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Inter and intra-specific diversity in *Alternaria* species infecting oilseed Brassicas in India

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Abstract

The work was initiated to determine diversity in pathogen population of Alternaria blight which results in up to 60% yield losses in the world. It is a most prevalent fungal disease of oilseed *Brassica* in North and North-West regions of India where approximately 80% of Brassicas are grown. Fifty five single spore isolates of *Alternaria* species (32 of *A. brassicae*, 20 of *A. brassicicola* and 3 of *A. alternata*) were isolated from infected leaf samples collected from 8 states of India. The purified isolates were evaluated for their morphological, cultural, biochemical and molecular characteristics, and also preserved in DMSO and glycerol. Significant variations ($P< 0.05$) were observed in sporulation intensity, spore morphology, radial growth and carbohydrate concentration among isolates of each species. Analysis of RAPD banding profiles also showed a high level of genetic diversity varying between 57-78%, 78-92% and 89-100%, among the *A. brassicae*, *A. brassicicola* and *A. alternata* isolates, respectively. Extensive variations were observed in isolates for all the parameters studied, but no correlation could be established. This study, thus indicates that a significant non-specific variation exist between isolates infecting different species and varieties of *Brassica* in India. This is the first report of comparative evaluation of the three *Alternaria* species from different locations, on three different media and cryopreservation.

Key words: *Alternaria blight, molecular diversity, morphological variability*

Introduction

The production of oilseed Brassicas is gravely hampered by several fungal diseases, Alternaria blight being one of the most devastating and pervasive diseases. It is reported over wide geographical areas in the world, causing an average yield loss of up to 60% (Kolte, 2002). *Alternaria brassicae*, *A. brassicicola* and *A. alternata*, have been widely reported to cause diseases in oilseed and vegetable *Brassicas*. Out of these three species, *A. alternata* has been reported as a seed borne (Tohyama and Tsuda, 1995) as well as leaf borne pathogen, which comes as an opportunistic pathogen or a secondary saprophyte on Brassicas (Sharma *et al*., 2013; Chauhan *et al*., 2009). *A. brassicae* is the most serious pathogen on oil yielding Brassicas (Verma and Saharan, 1994). All cultivated varieties of oilseed Brassicas lack durable resistance (Singh *et al*., 2006), although, efforts have been made to transfer resistance from related species (Gupta *et al*., 2010; Agnihotri *et al*., 2009).

Changes in temperature and relative humidity have been reported to modify the pathogen aggressiveness, host susceptibility and fungal sporulation intensity (Mitchell *et al*., 2003), thereby allowing the evolution of new strains by adaptation (Coakley *et al*., 1999). The intraspecific variability in *Alternaria* isolates with respect to the morphological and cultural aspects has been reported (Kumari *et al*., 2012; Khulbe *et al*., 2011; Reis and Boiteux, 2010). The *in vitro* culture conditions including temperature,

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pH, relative humidity and photoperiod have also been reported to affect such changes (Meena *et al*., 2012; Goyal *et al*., 2011). Vishwanath and Kolte, (1997) reported biochemical diversity in *A. brassicae* isolates with respect to their carbohydrate, lipid, protein, amino-acid and nucleic acid composition and revealed a positive correlation between the aggressiveness of the pathogen and its total carbohydrate content. Although, the pathogen can be differentiated based on its growth behavior, physiological characters and microscopic appearance, molecular characterization should be used to identify the genetic diversity.

There are only a few reports on molecular characterization of *Alternaria* species pathogenic to oilseed Brassicas. Chou and Wu (2002) and Berbee *et al*., (2003) studied the ITS regions of rDNA of *A. brassicae* and *A. brassicicola* to evaluate the phylogeny of the pathogen. The RAPD has been efficiently used to analyze inter and intraspecific genetic variations in *A. brassicae* (Kaur *et al*., 2007; Cooke *et al*., 1998; Sharma and Tewari, 1995), *A. brassicicola* (Avenot *et al*., 2005; Sharma and Tewari, 1998), *A. alternata* (Andreote *et al*., 2009), and also in some of the less prevalent species (Kumar *et al*. 2008; Pryor and Michailides, 2002). Studies suggest that RAPD enables the detection of single base changes that cannot be detected in plant pathogens with virulence analysis unless isogenic differential hosts are available (Williams *et al*., 1990).

Earlier studies have used only small number of isolates collected from a limited locations and from a very few *Brassica* hosts. In the present study, fifty-five isolates from three distinct *Alternaria* species were isolated from 6 different *Brassica* species; including *B. juncea, B. napus, B. carinata, B. nigra, B. rapa and B. oleracea* and 5 wide relatives i.e. *B. alba, B. chinensis, Sinapis alba, Eruca sativa* and *Crambe*, and were evaluated for their morphological, cultural, biochemical and molecular diversity.

Materials and Methods

Isolation, purification and identification

One hundred samples of Alternaria blight-infected leaves from various *Brassica* species were collected from the major North and North-Western regions in India (Table 1). The fungal cultures were isolated using leaf disc inoculation method (Verma and Saharan, 1994) on potato dextrose agar (PDA) and incubated in continuous diffused light conditions at 20 ± 2ºC for 15 ± 2 days. Pure cultures were obtained by repeated sub-culturing followed by single-spore isolation. The purified *Alternaria*

Table 1: Collection sites of *Alternaria* species: Geographical location and Climatic conditions

Location	State	Geographical Location*	Average temperature & Relative humidity (October-March)#
Pantnagar	Uttarakhand	29°.02'N - 79°.49'E	9.71°C and 66.2%
Meerut	Uttar Pradesh	28°.98'N - 77°.70'E	19.36°C and 62.2%
Alwar	Rajasthan	27°.56'N - 76°.62'E	19.36°C and 46.2%
Bharatpur	Rajasthan	27°.21'N - 77°.49'E	20.3°C and 57.6%
Kanpur	Uttar Pradesh	26°.40'N - 80°.33'E	20.7°C and 65.3%
Ludhiana	Punjab	30°.90'N - 75°.86'E	17.5°C and 73.3%
New Delhi	Delhi	28°.63'N - 77°.22'E	19.36°C and 56.4%
Kangra	Himachal Pradesh	32°.06'N - 76°.27'E	16.4°C and 69.5%
Hisar	Haryana	29°.15'N - 75°.72'E	19.25°C and 61.6%
Srinagar	Jammu & Kashmir	34°.08'N - 74°.80'E	6.78°C and 71.8%

Source - *http://www.worldatlas.com/aatlas/findlatlong.htm and #http://www.accuweather.com

Table 2: Details of identified *Alternaria* species isolates

Alternaria species	Location	Host plant	Year of collection	Isolate Code
A. brassicae	Delhi	*B. rapa*	2011	ABc-D01
A. brassicae	Delhi	*B. napus*	2011	ABc-D02
A. brassicae	Delhi	*B. juncea*	2011	ABc-D06
A. brassicae	Delhi	*B. rapa*	2011	ABc-D07
A. brassicae	Pantnagar	*B. nigra*	2010	ABc-P01
A. brassicae	Pantnagar	*B. carinata*	2010	ABc-P04
A. brassicae	Pantnagar	*B. napus*	2011	ABc-P05
A. brassicae	Pantnagar	*B. rapa*	2011	ABc-P06
A. brassicae	Pantnagar	*Sinapis alba*	2011	ABc-P07
A. brassicae	Pantnagar	*B. carinata*	2011	ABc-P08
A. brassicae	Pantnagar	*B. rapa*	2011	ABc-P09
A. brassicae	Pantnagar	*B. juncea*	2011	ABc-P10
A. brassicae	Pantnagar	*Eruca sativa*	2011	ABc-P11
A. brassicae	Pantnagar	*B. nigra*	2011	ABc-P12
A. brassicae	Ludhiana	*B. juncea*	2011	ABc-L01
A. brassicae	Ludhiana	*B. juncea*	2011	ABc-L02
A. brassicae	Ludhiana	*B. rapa*	2011	ABc-L05
A. brassicae	Ludhiana	*B. rapa*	2011	ABc-L07
A. brassicae	Ludhiana	*B. napus*	2011	ABc-L08
A. brassicae	Ludhiana	*B. rapa*	2011	ABc-L10
A. brassicae	Ludhiana	*B. juncea*	2011	ABc-L12
A. brassicae	Ludhiana	*B. rapa*	2011	ABc-L19
A. brassicae	Ludhiana	*B. rapa*	2011	ABc-L20
A. brassicae	Bharatpur	*B. juncea*	2012	ABc-B01
A. brassicae	Bharatpur	*B. juncea*	2012	ABc-B03
A. brassicae	Hisar	*B. rapa*	2012	ABc-H03
A. brassicae	Hisar	*B. rapa*	2012	ABc-H04
A. brassicae	Hisar	*B. rapa*	2012	ABc-H05
A. brassicae	Hisar	*B. rapa*	2012	ABc-H06
A. brassicae	Kangra	*B. rapa*	2012	ABc-Kg01
A. brassicae	Kangra	*B. rapa*	2012	ABc-Kg02
A. brassicae	Kanpur	*B. juncea*	2012	ABc-Kn01
A. brassicicola	Delhi	*B. nigra*	2011	ABo-D03
A. brassicicola	Delhi	*B. chinensis*	2011	ABo-D04
A. brassicicola	Delhi	*B. carinata*	2011	ABo-D05
A. brassicicola	Delhi	*Diplotaxis* sp.	2011	ABo-D08
A. brassicicola	Delhi	*B. nigra*	2011	ABo-D09
A. brassicicola	Delhi	*B. juncea*	2011	ABo-D10
A. brassicicola	Pantnagar	*B. alba*	2010	ABo-P02
A. brassicicola	Pantnagar	*B. rapa*	2010	ABo-P03
A. brassicicola	Ludhiana	*B. napus*	2011	ABo-L03
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L04
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L06
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L09
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L11

A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L13
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L15
A. brassicicola	Ludhiana	*B. rapa*	2011	ABo-L16
A. brassicicola	Ludhiana	*B. juncea*	2011	ABo-L17
A. brassicicola	Ludhiana	*B. juncea*	2011	ABo-L18
A. brassicicola	Hisar	*B. rapa*	2012	ABo-H01
A. brassicicola	Hisar	*B. rapa*	2012	ABo-H02
A. alternata	Ludhiana	*B. juncea*	2011	ABa-L14
A. alternata	Bharatpur	*B. juncea*	2012	ABa-B02
A. alternata	Kanpur	*B. juncea*	2012	ABa-Kn02

isolates were observed microscopically under 40x optical microscope and identified based on conidia shape and size, septation and beak size (Courtesy Dr. RP Awasthi, GBPUA&T, Pantnagar; Verma and Saharan, 1994; Table 2).

Growth Optimization and Maintenance of fungal cultures

The isolates were tested for optimum mycelial growth and sporulation intensity on three different culture media viz. PDA, Weak Acidified Potato Dextrose Agar (WAPDA) and V8-juice agar (VJA) under alternating 9hr diffused light and 15 hr dark photoperiod, and continuous diffused light conditions. The observation for radial growth, surface texture, colour, form, elevation, margin, sporulation intensity, spore, beak size and septation after seven days of incubation and quantitative data were statistically analyzed (Table 3). The pure cultures were maintained in PDA plates and slants at 4ºC, and also in 10% glycerol and 5% DMSO at -20ºC.

Table 3: Sporulation index of different isolates

Sporulation Intensity	Number of spores per microscopic view (40x)
Absent	Nil
Low	1-10
Moderate	10-30
High	>30

Morphological and cultural evaluation

Spore suspensions of isolates were prepared using sterile distilled water and fifty conidia from each slide were examined under digital 40x optical microscope to record the conidial characteristics, including sporulation intensity, spore and beak length, spore shape, and number of longitudinal and transverse septa. The colonies formed by the isolates on PDA were evaluated for radial growth, surface colour, texture, form, elevation, margin and presence of zonation.

Biochemical evaluation

The method of Vishwanath and Kolte (1997) with minor modifications was followed for evaluation of total carbohydrate content. Isolates were grown in 100 ml Czapek Dox broth on a rotary shaker at 70 rpm under continuous diffused light at 24 ±2ºC, for 21 days. The mycelia was harvested by filtering through three layered sterile muslin cloth, washed with sterile distilled water, wrapped in Whatman paper no. 100 and kept for drying overnight in an oven at 60ºC. The dried mycelia biomass was ground, fractionated and the supernatant was analyzed for total carbohydrates using anthrone reagent in a UV/Vis spectrophotometer (LabIndia) at 620 nm against a glucose standard and concentration of carbohydrates was calculated for individual isolates. The data was statistically analyzed using analysis of variance.

Genomic DNA isolation and PCR amplification

The mycelium of each of the 55 isolates was harvested after 10 days of growth on liquid PDA, lyophilized overnight and the freeze-dried mycelia were ground to fine powder in liquid nitrogen.

Modified CTAB method was used for DNA isolation (Weising *et al*., 1995), purified genomic DNA was stored at -20ºC, and quantification was done using Nano-Drop spectrophotometer.

Genetic variations in fifty five *Alternaria* isolates from different regions in India were studied using RAPD markers. Thirty four random DNA primers of 10 nucleotides each were selected for carrying out RAPD-PCR's. Thirty primers were procured form Eurofins mwg operon (Eurofins genomics Pvt. ltd, Bangalore, India) and four were custom synthesized by Infobio (New Delhi, India). PCR amplifications were performed in 50µl reaction volume containing 10X PCR Buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, 10 pM primer, 1.5U Taq DNA polymerase and 20 ng genomic DNA. Amplification was performed in a gradient thermal cycler (BIORAD) with lid heating set at 110 ºC and programmed as: one cycle of initial denaturation at 94°C for 5 min; 40 cycles of 94ºC for 30 s, 37ºC for 1 min, 72ºC for 2 min followed by a final extension for 10 min at 72ºC and a 10 min hold at 4 ºC. The amplification products were resolved along with 1kb DNA ladder by electrophoresis on 1.4% agarose gel in 1X TAE buffer. The RAPD banding profiles obtained with different primers were analyzed for percentage polymorphism among the isolates.

Phylogenetic analysis

A data record was constructed for all the isolates and the primers showing maximum polymorphism were selected for further analysis. The binary matrices of the selected primers were clubbed into one and used to develop similarity matrix and dendrograms using NTSys software. The data from the similarity matrix was then used to calculate the dissimilarity coefficients among all the isolates at both inter and intra-regional levels.

Results and Discussion

Morphological and cultural evaluation of *Alternaria* isolates

Among the three media tested, for *A. brassicae* and *A. brassicicola*, WAPDA yielded maximum average radial growth of 58.9 mm, followed by PDA (55.3 mm) and VJA (50.06 mm), but no significant difference was observed. *A. alternata* produced maximum growth on PDA (57.3 mm). All isolates showed maximum sporulation intensity on PDA followed by VJA and WAPDA (Fig.1).

Surface colour of the colonies of all isolates ranged

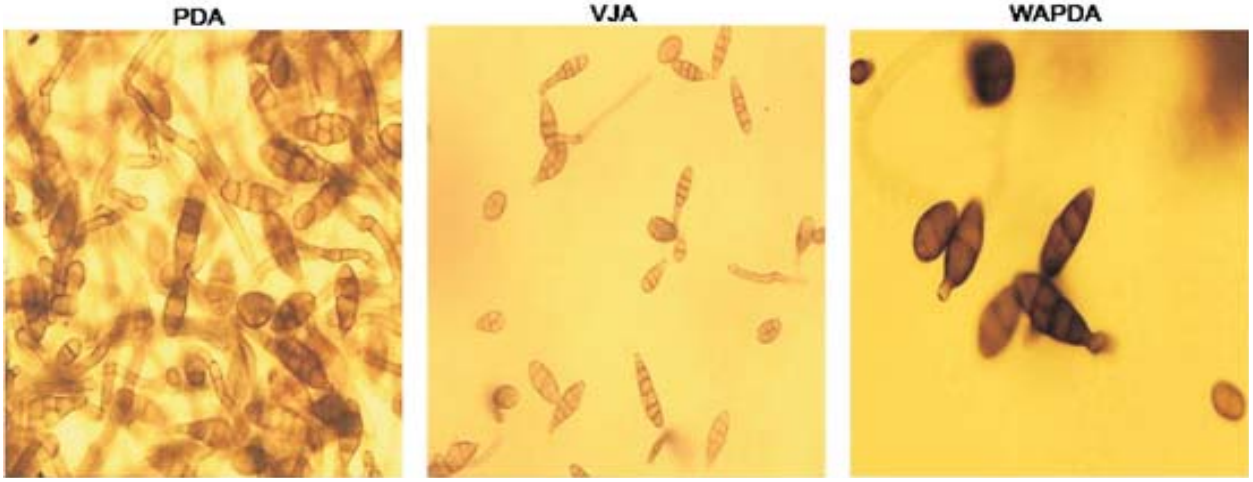

Fig. 1: Sporulation intensity of *A. brassicicola* isolate (ABo-D08) on different culture media [PDA – Potato Dextrose Agar, VJA – V_8 Juice Agar and WAPDA – Weak Acidified Potato Dextrose Agar]

from pale to dark olive green on PDA, pale grayish brown to dark brown on VJA and dark green on WAPDA. The isolates showed mixed response with respect to colony form, elevation and margin on different media. Concentric zones were observed for *A. brassicae* isolates on both PDA and VJA. Only one *A. brassicicola* isolate, ABo-L03, showed the formation of radiating furrows on PDA,

whereas none of the isolates showed any zonation pattern on WAPDA. The spore and beak lengths showed significant difference at P<0.05 (Figure 2). On all three media, septation pattern was similar for the isolates with 3-6, 2- 6, 2-4 transverse and 0-2, 0-1, 2-3 longitudinal septae in *A. brassicae*, *A. brassicicola* and *A. alternata*, respectively. The sporulation intensity was better under continuous diffused light as compared to alternating 9hr diffused light and 15 hr dark photoperiod.

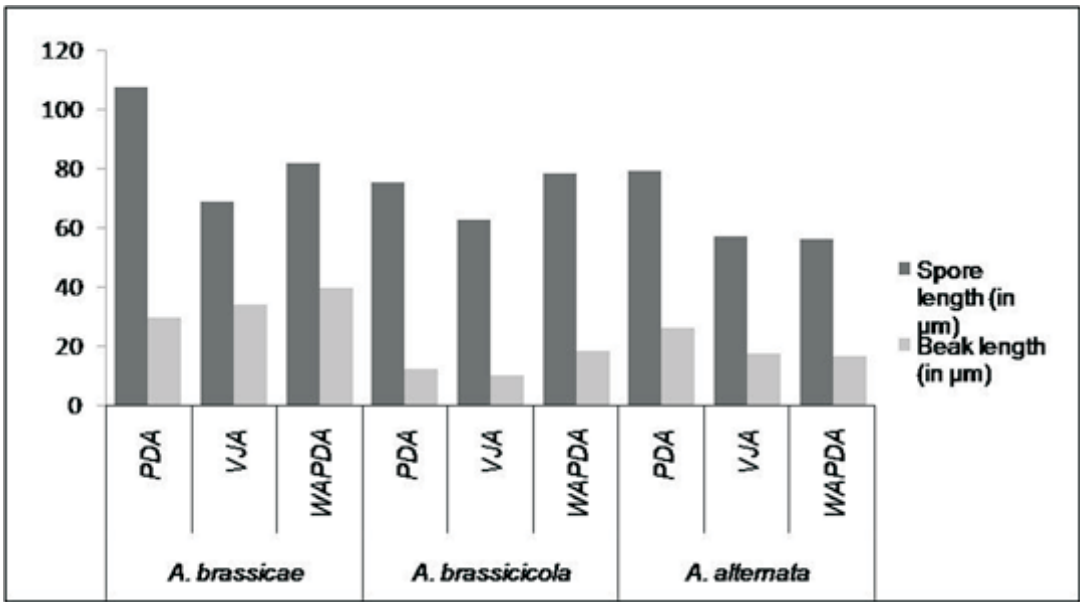

Fig. 2: Variability in spore and beak length of three *Alternaria* species (from Delhi, Ludhiana, Pantnagar, Bharatpur and Kanpur) on different culture media

Maintenance of isolates

The isolates maintained under different conditions (PDA plates and slants at 4°C; 10% glycerol and 5% DMSO at -20°C) showed similar germination and sporulation on PDA. Repeated sub-culturing on PDA plates and slants showed reduction in pathogenicity after three sub cultures. The isolates maintained in Glycerol and DMSO at -20°C showed revival on PDA medium after 6 months of storage.

Morphological diversity

Sporulation intensity varied with the production of a very few spores in some isolates to profuse sporulation in others. Among the *A. brassicae* isolates, maximum sporulation intensity was shown by Bharatpur isolates followed by, in decreasing intensity isolates from Pantnagar, Ludhiana, Kangra, Hisar, Kanpur and Delhi. In *A. brassicicola* isolates, maximum sporulation shown by isolates from Ludhiana, followed by Delhi, Pantnagar and Hisar. *Alternaria alternata* isolates from Bharatpur and Kanpur sporulated profusely whereas, low sporulation was observed in Ludhiana isolate.

The spore size varied from 36.7-257.6 ìm (mean 95.15±9.94) in *A. brassicae* isolates (Fig. 3), 35.9-

Fig. 3**:** Variability in spore morphology in some of the *A. brassicae* isolates [(a) ABc-D01; (b) ABc—D06; (c) ABc-P04; (d) ABc-P06; (e) ABc-P09; (f) ABc-P10; (g) ABc-L05; (h) ABc-B03; (i) ABc-H06; (j) ABc-Kn01; (k) ABc-Kg02; (l) ABc-L07 and (m) ABc-L02]

130.9 ìm (mean 69.11±6.67) in *A. brassicicola* isolates (Fig. 4) and 71.3-83.9 ìm (mean 76.23±7.34) in *A. alternata* isolates (Fig. 5). The beak size also showed variation within and among the species, with *A. brassicae* isolates with long beaks of 27.8-120.2 ìm (mean 50.9±2.14), followed by *A. alternata* isolates with 7.8-45.3 ìm (23.7±1.10) beak length and lastly by *A. brassicicola* having the smallest beaks of 0-42.2 ìm (8.1±0.67). The *A. brassicae* and *A. brassicicola* isolates were subsequently categorized into four groups on the basis of the variation observed in their spore lengths.

Fig. 4: Variability in spore morphology in some of the *A. brassicicola* isolates
[(a) ABo-D03; (b) ABo-D08; (c) ABo-D09; (d) ABo-D10; (e) ABo-P03; (f) ABo-L03; (g) ABo-L06; (h) ABo-L17; (i) ABo-H01 and (j) ABo-H02]

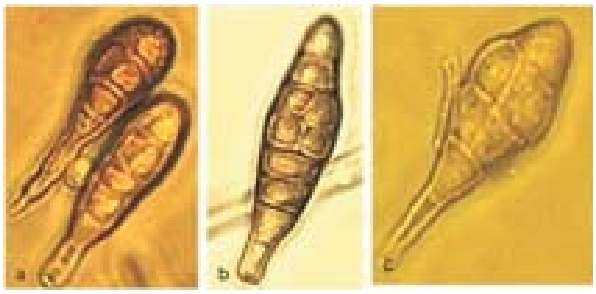

Fig. 5: Variability in spore morphology in some of the *A. alternata* isolates
[(a) ABa-L14; (b) ABa-B02 and (c) ABa-Kn02]

Cultural diversity

The radial mycelial growth varied between 30-80 mm for both *A. brassicae* and *A. brassicicola* isolates and 45-55 mm for *A. alternata* isolates. The *A. brassicae* isolates, ABc-L02 and ABc-P01, *A. brassicicola* isolates, ABo-L16 and ABo-L03, and *A. alternata* isolates, ABa-B02 and ABa-L14 showed minimum and maximum radial growth, respectively. The colonies exhibited differences with respect to colony surface and reverse colour mostly from pale olive to dark olive, grey to green, or light to dark brown. The colony form varied from irregular to filamentous and elevation from flat to raised. The colonies mostly had undulated margins, but a few also showed filiform margins. Most *A. brassicae* colonies showed concentric zones, depicting variable mycelium intensity; whereas, *A. brassicicola* isolates mostly formed colonies with overlapping /non-concentric zones (Fig. 6).

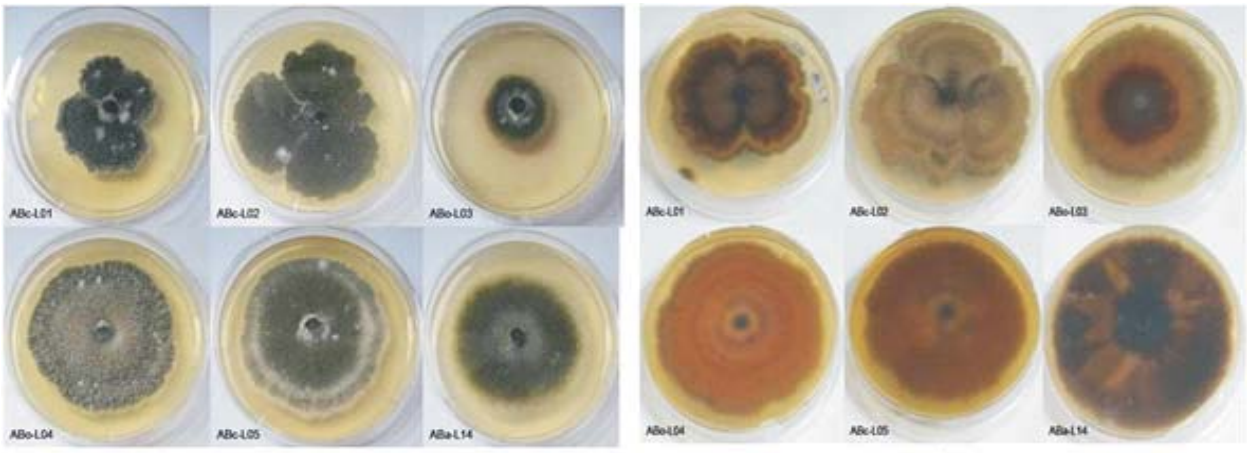

Fig. 6: Cultural diversity in *Alternaria* isolates [(a) Variability in radial growth, colour, form, elevation and surface , (b) Variability in zonation patterns observed in *A. brassicae* (ABc-L01, ABc-L02, ABc-L05), *A. brassicicola* (ABo-L03, ABo-L04, ABo-L05) and *A. alternata* (ABc-L14) isolates from Ludhiana]

Biochemical diversity

The total carbohydrate content from isolates of different regions revealed considerable variations at inter- as well as intra-specific levels (Table 4). The average concentration of carbohydrate content was found to be maximum in *A. alternata* followed by *A. brassicae* and *A. brassicicola* isolates. In *A. brassicae* isolates the concentration varied from 0.47 (ABc-P04) - 2.07 mg/l (ABc-H05), in *A. brassicicola* it ranged from 0.56 (ABo- P03) to 1.99 mg/l (ABo-D08) and in *A. alternata* it varied from 1.33 (ABa-B02) to 1.83 mg/l (ABa-L14).

Table 4: Mean carbohydrate concentration of *Alternaria* isolates from different regions

Region	Mean Carbohydrate concentration (mg/ l)#		
Isolates	*A. brassicae* (32)	*A. brassicicola* (20)	*A. alternata* (3)
Delhi	1.37 ± 0.012	1.73 ± 0.013	-
Pantnagar	1.226 ± 0.014	1.11 ± 0.014	-
Ludhiana	1.272 ± 0.036	1.13 ± 0.016	1.83
Bharatpur	1.18 ± 0.017	-	1.33
Hisar	1.717 ± 0.018	0.835 ± 0.014	-
Kangra	0.935 ± 0.014	-	-
Kanpur	1.85	-	1.44
	1.306 ± 0.021	1.295 ± 0.015	1.53 ± 0.015
CD (at P=0.05)	0.058	0.043	0.051
C.V.	2.733	1.976	1.641

Mean of three replicates of all the isolates; Figure in brackets indicate the number of isolates under each species

Molecular diversity

Of the thirty-four primers tested, six primers viz., FP1, FP2, RP1, OPG13, OPK07 and OPM13 produced good number of countable and reproducible PCR products. Out of these, the primer RP1 amplified maximum number of bands, followed by the primer OPK-07 (Fig. 7, 8).

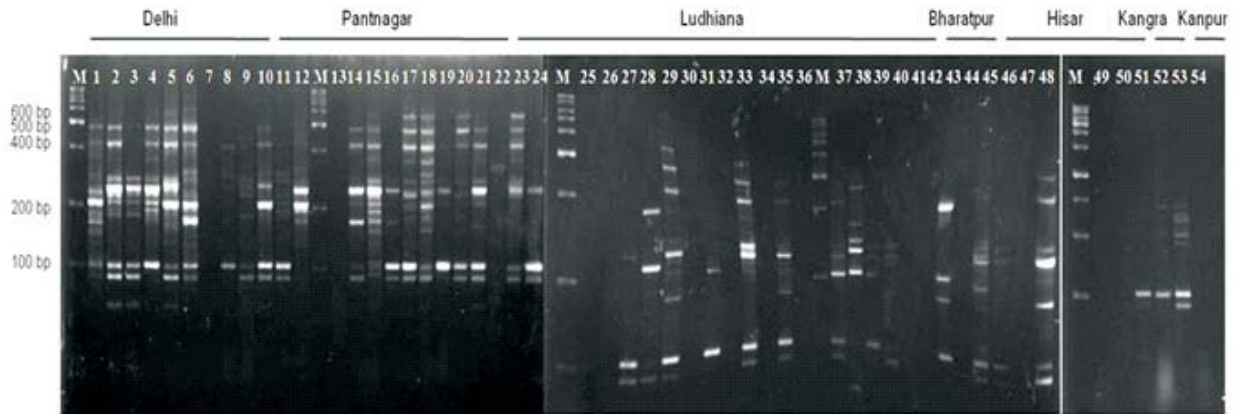

Fig. **7:** RAPD banding profile of 55 *Alternaria* isolates with primer RP1 [M is 1 kb DNA ladder; 1-10, 11-22, 23-42, 43-45, 46-51, 52-53 and 54-55 are isolates from Delhi, Pantnagar, Ludhiana, Bharatpur, Hisar, Kangra and Kanpur, respectively]

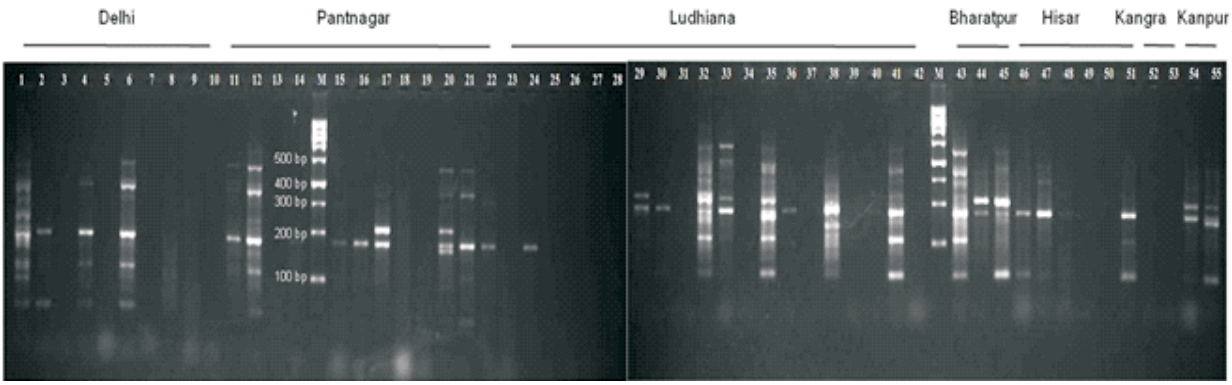

Fig. 8: RAPD banding profile of 55 *Alternaria* isolates with primer OPK-07 [M is 1 kb DNA ladder; 1-10, 11-22, 23-42, 43-45, 46-51, 52-53 and 54-55 are isolates from Delhi, Pantnagar, Ludhiana, Bharatpur, Hisar, Kangra and Kanpur, respectively]

Intraspecific diversity

A. brassicae isolates

The analysis of the similarity matrix data for *A. brassicae* isolates (Figure 9) revealed a high level of diversity (82.83%) among all the isolates from different regions. Maximum average genetic diversity among isolates from different *Brassica* species and subspecies within the same region was obtained for Delhi (78.8%), followed by isolates from Kangra (78%), Pantnagar (70.3%), Hisar (69.57%), Ludhiana (69.1%) and Bharatpur (57.3%). Significant difference in average genetic diversity has also been observed between isolates from different regions, with isolates from Ludhiana showing the maximum average diversity ranging from 79-100%. Isolate from Kanpur (ABc-Kn01) was found to be highly distinct with 100% dissimilarity with isolates from Ludhiana, Hisar and Kangra, and 97-98.5% genetic diversity with isolates from Bharatpur, Delhi and Pantnagar, respectively.

	ABc-D01	ABc-D02	ABc-D06	ABc-D07	ABc-P01	ABc-P04	ABc-P05	ABc-P06	ABc-P07	ABc-P08	ABc-P09	ABc-P10	ABc-P11	ABc-P12	ABc-L01	ABc-L02	ABc-L05	ABc-L07	ABc-L08	ABc-L10	ABc-L12	ABc-L19	ABc-L20	ABc-B01	ABc-B03	ABc-H03	ABc-H04	ABc-H05	ABc-H06	ABc-KN01	ABc-KG01	ABc-KG02
ABc-D01	1.00																															
ABc-D02	0.32	1.00																														
ABc-D06	0.42	0.42	1.00																													
ABc-D07	0.07	0.04	0.03	1.00																												
ABc-P01	0.23	0.09	0.19	0.31	1.00																											
ABc-P04	0.09	0.00	0.00	0.30	0.33	1.00																										
ABc-P05	0.41	0.33	0.30	0.17	0.32	0.28	1.00																									
ABc-P06	0.07	0.00	0.00	0.00	0.17	0.44	0.13	1.00																								
ABc-P07	0.24	0.22	0.20	0.06	0.25	0.29	0.30	0.27	1.00																							
ABc-P08	0.27	0.15	0.19	0.18	0.38	0.24	0.36	0.10	0.31	1.00																						
ABc-P09	0.16	0.17	0.12	0.08	0.25	0.31	0.23	0.40	0.64	0.23	1.00																					
ABc-P10	0.23	0.21	0.19	0.00	0.24	0.28	0.29	0.25	0.81	0.30	0.50	1.00																				
ABc-P11	0.09	0.10	0.14	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.00	1.00																			
ABc-P12	0.07	0.00	0.00	0.00	0.18	0.50	0.14	0.80	0.29	0.10	0.44	0.27	0.00	1.00																		
ABc-L01	0.47	0.45	0.50	0.10	0.29	0.09	0.35	0.06	0.26	0.27	0.23	0.26	0.03	0.06	1.00																	
ABc-L02	0.33	0.21	0.25	0.23	0.36	0.11	0.34	0.00	0.06	0.22	0.07	0.09	0.13	0.00	0.34	1.00																
ABc-L05	0.40	0.48	0.31	0.14	0.13	0.06	0.33	0.00	0.17	0.25	0.13	0.17	0.03	0.00	0.44	0.23	1.00															
ABc-L07	0.28	0.43	0.36	0.18	0.16	0.07	0.39	0.00	0.21	0.26	0.15	0.16	0.04	0.00	0.39	0.19	0.66	1.00														
ABc-L08	0.37	0.35	0.38	0.14	0.23	0.06	0.40	0.00	0.17	0.25	0.13	0.14	0.00	0.00	0.51	0.29	0.59	0.55	1.00													
ABc-L10	0.35	0.36	0.50	0.07	0.24	0.03	0.45	0.00	0.11	0.20	0.06	0.14	0.07	0.00	0.49	0.30	0.39	0.52	0.61	1.00												
ABc-L12	0.26	0.26	0.34	0.05	0.11	0.05	0.26	0.00	0.00	0.13	0.00	0.03	0.19	0.00	0.33	0.23	0.27	0.20	0.35	0.37	1.00											
ABc-L19	0.31	0.43	0.30	0.21	0.33	0.10	0.43	0.00	0.19	0.37	0.14	0.22	0.00	0.00	0.43	0.28	0.50	0.55	0.46	0.43	0.22	1.00										
ABc-L20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00									
ABc-B01	0.35	0.23	0.32	0.17	0.35	0.19	0.47	0.09	0.26	0.38	0.18	0.29	0.10	0.10	0.26	0.21	0.27	0.28	0.24	0.24	0.22	0.35	0.00	1.00								
ABc-B03	0.30	0.21	0.25	0.00	0.31	0.15	0.38	0.17	0.50	0.28	0.35	0.54	0.08	0.18	0.31	0.19	0.14	0.19	0.20	0.26	0.16	0.24	0.00	0.37	1.00							
ABc-H03	0.31	0.22	0.20	0.09	0.33	0.22	0.40	0.19	0.42	0.33	0.38	0.52	0.00	0.20	0.32	0.20	0.27	0.28	0.31	0.28	0.09	0.42	0.00	0.35	0.56	1.00						
ABc-H04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.99	0.00	0.00	0.00	1.00					
ABc-H05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.99	0.00	0.00	0.00	0.99	1.00				
ABc-H06	0.12	0.20	0.14	0.00	0.11	0.08	0.17	0.11	0.19	0.03	0.17	0.18	0.29	0.13	0.10	0.08	0.10	0.13	0.07	0.11	0.11	0.07	0.00	0.13	0.23	0.14	0.00	0.00	1.00			
ABc-KN01	0.02	0.00	0.04	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	1.00		
ABc-KG01	0.04	0.15	0.10	0.00	0.05	0.00	0.10	0.00	0.13	0.03	0.18	0.12	0.14	0.00	0.10	0.04	0.11	0.19	0.07	0.15	0.06	0.12	0.00	0.06	0.18	0.09	0.00	0.00	0.29	0.00	1.00	
ABc-KG02	0.24	0.28	0.21	0.21	0.48	0.18	0.42	0.10	0.28	0.28	0.29	0.22	0.10	0.10	0.31	0.33	0.22	0.30	0.25	0.29	0.14	0.40	0.00	0.26	0.43	0.31	0.00	0.00	0.28	0.00	0.22	1.00

Fig. 9: Similarity matrix for *A. brassicae* isolates from different regions

A. brassicicola isolates

The *A. brassicicola* isolates revealed 84.43% diversity among the isolates from different regions. Maximum average genetic diversity among isolates from different *Brassica* species and subspecies within the same region was obtained for Pantnagar (92.0%), followed by isolates from Hisar (89.0%), Ludhiana (84.5%) and Delhi (78.2%). Significant difference in average genetic diversity was also observed between isolates from different regions. The isolates from Delhi showed an average diversity ranging from 82.4-88.7% compared to isolates of other regions, with the highest dissimilarity of 88.7% with Hisar isolates, followed by Pantnagar (84.7%) and Ludhiana (82.4%). Similarly, in Pantnagar isolates the diversity with respect to other regions varied between 84.8-89.3%, with the highest differences observed for Hisar followed by Ludhiana. The Ludhiana isolates showed 82.4-89.7% variation in genetic diversity with other regions; Hisar isolates being highly distinct from the Ludhiana isolates with 89.7% dissimilarity. Overall, among all the *A. brassicicola* isolates, ABo-L03 was the most distinct as compared to the others.

A. alternata isolates

Among the three isolates sampled from Ludhiana (ABa-L14), Kanpur (ABa-Kn02) and Bharatpur (ABa- B02), the average genetic diversity was found to be 96.3%. The isolate ABa-Kn02 was highly distinct with 100% dissimilarity with ABa-L14 and ABa-B02, whereas, 89% diversity was found between ABa-L14 and ABa-B02 isolates.

Cluster analysis

A. brassicae isolates

UPGMA analysis for determining genetic relationship among 32 *A. brassicae* isolates revealed the clustering of isolates into four major clades (Fig. 10). Among different clades, the average genetic diversity varied from 0.45- 0.65. Based upon the similarity coefficients, clade I further segregated into sub-groups a, b and c;

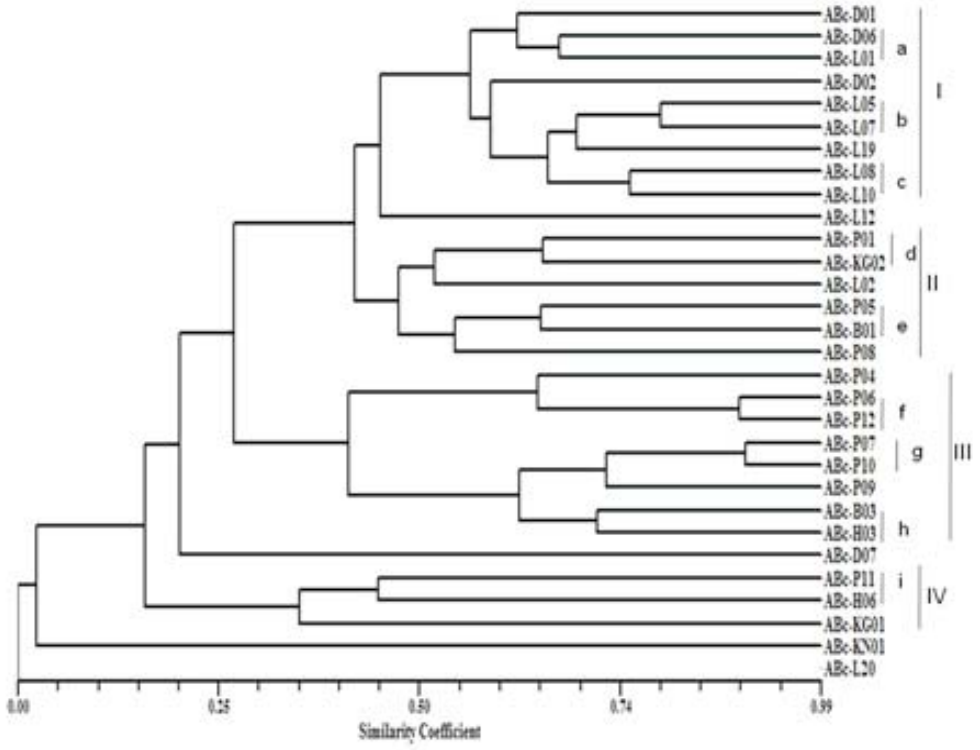

Fig. 10: UPGMA based dendrogram showing clustering pattern among *A. brassicae* isolates from different regions revealing four major clades

sub-group a showed close relationship among isolates with genetic similarity (GS) at a value of 0.68, sub-group b GS = 0.79 and sub-group c GS = 0.75. Isolate ABc-D01 and ABc-L19 were not the members of any sub-group but showed a close proximity to group a and b at GS value of 0.65 and 0.70, respectively. Isolates ABc-L12 showed relatedness with clade I, with GS value of 0.45. In clade II, members of the sub-group d and e were related with each other at a GS value of 0.65. Groups f, g and h within clade III showed low genetic diversity among themselves with GS values of 0.1, 0.09 and 0.28. Isolate ABc-D07 lying next to clade III was found to show high genetic diversity (GS = 0.20) with clades I, II and III. Only one sub-group, i was formed in clade IV showing genetic similarity at a value of 0.45. Isolates ABc-Kn01, ABc-L20, ABc-H04 and ABc-H05 were not found to be clustered in any of the clades and showed distinctness with very high genetic diversity (> 97%) at GS values ranging between 0.0-0.025. As observed from the clustering pattern, differential grouping by the isolates of the same region revealed a high genetic diversity among the isolates within that region.

A. brassicicola isolates

The twenty *A. brassicicola* isolates were broadly clustered into three major clades (Fig. 11). Clade I comprised a total of seven isolates from Delhi, Ludhiana and Pantnagar, clade II had six isolates from Delhi, Ludhiana and Hisar, whereas, clade III comprised a total of three isolates, one each from Pantnagar, Ludhiana and Hisar. Rest of the isolates (ABo-D03, ABo-L09, ABo-L15 and ABo-L03) did not form any cluster, although, isolates ABo-D03 and ABo-L09 showed relatedness to clade I with GS values varying from 0.47-0.49. Isolates ABo-

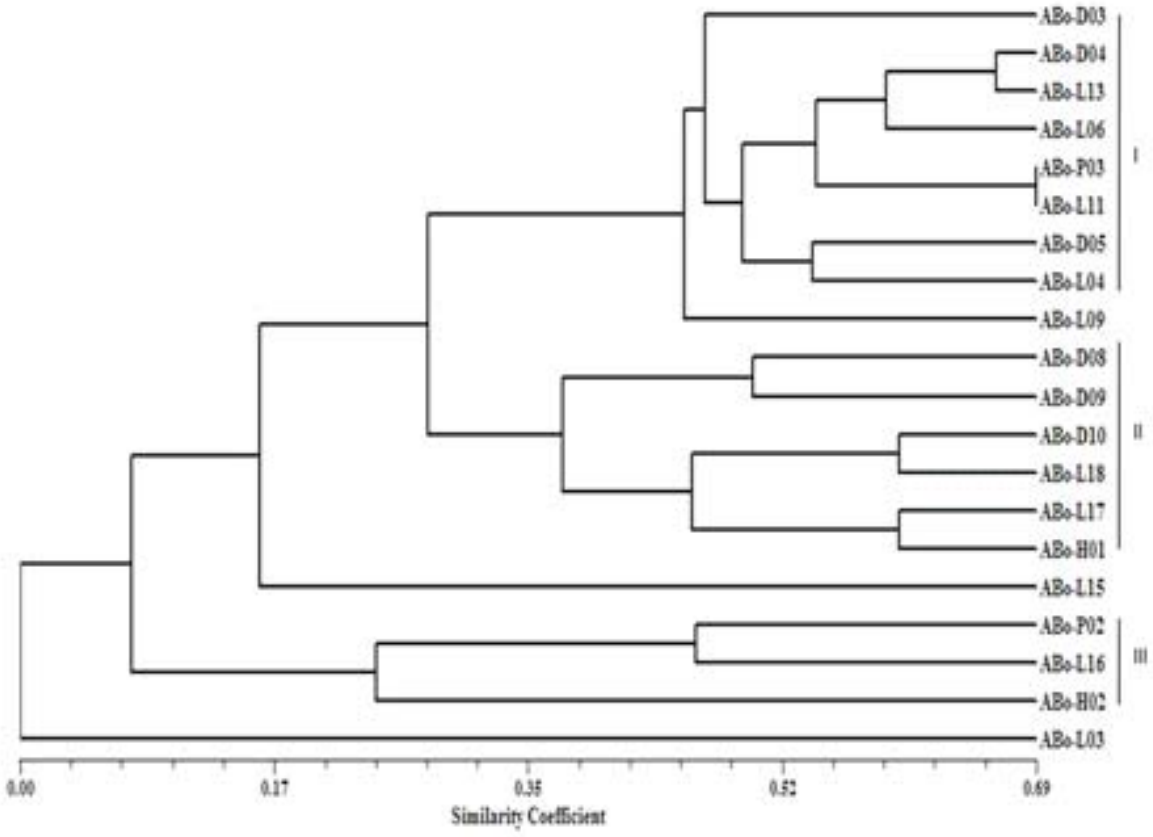

Fig. 11: UPGMA based dendrogram showing clustering pattern among *A. brassicicola* isolates from different regions revealing three major clades

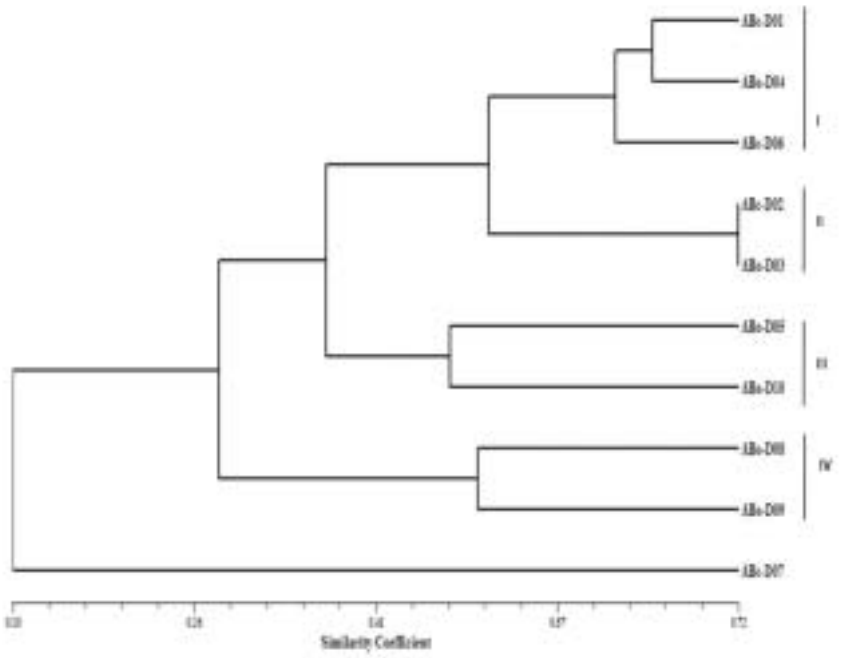

Fig. 12: UPGMA based dendrogram showing clustering pattern among *A. brassicae* and *A. brassicicola* isolates from Delhi

L15 and ABo-L03 on the other hand, were highly distinct with very low GS values with respect to clade I & II and clade I, II and III (0.17, 0.068). Among different clades, the average genetic diversity varied with a value of 0.51 in clade I, 0.63 in clade II and 0.76 in clade III. The similarity coefficients data further segregated clade I into sub-groups a, b and c; sub-group a showed close relationship among isolates with GS at a value of 0.66, sub- group b GS = 0.69, and sub-group c GS = 0.54. In clade II, members of the sub-group, d, e and f were related with each other at a GS value of 0.49 and 0.59 respectively. Group g showed a GS value of 0.47.

A. alternata isolates

Among the three *A. alternata* isolates, ABa-L14 from Ludhiana and ABa-B02 from Bharatpur showed considerable genetic diversity with GS value of 0.20, whereas, Kanpur isolate ABa-Kn02 was highly distinct from others (GS = 0).

Interspecific diversity

Considerable genetic diversity was observed between *A. brassicae, A. brassicicola and A. alternata* isolates at the interspecific level. Among *A. brassicae* and *A. brassicicola* isolates from the same region, the isolates from Hisar showed maximum average genetic dissimilarity of 95.5%. The *A. brassicicola* isolate ABo-H02 from Hisar showed highest dissimilarity with *A. brassicae* isolates ABc-H03, ABc-H04 and ABc-H05. Among the Pantnagar isolates, 79.6% dissimilarity was observed maximum for *A. brassicicola* isolate ABo-P03 with *A. brassicae* isolates ABc-P06 and ABc-P12. Among isolates of Ludhiana 79.3% dissimilarity was observed; *A. brassicicola* isolates ABo-L03, ABo-L15 and ABo-L16 being most distinct from all the *A. brassicae* isolates of the same region. Similarly, 78.9% genetic dissimilarity was observed in isolates of Delhi, highest for *A. brassicae* isolate ABc-D07 with *A. brassicicola* isolates ABo-D08 and ABo-D09. The *A. alternata* isolates from Bharatpur (ABa-B02), Kanpur (ABa-Kn02) and Ludhiana (ABa-L14) also showed very high genetic dissimilarity with *A. brassicae* and *A. brassicicola* isolates. Among *A. brassicae* and *A. alternata* isolates from Kanpur, the *A. alternata* isolate ABa-Kn02 showed maximum dissimilarity with 100% distinctness from *A. brassicae* isolate ABa-Kn01of

the same region. The *A. alternata* isolates from Ludhiana and Bharatpur exhibited 82% and 92% dissimilarity with *A. brassicae* isolates of the same regions, respectively. Similarly, among *A. brassicicola* and *A. alternata*, isolates from Ludhiana showed 78% dissimilarity, the highest being for *A. alternata* isolate ABa-L14 with *A. brassicicola* isolates ABo-L03, ABo-L15 and ABo-L16.

Slightest change in the environmental conditions is accompanied by multiple, small to large scale, adaptations in the pathogen population. The extent and rate of such adaptations brought about in a pathogen not only shapes its morphological and physiological characters, but also changes its behavior towards the host population. In the present study 55 *Alternaria* isolates were purified from 100 leaf samples of different *Brassica* species inhabiting 8 states of Northern and North-Western region of India. The variability observed among the three *Alternaria* species with respect to the conidial morphology is in agreement with the earlier reports (Meena *et al*., 2010; Verma and Saharan, 1994).

Ansari *et al.* (1988) have reported high level of growth and sporulation of *A. brassicae* on PDA. The influence of culture media has also been reported on conidia formation and its morphology in *A. alternata* (Anginyah *et al*., 2001). The three culture media used in this study have been compared, for the first time, for optimum growth and sporulation of *Alternaria* sp. In our study, *A.brassicae* isolates showed higher mean spore and beak lengths on PDA followed by WAPDA and VJA; for *A. brassicicola* isolates the mean lengths were best on WAPDA> PDA>VJA, whereas, *A. alternata* isolates were found to achieve higher lengths on PDA followed by VJA and WAPDA. Such kind of morphological plasticity in fungal cultures in response to growth conditions is a commonly observed phenomenon (Roberts *et al*., 2000).

Sporulation and pathogenicity of fungal plant pathogens has also been reported to be affected by periodical sub-culturing (Dhingra and Sinclair, 1995). Reduction in virulence/pathogenicity in *Alternaria* isolates after the 3rd subculture stage was also observed in the present study. As per our knowledge, this is the first report on affect of sub-culturing on pathogenicity of *Alternaria* isolates. Till date the maintenance of *Alternaria* isolates has been carried out only by sub-culturing on suitable media or on host plant. However, we report for the first time the use of 10% Glycerol and 5% DMSO for cryo-preservation and maintenance of *Alternaria* isolates. The cryo-preserved isolates were successfully revived on PDA medium after 6 months.

The *Alternaria* isolates of all the three species in this study showed extensive variations in their morphological and cultural characters. This is in agreement with the earlier reports on *A. brassicae* (Kumari *et al*., 2012; Goyal *et al*., 2011; Khulbe *et al*., 2011; Reis and Boiteux 2010; Singh *et al*., 2009). However, the mentioned studies are limited to only *A. brassicae* isolates, unlike the present study which reports inter and intraspecific variability between *A. brassicae, A. brassicicola* and *A. alternata* isolates.

Earlier studies have also revealed that pathogenic strains showing slightest differences in their morphology within the species may show a high degree of variation in physiological and biochemical processes. Quantitative analysis of the total carbohydrates of isolates from different regions used in the present study revealed considerable variations both at inter and intraspecific levels. These results are similar to those of Vishwanath and Kolte (1997) who also reported biochemical variations in three *A. brassicae* isolates viz., A, C and D from *B. carinata* cv. PPCS-1, sampled from Pantnagar.

Molecular techniques like RAPD have been used to study the genetic variations in fungi at genus, species and/or subspecies levels (Gherbawy and Abdelzaher, 2002). Although some work has been reported on diversity of *Alternaria* species in other crops in India (Goyal *et al*., 2011; Kumar *et al*., 2008), there exists only a single report on diversity of *A. brassicae* isolates pathogenic to *Brassica* species in India and that too with very small number of isolates (Kaur *et al*., 2007). In order to make a reliable inference for the pathogen behavior a detailed study using large numbers of isolates of different Alternaria species from various Brassica species spread over a wide geographical location

was lacking. In the present study, 55 *Alternaria* isolates from different regions were tested for their molecular diversity. High level of genetic diversity varying from 57-78% was observed among the *A. brassicae* isolates. The results showed that the *A. brassicae* isolates from Delhi region were most diverse. For the *A. brassicicola* isolates, genetic diversity (78-92%) was higher as compared to *A. brassicae* isolates, with the highest diversity shown by Pantnagar isolates. In case of *A. alternata* isolates, the diversity ranged from 89-100%, with ABa-Kn02 being the most distinct. As evident from the dendrogram, a few isolates from different regions of North and north-west India were clustered together. This indicated that no relationship existed between the isolates on the basis of their origin, and the observed variation at the molecular level may have been due to dispersal of the pathogen species, across different areas. The immense diversity observed in *Alternaria* isolates at all levels indicates pathogen's ability to adapt and survive under diverse agro-climatic conditions.

On the basis of the observed variations in morphology, cultural characteristics, carbohydrate concentration and molecular characteristics an attempt was made to characterize the isolates into different groups. However, consistent grouping with respect to all the studied characters was not possible due to the wide diversity obtained. Thus, it can be concluded that the diversity between and among *Alternaria* species infecting different *Brassica* species/variety in India is non-specific. Earlier studies based on symptoms, assumed that a quantitative form of resistance governed by polygenes or minor genes exists (Krishnia *et al*., 2000). The present study may also suggest similar inference, but conclusively showed that the diversity observed between and among three *Alternaria* species infecting *Brassica* crops in India are non-specific. Deployment of QTL based approach in future for developing durable resistance/tolerance to Alternaria blight in Brassicas may prove useful.

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References

Agnihotri A, Gupta K, Prem D, Sarkar G, Mehra VS and Zargar SM. 2009. Genetic enhancement in rapeseed- mustard for quality and disease resistance through *in vitro* techniques. In : *Proc. of the 16th ustralian Research Assembly on Brassicas*, Sep 14-16, Ballarat, Australia, pp. 28.

Andreote FD, Pietrobon VC, Andreote FD, Romao AS, Sposito MB and Araujo WL. 2009. Genetic variability of Brazilian isolates of *Alternaria alternata* detected by AFLP and RAPD techniques. *Braz J Microbio* **40**: 670-677.

Anginyah TJ, Narla RD, Carey EE and Njeru R. 2001. Etiology, effect of soil pH and sweetpotato varietal reaction to *Alternaria* leaf petiole and stem blight in Kenya. *Afric Crop Sci J* **9**: 287-292.

Ansari NA, Khan MW and Muheet A. 1988. Effect of Alternaria blight on oil content of rapeseed mustard. *Curr Sci* **57**: 1023-1024.

Avenot H, Dongo A, Simoneau NB, Iacomivasilescu B, Hamon B, Peltiers D and Simoneau P. 2005. Isolation of 12 polymorphic microsatellite loci in the phytopathogenic fungus *Alternaria brassicicola*. *Mol Eco Notes* **5**: 948-950.

Berbee ML, Payne BP, Zhang G, Roberts RG and Turgeon BG. 2003. Shared ITS DNA substitutions in isolates of opposite mating type reveal a recombining history for three presumed

asexual species in filamentous ascomycetes genus *Alternaria*. *Mycol Res* **107**:169-182.

Coakley SM, Scherm H and Chakraborty S. 1999. Climate Change and Disease Management. *Ann Rev Phytopath* **37**: 399-426.

Chauhan JS, Badoni A, Singh NI and Ali S. 2009. Effect of *Alternaria* on some members of family Brassicaceae of Garhwal Himalaya. *New York Sci J* **2**: 80-85.

Cooke DEL, Forster JW, Jenkins PD, Gareth DJ and Lewis DM. 1998. Analysis of intraspecific and interspecific variation in the genus *Alternaria* by the use of RAPD-PCR. *Annals App Bio* **132**: 197-209.

Chou HH and Wu WS. 2002. Phylogenetic analysis of internal transcribed spacer regions of the genus *Alternaria*, and the significance of filament-beaked conidia. *Mycol Res* **106**: 164-169.

Dhingra OD and Sinclair JB. 1995. In *Basic Plant Pathology Methods*. CRC Press. Boca Raton, Florida, USA. pp. 448.

Gherbawy YAMH and Abdelzaher HMA. 2002. Use of RAPD-PCR for separation of *Pythium spinosum Sawada* into two varieties: var. *spinosum* and var. *sporangiiferum*. *Cytol* **67**: 83-94.

Goyal P, Chahar M, Mathur AP, Kumar A and Chattopadhyay C. 2011. Morphological and cultural variation in different oilseed Brassica isolates of *Alternaria brassicae* from different geographical regions of India. *Indian J Agri Sci* **81**: 1052-1058.

Gupta K, Prem D and Agnihotri A. 2010. Pyramiding white rust and *Alternaria* blight resistance in low erucic *Brassica juncea* (L.) using *Brassica carinata. J Oilseed Brassica* **1**: 55-65.

Kaur S, Singh G and Banga SS. 2007. Documenting variation in *Alternaria brassicae* isolates based on conidial morphology, fungicidal sensitivity and molecular profile. In: *Proc. of the 12 Internl Rapeseed Cong,* Mar 26-30, Wuhan, China **4**: 87-90.

Khulbe A, Awasthi RP and Tewari AK. 2011. Morphological and cultural diversity in isolates of *Alternaria brassicae* infecting rapeseed and mustard. *Pant J Res* **9**: 206-209.

Kolte SJ. 2002. Diseases and their management in oilseed crops – new paradigm. In: *Oilseeds and Oils – Research and Development Needs*. Rai M, Singh H, Hegde DM, (Eds) Indian Society of oilseeds Research, Hyderabad, pp. 244-253.

Kumar V, Haldar S, Pandey KK, Singh RP, Singh AK and Singh PC. 2008. Cultural, morphological, pathogenic and molecular variability amongst tomato isolates of *Alternaria solani* in India. *World J Microbio Biotech* **24**: 1003-1009.

Kumari V, Kumar A, Chaudhary HK, Prasad R, Jambhulkar S and Sharma S. 2012. Variability in *Alternaria brassicae* and characterization of host response in Ethopian mustard (*Brassica carinata* A. Braun). In: *Abstracts of 1st National Brassica Conference*, CCSHAU, Hisar, pp. 45-46.

Krishnia SK, Saharan GS and Singh D. 2000. Genetics of Alternaria blight resistance in inter and intraspecific crosses of *Brassica juncea* and *B. carinata*. *Annals Bio* **16**: 212-216.

Meena PD, Awasthi RP, Chattopadhyay C, Kolte SJ and Kumar A. 2010. *Alternaria* blight: a chronic disease in rapeseed mustard. *J Oilseed Brassica* **1**: 1-11.

Meena PD, Rani A, Meena R, Sharma P, Gupta R and Chowdappa P. 2012. Aggressiveness, diversity and distribution of *Alternaria brassicae* isolates infecting oilseed brassica in India. *African J Microbio Res* **6**: 5249-5258.

Mitchell CE, Reich PB, Tilman D and Groth JV. 2003. Effects of elevated CO_2, nitrogen deposition, and decreased species diversity on foliar fungal plant disease. *Glob Change Bio* **9**: 438-451.

Pryor BM and Michailides TJ. 2002. Morphological, pathogenic and molecular characterization of *Alternaria* isolates associated with Alternaria late blight of pistachio. *Phytopath* **92**: 406-416.

Reis A and Boiteux LS. 2010. *Alternaria* species infecting Brassicaceae in the Brazilian neotropics: Geographical distribution, host range and specificity. *J Plant Path* **92**: 661-668.

Roberts RG, Reymond ST and Anderson B. 2000. RAPD fragment pattern analysis and morphological

segregation of small-spored *Alternaria* species and species group. *Myco Res* **104**: 151-160.

Singh HK, Singh RB and Bhajan R. 2006. Identification of resistant source against *Alternaria* blight of rapeseed-mustard. *Indian Phytopath* **59**: 385.

Singh D, Singh R, Singh H, Yadav RC, Yadav N, Barbetti M, Salisbury P, Nimbal S, Chattopadhyay C and Kumar A. 2009. Cultural and morphological variability in *Alternaria brassicae* isolates of Indian mustard [*Brassica juncea* (L.) Czern & Coss]. *J Oil*seed *Res* **26**: 134-137.

Sharma P, Deep S, Sharma M, Bhati DS and Chowdappa P. 2013. PCR based assay for detection of *Alternaria brassicicola* in crucifers. *Indian Phytopath* **66**: 263-268.

Sharma TR and Tewari JP. 1995. Detection of genetic variation in *Alternaria brassicae* by RAPD fingerprints. *J Plant Biochem Biotech* **4**: 105-107.

Sharma TR and Tewari JP. 1998. RAPD analysis of three *Alternaria* species pathogenic to crucifers. *Mycol Res* **102**: 807-814.

Tohyama A and Tsuda M. 1995. *Alternaria* on cruciferous plants. 4. *Alternaria* species on seed of some cruciferous crops and their pathogenicity. *Mycoscience* **36**: 257-261.

Verma PR and Saharan GS. 1994. In *Monograph on Alternaria diseases of Crucifers*. Canada Agriculture and Agri-Food Canada: Research Branch. pp. 1-146.

Vishwanath and Kolte SJ. 1997. Biochemical variation among three isolates of *Alternaria brassicae*. *Indian Phytopath* **50**: 437- 438.

Weising K, Nybom H, Wolff K and Meyer W. 1995. In: *DNA Fingerprinting in Plants and Fungi*. CRC Press, Boca Raton, Florida, USA. pp. 336.

Williams JGK, Kublelik AR, Livak KJ, Rafalski JA and Tingey SV. 1990. DNA polymorphism's amplified by arbitrary primers are useful as genetic markers. *Nuclic Acids Res* **18**: 6531-6535.