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Ph D Thesis Award

Studies on cultural, morphological, pathogenic and molecular variability of *Alternaria brassicae*, the causal agent of blight disease of rapeseed-mustard

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Abstract

In India, rapeseed-mustard is one of the most edible oilseed crops after groundnut and contributes around 26.1 per cent of the total oilseed production. Among Brassica oil seed crops, Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is commonly cultivated in most of the Indian states. Alternaria blight disease caused by *A. brassicae* has been reported from all the continents of the world causing up to 47 per cent yield losses with no proven source of resistance. The present investigation was carried out to study the cultural, morphological, pathogenic and molecular variability among *A. brassiace* isolates collected from different *Brassica* spp. (Pantnagar) and from different geographical locations of India (Karnal, Punjab, Bihar, Jammu and Kangra) to exploit them in breeding programme during screening for resistance sources and selection of host differentials. In the present studies *A. brassicae* isolates (20 nos.) showed the existence of genetic diversity. Significant variation in cultural, morphological, pathogenic and molecular variability was observed in *A. brassiace* isolates irrespective to geographical locations and *Brassica* spp. Maximum radial growth (82.0 mm) was in AB-*B. juncea* Pantnagar isolate, while minimum in AB- *B. caulorapa* (49.7 mm) on PDA. Variations were also observed in colony colour, appearance, margin and zonation number. Substantial variations were found in spore morphology in respect to conidial length, width and number of septa. Average conidial length and width were varied from 29.0-6.6 x 185.3-28.2µm. Maximum spore length and width was in AB-*B. carinata* isolate (185.3 x 25.6 µm), while minimum in AB-*B. caulorapa* (29.0 x 6.6µm). Number of horizontal and vertical septa ranged between 3.50-14.75 and 0.75-5.0 respectively. All the isolates were pathogenic to *Brassica* spp. AB-*B. juncea* Pantnagar isolate was most virulent, while AB-*S. alba* least virulent. RAPD (18 no.) and ISSR (4 no.) primers generated a total of 310 reproducible and scorable polymorphic bands ranged from 100 to 1350 bp which displayed genetic polymorphisms among the *A. brassicae* isolates. On the basis of cultural, morphological, pathogenic and molecular variability, the *A. brassiace* isolates has been categorized into 5 major groups. Group I: AB-*R. sativus*, AB-*B. pekinensis*, AB-*B. oleracea*, AB-*B. botrytis* and AB-*B. caulorapa*; Group II**:** AB-*B. juncea* Pantnagar, AB-Yellow sarson, AB-Toria, AB-*B. nigra*, AB-*R. oleifera*, AB-*B. juncea* Karnal, AB-*B. juncea* Punjab and AB-*B. juncea* Bihar; Group III**:** AB-*B. napus*, AB-*B. carinata*, AB-*E. sativa* , AB-*S. alba*; Group IV**:** AB-*B. juncea* Kangara and AB-*B. juncea* Jammu; Group V**:** AB-*B. rugosa*. The different *Brassica* spp. (08 nos.) used in the present investigation to differentiate different *A. brassicae* isolates (09 nos.) as host differentials has been categorized into 4 major groups on the basis of differential phenotypic disease reactions. Group I**:** Varuna, PT-303, PYS-6 and *B. nigra*; Group II**:** PBN-9501 and Kiran; Group III**:** *E. sativa*; Group IV**:** *S. alba*. Among different methods of culture preservation of *A. brassicae* isolates, 10 per cent glycerol at -20ºC was best in terms of sporulation (66.7%) followed by PDA at 4ºC (65.7%) and could be used for the preservation of *A. brassicae* isolates at least two years**.**

Key words: *Alternaria brassicae, cultural, morphological, pathogenic and molecular variability*

Introduction

In India, rapeseed-mustard is an important group of edible oilseed crops that contributes around 26.1 per cent of the total oilseed production. Indian mustard (*B. juncea*) is one of the major oilseed crops cultivated in India. Among various diseases Alternaria blight caused by *A. brassicae* has been reported from all the continents of the world. It is one of the most important diseases of mustard causing up to 47 per cent yield loss (Meena *et al.,* 2010) with no proven source of resistance till date. Studies on pathogen variability have to be the foundation for development of pre-breeding populations as strategic defense mechanism.

Severity of Alternaria blight on oilseed Brassica differs among regions and also Brassica crops within a region might be due to existence of variability among the isolates of *A. brassicae*. The existence of pathogen diversity among *A. brassicae* isolates has already been reported by earlier workers. Different isolates of *A. brassicae* showed variable response on different *Brassica* spp./genotypes. Significant tolerance was observed in *B.alba,* EC-399299, PAB 9511 (*B. juncea*), *Eruca sativa, B. carinata* and *B. napus*. Variation in tolerance and susceptibility on same host depending on aggressiveness of isolates revealed the existence of variability among *A. brassicae* isolates. PCR-based marker techniques viz. RAPD and ISSR have been used extensively for genetic variation in populations of *A. brassicae* isolates.

Since mid 90's, Alternaria blight disease of crucifers is being managed by the use of fungicides. Due to deleterious effects of fungicides, the focus now lays on the development of resistant varieties which is one of the most eco-friendly, economic, and effective method for the management of the disease. Sources of resistance has been reported but their utility and effectiveness in breeding for disease resistance cultivars is limited due to lack of information on the occurrence and distribution of pathotypes and suitable genotypes screening technique using different races of *A. brassicae*.

In view of the above facts the present investigation on study of cultural, morphological, pathogenic and molecular variability was undertaken to find out variability among *A.brassicae* isolates collected from different *Brassica* spp. and from different growing locations of India so that the isolates could be classified in different groups and further to be exploited for the testing of resistant sources in *Brassica* spp. Grouping of different *Brassica* spp. as host differentials may also be helpful to differentiate different *A. brassicae* isolates.

Materials and Methods

Collection, isolation and purification

A. brassicae isolates (20 no.) collected from different *Brassica* spp. (Pantnagar) during the crop season of 2013-14 and from different growing locations of India (Karnal, Punjab, Bihar, Jammu and Kangra) were cultured on PDA and further purification was done using single spore isolation. The culture was preserved in the refrigerator (4°C) for further studies (Table 1).

Cultural variability

Eight different media (Potato dextrose, Radish root dextrose, Cauliflower dextrose, Carrot potato dextrose, Mustard seed dextrose, Maize seed dextrose, Brassica leaf extract and Modified V-8 juice agar) were tested to determine their effect on growth of *A. brassicae* isolates. The observations on radial growth (mm), colony color, shape, margin, zonation number and appearance of each isolate on different media were recorded 10 DAI at 24±1°C.

Morphological variability

Ten conidia of each isolates were examined at 10X and 40 X under advance light microscope. The conidial length, width and beak (µm) were measured and number of horizontal and vertical septa and number of cells were recorded.

Pathogenic variability

The experiment was conducted in plastic pots under glasshouse during two consecutive seasons 2014-15 and 2015-2016. Forty days old plants (*B. juncea* var. Varuna) inoculated with conidial suspension ($4x10^4$conidia/ml) of each *A. brassicae* isolates separately by single drop inoculation method (10µl/leaf) were kept in moist chamber for 48 hrs under glasshouse. The observations on spot and periphery

Table 1: *Altenaria brassicae* isolates collected from different *Brassica* spp. and from different geographical locations

A. brassicae isolate	Host	Location	Latitude & Longitude	Plant part
AB- *B. juncea* Pantnagar	*B. juncea* cv. Varuna	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *B. napus*	*B. napus* cv. PBN-9501	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *B. carinata*	*B. carinata* cv. Kiran	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- Toria	*B. rapa var.* toria cv. PT-303	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- Yellow sarson	*B. rapa var.* yellow sarson cv. PYS-6	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *E. sativa*	*Eruca sativa* (local)	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *S. alba*	*Sinapis alba* (local)	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *R. oleifera*	*Raphanus sativus* var. *oleifera* (local)	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *B. nigra*	*B. nigra* (local)	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *B. rugosa*	*Brassica rugosa* (local)	Pantnagar, CRC	29^0N, 79.8^0E	leaf
AB- *R. sativus*	*Raphanus sativus*	Pantnagar, VRC[#]	29^0N, 79.8^0E	leaf
AB- *B. pekinensis*	*Brassica rapa* var. *pekinensis*	Pantnagar, VRC	29^0N, 79.8^0E	leaf
AB- *B. oleracea*	*Brassica oleracea* var. *capitata*	Pantnagar, VRC	29^0N, 79.8^0E	leaf
AB- *B. botrytis*	*Brassica oleracea* var. *botrytis*	Pantnagar, VRC	29^0N, 79.8^0E	leaf
AB- *B. caulorapa*	*Brassica caulorapa*	Pantnagar, VRC	29^0N, 79.8^0E	leaf
AB- *B. juncea* Karnal	*Brassica juncea*	Karnal	28^0N, 77^0E	leaf
AB- *B. juncea* Punjab	*Brassica juncea*	Punjab	31.1^0N, 75.3^0E	leaf
AB- *B. juncea* Bihar	*Brassica juncea*	Bihar	25^0N, 85.3^0E	leaf
AB- *B. juncea* Jammu	*Brassica juncea*	Jammu	32.4^0N, 74.5^0E	leaf
AB- *B. juncea* Kangara	*Brassica juncea*	Kangara	32^0N, 76.18^0E	leaf

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color, presence or absence of concentric rings, central point, yellow halo region and disease index (0-9 scale) were recorded at 50 DAI.

Molecular variability

DNA of twenty *A. brassicae* isolates was isolated by adapting Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method (Doyle and Doyle, 1990). Molecular variability among *A. brassicae* isolates were studied by using RAPD (20 no.) and ISSR primers (05 no.) manufactured by Eurofins mwg operon (Eurofins genomics Pvt. ltd, Bangalore, India).

In RAPD, the polymerase chain reaction (PCR) was performed in 20 µl volumes containing 1 µl DNA (50 ng/ µl), 1 µl primer (50 ng/ µl), 2.0 µl of 10X PCR buffer, 1.2 µl of 25 mM $MgCl_2$, 0.75 µl *Taq* polymerase (1 U) and 1.0 µl dNTPs mix (10mM). Forty PCR amplification cycles were carried out in PCR machine [Eppendorf, Germany; model: Mastercycler(R) family] by denaturation at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1 min. Each PCR amplification reaction was preceded by an initial denaturation at 94°C for 5 min. followed by final extension at 72°C for 7 min. The amplified products were separated by electrophoresis in 1.5% (w/v) agarose (Genei, Bangalore) gel with 1X TBE buffer, stained with ethidium bromide (0.5 µg/ml) at 90 V for 3.0 to 3.5 h and photographed using gel documentation system (Alpha Innotech, USA Alpha Innotech, USA; model: AlphaImagerTM 3400). The sizes of the amplification product were estimated using 100 bp to 3.0 kb ladder (Ô × 174 DNA/ BsuRI (Hae III), Fermentas.

In ISSR, the polymerase chain reaction (PCR) was performed in 20 µl volumes containing 1 µl DNA (50 ng/ µl), 1 µl primer (50 ng/ µl), 2.0 µl of 10X PCR buffer, 1.2 µl of 25 mM $MgCl_2$, 0.75 µl *Taq* polymerase (1 U) and 1.0 µl dNTPs mix (10mM). Forty PCR amplification cycles were carried out in PCR machine [Eppendorf, Germany; model: Mastercycler (R) family] by denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 2 min. Each PCR amplification reaction was preceded by an initial denaturation at 94°C for

5 min followed by final extension at 72°C for 7 min. The amplified products were separated by electrophoresis in 1.5% (w/v) agarose (Genei, Bangalore) gel with 1X TBE buffer, stained with ethidium bromide (0.5 ìg/ml) at 90 V for 3.0 to 3.5 h and photographed using gel documentation system (Alpha Innotech, USAAlpha Innotech, USA; model: Alpha Imager TM 3400). The sizes of the amplification product were estimated using 100 bp to 3.0 kb ladder (Ô × 174 DNA/ BsuRI (Hae III), Fermentas. All the reactions were repeated in at least two independent experiments. All the amplified bands were scored as present or absent for each DNA sample and further, to analyze the relatedness among the species, a similarity matrix was generated from the binary data using DICE similarities coefficient in SIMQUAL program of NTSYS-PC Package (Rohlf, 2000). Cluster analysis was done with the Unweighed Pair Group Method with Arithetic Mean (UPGMA) in the SAHN program of NTSYS-PC Package, and constructed dendrogram based on genetic distances.

Selection of host differentials

Eight different *Brassica* spp. viz. Varuna (*B. juncea),* PBN-9501(*B. napus*), Kiran (*B. carinata), Sinapis alba* (local), PT-303 (*B. rapa var*. toria), PYS-6 (*B. rapa var.* yellow sarson), *Eruca sativa* (local), and *B. nigra* (local) were cross inoculated with each nine *A. brassicae* isolates collected from different *Brassica* spp. *B. juncea, B. napus, B. carinata,* Toria, Yellow sarson, *B. nigra, E. sativa, S. alba* and *R.oleifera* by single drop inoculation method (10 µl/leaf) at 40 DAS. The plants were kept in glasshouse under suitable environmental conditions**.** The observations on phenotypic disease reactions and percent disease index on each *Brassica* spp. against each isolate were recorded at 50 DAI. Based on different phenotypic disease reactions, the different *Brassica* spp. will be categorized in different groups as host differentials.

Long term preservation methods of A. brassicae

One year old *A. brassicae* isolates preserved in PDA alone (4^0C), 10 per cent glycerol (-20ºC) and 5 per cent DMSO (-20ºC) collected from Amity University, Noida were used for the study of their sporulation *in vitro* and pathogenecity in glasshouse in two successive years. All thirty five *A. brassicae* isolates were re-cultured on PDA. The observations on mycelial growth and sporulation were recorded (0 days after incubation (DAI). The pathogenic virulence was tested on Varuna cultivar grown in pots under glasshouse by inoculating with the conidial suspension of each *A. brassicae* isolates separately and kept in glasshouse under suitable environmental conditions**.** The observations on disease severity against each isolates were recorded using 0-9 scale and per cent disease index was calculated.

Statistical analysis

Data obtained on various traits under laboratory and glasshouse experiments were assessed using STPR software and R-studio software.

Results and Discussion

Cultural variability

Twenty *A. brassicae* isolates showed variable cultural characteristics viz. radial growth, colony colour (olive green, whitish gray, grayish, dark green, olive gray, whitish black, dark gray and greenish), shape (circular and irregular), zonation number and appearance (compressed and fluffy) on PDA. On the basis of colony colour i.e. olive green, whitish gray, grayish, dark green, olive gray, whitish black, dark gray and greenish, the isolates were categorized into 8 groups viz. Group I (olive green): AB-*B. carinata*, AB-Toria, AB-*R. oleifera*, AB- *B. juncea* Karnal, AB-*B. juncea* Kangara and AB-*B. botrytis*; Group II (whitish gray): AB-Yellow sarson, AB-*B. rugosa*, AB-*B. juncea* Bihar and AB-*B. juncea* Jammu; Group III (grayish): AB-*B. juncea* Pantnagar, AB-*B. juncea* Punjab and AB-*B. pekinensis*; Group IV (dark green): AB-*S. alba* and AB-*B. caulorapa*; Group V (olive gray): AB-*B. napus* and AB-*E. sativa*; Group VI (whitish black) AB-*B. nigra*, Group VII (dark gray) AB-*R. sativus*; Group VIII (greenish) AB-*B. oleracia* (Table 2; Fig 1). The present results are in accordance with Kaur *et al.* (2007), Sharma *et al.* (2013), Pramila *et al*. (2014) and Saha *et al*. (2015) who reported cultural variability among different *A. brassicae* collected from different geographical regions of India on the basis of colony colour, shape, zonation number and appearance.

Table 2: Cultural characters of *A. brassicae* isolates on Potato dextrose agar medium

A. brassicae isolate	Colony colour	Growth	Shape	Margin	Zonation (No.)	Appearance
AB-*B. juncea* Pantnagar	grayish	fast	circular	smooth	2	fluffy
AB-*B. napus*	olive gray	medium	circular	smooth	2	fluffy
AB-*B. carinata*	olive green	medium	circular	smooth	2	compressed
AB-Toria	olive green	medium	irregular	smooth	3	compressed
AB-Yellow sarson	whitish gray	medium	irregular	smooth	4	fluffy
AB-*E. sativa*	olive gray	medium	irregular	smooth	2	compressed
AB- *S. alba*	dark green	medium	irregular	smooth	3	compressed
AB- *R. oleifera*	olive green	medium	circular	smooth	-	compressed
AB-*B. nigra*	whitish black	medium	circular	smooth	-	compressed
AB-*B. rugosa*	whitish gray	medium	irregular	smooth	-	compressed
AB-*B. juncea* Karnal	olive green	fast	circular	smooth	-	compressed
AB-*B. juncea* Punjab	grayish	medium	irregular	smooth	-	fluffy
AB-*B. juncea* Bihar	whitish gray	medium	circular	smooth	4	compressed
AB-*B. juncea* Jammu	whitish gray	medium	irregular	smooth	2	compressed
AB-*B. juncea* Kangara	olive green	medium	circular	smooth	-	compressed
AB-*R. sativus*	dark gray	medium	irregular	smooth	2	fluffy
AB-*B. pekinensis*	grayish	medium	irregular	smooth	-	fluffy
AB-*B. oleracea*	greenish	slow	circular	smooth	-	compressed
AB-*B. botrytis*	olive green	medium	irregular	smooth	2	compressed
AB-*B. caulorapa*	dark green	slow	irregular	smooth	2	compressed

Fig 1: Cultural characteristics of *A. brassicae* isolates on PDA medium

Table 3: Radial growth of *A. brassicae* isolates on different solid media

A. brassicae isolate	Radial growth (mm)								
	Potato dextrose	Radish dextrose	Cauli flower dextrose	Carrot Potato dextrose	Mustard seed leaf	Brassica dextrose seed	Maize extract dextrose	Modified V-8 juice	Mean
AB-*B. juncea* Pantnagar	82.0	72.7	71.7	70.3	74.3	64.3	68.7	60.7	76.9
AB-*B. napus*	68.0	61.3	63.3	42.0	65.0	48.3	45.0	47.0	64.3
AB-*B. carinata*	63.0	72.0	65.7	67.3	67.7	50.0	65.7	52.3	67.9
AB-Toria	80.0	73.3	67.3	54.7	71.0	69.0	57.7	65.3	76.34
AB-Yellow sarson	76.0	70.3	66.0	64.7	72.0	65.7	61.0	48.3	72.9
AB-*E. sativa*	63.3	68.3	62.0	62.7	60.3	63.3	50.3	49.7	66.1
AB- *S. alba*	62.3	44.7	48.0	59.7	57.7	62.7	46.7	59.7	52.6
AB- *R. oleifera*	68.0	57.7	57.3	60.7	52.7	60.3	52.7	51.0	62.3
AB-*B. nigra*	70.3	69.0	64.7	70.0	69.7	59.7	66.3	42.0	69.6
AB-*B. rugosa*	68.0	75.7	69.0	55.0	53.7	58.7	63.3	55.7	72.2
AB-*B. juncea* Karnal	78.7	72.7	69.7	60.3	64.0	65.7	64.0	62.3	75.4
AB-*B. juncea* Punjab	76.7	56.3	68.7	70.0	62.0	68.3	58.0	48.0	65.5
AB-*B. juncea* Bihar	70.7	62.7	54.7	69.0	68.3	59.7	42.0	62.7	66.3
AB-*B. juncea* Jammu	70.3	63.3	60.3	62.0	60.0	63.7	66.7	61.7	66.5
AB-*B. juncea* Kangara	66.0	67.7	42.0	53.3	55.0	47.7	57.7	59.7	66.9
AB-*R. sativus*	71.0	70.3	65.0	62.7	64.7	62.7	62.7	55.7	70.6
AB-*B. pekinensis*	67.7	66.7	43.3	64.7	68.3	60.0	58.7	61.7	67.1
AB-*B. oleracea*	53.3	66.3	64.0	61.7	62.7	68.0	46.3	62.0	60.5
AB-*B. botrytis*	69.3	62.3	66.3	62.0	55.0	62.7	47.7	53.0	65.5
AB-*B. caulorapa*	49.7	61.0	50.3	68.7	51.3	55.0	48.3	50.0	55.9
CD 5%	4.3	3.8	3.3	3.5	4.3	3.2	3.3	3.3	2.5
CV	3.7	3.5	3.3	3.4	4.2	3.2	3.5	3.6	3.8

Eight different nutrient media brought variation in the radial growth of twenty different *A. brassicae* isolates (Table 3). Significant maximum radial growth (82.0 mm) was observed in PDA followed by Brassica leaf extract (74.3 mm) and Radish root dextrose (72.7 mm), while significantly minimum growth in Maize seed dextrose (42.00 mm) medium.

On the basis of radial growth on different solid media the *A. brassicae* isolates were categorised into seven different groups Group I**:** AB-*B. juncea* Pantnagar (76.9 mm), AB-Toria (76.3 mm), AB-*B. juncea* Karnal (75.4 mm); Group II**:** AB-Yellow sarson (72.9 mm), AB-*B. rugosa* (72.2 mm) AB-*R. sativus* (70.6 mm); Group III**:** AB-*B. nigra* (69.6 mm), AB-*B. carinata* (67.9 mm), AB-*B. pekinensis* (67.1 mm); Group IV**:** AB-*B. juncea* Kangara (66.9 mm), AB-*B. juncea* Jammu (66.5 mm), AB-*B. juncea* Bihar (66.3 mm), AB-*E. sativa* (66.1 mm), AB-*B. juncea* Punjab (65.5µ mm), AB-*B. botrytis* (65.5 mm); Group V**:** AB-*B. napus* (64.3 mm), AB-*R. sativus* (local) (62.3 mm); Group VI**:** AB-*B. oleracea* (60.5 mm); Group VII**:** AB-*B. caulorapa* (55.9 mm), AB-*S. alba* (52.6 mm).

Earlier workers also reported variability on the basis of radial growth on different media. In the present findings PDA was found best in terms of radial growth of all the *A. brassicae* isolates followed by Radish dextrose agar and Brassica leaf extract agar media which are in accordance with the findings of Kumar and Singh (2003) and Singh *et al.* (2015). However**,** Selvamani *et al.* (2013) found Cauliflower Leaf Extract Agar as the best medium followed by Potato Dextrose Agar.

Morphological variability

The *A. brassicae* isolates purified through single spore isolation showed significant morphological variability in respect of conidial length, conidial width, conidial beak length, number of septa and number of cells (Table 4; Fig 2). Average conidial length

Table 4: Morphological variability among different *A. brassicae* isolates

A. brassicae isolate	Conidial length (µm)	Conidial beak length (µm)	Conidial width (µm)	Horizontal septa (No.)	Vertical septa (No.)	Number of cells (No.)
AB-*B. juncea* Pantnagar	61.0	17.6	9.1	5.3	1.5	9.5
AB-*B. napus*	58.5	20.1	10.0	6.8	2.0	9.3
AB-*B. carinata*	185.3	93.4	25.6	14.8	5.0	17.3
AB-Toria	107.8	31.9	15.1	8.8	4.0	11.3
AB-Yellow sarson	59.7	12.0	10.1	7.3	1.5	10.5
AB-*E. sativa*	91.4	31.8	11.7	8.3	2.8	10.3
AB- *S. alba*	175.8	78.2	22.8	12.5	5.0	16.5
AB- *R. oleifera*	51.8	15.0	10.6	6.3	1.8	9.5
AB-*B. nigra*	50.6	16.0	10.1	7.8	1.5	9.5
AB-*B. rugosa*	38.9	11.0	8.1	6.3	1.5	8.8
AB-*B. juncea* Karnal	131.8	41.2	28.2	10.0	4.8	13.5
AB-*B. juncea* Punjab	62.5	19.9	12.3	6.5	2.0	9.3
AB-*B. juncea* Bihar	57.9	18.4	10.0	8.0	2.3	11.5
AB-*B. juncea* Jammu	44.3	14.7	9.9	5.8	0.8	7.8
AB-*B. juncea* Kangara	45.4	15.6	8.3	6.3	1.0	8.5
AB-*R. sativus*	44.7	16.0	9.4	7.0	1.5	10.0
AB-*B. pekinensis*	39.1	10.8	8.7	5.5	0.8	8.5
AB-*B. oleracea*	30.9	8.7	8.8	5.8	1.3	8.0
AB-*B. botrytis*	40.5	5.5	9.0	6.0	0.8	8.8
AB-*B. caulorapa*	29.0	7.1	6.6	3.5	0.8	5.8
CD 5%	5.7	2.5	2.0	1.6	1.0	2.0
CV	5.8	7.3	11.4	15.5	34.4	13.7

and width in *A. brassicae* isolates varied from 29.0 to 182.3µm x 6.6 to 28.2µm. significantly maximum conidial length was in AB-*B. carinata* isolate (182.3µm) and minimum in AB-*B. caulorapa* (29.0µm). Maximum conidial width was in AB-*B. juncea* Karnal isolate (28.2µm), while minimum in AB-*B. caulorapa* (6.6 µm). Average conidial beak length varied from 5.5 to 93.4µm. Maximum conidial beak length was in AB-*B. carinata* isolate (93.4µm), while minimum in AB-*B. botrytis* (5.5µm). Average number of horizontal septa varied from 3.5 to 14.8 no. Maximum horizontal septum was in AB-*B. carinata* isolate (14.8 no.), while minimum in AB-*B. caulorapa* (3.5 no.). Vertical septa varied from 0.75 to 5.0 no. Maximum vertical septa were in AB-*B. carinata* isolate (5.0 no.), while minimum in AB-*B. caulorapa* (0.75 no.). The largest conidia and highest number of septa was observed in AB-*B. carinata* isolate followed by AB-*S. alba*, AB-*B. juncea* Karnal and AB-Toria isolates, while smallest conidia and lowest number of septa was in AB-*B. caulorapa* isolate. Earlier workers have also characterized different *A. brassicae* isolates on the basis of morphological characters viz. conidial length, conidial width, number of horizontal and vertical septa, beak length (Mehta *et al.*, 2003; Kaur *et al.*, 2007; Meena *et al*., 2012; Selvamani *et al.*, 2013; Saha *et al*., 2015). They reported conidia length from 51.4-481.2 µm, width from 6.9-285.9µm, beak length 16.3-266.9 µm, number of horizontal and vertical septa from 3.0-9.0 and 1-2 respectively.

In the present studies on the basis of conidial length *A. brassicae* isolates were categorized into 4 different groups viz. Group I (200-150 µm): AB-*B. carinata* (182.3µm), AB-*S. alba* (175.8µm); Group II (150-100 µm): AB-*B. juncea* Karnal (131.8µm), AB-Toria (107.8µm); Group III (100-50 µm): AB-*E. sativa* (91.4µm), AB- *B. juncea* Punjab (62.5µm),

AB-*B. juncea* Pantnagar (61.0µm), AB-Yellow sarson (59.7µm), AB-*B. napus* (58.5µm), AB-*B. juncea* Bihar (57.9µm), AB-*R. oleifera* (51.8µm), AB-*B. nigra* (50.6µm); Group IV (< 50) : AB-*R. sativus* (44.7µm), AB-*B. juncea* Jammu (44.3µm), AB-*B. juncea* Kangara (45.4µm), AB-*B. pekinensis* (39.1µm), AB-*B. rugosa* (38.90µm), AB-*B. botrytis* (40.5µm), AB- *B. oleracea* (30.9µm) and AB-*B. caulorapa* (29.0µm).

Pathogenic variability

The qualitative characters viz. spot colour, periphery colour, concentric rings, central point and yellow halo were recorded on leaves at 40 days after inoculation (DAI). The *A. brassicae* isolates showed variability in spot colour *i.e.* brown and gray in periphery colour *i.e.* brown, gray and green; presence or absence of concentric rings; presence or absence of central point and colour (black, brown, gray); presence or absence of yellow halo. The dendogram constructed (using statistical software R studio) for pathogenic variation among *A. brassicae* isolates (Fig. 3) was categorized in 5 groups. Group I: AB-*B. caulorapa*; Group II: AB-*B. rugosa*, AB-*E. sativa*, AB-*S. alba*; Group III: AB-*B. juncea* Kangara, AB-*B. juncea* Jammu; Group IV: AB-*B. juncea* Punjab, AB-*R. oleifera*, AB-*B. nigra*, AB-*B. juncea* Bihar, AB-*B. juncea* Karnal, AB-*B. juncea* Pantnagar, AB-Yellow sarson, AB-Toria; Group V: AB-*B. carinata*, AB-*B. pekinensis* AB-*B. botrytis*, AB-*R. sativus*, AB-*B. napus* and AB-*B. oleracea*.The quantitative character viz. per cent disease index was recorded during 2014-15 and 2015-16, 50 DAI revealed significantly maximum per cent disease index and AUDPC in Varuna with AB-*B. juncea* Pantnagar isolate (49.3 %, 44.8 % & 313.4, 278.3), followed by AB-*B. juncea* Karnal (42.6 %, 37.0% & 262.5, 220.8), AB-Toria (41.9%, 37.8% & 259.3, 226.4) and AB-Yellow sarson (39.6 %, 34.1 % & 244.9, 202.1), respectively and were at par with each other. Minimum per cent disease index and AUDPC was observed with AB-*S. alba* (18.9 %, 17.0% & 102.3, 81.5) followed by AB-*E. sativa* (20.4% ,18.89 %& 113.4, 93.5), respectively (Fig 4 & 5). In the present studies AB-*B. juncea* Pantnagar isolate was found most virulent, while AB-*S. alba* least virulent.

Studies on pathogenic variability are essential for the development of pre-breeding populations as strategic defense mechanism. The pathogenic variability among *A. brassicae* isolates on host genotypes, on the basis of qualitative and quantitative characters were also reported by earlier workers (Kolte *et al*., 1991; Kaur *et al*., 2007; Khan *et al*., 2007; Goyal *et al.*, 2013; Pramila *et al*., 2014 and Saha *et al*., 2015).

Molecular variability

RAPD markers

Fig 3: Dendogram on the basis of pathogenic qualitative characteristics of *A. brassicae* isolates (20 no.) on *B. juncea* var. Varuna

Fig 4: Percent disease index and AUDPC on *B. juncea* cv. Varuna 2014-15

Fig 5: Percent disease index and AUDPC on *B. juncea* cv. Varuna 2015-16

Twenty random decamer oligonucleotide primers were screened initially, of these two primers did not give any amplification. Eighteen primers therefore used in molecular diversity analysis of *A. brassicae* isolates. A total of 259 reproducible and scorable polymorphic bands ranging from 100-1350 bp were generated for all twenty *A. candida* isolates with 18 RAPD primers. The number of polymorphic bands produced were maximum (21) with the primers LC 106 and LC 79, while minimum (7) with primer LC 94.

The UPGMA (un-weighted pair group method with arithmetic mean) dendrogram was constructed using Jaccard's similarity coefficient of RAPD marker data of 18 polymorphic primers generated for twenty *A. brassicae* isolates employing the program NTSYS 2.11 (Table 5, Fig 6, Plate 1). RAPD marker based dendrogram categorised twenty *A. brassiace* isolates into two major groups i.e. A and B. Group A and Group B were differentiated to each other at similarity coefficient of 0.59. In Group A isolates were categorized into Cluster I, consisted of 5 isolates AB-*R. sativus*, AB-*B. pekinensis*, AB-*B. oleracea*, AB-*B. botrytis* and AB-*B. caulorapa*. Intra cluster similarity coefficient between pairs of isolates within Cluster I varied from 0.87-0.76. In Group B isolates were categorized into Cluster II, III, IV and V on the basis of similarity coefficient. Cluster II consisted of only one isolate AB-*B. rugosa* and differentiated from remaining of the clusters of Group B at similarity coefficient of 0.70. Cluster III consisted of 9 isolates viz. AB-*E. sativa*, AB-*S. alba*, AB-*R. sativus* (local), AB-*B. nigra*, AB- *B. juncea* Karnal, AB-*B. juncea* Punjab, AB-*B. juncea* Jammu, AB-*B. juncea* Bihar and AB-*B. juncea* Kangara and exhibited similarity coefficient of 0.70 with Cluster IV. Similarity coefficient between isolates of Cluster III varied from 0.76 to 0.91. Cluster IV consisted of only one isolate i.e. AB-Yellow sarson and established similarity coefficient of 0.7 with Cluster V. Cluster V consisted of 4 isolates viz. AB-*B. juncea* Pantnagar, AB-*B. napus*, AB-*B. carinata* and AB-Toria and pair wised similarity coefficient varied from 0.8 - 0.7.

Plate 1: RAPD profile of *A. brassicae* isolates with primer LC 80

ISSR markers

Five ISSR primers were screened initially, of these four ISSR primers gave distinct and reproducible marker profile against *A. brassicae* isolates. A total of 51 reproducible and scorable polymorphic bands ranging from 100-950 bp were generated with four ISSR primers for genetic polymorphisms among the isolates. The number of polymorphic bands were

Table 5: Similarity coefficient for *A. brassicae* isolates using RAPD primers

1.	1.000																			
2.	0.733	1.000																		
3.	0.791	0.810	1.000																	
4.	0.768	0.764	0.822	1.000																
5.	0.710	0.729	0.718	0.764	1.000															
6.	0.741	0.698	0.764	0.810	0.768	1.000														
7.	0.756	0.706	0.764	0.841	0.745	0.861	1.000													
8.	0.745	0.702	0.752	0.814	0.733	0.810	0.880	1.000												
9.	0.710	0.667	0.718	0.803	0.729	0.837	0.876	0.818	1.000											
10.	0.675	0.648	0.667	0.776	0.710	0.710	0.733	0.752	0.733	1.000										
11.	0.714	0.671	0.745	0.806	0.733	0.826	0.872	0.830	0.849	0.729	1.000									
12.	0.772	0.656	0.706	0.776	0.756	0.795	0.857	0.837	0.826	0.729	0.891	1.000								
13.	0.749	0.667	0.710	0.764	0.760	0.791	0.783	0.803	0.768	0.749	0.833	0.826	1.000							
14.	0.749	0.683	0.733	0.818	0.768	0.783	0.837	0.833	0.806	0.779	0.864	0.872	0.837	1.000						
15.	0.679	0.644	0.687	0.741	0.729	0.737	0.776	0.741	0.729	0.694	0.741	0.787	0.752	0.799	1.000					
16.	0.583	0.571	0.606	0.613	0.648	0.586	0.610	0.613	0.563	0.637	0.621	0.621	0.640	0.648	0.602	1.000				
17.	0.548	0.552	0.571	0.579	0.613	0.536	0.559	0.571	0.521	0.617	0.579	0.571	0.613	0.613	0.567	0.864	1.000			
18.	0.552	0.540	0.559	0.575	0.610	0.563	0.579	0.613	0.525	0.644	0.583	0.590	0.625	0.633	0.579	0.791	0.772	1.000		
19.	0.555	0.544	0.548	0.555	0.613	0.528	0.536	0.586	0.498	0.602	0.540	0.548	0.621	0.598	0.583	0.756	0.722	0.872	1.000	
20.	0.598	0.579	0.606	0.621	0.640	0.579	0.610	0.637	0.579	0.652	0.629	0.621	0.671	0.656	0.586	0.776	0.764	0.845	0.810	1.000
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Plate 2: ISSR profile of *A. brassicae* isolates with primer ISSR 2

Fig 6: Dendogram of *A. brassicae* isolates using RAPD primers

maximum (15) with ISSR 2 and ISSR 10, while minimum (9) with the ISSR 12 primers.

The ISSR markers were also used to determine level of genetic diversity among twenty *A. brassicae* isolates. The UPGMA dendrogram was constructed using Jaccard's similarity coefficient of ISSR marker and data were generated using four ISSR primers (Table 6, Fig 7, Plate 2). ISSR marker based dendogram categorised twenty *A. brassiace* isolates into two major groups i.e. A and B. Group A and B were differentiated at similarity coefficient of 0.67. Group A isolates were categorized into Cluster I, consisted of 5 isolates viz. AB-*R. sativus,* AB-*B. pekinensis,* AB-*B. oleracea,* AB-*B. botrytis* and AB-*B. caulorapa*. Intra cluster similarity coefficient between pairs of isolates within Cluster I varied from 0.90-0.79. Group B isolates were categorized into Cluster II, III, IV and V. Cluster II consisted only one isolate i.e. AB-*B. napus* and differentiated from remaining of the clusters of Group B at similarity coefficient of 0.74. Cluster III again had only isolate i.e. AB-*R. oleifera*, and exhibited similarity coefficient of 0.75 with Cluster IV. Similarity coefficient of Cluster III varied from 0.75 to 0.98. Cluster IV consisted of six isolates viz. AB-*S. alba,* AB-*B. juncea* Jammu, AB-*B. rugosa*, AB- *B.*

Fig 7: Dendogram of *A. brassicae* isolates using ISSR primers

Table 6: Similarity coefficient for *A. brassicae* isolates using ISSR primers

1.	1.000																			
2.	0.784	1.000																		
3.	0.882	0.784	1.000																	
4.	0.862	0.725	0.862	1.000																
5.	0.862	0.764	0.862	0.921	1.000															
6.	0.843	0.823	0.882	0.862	0.862	1.000														
7.	0.745	0.686	0.745	0.882	0.803	0.823	1.000													
8.	0.705	0.725	0.745	0.764	0.725	0.784	0.803	1.000												
9.	0.843	0.705	0.882	0.901	0.901	0.843	0.823	0.705	1.000											
10.	0.803	0.745	0.843	0.862	0.862	0.882	0.862	0.745	0.921	1.000										
11.	0.823	0.764	0.862	0.882	0.882	0.901	0.882	0.764	0.941	0.980	1.000									
12.	0.725	0.627	0.725	0.823	0.745	0.764	0.823	0.705	0.803	0.843	0.862	1.000								
13.	0.882	0.745	0.921	0.941	0.941	0.882	0.823	0.745	0.960	0.921	0.941	0.803	1.000							
14.	0.784	0.686	0.784	0.843	0.803	0.823	0.921	0.764	0.862	0.901	0.921	0.862	0.862	1.000						
15.	0.764	0.705	0.803	0.862	0.823	0.843	0.862	0.745	0.882	0.921	0.941	0.882	0.882	0.901	1.000					
16.	0.667	0.529	0.705	0.764	0.725	0.667	0.725	0.647	0.745	0.705	0.725	0.627	0.784	0.725	0.705	1.000				
17.	0.667	0.529	0.705	0.725	0.686	0.667	0.686	0.607	0.705	0.667	0.686	0.588	0.745	0.686	0.667	0.764	1.000			
18.	0.705	0.568	0.784	0.764	0.725	0.705	0.725	0.725	0.745	0.705	0.725	0.667	0.784	0.725	0.705	0.803	0.803	1.000		
19.	0.588	0.450	0.667	0.686	0.647	0.588	0.647	0.607	0.667	0.627	0.647	0.549	0.705	0.647	0.627	0.764	0.764	0.843	1.000	
20.	0.607	0.470	0.686	0.705	0.667	0.607	0.667	0.627	0.686	0.647	0.667	0.568	0.725	0.667	0.647	0.823	0.823	0.862	0.901	1.000
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

juncea Karnal, AB-*B. juncea* Kangara and AB-*B. juncea* Punjab and established similarity coefficient of 0.84 with Cluster V. Cluster V consisted of seven isolates viz. AB-*B. juncea* Pantnagar, AB-*B. carinata,* AB-Toria, AB-*B. nigra,* AB-*B. juncea* Bihar, AB-Yellow sarson and AB-*E. sativa* pair wised similarity coefficient varied from 0.96-0.86.

In the present study, molecular profile based on dendogram generated by 18 RAPD and 4 ISSR markers revealed genetic diversity among different *A. brassicae* isolates as both RAPD and ISSR primers successfully differentiated *A. brassicae* isolates into different clusters. Goyal *et al.* (2013), Pramila *et al.* (2014) and Saha *et al.* (2015) also studied genetic variability among *A. brassicae* isolates RAPtD primers. Sharma *et al.* (2013) characterized 32 Indian isolates using ITS primers between 550- 600 bp. Kumar *et al*. (2014) performed DNA fingerprinting using ISSR primers to study genetic diversity among 32 isolates of *A. brassicae* isolates.

Selection of host differentials

Based on phenotypic diseases reactions against nine different *A. candida* isolates, the *Brassica* genotypes were categorized into 4 different groups as host differentials viz., Group I: Varuna, PT-303, PYS-6 and *B. nigra* which showed susceptible disease reaction (S) i.e. coalescing larger brown spots with a distinct margin yellow halo, 26-50 per cent leaf area covered by the lesions, against all the isolates; Group II**:** PBN-9501 and Kiran which showed moderately resistant reactions (MR) i.e. small roundish slightly sporulating small brown necrotic spots, 11-25 per cent leaf area covered by lesions; Group III: *E. sativa* which showed resistant disease reaction (R) i.e. pinpoint size or small brown necrotic spots, less than 5-10 per cent leaf area covered by lesions: Group IV: *S. alba* which showed disease resistant reaction (R) *i.e.* pinpoint size or small brown necrotic spots, less than 5-10 per cent leaf area covered by lesions against AB-*B. juncea* Pantnagar, AB-Toria, AB-Yellow sarson and AB-*S. alba* isolates, while showed immune response against AB-*B. napus*, AB-*B. carinata*, AB-*B. nigra,* AB-*E. sativa* and AB-*R. oleifera* isolates (Table 7). Earlier workers (Sangwan and Mehta, 2007 and Meena *et al.,* 2012**)** used seventeen and six different Brassica genotypes respectively for the

Table 7: Grouping of *Brassica* spp. based on phenotypic disease reactions against different *A. brassicae* isolates

Brassica spp.	*A. brassicae* isolate								
	AB-*B. juncea* Pantnagar	AB-*B. napus*	AB-*B. carinata*	AB-Toria	AB-Yellow sarson	AB-*E. sativa*	AB- *S. alba*	AB- *R. oleifera*	AB-*B. nigra*
Group I									
Varuna	S	S	S	S	S	S	S	S	S
Toria	S	S	S	S	S	S	S	S	S
Yellow sarson	S	S	S	S	S	S	S	S	S
B. nigra	S	S	S	S	S	S	S	S	S
Group II									
B. napus	MR	MR	MR	MR	MR	MR	MR	MR	MR
B. carinata	MR	MR	MR	MR	MR	MR	MR	MR	MR
Group III									
E. sativa	R	R	R	R	R	R	R	R	R
Group IV									
S. alba	R	-	-	R	R	-	R	-	-

(-) no reaction

differentiation of different *A. brassicae* isolates on the basis of phenotypic disease reactions.

Long term preservation methods of *A. brassicae* culture

Mycelial growth was observed in all of the *A. brassicae* cultures (56 nos.) preserved in PDA (4ºC), 10 per cent glycerol (-20ºC) 5 per cent DMSO (-20ºC). However, sporulation was observed only in thirty five *A. brassicae* cultures. On PDA (4ºC) out of 35 isolates only 23 isolates sporulate well; On 10 per cent glycerol (-20ºC) out of 9 isolates only 6 isolates sporulate well; On 5 per cent DMSO (-20ºC) out of 12 isolates only 7 isolates sporulate well. The above findings revealed that culture preservation in 10 per cent glycerol at -20ºC was the best in terms of sporulation (66.7%) followed by PDA at 4ºC (65.7%) and could be used for preservation and storage of *A. brassicae* isolates at least for 2 years (Table 8). The present findings are in accordance with the work of Aneja *et al.* (2014) who reported that *A. brassicae* isolates, could be maintained by preserving in PDA at (4°C); 10% glycerol and 5% DMSO (-20°C) at least 6 months of storage.

Conclusion

In present studies significant variation in cultural, morphological, pathogenic and molecular variability was observed in *A. brassiace* isolates irrespective to geographical locations and *Brassica* spp. On the basis of cultural, morphological, pathogenic and molecular variability, the *A. brassiace* isolates are categorized into 5 major groups. Group I: comprised 5 isolates viz. AB-*R. sativus*, AB-*B. pekinensis*, AB-*B. oleracea*, AB-*B. botrytis* and AB-*B. caulorapa*; Group II: comprised 8 isolates viz. AB-*B. juncea* Pantnagar, AB-Yellow sarson, AB-Toria, AB-*B. nigra*, AB-*R. oleifera*, AB-*B. juncea* Karnal, AB-*B. juncea* Punjab and AB-*B. juncea* Bihar; Group III: comprised 4 isolates viz. AB-*B. napus*, AB-*B. carinata*, AB-*E. sativa*, AB-*S. alba*; Group IV: comprised 2 isolates viz. AB-*B. juncea* Kangara and AB-*B. juncea* Jammu; Group V: comprised 1 isolate *i.e.* AB-*B. rugosa*. These *A. brassicae* isolates could be used during screening of resistant sources in Brassica genotypes against Alternaria blight disease.

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Table 8: Effect of different preservation methods on the growth and sporulation of *A. brassicae* isolates

Observation	*A. brassicae* isolates		
	Methods of preservation		
	PDA (4°C)	10% glycerol (-20°C)	5% DMSO (-20°C)
Mycelia growth with sporulation	ABc-D01, ABc-D02, ABo-D03, ABo-P03, ABo-P04, ABc-P08, ABc-P10, ABc-P12, ABc-L06, ABc-L08, ABc-L11, ABc-L12, ABc-L13, ABc-L14, ABc-L20, ABc-L01, ABc-L04, ABc-L09, ABc-L16, ABc-L18, ABc-Kg02, ABc-B01, ABc-B03	ABc-D01, ABc-D02, ABo-D03, ABo-P03, ABo-P04, ABc-P08	ABc-D01, ABc-D02, ABo-D03, ABo-P03, ABo-P04, ABc-P10, ABc-P12
Mycelia growth without sporulation	ABa-Kn02, ABa-B02, ABo-D04, ABo-D05, ABc-D06, ABc-D07, ABo-P02, ABo-P09, ABc-P11, ABc-L02, ABc-L03, ABc-L05	ABo-D04, ABo-D05, ABo-P02	ABo-D04, ABo-D05, ABo-P02, ABc-P09, ABc-P11
Sporulation percentage		65.7%	66.7% 58.3%

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