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Morphological, Physiological and cultural variability in *Alternaria brassicae* isolates of Indian mustard, *Brassica juncea* L. Czern & Coss. collected from different agro-climatic regions of India

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Abstract
Indian mustard (*B. juncea)* is a crop of tropical as well as temperate zone, which is grown during *rabi*. Alternaria blight disease caused by *A. brassicae* (Berk.) Sacc. is one of the most important diseases of Indian mustard. Ten isolates of *A. brassicae* were collected from different geographical region in India for studied the morphological, physiological and cultural variation. All the isolates showed high level of variability *in vitro* in respect of size and septation of conidiophores and conidia, conidial beak length, effect of temperature and relative humidity on redial growth and different liquid medium on mycelia fresh weight. The length and width of conidiophores ranged from 36.82 to 63.45µm and 4.73 to 6.58 µm respectively. The average conidiophores septation ranged from 4.53 to 6.25 in different isolates of *A. brassicae*. The conidial length ranged from 104.0 to 142.47 µm, width 11.62 to 16.95 µm and beak length of conidia ranged from 43.35 µm to 70.57 µm in different isolates of *A. brassicae.* Number of transverse and longitudinal septa ranged from 6.0 to 8.3 and 0.25 to 2.75 respectively. The effect of different temperature on growth of ten isolates of *A. brassicae* indicates that maximum growth among all the isolates was noted at 25 ^{0}C followed by 30 ^{0}Cand 20 ^{0}C. Each isolates of *A. brassicae*, grown on Potato dextrose broth, Oatmeal broth, Host leaf broth, Czapek's broth and Carrot broth. Maximum fresh mycelial weight in the present study was obtained on 20 days of inoculation on potato dextrose broth, which is indicative of the optimum growth of the fungus. The fresh weight of mycelium ranged between 469.67 to 656.67 mg in Potato dextrose broth, 615.67 to 436.33 mg in Oatmeal broth, 441.67 to 628.67 mg in Host leaf extract broth, 389.33 to 571.33 mg in Czapek-dox broth and in Carrot broth, the fresh weight ranged from 425.00 to 611.33 mg in different isolates of *A. brassicae*.

Keywords: *Alternaria brassicae, Brassica juncea,* morphological, physiological, cultural, variability

Introduction
Indian mustard [*Brassica juncea* (L.) Czern & Coss.] is one of the most important oilseed crops which contribute about 80 per cent of total rapeseed-mustard produced in India (AICRP-RM, 2011). Mustard seed is the second largest produced oilseed in the world with an area of 37.0 m ha, with the production of 63.09 m tonnes and the productivity of 18.50 q/ha (Singh *et al.* 2014). In India it had the area of 6.3 m ha with production of 7.6 m tonnes and productivity of 11.90 q/ha. India contributes 28.3% and 19.8% in world acreage and production (Anonymous, 2013). Indian mustard (*B. juncea*) is cultivated in the states of Assam, Bihar, Gujarat, Haryana, Himachal Pradesh, Jammu & Kashmir, Madhya Pradesh, Orissa, Punjab, Rajasthan, Uttar Pradesh and West Bengal as a Rabi crop. A wide gap exists between the potential yield and the yield realized at the farmers' field, which is largely because of number of biotic and abiotic stresses to which the rapeseed-mustard crop is exposed. Among biotic diseases, Alternaria blight caused by Alternaria brassicae (Berk.) Sacc. is one of the most common and destructive disease of Indian mustard, which causes up to 47 per cent yield loss (Meena *et al.*, 2010). Lack of resistant varieties indicates the presence of several variants in the pathogen. Information on morphological, physiological and cultural variability of A. brassicae population in India is meagre. A comparative knowledge of the nutritional patterns and factors influencing its growth are prerequisite to any study leading to the understanding of host-pathogen relationship and specificity. *Alternaria* blight severity on rapeseed-mustard differs among seasons and regions as also between individual crops within a region. This may be due to existence of variability among isolates of *Alternaria* species. Some reports on the existence of morphological, physiological and cultural variability within the isolates of *Alternaria brassicae* have been reported by earlier workers (Meena *et al*. 2005, Goyal *et al*. 2011, Sharma *et al.* 2013, Pramila *et al.* 2014 and Singh *et al.* 2014). Special attention was focused holistic

study on morphological, physiological and cultural variability among ten isolates of *Alternara brassicae* collected from different geographical location of India.

Materials and Methods

Collection and maintenance of samples

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, Madhya Pradesh, Uttarakhand, Bihar, Jharkhand, West Bengal, Haryana, Rajasthan, Chhattisgarh and Gujarat. The samples were kept in sterile polythene bags and roughdry paper envelops, especially meant for the purpose. Each envelops was marked clearly to show details of the location, variety and date of collection. The collected samples were dried for 24 hours under shade in order to remove excess surface moisture in laboratory. After drying, the samples were repacked, kept in B.O.D. and maintained at 6-8 ^{0}C temperature for further study.

Isolation and purification of the pathogen

The diseased leaves showing characteristics symptoms were selected for the isolation of the pathogen and washed with fresh sterilized water in order to remove the dust particles and surface contaminants. The washed leaves samples were cut into small bits with some healthy portions with the help of flamed razor blade and forceps. The bits were surface sterilized by dipping them in 0.1 per cent mercuric chloride ($HgCl_2$) solution for 10-15 seconds under aseptic conditions followed by 3 to 4 washing with sterilized water to remove the traces of $HgCl_2$. These bits were de-moisturized by placing them between folds of sterilized filter paper. The bits were placed on each Petri-plates having PDA under aseptic conditions and incubated at 25^{o}C (Singh *et al.* 2014).

Pathogens were purified by using single spore isolation technique. 10 ml of clear, filtered two per cent water agar was poured into sterile Petri-plates and allowed to solidify. Dilute spore suspension was prepared in sterilized distilled water from 15 days old culture. One ml of such suspension was spread uniformly on agar plate. These plates were incubated at 25 ^{o}C for 12 hr. Then such plates were examined under microscope to locate single conidia and marked with permanent marker on the surface of the plates. Single conidium was transferred to Petri-plates having PDA with the help of cork borer under aseptic conditions and incubated at 25±1 ^{o}C. The different isolates of the *A. brassicae* were purified as maintain separately on PDA slants for further studies.

Morphological variability

Morphological variability of ten isolates was studied by calibrating ocular micrometry (Meena *et al*., 2005). The different characters *viz.* size and septation of conidiophores and conidia were studied on Potato Dextrose Agar (PDA) medium incubated at 25 ^{0}C for 15 days. Growth of each isolate was measured as colony diameter in mm. Total 30 conidia from each slide were examined at 40X magnification of light microscope and measured using ocular and stage micrometre. The average was used to calculate the radial growth of the fungus, conidial length, width, and number of transverse and longitudinal septation.

Physiological variability

Effect of temperature and relative humidity on radial growth of different isolates of *A. brassicae* was studied. Petri plates containing PDA medium were inoculated by 5 mm culture discs of 7 days old culture. Plates were incubated for 10 days at 20, 25, and 30°C in B.O.D. incubator at a constant 100% relative humidity (RH) maintained in separate lower lid of the plate which was fixed with sticky tape on the other lower lid of fungal culture plates by mixing different quantities of KOH and distilled water (Chattopadhyay and Appaji, 2000). Similarly, petri plates of different isolates were incubated at normal growth temperature *i.e.* 25 ^{0}C in B.O.D. incubator for 10 days at 80, 90 and 100% RH. Radial growth (mm) was measured in 4-5 directions from 10 days old culture with the help of small scale in mm and the average colony diameter was taken.

Cultural variability

Each isolates of *A. brassicae* were grown on Potato dextrose broth, Oatmeal broth, Host leaf broth, Czapek broth and Carrot broth, in four replications. Ten days old, mycelial discs of 5 mm diameter were transferred aseptically into sterilized 250 ml flasks containing 50 ml of different liquid medium. These flasks were incubated at 25 ^{0}C for 15 days. The fungal growth of each flask was harvested on Whatman filter paper No. 42 and fresh weight was taken as methods described by Meena *et al*. 2012.

Result and Discussion

Morphological variability among *A. brassicae* isolates

It is clear from the Table-1 that the length and width of conidiophores ranged from 36.83 to 63.45µm and 4.73 to 6.58 µm respectively. The conidiophores length was maximum in Ab7 of West Bengal (63.45µm) followed by Ab3 of Uttarakhand (62.50µm) and Ab6 of Haryana (55.03µm), whereas it was minimum in isolate Ab2 of Madhya Pradesh (36.83 µm). There was no significant difference between isolates Ab7 of Haryana and Ab3 of Uttarakhand; Ab8 of Rajasthan and Ab4 Ab4 of Bihar. Similarly, isolates Ab5, Ab1 of Uttar Pradesh, Ab9 of Chhattisgarh, Ab10 of Gujarat and Ab2 of Madhya Pradesh were at par to each other. However, Ab6 significantly differed from other isolates. The conidiophores width was maximum in Ab3 (6.58 µm) followed by Ab7 (6.30 µm) and Ab6 (6.25 µm) and minimum in isolate Ab1 (4.73 µm). No significant difference was recorded among isolates Ab3, Ab7 and Ab6. Isolates Ab8, Ab9, Ab5, Ab2 Ab10, Ab4 and Ab1 were also at par to each other. The septation of conidiophores ranged from 4.50 to 6.25 µm in different isolates of *A. brassicae*. Highest number of septa was shown by isolate Ab3 (6.25) followed by Ab7 (6.04), Ab6 (5.25), Ab8 (5.07), Ab5 (5.04), Ab2 (5.03), Ab9 (4.75), Ab1 (4.75), Ab10 (4.54) and was minimum in isolate Ab4 (4.50).There was no significant difference between Ab3 and Ab7. Similarly, among isolates Ab6, Ab8, Ab5 and Ab2; Ab9, Ab1, Ab10 and Ab4 were at par to each other.

The conidial length and width ranged from 104.0 µm to 142.47 µm and 11.62 to 16.95 µm respectively. The conidial length was maximum in Ab3 (142.47 µm) followed by Ab7 (138.05) and Ab6 (132.90), whereas minimum was recorded in isolate Ab2 (104.0 µm). The conidial width was maximum in isolates Ab3 (16.95 µm) followed by Ab6 and Ab7 (15.52 µm), Ab5 (14.57 µm), Ab4 (13.25 µm), Ab10 (12.50 µm), Ab1 (12.42 µm), Ab9 (12.32 µm), Ab8 (12.07 µm) and Ab2

(11.62 µm). Isolates Ab3, Ab7 and Ab6; and Ab5, Ab9, Ab10, Ab1, Ab8, Ab4 and Ab2 were statistically at par. Width of conidia ranged from 11.62 to 16.95 µm in different isolates of *A. brassicae.* There was no significant difference among isolates Ab3, Ab7, Ab6 and Ab5. Similarly, isolates Ab4, Ab10, Ab1, Ab9, Ab8 and Ab2 were at par to each other.

The beak length of conidia ranged from 43.35 µm to 70.57 µm. The maximum beak length was found in isolate Ab3 (70.57 µm) followed by Ab7 (66.80 µm) and Ab6 (62.45 µm) and minimum in isolate Ab10 (43.35). There was no significant difference among isolates Ab3, Ab7 and Ab6. Isolates Ab8, Ab5, Ab1, Ab2, Ab4, Ab9 and Ab10 were at par to each other.

The transverse septation of conidia ranged from 6.0 to 8.3 in different isolates of *A. brassicae*. The maximum transverse septation was noted in isolate Ab7 (8.3) followed by Ab6 (8.3), Ab9 (8.0) Ab4 (7.5), Ab3 (7.3), Ab5 (7.3), Ab1 (7.3), Ab10 (7.0), Ab8 (6.0) and Ab2 (6.0). There was no significant difference among isolates Ab7, Ab6 and Ab9; Ab4, Ab3, Ab5, Ab1 and Ab10. However, isolates Ab8 and Ab2 were at par to each other. The longitudinal septation of conidia ranged from 0.25 to 2.75 in different isolates of *A. brassicae*. The longitudinal septation was maximum in isolates Ab3 (2.75) followed by Ab5 (2.0), Ab7 (1.75), Ab6 (1.75), Ab9 (1.50), Ab1 (1.25), Ab10 (1.0), Ab8 (1.0), Ab4 (0.75) and Ab2 (0.25). No significant difference was noted among isolates Ab5, Ab7 and Ab6; Ab9, Ab1, Ab10 and Ab8. However, isolates Ab3, Ab4 and Ab2 differed significantly from other isolates.

Based on size and septation of conidiophores and conidia, isolates could be classified into three different groups as fallows. Group 1 (Ab3, Ab6 and Ab7), group 2 (Ab2, Ab5 and Ab8) and group 3 (Ab1, Ab4, Ab9 and Ab10). These results are in agreement with earlier workers (Awasthi and Kolte, 1989; Varma *et al*., 2006; Meena *et al.*, 2005; Kaur *et al.*, 2007; Singh *et al.*, 2007; Sharma *et al*., 2013 and Pramila *et al.* 2014), who observed morphological variability in different geographical isolates within *Alternaria* species.

Table: 1 Size and septation of conidiophores and conidia in different isolates of *Alternaria brassicae*

Isolates	Conidiophores			Conidial characters				
	Length (µm)	Width (µm)	Number of septa	length (µm)	width (µm)	Beak length (µm)	Transverse septa	Longitudinal septa
Ab_1	41.98	4.73	4.75	111.55	12.42	51.15	7.3	1.25
Ab_2	36.83	4.88	5.0	104.00	11.62	50.47	6.0	0.25
Ab_3	62.50	6.58	6.25	142.47	16.95	70.57	7.3	2.75
Ab_4	43.65	4.75	4.5	110.15	13.25	50.00	7.5	0.75
Ab_5	42.30	5.23	5.0	120.00	14.57	51.95	7.3	2.00
Ab_6	55.03	6.25	5.25	132.90	15.52	62.45	8.3	1.75
Ab_7	63.45	6.30	6.0	138.05	15.52	66.80	8.3	1.75
Ab_8	48.75	5.45	5.0	110.20	12.07	52.60	6.0	1.00
Ab_9	40.40	5.25	4.75	116.62	12.32	47.77	8.0	1.50
Ab_{10}	38.60	4.83	4.5	114.15	12.5	43.35	7.0	1.00
SEm±	2.11	0.25	0.08	4.72	0.97	3.12	0.10	0.10
CD at 5%	6.11	0.73	0.24	13.64	2.80	9.02	0.31	0.31

Table: 2 Effect of temperature and relative humidity (RH) on radial growth and fresh weight of *Alternaria brassicae* isolates

Isolates	Radial growth (mm)						Fresh weight (mg)				
	Temperature (^{0}C)			Relative Humidity (%)			Potato dextrose broth	Oat meal broth	Host leaf extract broth	Czapek dox broth	Carrot juice broth
	20	25	30	80	90	100					
Ab_1	50.53	80.77	59.40	53.10	64.80	71.23	548.00	520.00	525.67	481.33	512.00
Ab_2	56.03	68.43	62.30	53.63	61.53	69.00	551.00	526.67	530.33	482.67	520.00
Ab_3	69.00	88.43	82.10	60.57	70.03	82.23	656.67	615.67	620.67	571.33	611.33
Ab_4	61.33	85.90	64.00	55.43	62.00	70.97	533.33	505.33	512.67	418.00	482.00
Ab_5	58.10	81.43	64.87	49.53	59.70	71.37	572.33	516.33	527.33	445.67	511.00
Ab_6	67.27	84.13	80.53	57.07	66.57	81.60	622.33	606.33	619.33	514.00	595.67
Ab_7	66.27	88.40	81.33	59.63	68.00	82.70	641.67	613.00	628.67	542.67	610.00
Ab_8	58.27	82.83	71.43	52.80	59.50	76.70	514.33	484.33	495.33	418.67	476.00
Ab_9	63.90	78.73	70.00	54.33	59.53	75.13	481.67	462.33	473.67	415.67	448.00
Ab_{10}	54.50	76.60	69.60	50.30	57.60	70.87	469.67	436.33	441.67	389.33	425.00
SE m±	2.92	2.38	1.37	1.04	1.00	1.84	4.97	1.87	2.43	2.74	2.71
CD at 5%	8.61	7.03	4.05	3.09	2.95	5.45	14.67	5.51	7.17	8.09	8.00

Effect of temperature and Relative Humidity (RH) on mycelial growth

Mycelial growth of ten isolates of *A. brassicae* was favoured at 25 ^{0}C followed by 30 ^{0}C and 20 ^{0}C. Among isolates maximum growth was recorded in isolate Ab3 of Uttarakhand (88.43mm) followed by Ab7 of West Bengal (88.40), Ab4 of Bihar (85.90) and Ab6 of Haryana (84.13 mm). However, it was minimum in isolate in Ab2 of Madhya Pradesh (68.43 mm) at 25 ^{0}C. The growth was statistically at par among isolates Ab3, Ab7, Ab4, and Ab6. Similarly,

isolates Ab8, Ab5, Ab1, Ab9 and Ab10 were also similar. The colony diameter was minimum in isolate Ab2 which differed significantly from other isolates (Fig-1).

Among three different relative humidity levels 100% was favoured to be ideal and produced the maximum mean mycelial growth Ab7 of West Bengal (82.70) followed by Ab3 of Uttarakhand (82.23) and Ab6 of Haryana (81.60 mm) and was minimum in Ab2 of Madhya Pradesh (69.0 mm) isolate. However, significant difference was noted at all the relative humidity among all the isolates. The variation in mycelial growth of *A. brassicae* in present studies support the views of earlier workers (Ansari *et al*. 1989), observed growth and sporulation of *A. brassicae* influenced by temperature and RH. Growth and sporulation occurred at

23 ^{o}C. The pathogen grow at all RH levels tested but a gradual increase in RH enhanced mycelia growth and sporulation, reaching an optimum at 95-100% (Khan *et al*. 2007), studied the effects of temperature (10, 15, 20, 25 or 30 ^{0}C) and found maximum (47.0 mm) where radial growth was at 25 ^{0}C and lowest at 10 ^{0}C (20.2 mm). These studies indicated the existence of variability among the isolates of *A. brassicae.*

Effect of liquid media on mycilial growth

Each isolates of *A. brassicae*, grown on Potato dextrose broth, Oatmeal broth, Host leaf broth, Czapek's broth and Carrot broth. Maximum fresh mycelial weight in the present study was obtained on 20 days of inoculation on potato dextrose broth, which is indicative of the optimum growth of the fungus. The fresh weight of mycelium ranged between 469.67 to 656.67 mg in Potato Dextrose Broth. Maximum weight was found in isolate Ab3 (656.67) followed by Ab7 (641.67), Ab6 (622.33), Ab5 (572.33), Ab2 (551.00), Ab1 (548.00), Ab4 (533.33), Ab8 (514.33), and Ab9 (481.67). However, it was found minimum in isolate Ab10 (469.67). The fresh weight of isolates Ab1 and Ab4; Ab9 and Ab10 were at par while, fresh weight of Ab3, Ab7, Ab6, Ab5, Ab2 and Ab8 differed significantly from all other isolates. In Oatmeal broth, the mycelial growth was maximum in isolate Ab3 (615.67) followed by Ab7 (613.00), Ab6 (606.33), Ab2 (527.67), Ab1 (520.00), Ab5 (516.33), Ab4 (505.33), Ab8 (484.33), Ab9 (462.33) and Ab10 (436.33). No significant difference in mycelial weight was noted between isolates Ab3 and Ab7; Ab1 and Ab5. However, Ab6, Ab2, Ab4, Ab8, Ab9 and Ab10 showed significant difference from other isolates with respect to mycelial fresh weight. The fresh mycelium weight ranged from 441.67 to 628.67 mg in Host leaf extract broth medium. The mycelium weight was maximum in isolate Ab7 (628.67) followed by Ab3 (620.67), Ab6 (619.33), Ab2 (530.33), Ab5 (527.33), Ab1 (525.67), Ab4 (512.67), Ab8 (495.33) and Ab9 (476.67). However, isolate Ab10 (441.67) showed minimum fresh weight of *A. brassicae*. There was no significant difference between isolates Ab3 and Ab6. Similarly, isolates Ab2, Ab5 and Ab1 were at par to each other. However, significant difference in fresh weight was noted in isolates Ab7, Ab4, Ab8, Ab9, and Ab10. It ranged from 389.33 to 571.33 mg in Czapek-dox broth. The mycelium weight was maximum in isolate Ab3 (571.33) followed by Ab7 (542.67), Ab6 (514.00), Ab2 (482.67), Ab1 (481.33), Ab5 (445.67), Ab8 (418.67), Ab4 (418.00) and Ab9 (415.67). It was observed minimum in isolate Ab10 (389.33). In Carrot broth, the fresh weight ranged from 425.00 to 611.33 mg. The mycelial weight was maximum in isolate Ab3 (611.33) followed by Ab7 (610.00), Ab6 (595.67), Ab2 (520.00), Ab1 (512.00), Ab5 (511.00), Ab4 (482.00), Ab8 (476.00) and Ab9 (448.00), however, the minimum fresh weight was recorded in isolate Ab10 (425.00). Fresh weight did not differ significantly between M2 and M3, whereas, it was differed significantly in M1, M4 and M5 media. Cultural characteristics among 32 representative Indian geographical isolates of *A. brassicae*. All the isolates showed high level of variability *in vitro* in respect of mycelial growth and sporulation among these isolates in different nutrient media (Sharma, *et al.* 2013). All the isolates did not grow and sporulate abundantly on the same nutrient medium. However, Potato Dextrose Agar, Host Agar medium and Carrot Potato Agar were better for all the cultures.

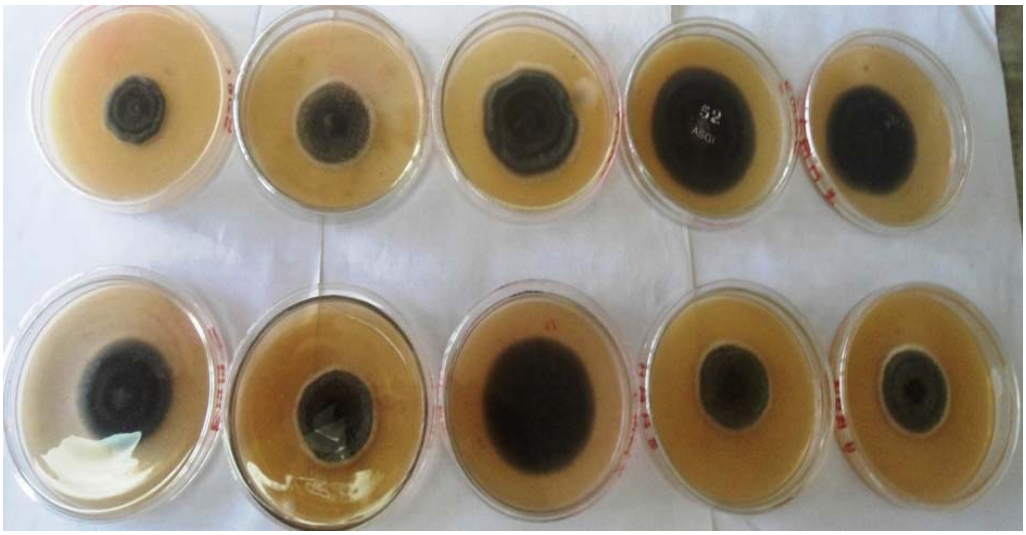

Fig: Mycelial growth of different isolates of *A. brassicae*

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