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Cultural, morphological and biochemical variations of *Alternaria* sp causing diseases on different vegetables

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Alternaria sp (Ellis and Martin) Jones and Grout is an economically important pathogen widely distributed and causing diseases on carrot, radish, bottlegourd, onion and other vegetables. The characteristic disease symptoms of this pathogen are dark brown to black concentric rings with a 'target board' or 'bull's eye' effect. Variation among isolates of *Alternaria* sp was determined based on conidial morphology and isozyme analysis. The conidia of *Alternaria* sp isolated from carrot, radish, bottlegourd and onion varied in length, breadth, beak size, septation within the host species. Cultural characteristics like colony size, colour of substrates, zonation, and growth characteristics were also different on different media. Isozyme analysis revealed considerable diversity among *Alternaria* sp isolates. Out of the two isozyme tested in native polyacrylamide gel electrophoresis (PAGE), α - and β - esterase produced well resolved electrophoretic phenotypes that could be used as markers for the isolates. Estimation of protein and phenol were also done. Bottlegourd and onion isolate were grouped together whereas carrot and radish were build up two different group in cluster analysis. The cluster analysis of morphological characters showed that Radish, Bottlegourd and Onion remained in same cluster whereas Carrot is different. So, it was concluded from the present experiment that *Alternaria* sp isolate infecting bottlegourd and onion were to some extent similar, whereas carrot and radish were different according to the isozyme, morphological and cultural characters.

Key words: *Alternaria* sp, gel electrophoresis, cultural, morphological and biochemical variability

INTRODUCTION

Alternaria sp (Ellis and Martin) Jones and Grout is an economically important pathogen widely distributed and causing diseases on carrot, radish, bottlegourd, onion and other vegetables. The characteristic disease symptoms of this pathogen are dark brown to black concentric rings with a 'target board' or 'bull's eye' effect. The variation in pathogenecity as well as genetic variability in *Al-*

ternaria sp. has been established by many workers (Tong Yunhui *et al*, 1994, Weir *et al*, 1998, Varma *et al*, 2007). Within the genus *Alternaria* species are defined primarily on conidium characteristics including size, septation, presence and size of a beak and pattern of catenation. There is a huge variation of *Alternaria* spp. in morphological plasticity that is dependent on cultural condition of substrate, temperature, light and humidity. With the advancement of molecular techniques, two isozyme tested in native polyacrylamide gel electrophoresis (PAGE), α - and β - esterase could be used as markers for the isolates and have been

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used to study variability of *Alternaria* sp isolates in different parts of the world (Morris *et al*, 2000; Van der Waals *et al*, 2004). However, little is known about the variability of *Alternaria* sp isolates in India as well as in West Bengal. Therefore, the purpose of this work is to provide information to the segregation of the species occurring in four crop plants like carrot, radish, bottle gourd, and Onion cultivated in Nadia district of West Bengal, based on morphology and biochemical diversity study by examining the morpho-diversity among *Alternaria* isolates, obtained from four crops to determine the temporal and spatial distribution of this pathogen.

MATERIALS AND METHODS

The pathogen *Alternaria* sp was isolated from infected leaves of carrot, radish, bottle gourd, and onion. Isolation was made by cutting a small section of infected portion along with healthy areas, which was surface, sterilized with 0.1% HgCl₂ solution and rinsed repeatedly in sterilized distilled water (Roy, 2013). It was then placed into the sterilized Petri plates containing solidified potato dextrose agar (PDA) medium, and incubated at 27±1°C. The pure cultures were maintained in PDA slants.

Morphological studies of the fungus

Five different media i.e., Czapek dox agar, potato carrot agar, carrot agar, potato dextrose agar and oatmeal agar were prepared and used for the cultural studies of the fungus. In 90 mm diameter Petri plates 20ml of each medium was poured. After solidification 5mm discs from 5-7 days old cultures of *Alternaria* sp. were cut out by using a sterilized cork borer and were placed aseptically at the centre of the plate. Each set of the experiment was replicated thrice and the plates were incubated at 27 ±1°C for 9 days. The colony diameter, colour of the colony, nature of colony margin and the zonation of the colony were recorded.

For morphological studies, a small amount of fungal culture obtained from nine days old culture growing on Potato Dextrose Agar medium, was placed on the slide and teased thoroughly with lactophenol to obtain uniform spread. Cover slip was placed over it and length, breadth, length of the beak and number of septations of the conidia was studied under phase contrast microscope. The photographs of the observed conidia were taken and the micrometric measurements of the conidia

were done.

Studies on isoenzyme

Alternaria isolates were grown in 250 ml conical flask containing 50 ml potato dextrose broth. Three flasks were used for each isolate. Each flask was inoculated with 2 discs each of 5mm diameter cut out from the periphery of actively growing 5 day old culture grown on PDA. The inoculated flasks were incubated at 27 ± 1°C for 10 days. Electrophoresis of esterase and isozyme was done in 7.5% gel according to the method proposed by Kahler and Allard (1970) and electrophoresis of native protein was also done in 7.5% gel according to the method proposed by Sadasivam and Manickam (1996). The Rm (Relative mobility) value of band(s) in gel was estimated.

$$\text{Rm value} = \frac{\text{Distance of the band from origin}}{\text{Distance of buffer front}}$$

Statistical analysis

Radial growth and other morphological characteristics, isozyme patterns of different isolates were statistically analyzed in a cluster analysis to find out the variations among these four *Alternaria* sp.

RESULTS AND DISCUSSION

Morphological variability

The different *Alternaria* sp isolate on different host species produces different morphological characters. The conidia of *Alternaria* sp isolated from carrot, radish, bottle gourd and onion varies in their length, breadth, beak size, septation within the host species. The morphological characters were different among the observation taken within the same host. The carrot isolate showed a varied length of conidia without beak 18.66- 36.57 µm, varied beak length 3.4-14.3 µm, varied length of conidia with beak 20.68- 47.8 µm, varied number of horizontal septation 3-4, vertical septation varied between 2-3 and oblique septation varied between 0-1. In case of radish, the length of conidia with beak varied between 59.09-45.67 µm, length of conidia without beak varied between 48.94-31.77 µm. Similarly, horizontal septation varied between 3-4, vertical septation varied between 1-2 and

Table 1 : Morphological characters and DMRT test of *Alternaria* sp.

<i>Alternaria</i> isolate on host	No. of horizontal septation	No. of vertical septation	No. of oblique septation	Length of conidia with beak (μm)	Length of conidia without beak (μm)	Beak length (μm)	Width of conidia (μm)
Carrot	(3-4) ^{ab}	(2-3) ^a	0-1	(20.68-47.8) ^a	(18.66-36.57) ^a	(3.4-14.3) ^a	(10.74-20.39) ^a
Radish	(3-4) ^a	(1-2) ^a	0-1	(59.09-45.67) ^b	(48.94-31.77) ^b	(3.67-10.4) ^b	(13.86-29.32) ^a
Bottlegourd	(2-3) ^b	(1-2) ^a	0-1	(29.17-48.87) ^{ab}	(20.36-40.20) ^{ab}	(3.83-7.45) ^{ab}	(11.34-23.67) ^a
Onion	(2-3) ^{ab}	(1-2) ^a	0-1	(43.44-56.80) ^b	(30.37-49.77) ^b	(4.48-8.75) ^a	(10.56-18.08) ^a

Table 2 : Relative mobility (Rm) values of α and β Esterase isozyme, protein and total phenol in different isolates of *Alternaria* sp.

Isolates	α -Esterase		β -Esterase		Rm value of native protein	Amount of total phenol (mg/g)
	Rm value	No. of bands	Rm value	No. of bands		
Carrot	0.29	2	0.38	2	0.276	0.1410
	0.45		0.47			
Radish	0.29	2	0.21	2	0.291	0.0699
	0.42		0.30			
Bottlegourd	0.53	4	0.63	2	0.276	0.0796
	0.59		0.60			
	0.56					
Onion	0.62	2	0.29	2	0.353	0.1158
	0.30		0.30			
	0.31					

oblique septation 0-1. In case of bottle gourd, length of conidia with beak varied between 29.17-48.87 μm , length of conidia without beak varied between 20.36- 40.20 μm , beak length varied between 3.83- 7.45 μm , horizontal septation varied between 2-3, vertical septation varied between 1-2 and oblique septation varied between 0-1. Similarly, in onion, different morphological characters showed among the 30 observations were taken. Length of conidia with beak varied between 43.44-56.80 μm , length of conidia without beak varied between 30.37-49.77 μm , beak length varied between 4.48-8.75 μm , horizontal septation varied between 2-3, vertical septation 1-2 and oblique septation 0-1 was observed. So, *Alternaria* sp. effect on different host produced different types of morphological variability. It was observed that *Alternaria* sp. affecting different host produces morphological variability (Table 1). So it was concluded that the conidial dimensions of the strains of *Alternaria* sp. are different on different host species. This conidial morphology of *Alternaria* sp. isolates

in different hosts was in accordance with those described by Ellis and Ellis (1985). However, Kaul and Saxena (1989) concluded that spore dimensions were not useful in distinguishing *A. solani* strains. The DMRT (Duncan Multiple Range Test) analysis was done among the four different *Alternaria* sp. infecting (carrot, bottle gourd, radish and onion) based on their morphological characters. It was observed that carrot and onion were similar in case of number of horizontal septation whereas bottle gourd and radish were different. In case of number of vertical septation all the four isolates were significantly same. In case of beak length, carrot and onion produced similar character and radish showed the different pattern whereas, bottle gourd was in between carrot or onion and radish. Onion and radish also produced significantly similar type of total length of conidia and carrot was different whereas, bottle gourd isolate was in between carrot and radish or onion. Length of conidia without beak for all the four isolates was similar to that of total length of conidia. Similarly, breadth of conidia

Table 3 : Growth and cultural characteristics of the isolates of *Alternaria* sp. from tested vegetables on different media

Crop	Media	Colony size (Diameter) (mm)	Colony colour	Mycelial growth	Margin of colony	Zonation
Carrot	Czapek's dox	11-27	Dark brown to black	Cottony	Round with blackish margin	Concentric zonation
	Potato Dextrose Agar	11.00-38.00	Whitish- brown with grey colouration	Cottony	Round with white margin	Concentric zonation
	Carrot Agar	11.00-32.00	Whitish- Brownish or green	Cottony	Irregular with white margin	Concentric zonation
	Potato carrot agar	9.00-27.00	Dark greenish	Cottony	Irregular with white margin	Concentric zonation
Radish	Oatmeal agar	12.00-36.00	Dark green to black	Cottony	Irregular with blackish margin	Concentric zonation
	Czapek's dox	11-26.00	Whitish-Dark greenish	Cottony	Roundish with white margin	Concentric zonation
	Potato Dextrose Agar	12.00-32.00	Whitish- grey	Cottony	Round with white margin	Concentric zonation
	Carrot Agar	9.00-31.00	Whitish- greenish	Cottony	Roundish with white margin	Concentric zonation
	Potato carrot agar	9.00-30.00	Whitish- Greenish or dark green	Cottony	Round with white margin	Concentric zonation
	Oatmeal agar	7.00-31.00	Whitish-dark greenish to black	Fluffy	Roundish with white margin	Concentric zonation
Bottle gourd	Czapek's dox	11.00-90.00	Greyish white	Thread like	Radiating white margin	No zonation
	Potato Dextrose Agar	16.00-90.00	Slight greenish white	Thread like	Roundish with white margin	No zonation
	Carrot Agar	12.00-90.00	Whitish-Greyish- green	Thread like	Roundish with white margin	No zonation
	Potato carrot agar	11.00-90.00	White-Greyish green	Thread like	Roundish with white margin	No zonation
Onion	Oatmeal agar	12.00-90.00	White-Greyish white	Thread like	Radiating white margin	No zonation
	Czapek's dox	7.00-26.00	brownish	Thread like	Irregular with white margin	Concentric zonation
	Potato Dextrose Agar	5.00-42.00	Whitish- Dark green to black	Submerged thread like	Round with white margin	Concentric zonation
	Carrot Agar	5.00-38.00	Whitish- Greenish white	Thread like	Round with white margin	Concentric zonation
	Potato carrot agar	5.00-39.00	Whitish- Dark green	Thread like	Round with white margin	Concentric zonation
	Oatmeal agar	11.00-36.00	Greenish white- brownish	Thread like	Round with white margin	Concentric zonation

was statistically similar among the four isolates. (Table 1).

Cultural variability

Alternaria sp. isolates showed variability in colony growth of the fungus on different solid media viz., Potato Dextrose Agar, Czapek's Dox Agar, Carrot Agar, Oatmeal Agar and Potato Carrot Agar. The diversity in growth was studied on different days after inoculation up to 6 days. Four different isolates produced different colony size on different media. The size of the colony was increased with increase in incubation period. Carrot isolate showed maximum colony diameter on Potato Dextrose Agar medium, carrot and radish isolates showed similar type of colony size in every days after inoculation among the five media. Maximum growth was observed on Potato Dextrose agar and later on, on Oat meal agar. Colour of the colony showed different colours on different media particularly whitish, greenish and brownish which was changed to dark green or dark brown with increase in the age of the fungal cultures in every isolate. Carrot, radish and onion produced cottony growth in every media, whereas bottlegourd produced non-cottony growth. Different media showed different margin of the colony of different isolates. *Alternaria* sp produces concentric zonation in all the media or substrate by the four isolates from 48 h after inoculation (Table 3).

Isozyme variability

In molecular variability, only isozyme variability of four isolates of *Alternaria* sp. differing in their effect on four different host i.e., carrot, radish, bottle gourd and onion and their UPGMA cluster analysis were done. For the extraction of enzyme from mycelial mats, the method given by Reddy and Stahman (1972) was used. Two different enzymes i.e., α - and β - esterase was examined and they all showed Positive activity was observed for both α - and β - esterase. β - Esterase enzyme showed the highest enzyme activity in terms of maximum numbers of banding loci among all the two isozyme tested. In case of Isolates of α -esterase carrot and radish exhibited isozyme pattern with two banding loci sharing only one locus of Rm value 0.29 in all the isolates. Among the four bands of bottle gourd, one locus showing maximum Rm value is 0.62. Carrot and radish isolate share the similar banding pattern with the minimum Rm value of

0.29, whereas onion produces two banding patterns 0.30 and 0.31 which is near the Rm value of radish. In case of β - Esterase, carrot and radish produces two bands, bottle gourd and onion one band. Carrot and radish isolate though produces two bands but their Rm value is different. Although, one moving band of Rm value 0.30 was similar to onion and radish. Highest moving bands 0.63 was observed in bottle gourd, whereas lowest band of Rm value 0.21 was observed in radish isolate. (Table 2)

Identical protein profiles were obtained for all the four isolates with respect to major bands. Highest band was observed on carrot isolate followed by onion isolate, bottle gourd isolate and lowest in radish isolate (Table1).

Identification phenol profiles were obtained for all the four isolates with respect to amount of total phenol present in the mycelia mat of the isolate. The biochemical analysis of phenol shows that the highest amount of total phenol was found in carrot isolate (0.1410 mg/g) followed by onion isolate (0.1158 mg/g) and bottlegourd (0.0796 mg/g) and minimum in radish isolate (0.0699mg/g) among the four isolates tested (Table 2).

Clustering analysis of these isozyme profiles showed three clusters among the four isolates. Radish isolate belonged to the first cluster and carrot isolate belonged to the second cluster whereas, bottle gourd isolate and onion isolate were grouped together into third cluster. In this study, four isolates of *Alternaria* sp. were analyzed using various parameters for understanding the extent of variability/diversity prevalent among them. The result gave an indication that the isolates varied significantly with regard to more than one parameters, when subjected to different growth pattern on media, sporulation behaviour and variability on host differentials for virulence and distinct polymorphism even at molecular level, indicating the existence of variability among the isolates under study.

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