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Pathogenic and molecular variability among *Brassica* isolates of *Alternaria brassicae* collected from different agro-climatic regions of India

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ABSTRACT

The Alternaria blight is one of the most destructive fungal disease of Indian mustard causes severe damage to the crop. Ten isolates of *A. brassicae* were collected from various agro-climatic location of India *viz.*, Uttar Pradesh (Ab_1), Madhya Pradesh (Ab_2), Uttarakhand (Ab_3), Bihar (Ab_4), Jharkhand (Ab_5), West Bengal (Ab_6), Haryana (Ab_7), Rajasthan (Ab_8), Chhattisgarh (Ab_9) and Gujarat (Ab_{10}) and characterized for pathogenic and molecular variations. All the isolates showed high level of variability. The incubation period of the isolates was recorded on *B. juncea* 3 to 4 days, *B. carinata* 6.17 to 6.83 days, *B. napus* 5.17 to 6.00 days, *B. nigra* 4.17 to 5.17 days and in *B. campestris* it was ranged from 3.17 to 4.00 days. The results revealed that the maximum PDI was noted on *Brassica juncea* followed by *B. campestris* var yellow *sarson*, *B. nigra*, *B. napus* and *B. carinata*. Based on PDI ten isolates could be classified into three groups in which group one consist of isolates Ab_3, Ab_6, Ab_7 and Ab_5. Isolates Ab_8, Ab_2 and Ab_4 fall in second group; while group three include isolates Ab_1, Ab_9 and Ab_{10}. The dendrogram analysis identified two major clusters with 82 per cent similarity. One cluster (group I) comprised of 3 isolates (Ab_1, Ab_{10} and Ab_2). Whereas, another cluster (group II) comprised of Ab_3, Ab_6, Ab_7, Ab_5, Ab_4, Ab_8 and Ab_9 at 86 per cent similarity. The three isolates (Ab_3, Ab_6 and Ab_7) of group II showed 100 per cent similarity based on molecular basis.

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INTRODUCTION

Alternaria blight disease of mustard caused by *Alternaria brassicae* (Berk), Sacc., and *A. brassicicola* (Schw.) Wilt., is one of the most destructive fungal disease causing significant qualitative and quantitative yield loss. The disease is more predominant in subtropical

and temperate countries. In India it is mostly cultivated in Rajasthan, Uttar Pradesh, Madhya Pradesh, Haryana, Gujarat, West Bengal and Assam (Anonymous, 2010). The yield loss due to this pathogen is upto 47 per cent (Singh *et al*., 2015). Alternaria blight severity and incidence on rapeseed -mustard differs between season to season, region to region and also individual crop to crop in (Sharma *et al.*, 2013 and Singh and Singh, 2014). The pathogen of rapeseed-mustard is very difficult to differentiate from other *Alternaria* spp., due to their similarity in morphological, cultural and pathogenic nature (Singh *et al*., 2014). Therefore, the present study was conducted to study the variability among *A. brassica* isolates using molecular markers to assess the genetic relationships among different isolates. The variability in *Alternaria* is very well known phenomenon and it may be noticed as changes in spore shape and size, growth and sporulation, pathogenicity, etc. Several researchers also studied about pathogenic and the genetic variation between *Alternaria* species by random amplified polymorphic DNA (RAPD) molecular marker (Kumar *et al*., 2011; Meena *et al*., 2011 and Bind *et al*., 2014).

MATERIAL AND METHODS

Pathogenic variability:

Pathogenic variability of ten isolates of *A. brassicae* were carried out in glass house of the Department of Plant Pathology, on five mustard verities (Table A) to study incubation period, number of spots on leaves and pods, size of spots and disease severity.

Table A : List of *Brassica* spp. for pathogenic variability

Common name	Botanical name	Local name
Indian mustard	*Brassica juncea*	Rai, Laha
Karan rai	*B. carinata*	Ethiopian mustard
Black mustard	*B. nigra*	Banarsirai
Gobhi*sarson*	*B. napus*	Gobhi*sarson*
Turnip rape	*B. campestris*	Yellow *sarson*

The seeds of five varieties of mustard were treated with Mancozeb @ 2.5 g per kg and sown in 15 cm diameter earthen pots containing sterilized soil @ 10 seeds/pot. They were allowed to grow for a month. In each pot, three plants were maintained in three replications. Spores from the colony were scraped in autoclaved distilled water separately from different isolates. Spore suspension having 20-25 conidia per microscopic field were inoculated with the help of automizer on 40 days old plants.

Incubation period:

The appearance of first symptoms was noted regularly after inoculating the plants by different isolates, subtracted from appearance of symptoms and date of inoculation was noted in days. The average number, size of spots/leaf from five leaves and disease intensity were alsorecorded at 15 days intervals (Fig. A and B).

Disease intensity:

For recording intensity of the disease, three plants were selected from each pot in three replications. Five leaves of selected plants were scored individually by using 0-6 scale, as suggested in the Proceedings of All India Co-ordinated Research Project on Rapeseed-Mustard Pathology, Planning and Review session-2009-10 given below (Anonymous, 2010). Per cent disease intensity (PDI) was calculated by using the following formula:

$$\text{PDI} = \frac{\text{Sum of total numerial ratings}}{\text{Total number of leaves examined x Maximum grade}} \times 100$$

Molecular variability amongdifferent isolates of *A. brassicae*:

Leaves of Indian mustard (*Brassica juncea* L.) infected by *Alternaria* spp. showing the characteristic symptoms were collected from various agro-climatic location of India *viz.*, Uttar Pradesh (Ab_1), Madhya Pradesh (Ab_2), Uttarakhand (Ab_3), Bihar (Ab_4), Jharkhand (Ab_5), West Bengal (Ab_6), Haryana (Ab_7), Rajasthan (Ab_8), Chhattisgarh (Ab_9) and Gujarat (Ab_{10}). Mycelium production was carried out by culturing the fungi in 100 ml of potato dextrose broth in 500-ml Erlenmeyer flasks and incubating them at 28°C for 6 days. Mycelial mats were then filtered under vacuum

Fig. A: Experimental overview

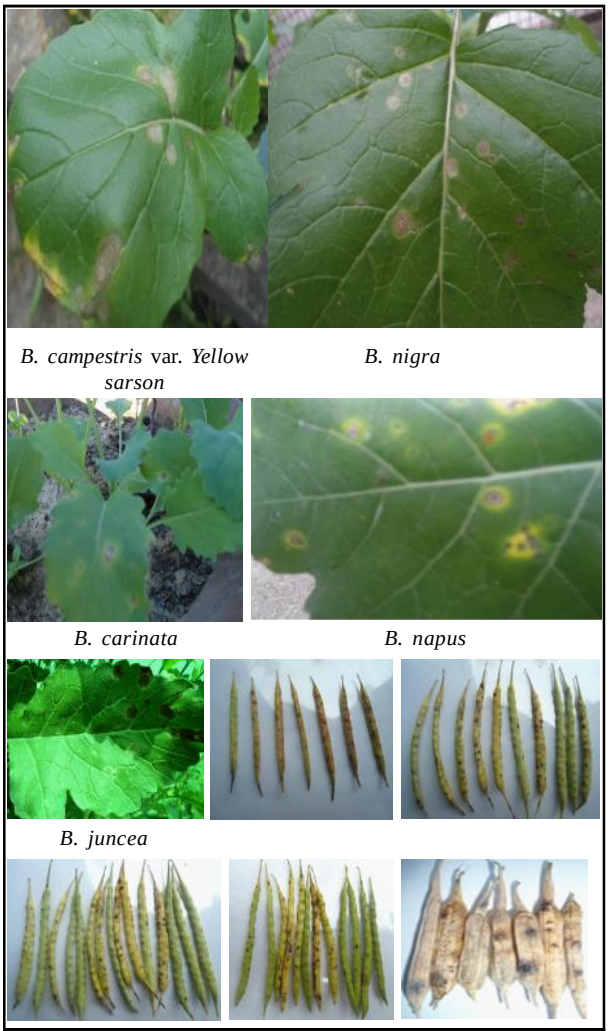

Fig. B: Spots on leaves and pods of different *Brassica* spp.

on a Buchner funnel, rinsed twice with distilled water and blotted dry. Mycelium to be used for isozyme and protein analyses was freeze-dried and stored at -20°C.

Preparation of protein extracts:

Soluble proteins were extracted by grinding 100 mg freeze-dried mycelium with pestle and mortar in liquid nitrogen and 5 ml buffer solution (0.1M Tris-HCl, pH 6.8). The mixture was centrifuged for 20 min at 6000 rpm and the supernatant collected. The protein content in supernatant was estimated with bovine serum albumin as standard protein. Protein content was adjusted to 2 mg/ml per sample (Kaur *et al*., 2015).

Preparation of stock solution, Buffer for gel electrophoresis:

Stock solution:

Acrylamide solution:

Acylamide 30%	=	29.2 g
Bis-acrylamide	=	0.8 g
Distilled water	=	100ml

10% SDS (Sodium dodecyl sulphate) solution:

SDS	=	1.0 g
Distilled water	=	10 ml

Staining solution *:*

Coomassie brilliant blue (R – 250)	=	1.25 g
Methanol	=	200 ml.
Acetic acid	=	50 ml.
Distilled water	=	250 ml.

Note: Coomassie dye dissolved in methanol first and stir up to dissolved then acetic acid.

De-staining solution:

Methanol	=	200 ml.
Acetic acid	=	50 ml.
Distilled water	=	250 ml.

Buffer solution :

Separating gel buffer (pH-8.8):

1.875 M Tris	=	22.7 g
Distilled water	=	250 ml.

pH was adjusted by adding NaOH / HCl and made the final volume 100 ml. Buffer was stored in dark bottle at 4^0C

Stacking gel buffer (pH- 6.8):

0.6 M Tris	=	7.26 g
Distilled water	=	100 ml.

pH was adjusted by adding NaOH / HCl and made the final volume 100 ml. Buffer was stored in dark bottle at 4^0C.

Electrode buffer (pH- 8.2):

0.025 M Tris buffer	=	3.0 g
0.192 M Glycine	=	14.0 g
0.1% SDS	=	1.0 g
Distilled water	=	1000 ml.

Sample loading dye (pH- 6.8) :

10.0% SDS	=	0.5 g
β- Mercaptoethnol	=	1.0 ml.

Stacking gel buffer = 5.0 ml.
Glycerol = 10 ml.
Bromophenol blue = 20 mg.

Dissolve the ingredients and made total volume of 100 ml. stored at a 4^0C.

Agar solution- 2 per cent:

Aga = 20 g
Distilled water = 1000 ml.

Agar was added in water and boiled until dissolved.

Gel electrophoresis of the pathogen:

Preparation of sample for gel electrophoresis:

The fungus (*Alternaria brassicae*) was grown in potato dextrose broth in 500 ml flasks at room temperature in darkness. The mycelium was harvested after 7 days, washed four times with autoclaved water, blotted to remove excess water, lyophilized and stored at 4^0 C until further processing.

Protein extraction:

Total proteins from mycelia of *Alternaria* spp. were extracted in 5ml. phosphate buffer (pH- 7.0). Took 2 gram dried mycelium and macerated in pestle mortar. Crude sap was filtered through muslin cloth and filtrate was centrifuged at 6000 rpm for 20 minutes at 4^0 C until for further use.

Loading of gel:

Prior to the loading of sample on gel, the sample loading dye was added to the sample were denatured by putting on water bath for 6 minutes and cooled at room temperature and finally chilled on ice prior to loading on gel.

Casting of gel:

One (8×7 cm) vertical gel system (Genie) was used for carrying out the gel electrophoresis experiment.

The gel plate and spacers were cleaned using ethanol, dried and then assembled properly. The chambered was sealed using 2 per cent water agar.

The separating gel mixture (10%) was prepared by mixing the following compound one by one carefully-

– 30 per cent Acrylamide solution 3.3ml.
– 1.5 M Tris buffer (pH- 8.8) 2.5ml.
– 10% SDS (Sodium dodecyl sulphate) 100µl.
– 10% APS (Ammonium per sulphate) 100µl.
– Distilled water 40µl.
– Temed 15µl.

Gel solution was poured in the chamber between glass plates without generating air bubbles and leaving space from the top and allowed to polymerization for 1.5 hours.

After polymerization 4% stacking gel (3ml.) mixture was prepared by mixing the following compound.

– 30% Acrylamide solution 0.50ml.
– 1.5 M Tris buffer (pH- 6.8) 0.38ml.
– 10% SDS (Sodium dodecyl sulphate) 100µl.
– 10% APS (Ammonium per sulphate) 100µl.
– Distilled water 40µl.
– Temed 15µl.

The stacking gel solution was shaken well and remaining portion of the chamber was filled with stacking gel solution. The comb was immediately placed in the stacking and allowed to polymerization for 1 hour. After polymerization of stacking gel, comb was removed carefully without distorting the shape of the well. The buffer tank was then filled with electrode buffer (Sharma *et al.,* 2013).

Running of gel:

The standard protein marker (Genie, 14.3- 97 kDa) and loading dye was mixed at 1:2 ratio. Marker and sample were loaded separately with the help of micro pipettes. After proper loading the electrophoresis unit was connected with power supply and gel was run at 40 V and after one hour the voltage was increase to 80 V. When the bromophenol blue reached the end of the gel, the power supply was turned off.

Staining and de-staining of gel:

After the run was completed, the gel was separated carefully from the gel plates and immersed in staining solution for five hours on slowly continuous shaker and then transferred in a tray containing de-staining solution.

De-staining solution was frequently changed until the background of the gel become colorless; the gel was photographed and documented with the help of densitometer. The gel stored storage solution for future use.

RESULTS AND DISCUSSION

The interaction between predator and prey mite was presented under the following headings:

Pathogenic variability of different isolates of *A. brassicae:*

Incubation period on *B. juncea* ranged from 3 to 4 days. It was maximum in isolate Ab_{10} (4.00) followed by Ab_9 (3.83), Ab_8 (3.83), Ab_5 (3.67), Ab_2 (3.67), Ab_1 (3.67), Ab_4 (3.5), Ab_6 (3.17), Ab_7 (3.00) and Ab_3 (3.00). Isolates Ab_9, Ab_8, Ab_5, Ab_2, Ab_1 and Ab_4 and Ab_6, Ab_7 and Ab_3 were at par with respect to incubation period. However, it was differed significantly by isolate Ab_{10} to other isolates. In *B. carinata* Incubation period ranged from 6.17 to 6.83 days. The incubation period was maximum in isolate Ab_{10} (6.83) followed by Ab_9 (6.83), Ab_8 (6.67), Ab_5 (6.67), Ab_4 (6.67), Ab_1 (6.67), Ab_2 (6.5), Ab_6 (6.33), Ab_7 (6.17) and Ab_3 (6.17). Incubation period non significantly differed among isolates Ab_{10}, Ab_9, Ab_8, Ab_5, Ab_4 and Ab_1 and Ab_2, Ab_6, Ab_7 and Ab_3. In *B. napus*it was ranged from 5.17 to 6.00 days. It was maximum in isolate Ab_{10} (6.00) followed by Ab_5 (5.83), Ab_9 (5.67), Ab_8 (5.67), Ab_4 (5.67), Ab_1 (5.67), Ab_2 (5.5), Ab_6 (5.33), Ab_7 (5.17) and Ab_3 (5.17). Incubation period did not differ among isolates Ab_{10}, Ab_5, Ab_9, Ab_8, Ab_4 and Ab_1 and Ab_2, Ab_6, Ab_7 and Ab_3. In *B. nigra* incubation period ranged from 4.17 to 5.17 days. The incubation period was maximum in isolate Ab8 (5.17) followed by Ab_{10} (4.83), Ab_9 (4.83), Ab_5 (4.83), Ab_4 (4.67), Ab_1 (4.67), Ab_2 (4.5), Ab_6 (4.33), Ab_7 (4.17) and Ab_3 (4.17). There

Table 1 : Incubation period of different isolates of *A. brassicae* on *Brassica* spp.

Isolate	Incubation period (days)							
	B. juncea	*B. carinata*	*B. napus*	*B. nigra*	*B. campstris* var. Yellow *sarson*	S.E.±	C.D. (P=0.05)	CV
Ab_1	3.67	6.67	5.67	4.67	3.83	0.16	0.52	5.89
Ab_2	3.67	6.50	5.50	4.50	3.83	0.10	0.33	3.80
Ab_3	3.00	6.17	5.17	4.17	3.17	0.14	0.46	5.95
Ab_4	3.50	6.67	5.67	4.67	3.67	0.14	0.46	5.34
Ab_5	3.67	6.67	5.83	4.83	3.67	0.16	0.52	5.85
Ab_6	3.17	6.33	5.33	4.33	3.33	0.16	0.52	6.41
Ab_7	3.00	6.17	5.17	4.17	3.17	0.14	0.46	5.95
Ab_8	3.83	6.67	5.67	5.17	3.83	0.16	0.52	5.73
Ab_9	3.83	6.83	5.67	4.83	4.00	0.14	0.46	5.12
Ab_{10}	4.00	6.83	6.00	4.83	3.83	0.12	0.40	4.38
S.E.±	0.13	0.16	0.15	0.16	0.16	-	-	-
C.D. (P=0.05)	0.38	0.47	0.44	0.47	0.47	-	-	-
CV	6.33	4.18	4.64	5.94	7.54	-	-	-

Table 2 : Per cent disease intensity of different isolates of *A. brassicae* on *Brassica* spp.

Isolates	Percent Disease Intensity (PDI)							
	B. juncea	*B. carinata*	*B. napus*	*B. nigra*	*B. campestris* var. *Yellow sarson*	S.E.±	C.D. (P=0.05)	CV
Ab_1	46.90	14.76	19.50	46.16	54.60	1.25	3.95	6.46
Ab_2	42.90	16.40	20.03	44.23	57.23	0.77	2.44	4.16
Ab_3	62.60	20.76	26.10	54.06	60.36	2.36	7.45	9.15
Ab_4	50.26	17.20	19.30	46.83	52.66	1.49	4.71	7.06
Ab_5	50.76	17.73	19.93	50.26	54.56	1.33	4.21	6.24
Ab_6	59.46	20.10	22.43	58.26	60.30	1.14	3.61	4.63
Ab_7	58.00	18.23	22.90	56.56	62.31	2.06	6.49	8.36
Ab_8	47.00	14.83	21.00	49.50	56.73	0.50	1.57	2.47
Ab_9	51.50	15.86	18.86	42.76	54.33	2.13	6.73	10.29
Ab_{10}	47.50	16.20	18.56	44.76	53.91	0.88	2.79	6.73
S.E.±	0.99	0.83	0.99	1.26	1.64	-	-	-
C.D. (P=0.05)	2.92	2.45	2.92	3.74	4.85	-	-	-
CV	3.73	5.90	6.32	4.92	5.82	-	-	-

was no significant difference in terms of incubation period among isolates Ab_{10}, Ab_9, Ab_5, Ab_4 and Ab_1 and Ab_2, Ab_6, Ab_7 and Ab_3. However isolate Ab_8 significantly differed to other isolates. Incubation period in *B. campestris* ranged from 3.17 to 4.00 days. The incubation period was maximum in isolate Ab9 (4.00) followed by Ab_{10} (3.83), Ab_8 (3.83), Ab_2 (3.83), Ab_1 (3.83), Ab_5 (3.67), Ab_4 (3.67), Ab_6 (3.33), Ab_7 (3.17) and Ab_3 (3.17). Incubation period did not differ among isolates Ab_9, Ab_{10}, Ab_8, Ab_2, Ab_1, Ab_5 and Ab_4 and Ab_6, Ab_7 and Ab_3. The incubation period was maximum in, *B. carinata,* followed by, *B. napus*, *B. nigra*, *B. campestis* var. yellow sarson and *Brassica juncea*. These results are in agreement with earlier workers.Mehta *et al.* (2003) observed that pathogenic variability among *A. brassicae* on rapeseed and mustard isolates from different agro-climatic zones in India. The isolates differed in incubation period varied from 3- 13 days in isolates but the majority generally took 3-5 days to cause infection. Meena *et al.* (2011) have also reported isolates of *A. brassicae* showed variable response on host differentials of *Brassica* species.

Molecular variability with protein profiling (SDS-PAGE):

The 10 different isolates were electrophoresed on SDS- PAGE (12%). The various types of bands visible on gel, the dendrogram (Fig. 1) were generated by using NTSYS PC (1.02) version. The dendrogram identified two major clusters with 82 per cent similarity. One cluster (group I) comprised of 3 isolates (Ab_1, Ab_{10} and Ab_2). Whereas, another cluster (group II) comprised of Ab_3, Ab_6, Ab_7, Ab_5, Ab_4, Ab_8 and Ab_9 at 86 per cent similarity (Fig. 2). The three isolates (Ab_3, Ab_6 and Ab_7) of group

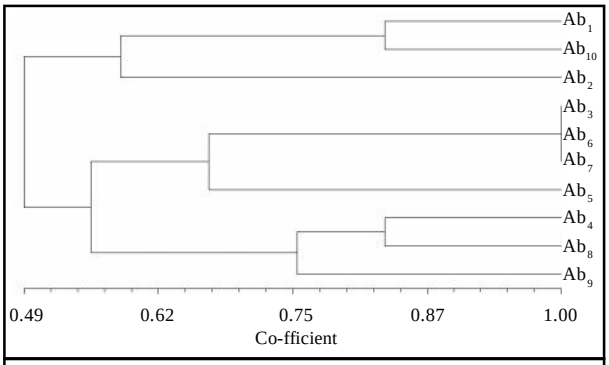

Fig. 1: Dendrogram generated based on banding pattern of SDS- PAGE of different isolates of *Alternaria brassicae*

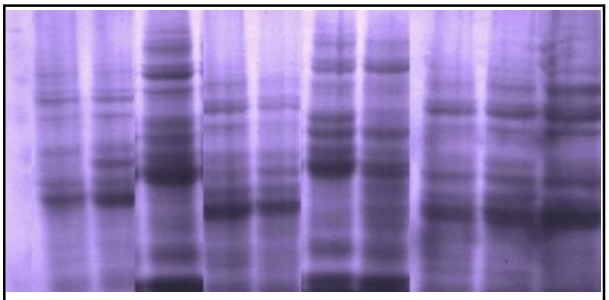

Fig. 2: SDS-PAGE protein bands of different isolate of *Alternaria brassicae*

II showed 100 per cent similarity. Molecular variability was also observed by Sharma *et al.* (2013) studied the cluster analysis of data on molecular characteristicamong thirty two *A. brassicae* isolates found a close relationship among isolates. These isolates characterized by using internal transcribed spacer region where all the isolates were found 56 per cent similar to each other and 99 per cent similar to the *A. brassicae* isolates presented in NCBI database. Pramila *et al*. (2014) studied molecular variability of ten isolates of *A. brassicae* based on dendrogram analysis.

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