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Morphological, cultural, pathogenic and molecular variability amongst Indian mustard isolates of *Alternaria brassicae* in Uttarakhand

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***Alternaria* blight (*Alternaria brassicae*) causes severe foliar damage to Indian mustard in Uttarakhand. Ten (10) isolates of *A. brassicae* were collected from different hosts and characterized for cultural, morphological, pathogenic and molecular variations. *A. brassicae* colonies varied in their cultural behaviour ranging from cottony, flurry to feathery, with smooth to rough margins. Colour of colonies ranged between white, off white to light brown. Colony growth varied from slow, medium to fast with fast growth in isolate KM and least in JD on the potato dextrose agar (PDA) medium. Significant morphological variations in conidia length, conidia width, and number of horizontal septa were observed in the isolates. Average conidial size ranged from 105 to 135 × 10 to 20 µm. Isolates exhibited variations in disease index, number and size of the lesions. The dendrogram analysis, based on molecular (random amplification of polymorphic DNA, RAPD) basis revealed two groups at 15% similarity coefficient. Group I was composed of seven isolates namely, VR, DV, P7, LM. P10, KR and ND with 18% similarity (82% dissimilarity) while group II was composed of only three isolates namely, JD, KA and AS with only 24% similarity (76% dissimilarity).**

Key words: Pathogenic variability, *Brassica*, *Alternaria brassicae*, RAPD-PCR.

INTRODUCTION

Alternaria brassicae (Berk.) Sacc., is an important necrotrophic pathogen causing *Alternaria* blight disease in Indian mustard [*Brassica juncea* (L.) Czern and Coss.]. It is very difficult to manage the disease, due to no proven source of resistance reported till date in any of the hosts (Meena et al., 2010b). The yield loss due to this pathogen is up to 47% in the entire mustard growing area (Meena et al., 2010a). One of the significant aspects of biology of an organism is the morphological and physiological characters of an individual within a species, which are not fixed. This holds true with fungi also, although it is not frequent in asexually produced individuals of the progeny. Variability studies are important to document the changes occurring in populations and individuals as variability in morphological and physiological traits indicate the existence of different pathotypes. *Alternaria* blight severity on oilseed Brassicas differ season to season, region to region and also individual crop to crop in India (Chattopadhyay et al., 2005). This might be due to the existence of variability among geographically similar isolates of *A. brassicae*. The variability is a well known phenomenon in genus *Alternaria* and may be noticed as changes in spore shape and size, growth and sporulation, pathogenicity, etc. Diversity appears even in single spore isolates.

Many reports on the existence of variability among different *Alternaria* species from different hosts have been reported by earlier workers (Pryor and Gilbertson,

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Table 1. *Alternaria brassicae* isolates infecting *Brassica* cultivars.

A. brassicae isolate	Host	Date of collection	Location	Latitude and longitude	Plant part
BJABI-1 (VR)	*B. juncea* (Varuna)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-2 (DV)	*B. juncea* (Divya)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-3 (LM)	*B. juncea* (local mustard)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-4 (P7)	*B. juncea* (Pre 2007)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-5 (P10)	*B. juncea* (Pre 2010)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-6 (KR)	*B. juncea* (Kranti)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-7 (ND)	*B. juncea* (NDRE4)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-8 (JD)	*B. juncea* (JD-6)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-9 (KA)	*B. juncea* (Kanti)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf
BJABI-10 (AS)	*B. juncea* (Ashirwad)	5th January, 2011	Pantnagar, Uttarakhand	29°N, 79.3°E	Leaf

BJABI, *Brassica juncea Alternaria brassicae* isolates.

2002; Pryor and Michailides, 2002; Quayyum et al., 2005; Kumar et al., 2008) as also within *A. brassicae* species (Kaur et al., 2007). Recently, Meena et al. (2012) studied the aggressiveness, diversity and distribution of *A. brassicae* isolates infecting oilseed *Brassica* in India. Variation in pathogen populations can generally be detected with methods like morphological, cultural, pathogenic and molecular specificity.

DNA markers have become a powerful tool to study taxonomy and molecular genetics of a variety of organisms. The Random Amplified Polymorphic DNA (RAPD) allows quick assessment of genetic variability, and has been used to study inter- and intra-specific variability amongst the isolates of several fungal species. Reports are available who studied the genetic variation within and between *Alternaria* species by random amplified polymorphic DNA (RAPD) molecular marker (Sharma and Tewari, 1995, 1998; Pryor and Michailides, 2002; Kumar et al., 2008). Since the crop and disease are of paramount importance to the Uttarakhand state and no studies on pathogenic and genetic variability have been conducted. Keeping this in mind, the present investigation focused on morphological, cultural, pathogenic and molecular variability of ten mustard isolates of *A. brassicae* in Uttarakhand.

MATERIALS AND METHODS

Collection of *A. brassicae* isolates

Plant material infected with *A. brassicae* was sampled randomly from different cultivars of *B. juncea* grown in the field of Crop Research Centre of G. B. Pant University of Agriculture and Technology, Pantnagar Uttarakhand, India. The isolates of *A. brassicae* were collected and designated as BJABI stands for *Brassica juncea Alternaria brassicae* isolates (Table 1). These selected infected spots were washed 3 to 4 times in sterilized distilled water and then surface sterilized by dipping in 4% NaOCl solution for 1 min, followed by washing with sterilized water 3 to 4 times. Surface sterilized leaf spot pieces were then aseptically transferred into 9 cm Petri dishes containing potato dextrose agar (PDA) and incubated at 25±2°C for seven days. Thereafter, growing mycelia from margin of apparently distinct colonies of the leaf spot pieces on the medium were aseptically transferred into another Petri plate containing PDA medium, where it was grown for 15 days at 23±2°C in the BOD incubator. On the basis of their conidiophore and conidial morphology as described by Simmons (2007), the pathogen was identified as *A. brassicae* (Berk.) Sacc. and purified by single spore isolation method. The isolated fungal pathogen cultures were maintained on PDA slants at 4°C.

Morphological variability of different isolates of *A. brassicae*

Ocular micrometer was calibrated and by use of micrometry (Meena et al., 2005), morphological variability among the 10 isolates of *A. brassicae* was studied in 2010 to 2011. Total of thirty conidia from each slide were examined at 40X magnification of light microscope and measured using ocular and stage micrometer. The average was used to calculate the conidial length, width and number of horizontal septa.

Cultural characteristics of different isolates of *A. brassicae*

The culture character was recorded on day 10 of inoculation of all isolates of *A. brassicae*. Characters like colony color, appearance, growth, shape, margin, sporulation and zonation were recorded by direct observation of culture-grown Petri plate on PDA which was incubated in B.O.D. incubator at 25°C temperature and 100% relative humidity.

Pathogenic variability of different isolates of *A. brassicae*

In order to confirm the identification of the disease and its causal agent, the pathogenicity test was conducted under polyhouse conditions in pot experiments using *B. juncea* cultivar Divya. Seedlings were raised in pots filled with sterilized soil. Spores from the colony were scraped in autoclaved distilled water and spore suspension of 2×10^3 spores/ml concentration was prepared (Giri et al., 2013). Such spore suspension of pathogenic inoculum of the isolates (AS, KA, LM, ND, P7, P10, and VR) was sprayed on 3rd/4th true leaves of 30 days old plant of *B. juncea* cultivar Divya by drop plus agarose artificial inoculation method (Giri et al., 2013). Three quantitative characters namely, disease index, average number of spot/10 cm^2 and average spot size (cm) were recorded on leaves at different time intervals after pathogen inoculation.

Table 2. List of RAPD primers sequences used for the study.

Name of primer	Base sequence (5'-3')
LC 68	AATCGGGCTG
LC 78	GTGATCGCAG
LC 79	CAACGCCGT
LC 80	CAGCACCCAC
LC 82	TTCCGAACCC
LC 86	GTTGCGATCC
LC 87	AGGTGACCGT
LC 90	GTGAGGCGTC
LC 94	GTCGCCGTCA
LC 95	TGAGCGGACA
LC 96	TTGGCACGGG
LC 97	GTGTGCCCCA
LC 99	AGCGCCATTG
LC 103	AGGGCGTAAG
LC 106	GTGACATGCC
LC 109	ACGCACAACC
LC 110	GGACTGCAGA
LC 111	CTCTCCGCCA
LC 113	AGACGTCCAC
LC 114	GACGCCACAC
LC 118	TCAGAGCGCC
LC 130	CCTGGGTTCC
LC 131	CCTGGGCCTA
LC 132	CCTGCGCTTA
LC 133	CCTGGGTCCA
LC 135	CCGGCCTTAG

Molecular variability of different isolates of *A. brassicae*

Molecular variability among ten single spore cultures of *A. brassicae* was analysed by RAPD molecular marker. Genomic DNA of ten single spore cultures of *A. brassicae* was isolated separately by using standard cetyl trimethyl ammonium bromide (CTAB) extraction method of Doyle and Doyle (1990). Molecular variability among *A. brassicae* isolates were studied by using twenty six RAPD primers from Life Tech Company (Table 2). The polymerase chain reaction (PCR) master mix was prepared with 1X *Taq* polymerase buffer, 1.8 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 pM primers and 1.5 U of *Taq* polymerase. Thereafter, 20 µl of master mix was added with 5 µl (50 ng) DNA in PCR tubes. 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 4 min followed by final extension at 72°C for 10 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: AlphaImager^{TM} 3400). The sizes of the amplification product were estimated using 100 bp to 3.0 kb ladder (Φ × 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, the RAPD reaction results were analyzed using software Gene Profiler. In order to analyze the relatedness among the species, a dendrogram based on unweighted pair group method with arithmetic average (UPGMA) and Nei and Li genetic distance matrix (Nei and Li, 1979) value was obtained.

RESULTS AND DISCUSSION

Morphological variability among isolates

The 10 single-spore isolates of *A. brassicae* showed significant ($P<0.05$) morphological variability (Figure 1) in respect of conidia length, conidia width and number of horizontal septa (Table 3). It was revealed that out of the 10 isolates, ND, KA, and JD showed maximum spore length (135 µm) followed by LM, VR, KR (128 µm), DV, P7, P10 (114 µm), and AS showed lowest spore length (105 µm). Whereas, in terms of conidia width maximum was shown by P7 (20 µm) followed by ND, LM, KA, P10, VR, AS (15 µm), DV, KR (12 µm) and JD showed minimum conidia width (10 µm). Highest number of horizontal septa was shown by KR (9.4) followed by JD (9.2), ND (9.1), DV (8.5), P10 (8.4), VR (8.2), LM (7.4), AS (7.3), KA (7.1) and P7 (6.9) showed minimum number of horizontal septa. 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), who observed morphological variability in different geographical isolates within *Alternaria* species.

Cultural characteristic of *A. brassicae* isolates

Isolates of *A. brassicae* showed variable cultural characteristics like colony color varied from white, off white to light brown, appearance of the colony from cottony, flurry to feathery, colony growth varied from slow, medium to fast, colony margin from wavy, smooth to rough (Figure 2). Based on these characteristics, all *A. brassicae* isolates could be grouped into three colony types. Group 1 isolates (DV and P7) produced white colonies with a fluffy appearance.

The colony was circular in shape with smooth margins. Group 2 isolates (KA, LM, ND, P10 and VR) produced off white colonies with a cottony and feathery appearance. The colony was circular in shape with all types of margins. Group 3 isolates (AS, JD and LR) produced light brown colonies with cottony appearance. The colony was circular in shape with wavy and rough margins (Table 4). Such kind of variability among the different *A. brassicae* isolates were also reported by Vishwanath (1999) and Meena et al. (2012).

A. brassicae colony size was also measured by the measurement of colony diameter. *A. brassicae* isolates also showed variability in their colony size. On the PDA medium, KA (3.55 cm) showed highest colony size followed by DV (3.27 cm), ND (3.27 cm), VR (3.27 cm), P7 (3.20 cm), AS (3.07 cm), LM (2.90 cm), KR (2.82 cm),

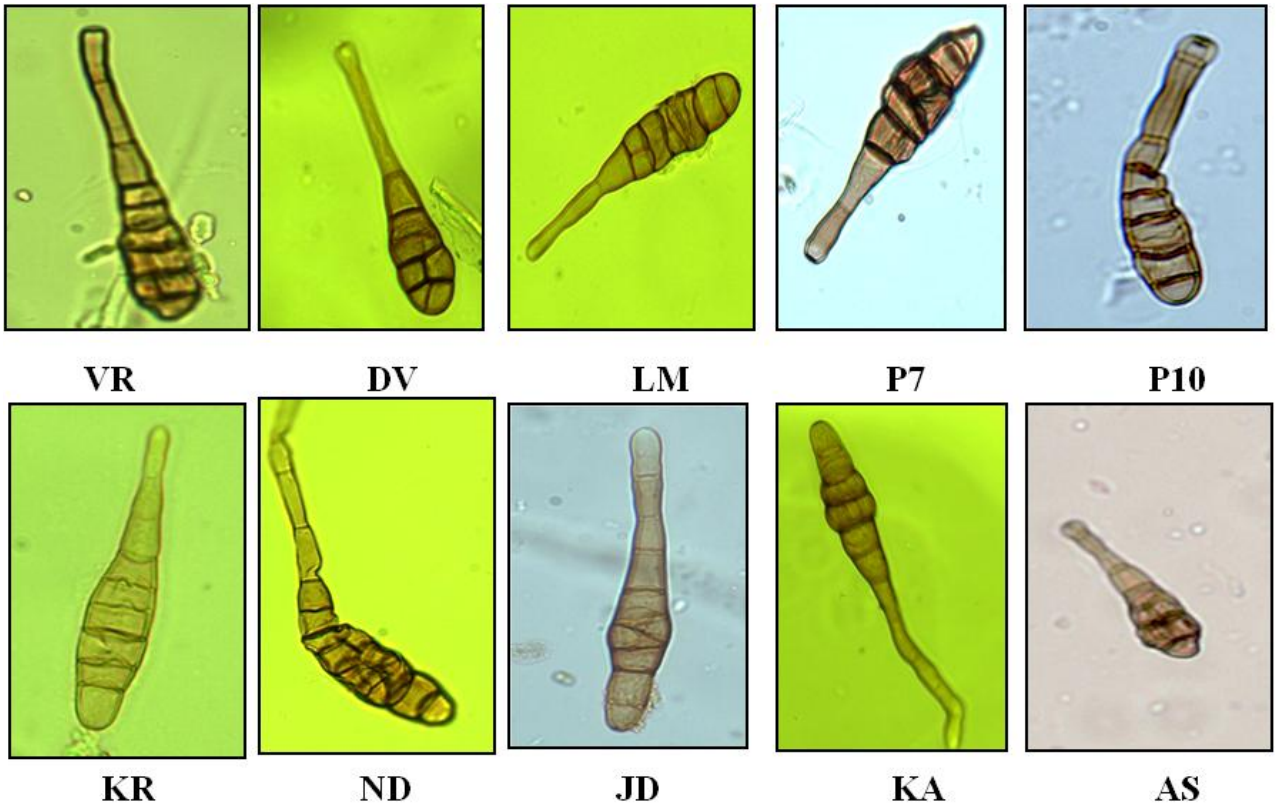


Figure 1. Conidia of different *A. brassicae* isolates.

Table 3. Conidial size of *A. brassicae* isolates.

Different isolates of *A. brassicae*	Average length (µm)	Average width (µm)	Average number of horizontal septa
AS	105	15	7.3
DV	114	12	8.5
JD	135	10	9.2
KA	135	15	7.1
KR	128	12	9.4
LM	128	15	7.4
ND	135	15	9.1
P7	114	20	6.9
P10	114	15	8.4
VR	128	15	8.2
Mean	123.6	14.4	8.15
CV %	26.06	14.62	28.06

*Average of 30 conidia in each isolates.

P10 (2.80 cm); while JD (2.40 cm) showed lowest colony size (Figure 3).

Pathogen aggressiveness of *A. brassicae* isolates

Different isolates of *A. brassicae* showed variable response on host *B. juncea* cultivar Divya. Variation in the disease index (Figure 4a), average number of spots/10 cm^2 (Figure 4b) and average size of spot (cm) (Figure 4c) on same host depending on aggressiveness of isolates revealed that the variability exist among *A. brassicae* isolates. ND was found to be the most aggressive whereas AS was found to be the least aggressive isolate.

A similar study was conducted by Michereff et al. (2003) who studied 38 isolates of *Alternaria brassicicola*

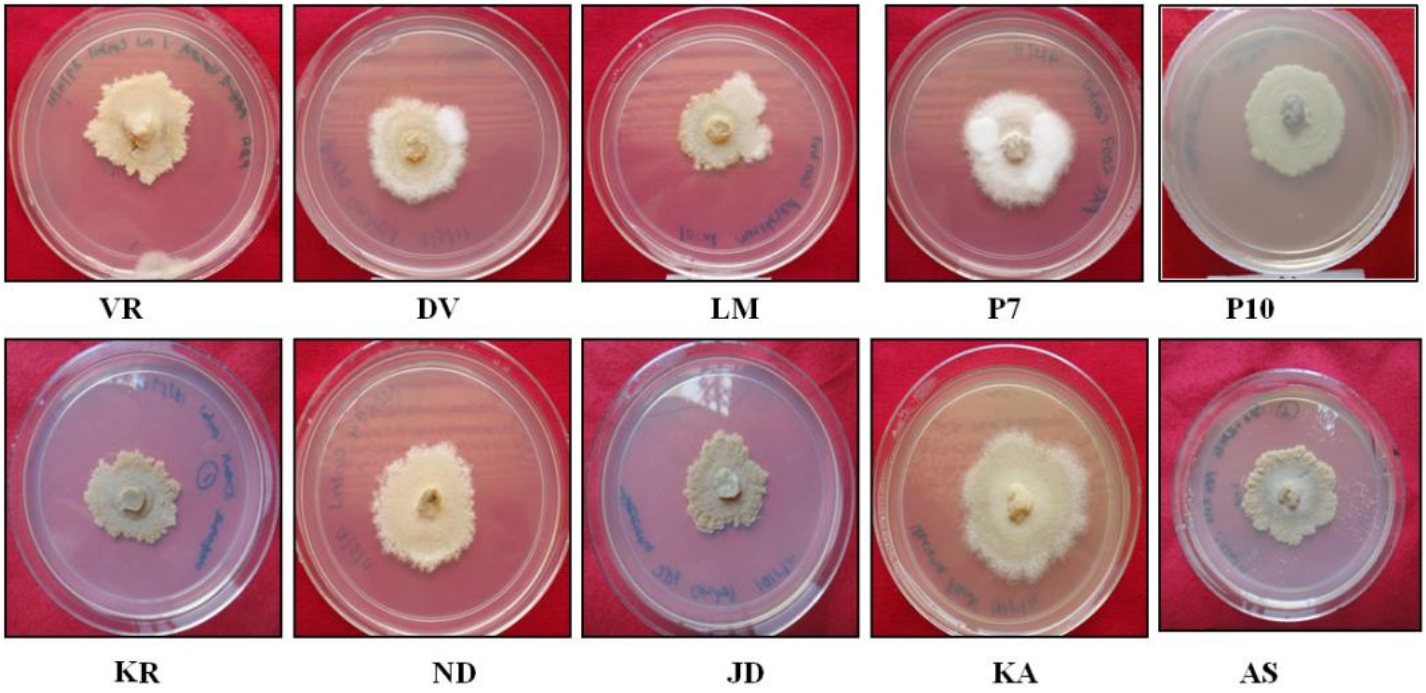


Figure 2. Colony morphology of *Alternaria brassicae* isolates.

Table 4. Colony morphology of *Alternaria* isolates on PDA medium.

Isolate	Color	Appearance	Growth	Shape	Margin	Sporulation	Zonation
AS	Light brown	Cottony	Medium	Circular	Wavy	Late	Present
DV	White	Fluffy	Fast	Circular	smooth	Medium	Present
JD	Light brown	Cottony	Slow	Circular	Rough	Late	Absent
KA	Off White	Cottony	Fast	Circular	Rough	Medium	Present
KR	Light brown	Cottony	Fast	Circular	Rough	Late	Present
LM	Off White	Cottony	Medium	Circular	Wavy	Early	Present
ND	Off White	Cottony	Medium	Circular	Rough	Late	Absent
P7	White	Fluffy	Medium	Circular	Smooth	Early	Present
P10	Off White	Feathery	Medium	Circular	Smooth	Late	Absent
VR	Off White	Cottony	Medium	Circular	Rough	Late	Absent

and estimates variability based on disease development and pathogen physiology and found that *A. brassicicola* isolates were highly variable. In another study, Kaur et al. (2007) reported the pathogenic variability among *A. brassicae* isolates considering only percent disease severity.

Molecular variability of *A. brassicae* isolates

Analysis by 26 RAPD primers revealed a high level of genetic variability among ten isolates of *A. brassicae* of different cultivars of *B. juncea*. Amplification of DNA of all the *A. brassicae* isolates produced 1014 scorable and reproducible RAPD markers. On an average, 39 bands were produced. The dendrogram prepared by using the similarity coefficients (Figure 5) clustered the ten representative isolates into two major groups that is, Groups I and II at only 15% similarity coefficient (85% dissimilarity). Group I was composed of seven isolates namely, VR, DV, P7, LM. P10, KR and ND with 18% similarity (82% dissimilarity) while group II was composed of only three isolates namely, JD, KA and AS with only 24% similarity (76% dissimilarity).

Seven isolates of the group I were sub-clustered into two minor clusters, of which one was composed of six isolates namely, DV, P7, LM. P10, KR and ND with 24% similarity (76% dissimilarity) while another was composed

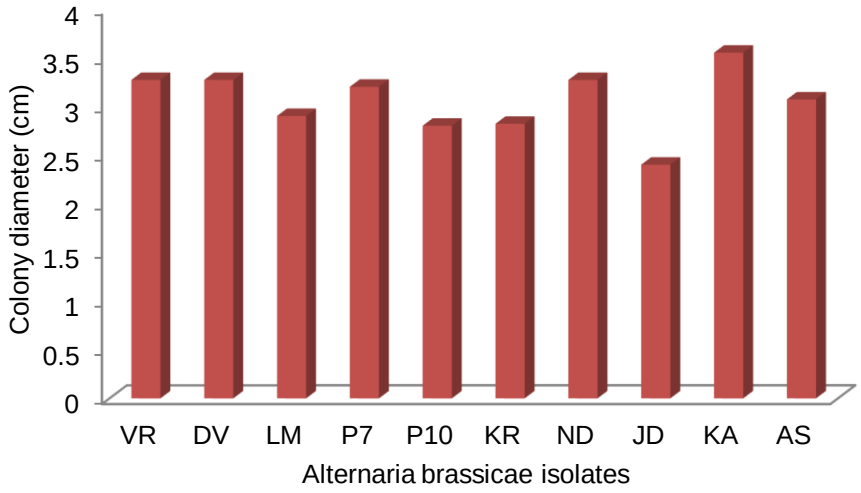
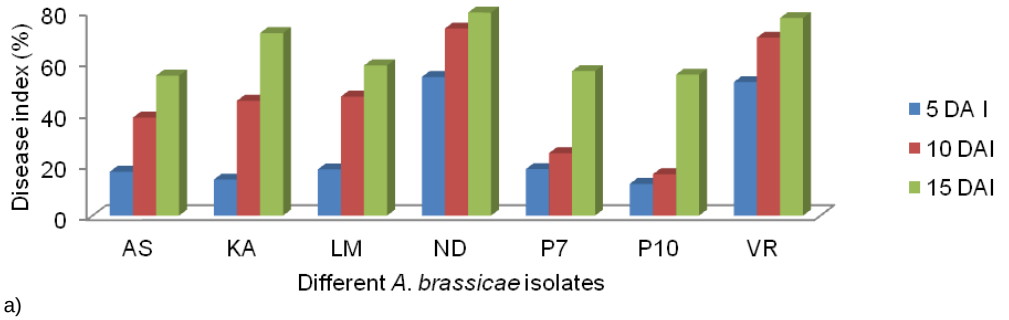
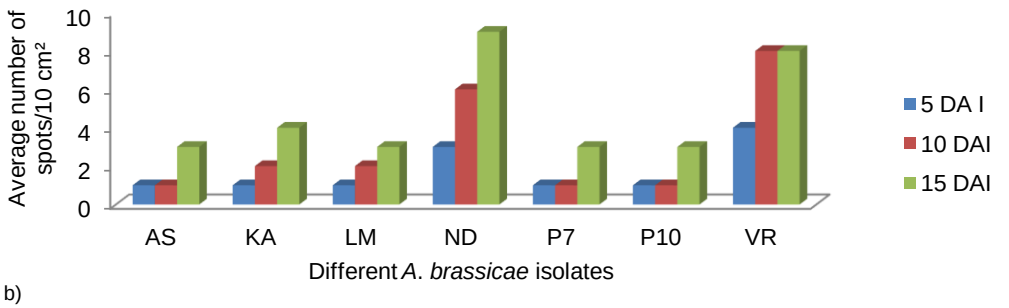


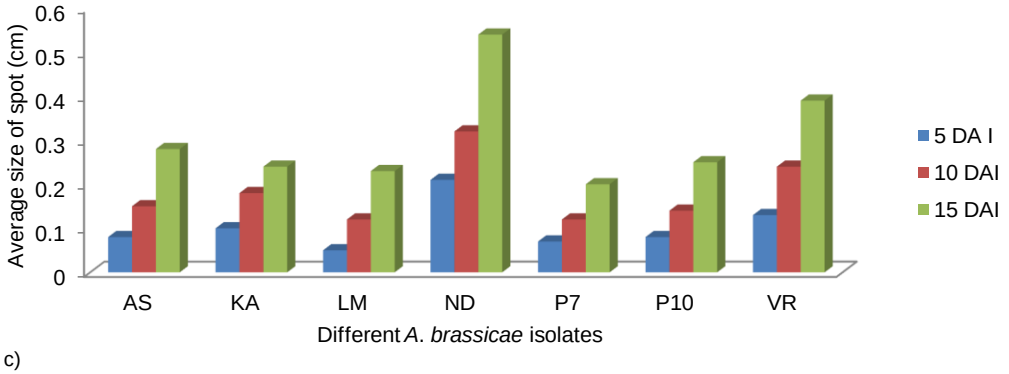
Figure 3. Growth of *A. brassicae* isolates on PDA.



a)



b)



c)

Figure 4. a) Disease index, b) average number of spots/10 cm^2, c) average size of spot (cm) on leaves of Divya inoculated by isolates grown on the PDA medium on 5, 10 and 15 days after inoculation (DAI).

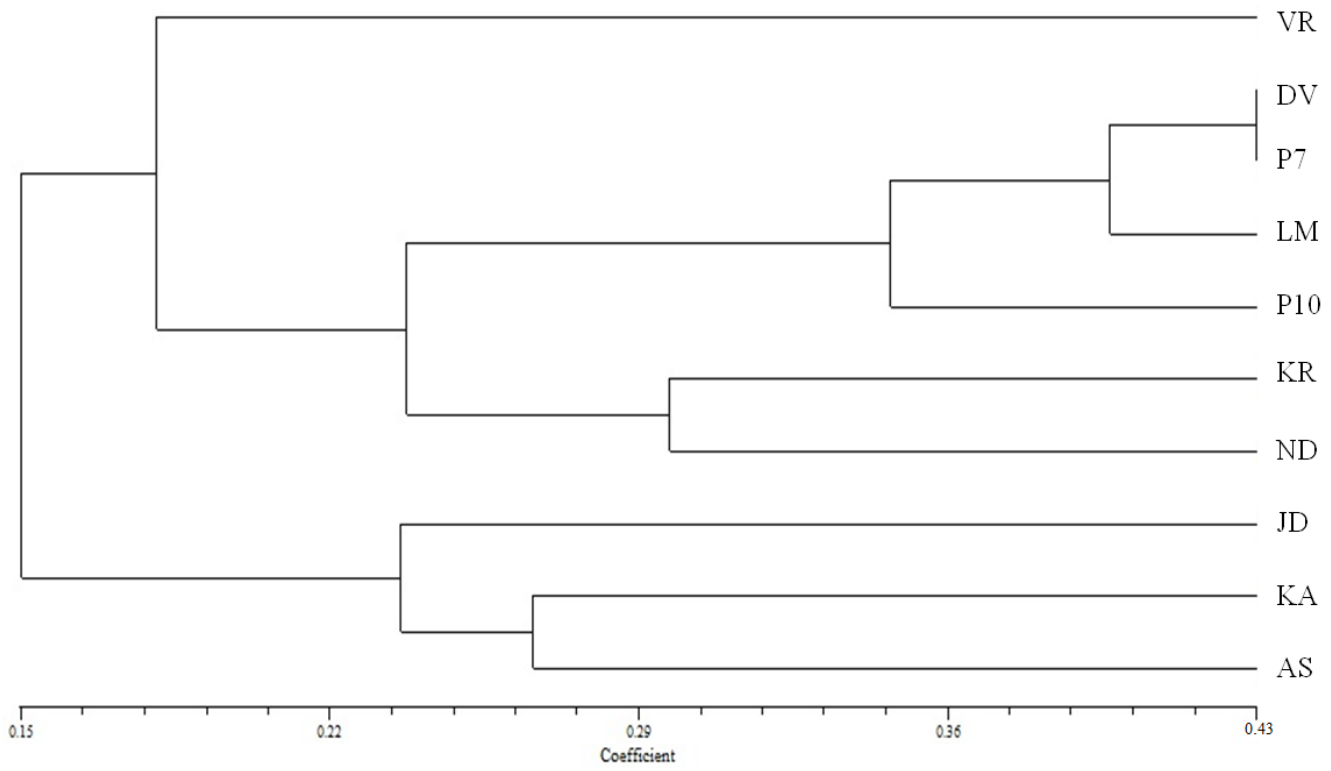


Figure 5. Genetic divergence among 10 isolates of *A*. *brassicae* based on UPGMA cluster analysis.

of remaining one isolates VR 18% similarity (82% dissimilarity). Likewise, group II was sub-clustered into two minor clusters, of which one was composed of two isolates namely, KA and AS with 27% similarity (73% dissimilarity) while another was composed of remaining one isolates JD with 23% similarity (87% dissimilarity).

The present results indicated high genetic divergence among the 10 isolates of *A. brassicae.* Polymorphism within an *Alternaria* species by RAPD molecular marker has been described by many workers (Sharma and Tewari, 1995, 1998; Kumar et al., 2008). Sharma and Tewari (1995) observed polymorphism among *A. brassicae* isolates from different geographical regions of the world. However, in 1998 they found low intra-regional variation among Indian and Canadian isolates of *A. brassicae* with 75% similarity among them. Although, the genus *Alternaria* is known as an imperfect fungus, it shows genetic variability within a species and this variability might be due to the existence of mutation, somatic hybridization, heterokaryosis, uniform host selection, extensive dispersal or of a cryptic sexual stage. High degree of genetic variability was observed among only ten isolates of *A. brassicae* from different *B*. *juncea* cultivars growing in Pantnagar region of Uttarakhand. This could be the probable possible reason behind extreme and different disease reaction of germplasm at Pantnagar from observations at most of other locations. In order to provide a better picture of the pathogenic as well as genetic divergence among *A. brassicae* populations of India, there is need to conduct similar holistic investigation among higher number of *A. brassicae* isolates which could be helpful to generate resistant material against *Alternaria* blight in oilseed Brassicas.

Conclusion

The variation in cultural, morphological, pathogenic and molecular characters of isolates observed indicated the existence of different strains of pathogen. Similar characters have formed the basis for defining the existence of different strains among the species of fungi imperfection.

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