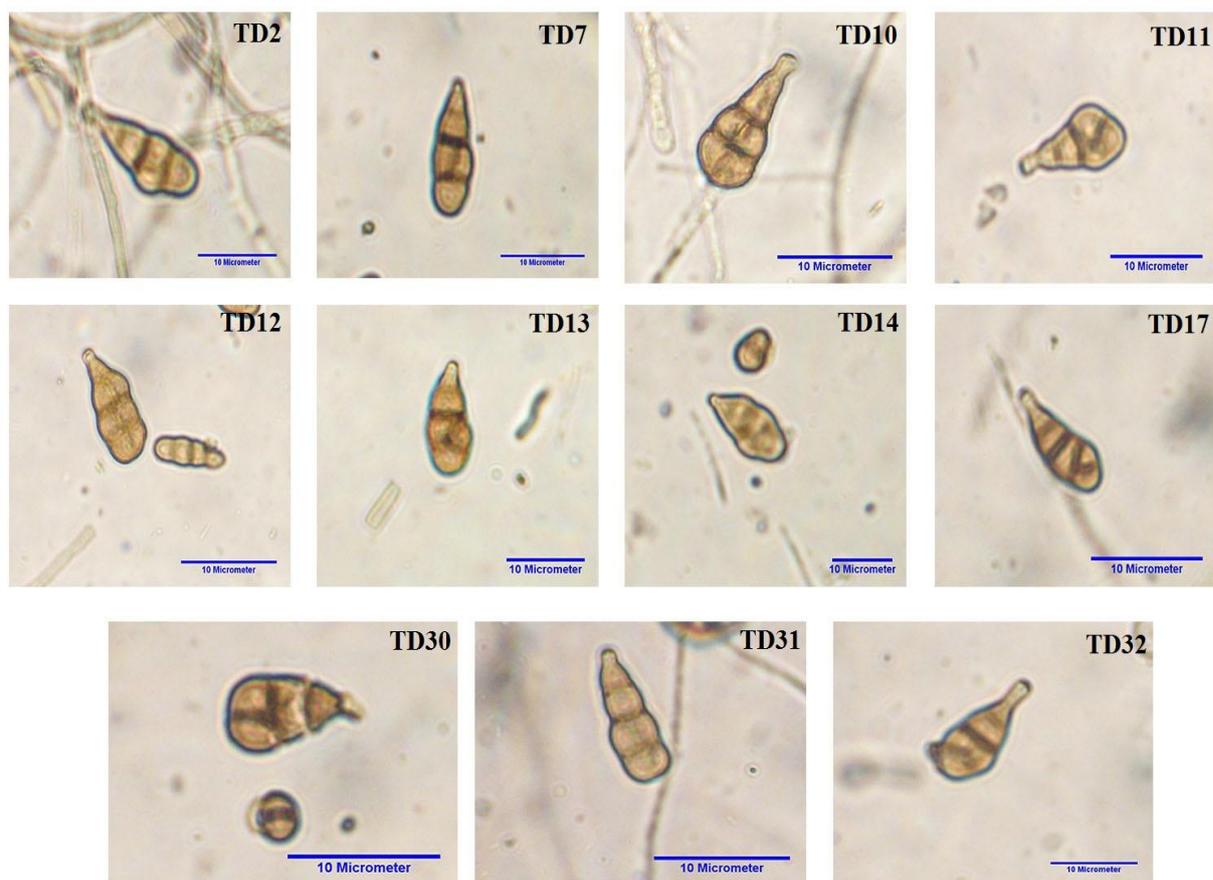


Fig.1: Spore morphology of *Alternaria* isolates

Table 4. Colony characters of the *Alternaria* isolates

Isolate	Colony colour	Margin of colony	Colour of margin	Mycelial growth
TD2	Blackish	Irregular	Creamy white	Flat
TD7	blackish	Roundish	Dull white	cottony
TD10	Grayish black	Irregular	Creamy white	Flat
TD11	Blackish gray	Irregular	White grey	Raised
TD12	Grayish black	Irregular	Creamy white	Raised
TD13	Brownish black	Irregular	Dull white	Raised
TD14	Blackish gray	Round	Creamy white	Cottony
TD17	Grayish black	Irregular	white	Cottony

TD30	Blackish gray ash	Round	White	Cottony
TD31	Blackish	Round	White	Cottony
TD32	Blackish gray	Roundish	Dull white	cottony

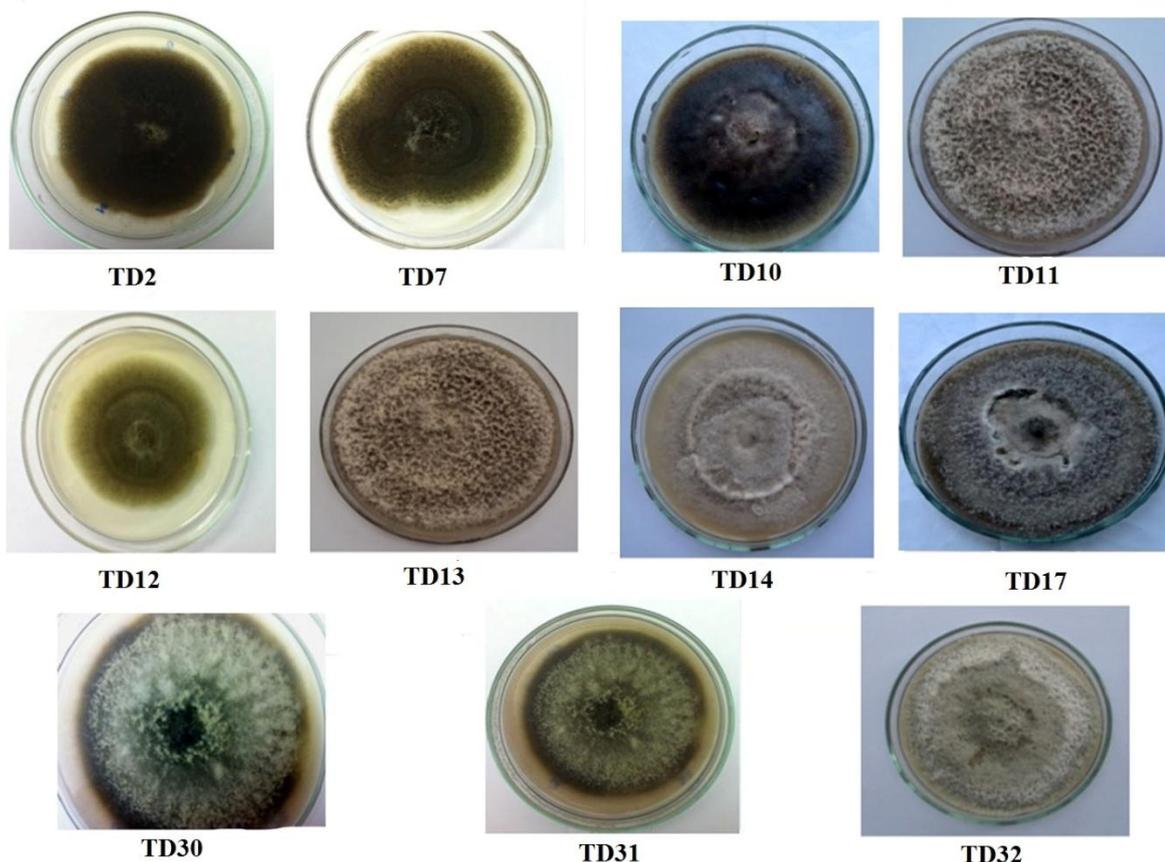


Fig. 2: Colony morphology of the *Alternaria* isolates.

The phylogenetic analysis of the eleven isolates showed notable genetic variability (Fig.3). TD10, TD30, TD14 and TD31, pathogenic on different host belonging to diverse families, belonged to a monophyletic cluster on the basis of RAPD primer1 and 3 (Fig 4A &C). The morphological features were also dissimilar i.e., TD10 and 14 formed large conidia while TD30 and 31 were small-spored isolates. These results indicated that variability study on the basis of morphological features and molecular markers differed and each parameter must be treated separately (Jadhav et al, 2011; Naik et al., 2010). 100% similarity was observed between TD7 and 2 in case of primer 1 and in TD12 and 17 in case of primer 3. TD12 and 17 were found to be infect plants from the same family Brassicaceae but TD7 and TD2 were isolated from plants of different families. TD17 and 12 were morphologically similar i.e., small sized spore with short beak, just as TD2 and 7 shared similar morphological features (long sized spore with short beak), the hosts of the latter two belonged

to different families. Thus no distinct relationship could be drawn between morphology, genetical and pathogenic variability.

Three monophyletic clusters could be recognized on the basis of primer 2 viz TD10 and 2 (Group 1), TD11, 32, 14, 13 (Group 2) and TD30, 7, 17, 31, 12 (Group 3) (Fig.4B). No relation between host specificity, morphological and genetic characters was found. The three RAPD primers presented three phylogenetic trees and all trees were dissimilar. Thus phylogenetic analysis reveals that the genus *Alternaria* is genetically highly variable. This has been reported by earlier workers also (Pryor & Michailides, 2002). From the above results we can conclude that though different *Alternaria* may be morphologically similar they may differ genetically while genetically similar isolates may be different in their morphology. However, for 100% genetical similarity the isolates must share morphologically similar features.

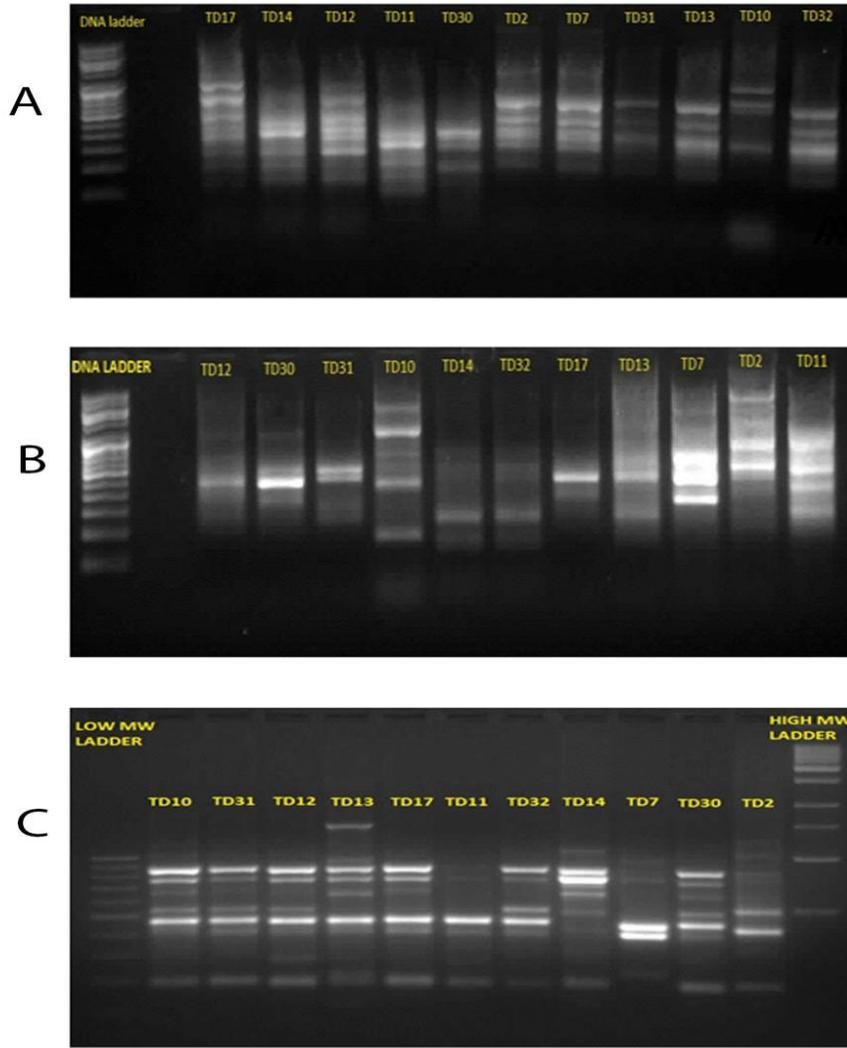


Fig.3: Gel electrophoresis of amplified products by three RAPD primers of *Alternaria* isolates. (A) Primer 1 (B) Primer 2 (C) Primer 3.

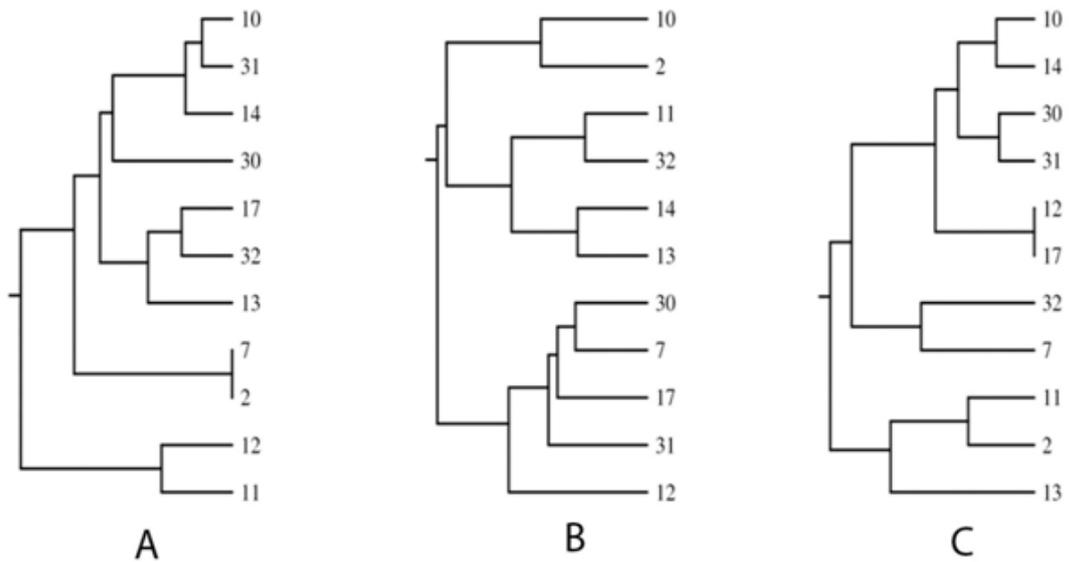


Fig.4: Phylogenetic trees constructed from the RAPD gel electrophoresis. (A) Primer 1 (B) Primer 2 (C) Primer 3.

CONCLUSION

Thus from this study we can conclude that seven *Alternaria* isolates show notable variability. Among the other four, the pairs TD7 & TD2 and TD17 & TD12 are genetically and morphologically similar. However, no significant relationship between morphological and genetical characteristics and host specificity of the seven isolates could be established. The genus *Alternaria* was morphologically and genetically highly variable.

ACKNOWLEDGMENT

The study was supported by the grants from the University Grants Commission (UGC), New Delhi. The authors also wish to thank Department of Science and Technology, Govt. of India, for its infrastructural support (FIST and PURSE) available to the Department of Botany, Kalyani University.

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