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Variability and growth response among *Alternaria brassicae* isolates causing black spot disease in oilseed Brassica

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Abstract

Alternaria brassicae (Berk.) Sacc., the most common and destructive fungus of oilseed Brassica worldwide, generally sporulates poor with slow growth on common media. A comparative analysis among 30 Indian geographical isolates of *A. brassicae* was undertaken to observe the variability as well as better growth conditions. Among 6 natural liquid media, the profound growth with maximum mycelial biomass of *A. brassicae* isolates was found of BAB-08, BAB-19, BAB-23, BAB-29, BAB-40, BAB-41, BAB-44, BAB-45, BAB 50, BAB 54, and BAB-56 on tomato broth, Brassica leaf extract, alfa-alfa seed decoction, and potato carrot broth. Tomato agar and Brassica agar medium were found suitable for the growth of *A. brassicae* isolates, whereas, Brassica seed agar medium does not favour the growth of *A. brassicae* due to presence of glucosinolate content responsible for antifungal activity. Brassica leaf extract supplemented with peptone as inorganic nitrogen source produced maximum growth among the tested natural media. Brassica leaf extract supplemented with maltose and yeast extract was found to be the most suitable for mycelial growth, and sporulation of *A. brassicae*. Variation in mycelial growth was also observed due to optimum pH, and light condition. In SDS-PAGE, a high genetic divergence among *A. brassicae* isolates was observed with 55 bands of 0.029 to 0.84 Rm value. Our results confirmed the existence of variability among isolates indicating that isolate BAB-18, BAB-29, and BAB-50 were more aggressive while tomato followed by Brassica leaf broth were found best medium for their growth, and sporulation of *A. brassicae*.

Key words: *Alternaria brassicae, culture media, isolates, oilseed Brassica, variability*

Introduction

Among the biotic stresses, Alternaria blight caused by *Alternaria brassicae* (Berk.) Sacc. is one of the most common, and destructive pathogen of Indian mustard [*Brassica juncea* (L.) Czern & Coss.] responsible to cause 47% seed yield losses (Meena *et al*., 2010). *A. brassicae* usually sporulates poorly, and gave slow growth in common media particularly on PDA (Meena *et al*., 2012). It makes difficult, the maintenance of culture for long period in any of the laboratory or repository. Continuous maintenance of *A. brassicae* isolates is still difficult due to non-availability of specific medium. Cultures reproduce the whitish and irregular growth after few days, and difficult to subculture again. However, available media in support of culture collections is a serious need of improvement, and plant pathologists continually face the risk of losing many of these collections.

Preliminary reports on variability in *A. brassicae* species were made from Awasthi and Kolte (1989), they distinguished three isolates viz., A, C, and D on the basis of morphology, sporulation, growth, and cultural characteristic. Variation in morphology, and cultural characteristics among 22 Indian geographical isolates of *A. brassicae* were analyzed by Meena *et al*. (2012). All the isolates did not grow, and sporulate abundantly on the same nutrient medium. Different synthetic media shows profound variation

in mycelial growth of *A. brassicae* isolates, and poor sporulation indicates that fungus requires some organic sources of nutrition for better growth, and sporulation. It seems that the degree of sporulation of *A. brassicae* isolates was a function of nutrition. Some researchers have found on substantial variation in *Alternaria* species in respect of mycelial growth, and sporulation on different temperature, relative humidity, hydrogen ion concentration (Ansari *et al.*, 1989), media (Sharma *et al*., 2013), and light (Ansari *et al*., 1989). Variability among *A. brassicae* isolates on the basis of morphology, sporulation, growth, and other cultural characteristics also have been reported earlier (Kaur *et al.*, 2007; Meena *et al*., 2012; Pramila *et al*., 2014).

Sporulation of a pathogen is a major factor in studies involving disease dynamics, and assaying host resistance. Earlier the effect of different carbon, and nitrogen sources were also studied by Mehta *et al.* (2003), reported that 14 *A. brassicae* isolates from Haryana state in India behaved differentially in growth, and sporulation. However, the systematic study on different natural media, and other conditions were not reported earlier. Further, *A. brassicae* is sensitive to nutritional, and environmental factors, and their growth as well as sporulation, therefore, greatly influenced by the composition of the nutrient media. The present study was undertaken to investigate the effect of these factors on the growth as well as variability among Indian population of *A. brassicae*.

Materials and Methods

Media

A total of six natural liquid media including Brassica leaf extract (fresh *B. juncea* leaf 200g, sucrose 20), tomato extract (tomato 200g, sucrose 20g), alfa-alfa seed decoction (alfa-alfa seed 100g, sucrose 20g), radish root extract (radish root 200g, mannitol 50g), potato carrot broth (Potato 200g, carrot 200g) and potato dextrose broth (PDB powder 24g; HIMEDIA), were used under study. These media were prepared by dissolving component for particular media in 1000ml distilled water and sterilized.

Isolation

To obtain isolates from host plant tissue, *Alternaria* infected plant bits were placed in 4% NaOCl (Merck) for 30 second followed by subsequent washing thrice in sterilized distilled water. Infected parts of leaf/ siliqua/ seed/ or stem tissues plated onto Petri dishes containing potato dextrose agar (PDA), and incubated for 7-days at 25+ 2°C. Approximately five putative *Alternaria* colonies were subcultured onto dishes containing PDA until pure cultures were obtained. *Alternaria* isolates from cultivated Brassicaceous hosts were obtained from commercial varieties, and germplasm lines. Total 30 geographical isolates of *A. brassicae* were attempted for revival, and purification (Table 1).

Purification of pathogen

After incubation, conidial suspension was obtained by flooding dish with 10 ml of sterile distilled water, and dislodging conidia with a pipette tip. Three drops of conidial suspension were pipetted onto the surface of a Petri dish containing water agar (15 g of Bacto agar liter^{-1} of distilled water), and the dishes were shaken horizontally on the laminar air flow bench to disperse the drops containing conidia. Water agar dishes were incubated for 2 - 4 h to allow for conidia germination. Single spore was picked with the help of needle under stereoscopic microscope (OLYMPUS SZX12) on suitable magnification, and transferred on dishes containing PDA. All the isolates were maintained in culture tubes of potato dextrose agar medium at 4 °C in refrigerator. Total 22 isolates have also been submitted to the ICAR-National Bureau for Agriculturally Important Microorganisms, Mau Nath Bhanjan (Uttar Pradesh), India (NAIMCC-F-02699-02620).

Effect of different liquid media

Thirty *A. brassicae* isolates were studied for the mycelial growth, and sporulation in six different natural liquid culture media viz., potato carrot extract (PCE), Brassica leaf extract (BLE), tomato extract (TE), alfa-alfa seed decoction (ASD), radish root extract (RRE), and potato dextrose broth (PDB), were taken under study. A 5 mm diameter mycelium disc from 15-day-old culture grown on PDA was transferred into each conical flask containing 25 ml test media. The flasks were incubated at 25±2^{0}C for 20 days. The fungal growth of each flask was

Table 1. *Alternaria brassicae* isolates infecting *Brassica* species in India

Isolate Code	NBAIM Accession number	Place and location of collection	Host plant	Plant part	Date of collection
BAB-02	NBAIMCC-F-02600	Jammu, J&K (32° 44' N, 74° 54' E)	*B. napus*	Leaf	15-Feb-05
BAB-03	NBAIMCC-F-02601	Mohanpur, WB (23°6'N, 88°30'E)	*B. juncea*	Leaf	04-Mar-05
BAB-04	NBAIMCC-F-02602	Kamroop, Assam (25° 74' N, 93° 85' E)	*B. rapa* sp. Toria	Silique	02-Mar-05
BAB-05	NBAIMCC-F-02603	Mau, UP	*B. juncea*	Leaf	15-Mar-05
BAB-06	NBAIMCC-F-02604	Golaghat, Assam (22° 07' N, 92° 06' E)	*B. juncea*	Leaf	03-Mar-05
BAB-08	-	Badh Dhamsya, Jaipur (26° 88' N, 76°15' E)	*B. juncea*	Leaf	08-Mar-06
BAB-09	-	GBPUAT, Pantnagar (29°03' N, 79°31' E)	*B. juncea*	Leaf	28-Dec-05
BAB-12	NBAIMCC-F-02605	Jagadhary, Haryana (30°17' N, 77°30' E)	*B. juncea*	Leaf	28-Dec-05
BAB-13	-	Wajirpur, Haryana (30° 6' N / 77° 17' E)	*B. juncea*	Leaf	28-Dec-05
BAB-18	NBAIMCC-F-02606	Pantnagar, Uttarakhand (29°03' N, 79°31' E)	*B. juncea*	Leaf	18-Jan-08
BAB-19	-	Bharatpur, Rajasthan (27°15' N, 77° 30' E)	*B. juncea*	Seed	22-Jan-09
BAB-20	-	Navgaon, Alwar (27° 34' N, 76° 38' E)	*B. juncea*	Silique	28-Feb-05
BAB-23	-	Baharampur, WB (24° 06' N, 88° 19' E)	*B. carinata*	Leaf	23-Feb-05
BAB-28	NBAIMCC-F-02607	Ri-Bhoi, Meghalaya (25° 50' N, 90° 55' E)	*B. juncea*	Leaf	02-Mar-05
BAB-29	NBAIMCC-F-02608	Dimapur, Nagaland (25° 55' N, 93° 44' E)	*B. juncea*	Leaf	05-Mar-05
BAB-39	NBAIMCC-F-02610	Kangra, HP (32° 05' N, 76° 18' E)	*B. carinata*	Leaf	01-Feb-10
BAB-40	NBAIMCC-F-02611	Kangra, HP (32° 05' N, 76° 18' E)	*B. juncea*	Leaf	01-Feb-10
BAB-41	NBAIMCC-F-02612	Kangra, HP (32° 05' N, 76° 18' E)	*B. napus*	Leaf	01-Feb-10
BAB-42	NBAIMCC-F-02613	Jhansi, UP (25° 27' N, 78° 37' E)	*B. juncea*	Leaf	10-Feb-10
BAB-43	NBAIMCC-F-02614	Hazaribag, Jharkhand (23° 59´N, 85° 25´E)	*B. juncea*	Leaf	21-Jan-10
BAB-44	NBAIMCC-F-02615	Bijnor, Uttar Pradesh (29° 27´N, 78° 29´E)	*B. juncea*	Leaf	30-Jan-10
BAB-45	NBAIMCC-F-02616	Jodhpur, Rajasthan (26° 18' N, 73° 04' E)	*B. juncea*	Leaf	26-Jan-10
BAB-47	NBAIMCC-F-02617	Tonk, Rajasthan (26° 11' N, 75° 50' E)	*B. juncea*	Leaf	10-Feb-10
BAB-48	NBAIMCC-F-02618	Kanpur, UP (26° 28' N, 80° 24' E)	*B. juncea*	Leaf	04-Feb-10
BAB-49	NBAIMCC-F-02619	Jobner, Rajasthan (26° 95' N,75° 34' E)	*B. juncea*	Leaf	25-Jan-10
BAB-50	NBAIMCC-F-02620	Jhansi, UP (25°27' N, 78°37' E)	*B. juncea*	Leaf	10-Feb-10
BAB-52	-	Pantnagar, Uttarakhand (29°03' N, 79°31' E)	*B. juncea*	Leaf	18-Jan-08
BAB-53	-	Pantnagar, Uttarakhand (29°03' N, 79°31' E)	*B. juncea var rugosa*	Leaf	18-Jan-08
BAB-54	-	Datia, MP (25°67' N, 78°47' E)	*B. juncea*	Leaf	08-Feb-05
BAB-55	-	Jhansi, UP (25°27' N, 78°37' E)	*B. juncea*	Leaf	10-Feb-10
BAB-56	-	Datia, MP (25°67' N, 78°47' E)	*B. juncea*	Leaf	08-Feb-05

separated on whatman filter paper No. 1, and subsequently oven dried for 72 hr at 60^0C to obtain the dry weight of mycelial mat. The dry weight of the fungal growth was obtained by subtracting the weight of filter paper using four replications.

Effect of different solid media

Experiments were conducted to study the effect of five different solid natural media including Brassica leaf agar (Fresh leaf 200g, agar 20g, sucrose 20, DW 1000ml), tomato agar (tomato 200g, agar 20g, sucrose 20g, DW 1000ml), Brassica seed agar (Indian mustard seed 100g, sucrose 20g, agar 20g, DW 1000ml), radish root agar (radish root 200g, mannitol 50g, agar 20g, DW 1000ml), and potato carrot agar (potato 200g, carrot 200g, agar 15g, DW 1000ml) on the mycelial growth, and sporulation of 28 *A. brassicae* isolates.

Effect of different carbon and nitrogen sources

To observe the effect of carbon sources, cellulose from the basal media was replaced by similar concentration of synthetic sources (dextrose, maltose, fructose, sucrose, and mannitol). Similarly to observe the effect of nitrogen sources on cellulose (exo-â- 1,4- glucanse, and endo-â- 1, 4-glucanse) production by *A. brassicae* isolates the basal media was replaced with other nitrogenous substances including peptone, yeast extract, KNO_3, asparagines, and $NaNO_3$. After inoculation flasks were incubated at 25±2 °C for 15 days in a rotary shaker (Biogentek Pvt. Ltd.) at 150 rpm, and culture filtrate was collected.

Mycelial growth

The mycelial growth of *A. brassicae* observed on five different solid media after 10-days of incubation. The inoculated plates were kept at 25±2°C. Experiments were replicated thrice. On the basis of mycelia growth, isolates categorized as Group A (> 35 mm), Group-B (30-35 mm), Group-C (25-30 mm), and Group-D (< 25 mm).

Effect of light and dark

The culture plates of different isolates on PDA were exposed to continuous light, and dark in an environment chamber maintained at 25±2°C. Inoculated plates were kept in environment chamber, and light intensity was adjusted to required level. The mycelia growth was measured perpendicular in each case 10-day-after-inoculation.

Effect of hydrogen ion concentration (pH)

The effect of pH on the growth of the pathogen was studied followed the method described by Norman *et al.* (1956) using radish root mannitoal agar medium. The pH of the broth was adjusted to 5.0, 6.0, 7.0, and 8.0 with the help of a digital pH meter using 0.1 N hydrochloric acid, and 0.1 N sodium hydroxide. The required quantity of agar was added to each flask, and sterilized. The sterilized media of different pH levels were poured in the sterilized Petri plates, and inoculated 10-days-old culture of different isolates, and incubated at 25±2 °C for 10 days then the mycelia growth diameter was measured.

Extraction of protein and protein profiling

Total 10 *A. brassicae* isolates were cultured on potato dextrose agar media (PDA; Difco) after 14 days incubation at 25 ºC. The mycelial mat was drained through a sterile Whatsman paper No. 1 for protein extraction which was lyophilized (LABCONCO 25), and stored at -80 ºC. Total soluble protein was extracted by a modification of the procedure of Osherov and Gregory (1998). The lyophilized fungal mycelia power, 0.1 gm of each isolate was defatted twice using defatting solution (Chloroform:Methanol:Acetone:: 2: 1:1) at 25 ºC. Extraction buffer (1 % SDS, 15 % Glycerol, 25 mM Tris-HCl pH 6.8, 1 mM EDTA disodium salt, 0.7 M â-Mercaptoethanol) 1 ml was added to each dried defatted material, and crushed with the help of mortar pestle. The crushed material was boiled for 2 minute at 100 ºC, vortexed for another minute, then again boiled for another minute. After boiling, the samples were centrifuged at 14000 rpm at 4 °C for 15 min, supernatant as the crude protein extract, and kept at -20 °C for further use.

Extracted soluble protein was fractioned by one dimensional SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) (Genei make) conducted on a 13% resolving and 6% stacking gel gradient polyacrylamide gel (Daiichi Pure Chemical, Japan) in the buffer system. Then 5µl of stacking dye (1%

Bromophenol blue in methanol) was added in 50µl of crude protein extract. Molecular weight markers (Unstained protein molecular weight marker, fermantas) were used to estimate the molecular weight of the protein. The gel was stained with Coomassie brilliant blue (Hi-Media) for 24 hours, and de-stained in 3% NaCl solution. An amount of 50µl of each extracted sample was loaded in well with protein molecular weight marker (Feramantas). The gel run was carried out at constant voltage at 4.6V per well. Protein banding pattern having unambiguous resolution were coded 0 or 1, respectively, depending on their absence or presence. Percent genetic similarities between pairs of isolates was derived by the Nei and Li (1979) similarity index, and the simple matching coefficient. Only reproducible bands occurring in high frequency in independent runs were used for scoring, and analysis. A dendrogram based on the genetic distance matrix was constructed by the un-weighed par group method with arithmetic averages (UPGMA) cluster analysis. The distance matrix, and dendrogram were both constructed using the NTSYS-pc version 1.8 (Rohlf, 1992).

Data analysis

The data recorded in the present investigation were statistically analyzed using ANOVA, average (x), and relative dispersion (CV %) from the values obtained were calculated. Cluster analysis is more primitive technique, thus, in the case of cluster analysis the inputs are similarity measures or the data from which these can be computed. The objective of cluster analysis is to group observations into clusters such that each cluster is as homogenous as possible with respect to the clustering variables (Sharma, 1996). A great deal of subjectivity is involved in the choice of similarity measures. Important considerations are the nature of the variables i.e., discrete, continuous or binary or scales of measurement (nominal, ordinal, interval, ratio etc.), and subject matter knowledge. If the items are to be clustered, proximity is usually indicated by some sort of distance. The variables however, grouped on the basis of some measure of association like the correlation co-efficient etc. (Chatfield and Collins, 1990).

Quantitative Variables

In case of k quantitative variables recorded on n cases, the observations can be expressed as:

$$\begin{array}{cccc} X_{11} & X_{12} & X_{13} \ldots & X_{1k} \\ X_{21} & X_{22} & X_{23} \ldots & X_{2k} \\ . & . & . & . \\ . & . & . & . \\ X_{n1} & X_{n2} & X_{n3} \ldots & X_{nk} \end{array}$$

Similarity r_{ij} (i,j = 1,2…..n)

Correlation between X_{ik} ‘s with X_{jk} ‘s

(Not the same as correlation between variables)

Distance $d_{ij} = \sqrt{\sum_k (X_{ik} - X_{jk})^2}$

Euclidean distance

X’s are standardized. It can be calculated for one variable.

Hierarchical clustering technique

Hierarchical clustering technique begins by either a series of successive mergers or of successive divisions (Johnson and Wichern, 1996). The analysis has been done by using SAS Version 9.1 available at ICAR-Indian Agricultural Statistics Research Institute, New Delhi, India.

Results and Discussion

Mycelial biomass on different liquid media

Among the six different natural liquid media, potato carrot broth, alfa-alfa seed decoction, Brassica leaf extract, and tomato broth showed profound growth with higher fresh mycelial weight *A. brassicae* isolates (BAB-08, BAB-23, BAB-40, BAB-41, BAB-44, BAB-45, BAB-50, BAB-54, and BAB-56). Maximum wet mycelial biomass was recorded of 10 isolates in each of tomato broth, and Brassica broth (Table 2).

However, the dry mycelial weight was observed

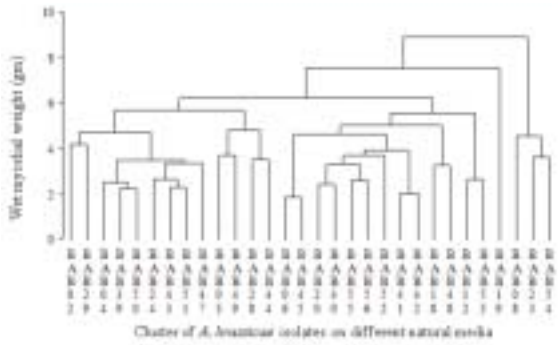

Figure 1: Cluster of *A. brassicae* isolates based wet mycelial growth on different liquid media

maximum in radish root broth media from all 30 isolates with a quantity of variations (Fig. 1). Although, the least wet, and dry mycelial weight was observed in potato carrot medium but was better than the commonly used potato dextrose broth media. The existence of variability may be due to the unusual preference of nourishment among the isolates. Natural medium also played an important role to persuade the sporulation of *A. brassicae* isolates. Results indicated that the use of tomato broth, Brassica broth media make the culture maintenance easy (Table 2). Continuous

Table 2: Dry mycelial weight (gm) of *A. brassicae* isolates on different natural media

A. brassicae isolates	Potato carrot broth	Alfa-alfa seed decoction	Brassica leaf extract	Potato dextrose broth	Tomato broth medium	Radish root broth
BAB-02	0.133	0.151	0.027	0.107	0.507	1.08
BAB-03	0.071	0.197	0.034	0.194	0.307	0.98
BAB-04	0.171	0.174	0.144	0.067	0.310	1.03
BAB-06	0.244	0.127	0.127	0.091	0.507	1.02
BAB-08	0.058	0.166	0.281	0.074	0.423	1.05
BAB-12	0.154	0.191	0.161	0.037	0.367	0.94
BAB-18	0.181	0.131	0.244	0.144	0.507	0.94
BAB-19	0.211	0.161	0.181	0.091	0.243	0.86
BAB-20	0.224	0.181	0.087	0.074	0.453	0.95
BAB-23	0.049	0.175	0.197	0.091	0.223	0.93
BAB-24	0.155	0.218	0.024	0.159	0.277	0.95
BAB-28	0.079	0.166	0.064	0.187	0.460	0.91
BAB-29	0.224	0.151	0.013	0.047	0.287	1.10
BAB-39	0.167	0.191	0.035	0.187	0.330	0.95
BAB-40	0.150	0.150	0.177	0.011	0.500	0.94
BAB-41	0.194	0.162	0.174	0.151	0.377	0.86
BAB-42	0.274	0.164	0.067	0.064	0.327	1.02
BAB-43	0.131	0.181	0.052	0.037	0.553	1.09
BAB-44	0.115	0.106	0.154	0.134	0.207	0.95
BAB-45	0.153	0.276	0.127	0.144	0.213	1.00
BAB-47	0.242	0.164	0.030	0.021	0.230	0.90
BAB-48	0.137	0.162	0.199	0.017	0.240	0.91
BAB-49	0.254	0.197	0.057	0.167	0.390	0.93
BAB-50	0.105	0.263	0.137	0.181	0.310	1.06
BAB-51	0.043	0.150	0.040	0.067	0.303	0.96
BAB-52	0.152	0.142	0.191	0.034	0.430	1.06
BAB-53	0.055	0.211	0.087	0.077	0.300	1.02
BAB-54	0.304	0.133	0.117	0.121	0.423	0.74
BAB-55	0.221	0.081	0.077	0.157	0.223	0.93
BAB-56	0.183	0.065	0.197	0.094	0.657	0.99
LSD (P=0.01)	0.068	0.087	0.835	0.180	0.170	0.165

maintenance of cultures on PDA resulted in low virulence, and less sporulation in long term maintenance of cultures.

Sporulation of *A. brassicae* isolates

Maximum sporulation was observed in BAB-02, BAB-08, BAB-28, BAB-44, and BAB-50 isolates were kept in group 1. Isolte BAB-03 sporulated well in Brassica leaf extract medium followed by alfa-alfa seed extract, and isolate BAB-42 in potato dextrose browth medium. Potato carrot extract showed less sporulation in all isolates. However, isolates including BAB -12, BAB -39, BAB -47, BAB -51, and BAB -53 found poorly sporulated were kept in group 5. Good sporulation of *A. brassicae* in different natural media indicated that the fungus provided some organic sources of nutrition for better growth and sporulation (Table 3). Based on number of spore in 10^{-1} ml spore dilution, the different isolates were categorized in five groups as: Group 1: > 30 spores; Group 2: > 20 spores; Group 3: > 10 spores; Group 4: > 10 spore; Group 5: No sporulation. On the basis of sporulation the different isolates are

Table 3: Sporulation of *Alternaria brassicae* isolates on different natural liquid media

Isolates	PCM	AA	BLE	PDB	TBM
BAB-02	++	+	++++	++	+
BAB-03	+	++++	++	++	++
BAB-04	+	+	+	++	+
BAB-06	+	+++	++	++	++
BAB-08	+	++	+++	++	+
BAB-12	+	+	+	+	-
BAB-18	+	++	+++	++	+
BAB-19	++	+++	++	++	++
BAB-20	+	+	++	++	+
BAB-23	+	+	+++	++	+
BAB-24	-	-	+	++	-
BAB-28	+	++	++++	+	+
BAB-29	+	-	+	+++	+
BAB-39	-	+	+	+	+
BAB-44	+	+	+	+++	++
BAB-56	-	+	++	++	+
BAB-40	+	+	++	++	+
BAB-41	+	+	++	++	++
BAB-42	+	+	+	++++	+
BAB-43	+	+	+	++	-
BAB-45	++	++	++	+++	-
BAB-47	+	-	+	+	+
BAB-48	+	+	++	+++	++
BAB-49	+	+	+	+++	+
BAB-50	+	+++	+++	++	+
BAB-51	-	-	+	+	+
BAB-52	+	+	+	++	-
BAB-53	+	-	+	+	-
BAB-54	++	-	+	++	+
BAB-55	+	+	+	++	+

Group 1 (++++): > 30 spores; Group 2 (+++): > 20 spores; Group 3 (++): > 10 spores; Group 4 (+): < 10 spores; Group 5 (-): No sporulation

categorized in different groups.

Mycelial growth on different solid media

Among five natural solid media, tomato agar, and Brassica leaf extract agar medium gave the maximum mycelia growth of 26 isolates of *A. brassicae*. Different isolates showed varying mycelial growth behavior on different test media. Data in table 4 revealed that the highest mycelial growth (77.7 and 75.3mm) was recorded in tomato agar media of isolate BAB-12, and BAB-40. However, the minimum growth was observed of isolate BAB-49 (44.7mm). Significant difference was observed among the isolates on same media showed the existence of variability among *A. brassicae* population infecting oilseed Brassica in India. Though, the Brassica seed agar medium does not favour mycelia growth of *A. brassicae* isolates except BAB-05, BAB-18, BAB-40, BAB-44, and BAB-50, which could be due to availability of glucosinolate content in Brassica seed caused antifungal effect. Some researchers found superior growth and sporulation on PDA (Ansari *et al*., 1988), while Mukadam and Deshpande (1977) found that *A. brassicae* not only grew, and sporulated poorly, it also lost its capability to grow and sporulate with successive subculturing.

Effect of different carbon and nitrogen source

The growth rate of 29 *A. brassicae* isolates was studied on Elliot's agar media using different carbon sources to find out the favourable carbon source for the growth of *A. brassicae*, and variability among the geographical isolates. Different geographical isolates of *A. brassicae* were characterized by their responses to various carbon, and nitrogen sources. All the isolates behave differentially in growth, and sporulation in relation to the different carbon, and nitrogen sources. Isolates BAB-50, BAB-29, and BAB-49 showed significantly higher growth on all the carbon sources whereas isolate BAB-54 have minimum growth, and responded differentially to different carbon sources (Fig. 2). Elliot's agar media supplemented with fructose, and maltose were found suitable for vigorous growth of *A. brassicae* followed by sucrose, dextrose, and D-mannitol.

Taber *et al*. (1968) reported that *A. brassicae* was

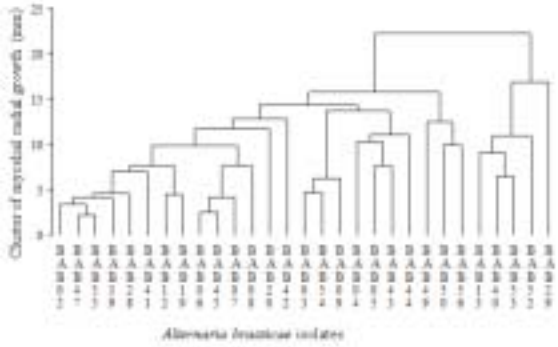

Figure 2: Cluster of *A. brassicae* isolates in response to growth on different carbon source

very in poor carbon source. However, the delayed growth was observed in case of maltose but sporulation of isolates was higher than the other sources of carbon in Elliot's agar media. In all carbon sources, the growth of *A. brassicae* isolates started with a mild peak after that increased. Isolates showed varied growth, and sporulation on different sources of carbon indicated existence of significant

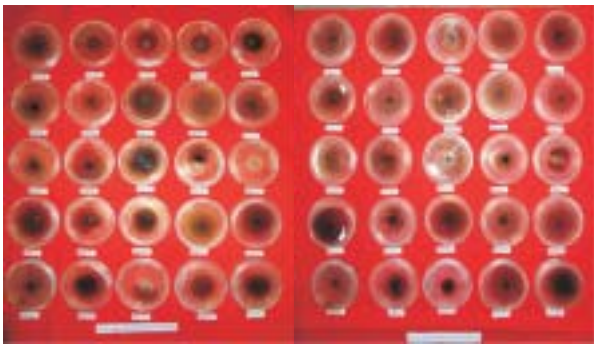

Plate 1: Growth behavior of *A. brassicae* isolates on Elliot's agar media supplemented with fructose and D-mannitol as carbon source

variability among the isolates. Among 29 isolates, BAB-13 from Haryana was found more aggressive in terms of growth, and sporulation with reference to different carbon sources. Our results demonstrated the existence of variation among the isolates of *A. brassicae* population, and supported with the results of Mehta *et al*. (2003) studied in a small population of *A. brassicae*.

Among the nitrogen sources evaluated, growth was maximum on yeast extract followed by peptone, and potassium nitrate. Isolates BAB-50, BAB-42, and BAB-07 gave maximum growth while BAB-53, and BAB-52 has minimum mycelial growth irrespective

Table 4: Effect of different media on radial growth of *A. brassicae* isolates

A. brassicae isolates	Mycelial radial growth (mm)				
	Tomato agar	Potato carrot agar	Brassica leaf agar	Brassica seed agar	Radish root agar
BAB-04	72.3	29.9	33.9	5.0	30.9
BAB-05	47.3	30.3	23.8	27.8	30.5
BAB-06	70.0	30.3	34.3	5.0	31.9
BAB-07	53.7	24.3	29.3	5.0	37.8
BAB-08	65.7	25.3	25.1	5.0	36.6
BAB-09	65.0	27.8	32.6	5.0	40.7
BAB-12	77.7	29.4	32.3	5.0	39.9
BAB-13	61.3	18.1	30.7	5.0	32.9
BAB-18	66.7	29.3	33.1	28.9	37.8
BAB-19	60.0	33.4	24.8	5.0	26.3
BAB-20	64.7	20.8	30.1	5.0	34.8
BAB-28	61.0	24.4	31.4	5.0	28.4
BAB-29	65.7	28.2	35.9	5.0	41.5
BAB-40	75.3	29.9	29.6	15.1	32.5
BAB-41	66.7	21.3	24.2	5.0	32.4
BAB-42	63.3	15.7	25.4	5.0	36.4
BAB-43	47.3	18.8	27.1	5.0	27.2
BAB-44	47.0	17.7	27.3	29.8	37.6
BAB-45	61.7	20.8	34.7	5.0	30.3
BAB-47	57.0	19.0	32.5	5.0	26.8
BAB-49	44.7	23.7	31.4	5.0	33.8
BAB-50	63.7	19.8	29.8	31.3	38.6
BAB-52	53.3	21.3	25.3	5.0	34.7
BAB-54	52.7	15.2	19.4	5.0	35.2
BAB-55	53.0	21.7	24.7	5.0	35.5
BAB-56	52.3	20.0	33.9	5.0	33.5
LSD (p=0.01)	3.9	3.5	1.6	1.5	4.2

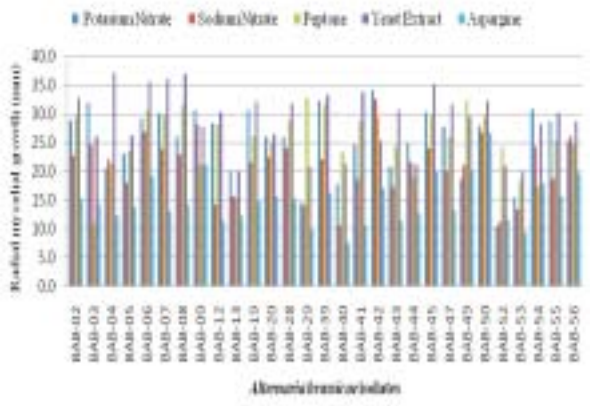

Figure 3: Effect of nitrogen sources on mycelial growth of *A. brassicae* isolates in Elliot's medium

of different nitrogen sources (Fig. 3). Isolates BAB-08, BAB-07, BAB-06, BAB-04, and BAB-45 respond maximum to yeast extract amended medium whereas, all isolates responds poorly to asparagine.

On the basis of myceial growth the different isolates were categorized in different groups. On Elliot's dextrose agar, BAB-49, BAB-50 were in Group-A. Maximum number of isolates stand in Group-B, 15 isolates BAB-02, BAB-04, BAB-05, BAB-08, BAB-09, BAB-12, BAB-13, BAB-19, BAB-28, BAB-29, BAB-45, BAB-47, BAB-52, BAB-55, and BAB-56. While, 7-isolates BAB-06, BAB-7, BAB-20, BAB-40, BAB-41, BAB-43, and BAB-44 were observed in Group-C. As evaluated above the isolate

BAB-54 showed minimum growth rate due to delay in initiation of growth was counted in Group-D (Fig. 3).

Effect of Hydrogen-ion (pH) concentraion

Hydrogen-ion concentration of the medium can affect the growth of the fungus in two ways. Externally, it can control the degree of dissociation of the inorganic ions in the culture solution. Since, dissociation plays a part in the movement of ions in the fungus, degree of dissociation will affect fungus growth. Internally, it can cause changes in pH in the mycelium. It is clear from the table 5 that all the isolates grew well within a range of pH 5 to 8, however, seven isolates including BAB-05 (8.4 mm), BAB-07 (6.3 mm), BAB-09 (14.4 mm), BAB-10 (14.8 mm), BAB-13 (11.1 mm), BAB-18 (9.4 mm), and BAB-24 (10.7 mm) were found sensitive to alkaline at pH 8 with poor growth. Although, the maximum mycelial growth was observed at pH 6 in isolates BAB-02 (41.5 mm), BAB-09 (40.7 mm), and BAB-11 (40.6 mm). Fungi differ considerably in their tolerance to different pH values (Table 5). The growth of fungi may be completely inhibited in media, which are either too acidic or too alkaline. Most of the fungi, however, tend to grow better on the acidic side. Singh (1980) studied the effect of pH on growth behavior of *A. brassicae*. Gupta *et al*. (1969) observed that mycelial growth of *A. brassicae* isolates occurred on a wide range of pH, i.e., 3.0-9.0. However, Lapis and Ricaforte (1974) observed profuse mycelial growth of *A. brassicae* at pH 6.0–10.0. During the present investigation, it was found that a wide range of pH, i.e. 5.0–8.0 supported mycelial growth of 24 *A. brassicae*

Table 5: Effect of different pH on mycelia growth of *A. brassicae* isolates on radish-root-mannitol agar medium

A.brassicae isolates	Mycelial growth (mm)			
	pH-5	pH-6	pH-7	pH-8
BAB-01	39.2	37.2	28.4	26.7
BAB-02	39.5	41.5	38.8	27.7
BAB-03	21.9	38.0	32.5	29.2
BAB-04	29.2	39.7	34.8	30.9
BAB-05	38.7	37.6	30.5	8.4
BAB-06	38.8	38.5	32.2	31.9
BAB-07	37.3	35.9	37.8	6.3
BAB-08	38.5	35.9	36.6	29.2
BAB-09	38.5	40.7	37.7	14.4
BAB-10	37.1	30.2	35.2	14.8
BAB-11	37.3	40.6	37.3	29.8
BAB-12	38.3	39.9	38.5	23.0
BAB-13	35.3	34.8	32.9	11.1
BAB-14	38.3	35.7	39.8	24.4
BAB-15	28.3	37.0	34.2	26.3
BAB-16	22.1	37.4	33.3	32.9
BAB-17	30.3	35.9	34.1	30.3
BAB-18	38.5	38.7	37.8	9.4
BAB-19	24.7	36.0	26.3	26.8
BAB-20	39.2	37.8	34.8	33.8
BAB-21	39.5	37.1	32.4	35.6
BAB-22	37.6	38.4	36.4	34.7
BAB-23	38.2	38.0	37.2	35.2
BAB-24	38.5	37.6	35.5	10.7
LSD (p=0.01)	3.6	3.6	4.2	3.3

isolates, respectively, while some isolates poorly grow at pH 8.0.

Effect of light

The light regime (4.5K lux) had a critical effect on culture growth, and produced the good amount of growth. Under continuous darkness, the cultures produced least mycelial growth. The cultures exposed to diurnal light showed zones with darker colour alternating with lighter colour. Highest growth under dark condition was recorded of BAB-29, BAB-56, and BAB-04 isolates, however least was in BAB-05, BAB-19, BAB-06, BAB-41, BAB-44, and BAB-55 isolates. Although, maximum growth under light regime was observed in BAB-45, BAB-06, BAB-09, BAB-29, and BAB-56 isolates (Fig. 4). Our findings supported with the earlier workers (Lapis and Ricaforte, 1974), but differ from the findings of Mukadam and Deshpande (1979).

Figure 4: Effect of light and dark on mycelial growth of different *A. brassicae* isolates

SDS-PAGE

In SDS-PAGE, total 55 bands (Plate 2) were observed of 10 isolates with Rm value ranged from 0.029 to 0.840. Band 1 was present only in BAB-41 which had very low intensity with lowest Rm value 0.029. Maximum numbers of band were observed in lane 12 of isolate BAB-54. Band 2 (Rm 0.040) was present in BAB-41 and BAB-47. Band 3 (Rm 0.066) was observed into the isolate BAB-41, BAB-44, and BAB-54. Band 4 (Rm 0.080) was present 6 isolates BAB-02, BAB-41, BAB-45, BAB-47, BAB-49, BAB-54. Band 5 (Rm 0.110) was present only in BAB-54, that showing monomorphic character of isolate BAB-54. Band 6 (Rm 0.139) was present in 6 isolates except BAB-40, BAB-41, BAB-47, BAB-49. Band 7

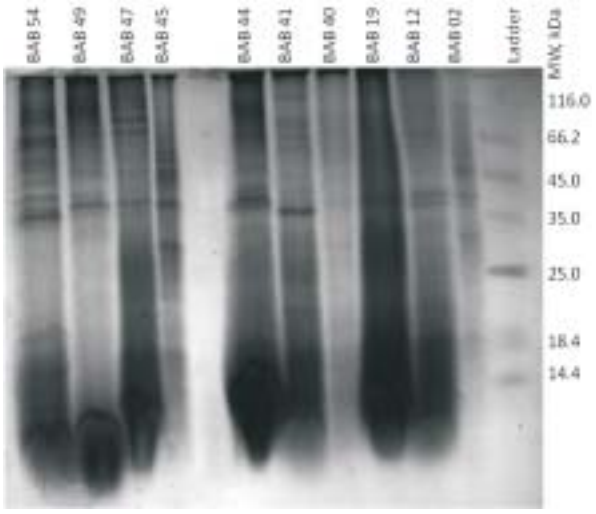

Plate 2: Protein profiling of *A. brassicae* isolates using SDS-PAGE

(Rm 0.176) was present in BAB-44, BAB-45, BAB-47, BAB-49, and BAB-54, that is showing the similarity in the isolates (Fig. 5).

Band 8 (Rm 0.205) was observed in BAB-2, BAB-12, BAB-19. Band 9 (Rm 0.228) was also present in 8 isolates viz., BAB-02, BAB-12, BAB-19, BAB-44, BAB-45, BAB-47, BAB-49, and BAB-54. Band 10 was (Rm 0.257) present in BAB-41, BAB-47, BAB-49, and BAB-54. Band 11 (Rm 0.038) was present only in 2 isolates BAB-02, and BAB-45. Band 12 (Rm 0.507) was present in 3 isolates BAB-12, BAB-40, BAB-41.

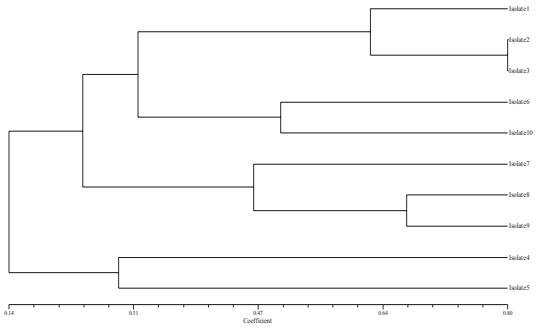

Figure 5: Unabridged dendrogram of the *A. brassicae* isolates based on similarity of banding patterns produced by cellular crude protein. Isolates 1-10 were BAB-02, BAB-12, BAB-19, BAB-40, BAB-41, BAB-44, BAB-45, BAB-47, BAB-49, and BAB-54.

Band 13 was (Rm 0.573) also unique band of isolate BAB-54, and showing the differentiation from other isolates. Band 14 was (Rm 0.602) most common band in pathogen isolates, and present in all isolate except BAB-45, BAB-47, and BAB-49. Band 15 was (Rm 0.647) present in 3 isolates of pathogen BAB-45, BAB-47, and BAB-54. Band 16 was (Rm 0.698) present in 2 isolates BAB-41, and BAB-54. Band 17 was (Rm 0.779) also present in only two isolates of pathogen BAB-47, and BAB-49. Band 18 was (Rm 0.846) also a unique band of BAB-54 of Rm 0.84. According to the results, 3 unique bands were present in isolate BAB-54, and a band was present in BAB-41. A high genetic divergence among *A. brassicae* isolates was also observed (Fig. 6). Results matched with the polymorphism within an *Alternaria* species by RAPD molecular marker has been described by many workers (Sharma and Tiwari, 1995, 1998; Kumar *et al*., 2008). Sharma and Tiwari (1995) observed polymorphism among *A. brassicae* isolates from different geographical regions of the world. However, Sharma and Tiwari (1998) found low intra-regional variation among Indian, and Canadian isolates of *A. brassicae* with 75% similarity.

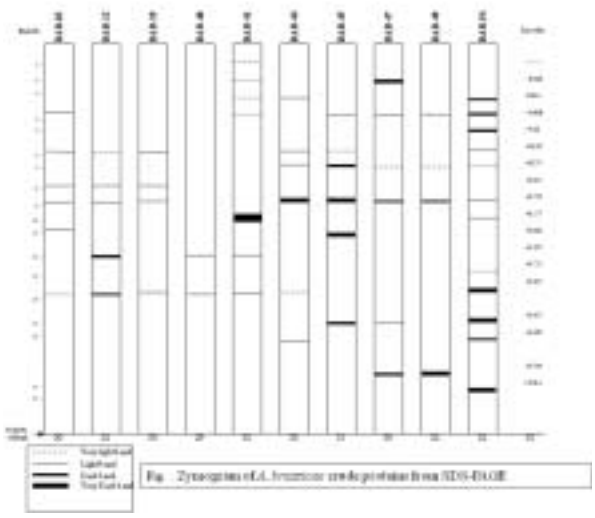

Figure 6: Zymogram showing cellular protein results of *A. brassicae* isolates

Conclusion

The comprehensive, and holistic study on the effect of diverse natural culture media, and different carbon as well as nitrogen sources on mycelial growth, pH, and light/dark demonstrated the existence of variability among isolates of *A. brassicae* from across the rapeseed-mustard growing region in India by clustering in groups is reported first time in this study. A high genetic divergence within *A. brassicae* isolates was proved by banding pattern recorded using SDS-PAGE. The identified aggressive isolates could be utilized for developing the resistance/ tolerance against Alternaria blight pathogen in Indian mustard.

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