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Morphological, cultural, pathogenic and molecular studies of *Alternaria brassicae* infecting cauliflower and mustard in India

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Dark leaf spot *(Alternaria brassicae*) is one of the important diseases in crucifers causing serious yield and quality loss in production due to the seed borne nature of the pathogen. Variation in morphology and cultural characteristics among 32 representative Indian geographical isolates of *Alternaria brassicae*, the causal agent of Alternaria blight of cauliflower (Vegetable) rapeseed-mustard (Oil seed), was studied. All the isolates showed high level of variability *in vitro* in respect of conidial length, width, and number of septa. Conidia of Uttar Pradesh isolate (CaAbU4) were smallest in size with lowest number of septa. Substantial variation was found in mycelial growth, sporulation among these isolates in different nutrient media. All the isolates did not grow and sporulate abundantly on the same nutrient medium. However, Potato Dextrose Agar, Cauliflower (Host) Agar medium and Carrot Potato Agar were good for all the cultures. Variation in mycelial growth, sporulation was also observed. Cluster analysis of data on cultural variability among thirty two *A. brassicae* isolates found a close relationship among isolates of both origins viz, from Cauliflower and mustard. Isolates from Uttar Pradesh, Delhi, Haryana and West Bengal were found to be more similar to each other whereas the Rajasthan isolates along with Tamil Nadu and Kerala isolate were distantly related to others. All the isolates were pathogenic in nature but not directly related to the cultural and the morphological characteristics. These isolates were further molecularly characterized by using internal transcribed spacer region where all the isolates were found 56% similar to each other and 99% similar to the *A. brassicae* isolates present in NCBI database.

Key words: Dark leaf spot, *Alternaria brassicae*, morphology, cultural variability, pathogenicity, ITS analysis.

INTRODUCTION

Among several fungal diseases, severe damage of the foliage or seed germination in crucifers occurs due to Alternaria blight caused by *Alternaria brassicae* (Berk) (Tewari, 1983; Weiss, 1983; Kolte, 1985; Tewari, 1991; Verma and Saharan, 1994). Black spot of different crucifers viz-oil seed rape, cabbage, cauliflower and mustard have been reported in many countries; Italy (Tosi and Zazzerini, 1985), USA, UK and several other European countries (Gladers, 1987), Canada (Berkenkamp and Kirkham, 1989; Conn and Tewari, 1990), Iran (Nourani et al., 2008) including India (Meena et al, 2010). Cauliflower (*Brassicae oleracea* var botrytis) and mustard (*Brassicae juncea*) are the two important crucifer crops of India which are facing serious yield and quality loss in production due to *Alternaria brassicae* (Berk) Sacc. causing dark leaf spot disease (Sharma et al., 2013).

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This species have the ability to survive in seeds for several months at different temperatures and relative humidity (Kumar and Gupta, 1994; Abul-Fazal et al., 1994). Losses up to 30% and 47% were caused by *Alternaria brassicae* in cauliflower (*Brassica oleracea* var *botrytis*) (Tamayo et al., 2001) and Indian mustard (*Brassica juncea*) (Chattopadhyay, 2008) respectively. Morphological characteristics of conidia and conidiophores and sometimes host plant association, provide the major taxonomic criteria for delimitation of fungal species (David, 1991). However, the classification of small spored species, including host-specific toxin producing fungi, has been particularly confused, because of the simple and convergent morphology of conidia and facultative parasitism, resulting in an ambiguous host range (Simmons, 1999). However, according to the study of Pattanamahakul and Strange (1999), the taxonomy of *Alternaria* on brassicas has been based principally on morphology and sometimes host plant association of each of the species occurring *(A. brassicicola, A. brassicae and A. raphani)* has a distinct morphology considering the diversity of conidium shapes and sizes among *Alternaria* spp. All commercial cultivars of brassicas are susceptible to this pathogen (Tewari, 1991). Till now no resistance are found among the crucifers against *Alternaria brassicae*. Development of resistant cultivars requires knowledge of pathogen variation present in different regions where these crucifers are grown. Severity of *Alternaria* blight on Brassicas 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. Many reports on the existence of morphological variability within the isolates of other *Alternaria* species have been reported by earlier workers (Verma and Saharan, 1994; Varma et al., 2006). Variability in the morphological characteristics in *A. brassicae* isolates of different regions of India have been reported (Meena et al., 2005; Kaur et al., 2007; Singh et al., 2007; Goyal et al., 2011).

Some researchers have worked on cultural variability in *Alternaria* species in respect of mycelial growth and sporulation (Ansari et al., 1989), media (Patni et al., 2005). Variability on the basis of morphology, sporulation, growth and other cultural characteristics also have been reported earlier (Kaur et al., 2007). Morphological and cultural variability among the oilseeds *Brassica* isolates of *A. brassicae* from different geographical regions of India were reported from across the rapeseed-mustard growing region of India (Goyal et al., 2011). However variability among the *A. brassicae* isolates from vegetable crop like Cauliflower is still missing.

The molecular approaches have been used increasingly in taxonomy and systematics of filamentous fungi including phytopathogens at the species and subspecies level (Benali et al., 2011). Variation in nuclear ribosomal DNA sequences among *Alternaria* species pathogenic to crucifers has recently been reported from *A. brassicae*, *A. brassicicola* (Schwein) Wiltshire, *A. raphani* J. W. Groves & Skolko and *A. alternata* (Fr.) Keissl. (Jasalavich et al., 1995).

The objective of this study was to evaluate isolates of *A. brassicae* collected from both crucifer crops cauliflower (vegetable) and mustard (oilseed) at morphological and cultural level and further relate at molecular level.

MATERIALS AND METHODS

Collection and maintenance of *Alternaria brassicae* isolates from black spot infected leaf samples

Alternaria brassicae isolates were obtained from black leaf spot infected leaf samples of both cauliflower and mustard (Table 1) collected during winter season in 2009 to 2011 from seven different regions of India namely Delhi, Uttar Pradesh, Rajasthan, Haryana, West Bengal, Tamil Nadu and Kerala. To obtain isolates from infected cauliflower and mustard leaves and other plant tissues, blighted leaf and stem pieces (2mm) were surface sterilized with 0.1% Mercuric Chloride ($HgCl_2$) for one minute, rinsed in sterile water 3x for 10 min and placed on Potato Dextrose Agar (PDA) plates. Fungal growth was observed after 5-7 days of incubation at 25°C. *A. brassicae* isolates growing on the leaf/stem pieces were transferred onto other PDA plates until purification by single spore technique. These isolates were purified and preserved as PDA slants at 4°C.

Single spore isolation

A total of 32 putative single spore *Alternaria* colonies were randomly picked from the lesions on infected plant parts under binocular microscope, and seeded onto the surface of water agar using tip of a sharp sterile inoculating needle. Inoculated plates were incubated on laboratory bench at room temperature (20-24°C). Conidial germination on the plates was checked daily and upon germination, agar blocks bearing single germinated conidia were cut out and aseptically seeded on PDA plates. The plates were incubated for 14 days at room temperature and natural lighting conditions (20-24°C and 12-h light). Plates of primary media were centrally inoculated with 2-mm diameter plugs taken from the edge of actively growing 4 day old cultures, and then incubated at 25°C after 72 h, the colony diameters were measured and agar blocks were transferred to the fresh media. Spore yields were determined with a haemocytometer after 24 h of incubation on different media.

Evaluation of morphological characters

All the isolates were microscopically identified based on their morphology on PDA using light microscope (Carl Zeiss, Germany) and available literature (Ellis, 1971). For all isolates, morphological characteristics of the colony and sporulation apparatus were determined from single-spored colonies as described earlier. The nature of mycelia growth, shape of conidia was noted. The size and shape of conidia (length and width) was determined using ocular and stage micrometer. Numbers of septa were also recorded.

Effect of different types of media type on growth and sporulation

The objective of this experiment was to establish the suitable growth conditions for the *in vitro* growth and sporulation of Alternaria pathogen. Investigation was done on growth rate and conidia formation of the Alternaria pathogen. Seven types of media

Table 1. Morphological and cultural characteristics of *Alternaria brassicae* isolates (32) collected from Cauliflower and Mustard grown in different states of India.

Isolate code	Plant Part	Place of Collection	State	Geographical Data	Accession Nos. of ITS sequences	Culture appearance on PDA plate	Myceliu m colour	Conidia			
								Colour	Surface	Shape	Beak
CaAbU2	Cauliflower Leaf	Ghaziabad	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439438	Light Olive Gray	Golden	Golden	Rough	Long Obpyriform	Long
CaAbU3	Cauliflower Leaf	Alipur	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439439	Olivaceous black	Light golden	Golden	Rough	Long Obpyriform	Long
CaAbU4	Cauliflower Leaf	Noida	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439440	Olivaceous black	Golden	Golden	Smooth	Obpyriform	Long
CaAbU5	Cauliflower Leaf	Meerut	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439441	Dark Olive gray	Golden	Golden	Smooth	Pyri Obpyriform	Long
CaAbU6	Cauliflower Leaf	Bagpat	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439442	Olive gray	Golden	Golden	Rough	Long Obpyriform	Long
CaAbU7	Cauliflower Leaf	Saharanpur	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JN108902	Dark olive gray	Light brown	Light brown	Smooth	Obpyriform	Long
CaAbU8	Cauliflower Leaf	Kanpur	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439443	Olive gray	Brown	Brown	Smooth	Long Obpyriform	Long
CaAbU9	Cauliflower Leaf	Lucknow	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JF439444	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
CaAbD1	Cauliflower Leaf	Vegetable fields, IARI	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JF439431	Dark olive gray	Brown	Brown	Smooth	Long Obpyriform	Long
CaAbD2	Cauliflower Leaf	Najafgarh	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JF439432	Olivaceous black	Brown	brown	smooth	Long Obpyriform	Long
CaAbD3	Cauliflower Leaf	Vegetable fields, IARI	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108900	Dark olive gray	Brown	Brown	Rough	Obpyriform	Long
CaAbD4	Cauliflower Leaf	Sarai Kala Khan, NCR	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JF439433	Dark olive gray	Brown	Golden brown	Smooth	Obpyriform	Long
CaAbD5	Cauliflower Leaf	Mother Dairy fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JF439434	Brownish black	Golden	Golden	Smooth	Long Obpyriform	Long
CaAbD6	Cauliflower Leaf	Pusa Campus	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JF439435	Brownish black	Brown	Dark brown	Smooth	Long Obpyriform	Long
CaAbR3	Cauliflower Leaf	Bansa, Jaipur	Rajastha n	Latitude : 25° 14' N Longitude: 75° 49' E	JF439436	Iron gray	Deep brown	Brown	Smooth	Obpyriform	Long
CaAbR4	Cauliflower Leaf	Samode, Jaipur	Rajastha n	Latitude : 25° 14' N Longitude: 75° 49' E	JF439437	Iron gray	Brown	Brown	Rough	Obpyriform	Long
CaAbH1	Cauliflower Leaf	Kalyani	West Bengal	Latitude : 22° 36' N Longitude: 88° 24' E	JF439448	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long

Table 1. Contd.

CaAbK1	Cauliflower Leaf	Hoogli	West Bengal	Latitude : 22° 36' N Longitude: 88° 24' E	JF439449	Olive gray	Brown	Brown	Rough	Obpyriform	Long
CaAbW1	Cauliflower Leaf	Kolkata	West Bengal	Latitude : 22° 36' N Longitude: 88° 24' E	JF439450	Dark olive gray	Brown	Brown	Smooth	Obpyriform	Long
CaAbW2	Cauliflower Leaf	Sonepat	Haryana	Latitude : 28° 59'N Longitude: 77° 0'E	JF439445	Dark olive gray	Brown	Brown	Rough	Obpyriform	Long
CaAbW3	Cauliflower Leaf	Pallakkad	Kerala	Latitude : 10° 00' N Longitude : 76° 25' E	JF439446	Black	Brown	Brown	Smooth	Obpyriform	Long
CaAbT5	Cauliflower Leaf	Coimbatore	Tamil Nadu	Latitude : 11° 00' N Longitude : 78° 00' E	JF439447	Olivaceous black	Brown	Brown	smooth	Obpyriform	Long
MAb1	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108903	Dark Olive gray	Brown	Dark brown	Smooth	Obpyriform	Long
MAb2	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108904	Dark Olive gray	Brown	Brown	Smooth	Obpyriform	Long
MAb3	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108905	Olivaceous black	Brown	Dark brown	Smooth	Obpyriform	Long
MAb4	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108906	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb5	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108907	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb6	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108908	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb7	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108909	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb8	Mustard Leaf	IARI fields	Delhi	Latitude : 28° 36' N Longitude: 77° 12' E	JN108910	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb10	Mustard Leaf	Katyali	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JN108911	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long
MAb11	Mustard Leaf	Katyali	Uttar Pradesh	Latitude: 29° 58' N Longitude: 77° 23' E	JN108912	Olivaceous black	Brown	Brown	Smooth	Obpyriform	Long

Ca: Cauliflower, M: Mustad, Ab: *Alternaria brassicae,* U: Uttar Pradesh, D: Delhi, R: Rajasthan, W: West Bengal, H: Haryana, K: Kerala, T: Tamil Nadu. All the morphological characters such as culture appearance on PDA plate, Mycelial colour, conidia appearance were observed and data were collected. Accession numbers of ITS sequences of the *A. brassicae* isolates deposited to NCBI database.

were tested to determine their effect on growth and sporulation of the pathogen. These were potato dextrose agar (PDA), Cauliflower agar media (CAM), Carrot Potato Agar (CPA), Oat Meal Agar (OMA), Czapex Dox Agar (CDA), V8-Juice Agar (VJA) and Corn Meal Agar (CMA). The different media served the purposes of growing of cultures, cleaning of cultures and sporulation of cultures.

Growth media for *Alternaria* species was i) Potato dextrose agar (PDA) made of Dextrose (20 g), agar (15 g) and Potatoes infusion from 200 g in 1000 ml water, ii**)** Cauliflower Agar Media (CAM) made of Dextrose (20 g), agar (15 g) and Cauliflower infusion from 200 g in 1000 ml water, iii) Carrot Potato agar (CPA) 24 g in 1000 ml distilled water, iv)Oat Meal Agar (OMA), 38 g in 1000 ml distilled water, v) Czapex Dox Agar (CDA) 49 g in 1000 ml distilled water, vi) V8 Juice Agar (VJA) 44.3 g in 1000 ml distilled water and vii) Corn Meal Agar (CMA) 17 g in 1000 ml distilled water. All synthesized media were of Himedia. The agar plates were autoclaved at 121°C for 15 min and approximately 20 ml were dispensed into sterile 90 mm plastic Petri dishes to form a layer of 2 mm deep. The media was inoculated using a uniform culture plug of 4 mm in diameter obtained from 14-day-old culture plates and placed in the centre of each dish. The plates were incubated at room temperature. The experiment was arranged in a completely randomized design with 3 replicates. Fungal growths (Radial growth in cm) of different isolates were studied. Measurements on radial colony diameter were taken on 7th day after inoculation whereby six plates were sampled each time for each media treatment.

Measurement of fungal sporulation

To determine conidial concentration of each isolates, cultures grown in the seven different media plates were considered. Ten milliliter (10 ml) of sterile distilled water was added to culture plate and using a sterile glass slide, the culture surface was gently scrapped to make a conidial suspension. Conidial concentration was determined using a haemocytometer.

Pathogencity testing of *A. brassicae* isolates

Twenty-two *A. brassicae* isolates from Cauliflower were tested for symptom production on a susceptible cultivar of Cauliflower viz., DC-23000. Similarly ten *A. brassicae* isolates from Mustard were tested on a susceptible variety of mustard viz., Pusa Jagganath. The seeds of cauliflower were sown in nursery beds during October, 2009-2010 and 2010-2011 seasons. Cauliflower Seedlings were transplanted after two weeks in fields with a spacing of 30 cm x 40 cm. Similarly mustard seeds were sown on fields. After 60 days leaves from both the cauliflower and mustard plant were taken for the detached leaf inoculation method. Test Leaves were properly washed under running tap water and then surface wiped off with 70% alcohol and 2 microlitres of 4 $x10^4$ spores ml^{-1} spores were inoculated with a fine needle (Dispovan, 2.5 ml) while sterile distilled water was applied on control. The leaves were placed inside moist chambers in green house conditions and were observed for appearance of disease symptoms on 7 day after inoculation. Experiments were conducted in completely randomized design (CRD). Each treatment consisted of three replicates. Symptoms observed were ranked as minus (-) for no symptom and plus (+) for black leaf spot with yellow halos on inoculated leaves. Appearance of symptom again divided into three groups viz., black spot with diameter 0.2-0.5cm ranked as single plus (+), spot with diameter 0.6-1.0cm ranked as double plus (++) and spot diameter more than 1cm were ranked as three plus sign (+++).

DNA extraction and purification

All the 32 *A. brassicae* (22 from cauliflower and 10 from mustard) isolates were grown on Potato Dextrose Broth (PDB) medium in 100 ml capacity conical flask for 7 days at 28°C. Mycelial mats were harvested by Whatman No.1 filter papers and lyophilized. DNA was isolated according to a modified Cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). Around 300 mg of lyophilized fungal material was ground in liquid nitrogen, dispersed in 800 µl of 2% CTAB extraction buffer at 65°C in water bath for 30 min. An equal volume of Chloroform: isoamyl alcohol (24:1, v/v) was added, mixed well and centrifuged (4000 rpm, 10min). The upper aqueous phase was transferred to a fresh tube. Nucleic acids were precipitated by adding 0.6 volume of ice cold isopropanol, and collected by centrifugation (14000 rpm for 30 min). Pellet was washed twice with 70% ethanol and then air dried by putting the tube face down on a paper towel. Then pellet were solubilized in 200 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH- 8.0). DNA extracts were then purified by treating with 10U of DNase- free RNase (Biomatrix Corp) for one hour at 37°C. RNase was removed by Phenol: Chloroform: Isoamyl alcohol (25:24:1) extraction step. DNA was treated with 3 M Sodium Acetate and ethanol precipitated, centrifuged, dried and dissolved in an appropriate volume of TE buffer.

Internal transcribed spacer (ITS) region analysis

Thirty two *A. brassicae* isolates were analyzed by amplifying the regions of the rDNA repeat from the 3'end of the 18s and the 5' end of the 28s gene using PCR conditions with the two universal primers, ITS1-3' TCC GTA GGT GAA CCT GCG G 5' and ITS4-3' TCC TCC GCT TAT TGA TAT GC 5' which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White et al., 1990; Jasalavich et al., 1995). The PCR- amplification reactions were performed in a 25 µl mixture containing 50 mM KCl, 20 mM Tris HCl (pH 8.0), 2.0 mM $MgCl_2$, 20 µM of each of the four deoxynucleotide triphosphates, 20 pmol of each primer, 50 ng/µl of template and 2.5 U of Taq polymerase. These reactions were subjected to an initial denaturation hot start at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 35 s, primer annealing at 60°C for 1 min 30 s and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C in a thermal cycler. Aliquots (4 µl) of the amplified products were analysed by electrophoresis in 1.5% (wt/vol) agarose gel in 1X TAE buffer (40 mM Tris, 20mM acetic acid, 1mM EDTA [pH8]), stained with ethidium bromide (1 µg/ml) and electrophoresis was carried out at 70 volts for 2 h in TAE buffer. The molecular marker was 100 bp ladder (Biomatrix Co. Ltd.). The desired bands were cut from the gel with minimum quantity of gel portion and the amplified PCR product was eluted using QIAGEN DNA gel extraction kit.

Nucleotide sequencing and *in silico* analysis

The sequencing of the PCR product was carried out in automated Sequencer at Xcelris Lab., Bangalore, India. Related sequences were searched for homology using BLAST bioinformatic search tool available at the Gen-Bank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997). Sequences used for comparison were obtained from NCBI database (http://www.ncbi.nlm.nih.gov) and the details are given in the results.

The multiple sequence alignment and pair wise alignment were performed using the Clustral W algorithm in Bioedit (Hall, 1999) and Phylogenetic analysis was done using MEGA5.0 (Tamura et al., 2011) software. To assess the possible phylogenetic relationship neighborhood-joining bootstrap tree was created using CLUSTAL W 1.6 matrix by the CLUSTAL X programme ver. 1.81 (Thompson et al., 1997). The ITS sequences were submitted to GenBank. Sequences used for comparison were obtained from NCBI database (http://www.ncbi.nlm.nih.gov) and the details are given in the results.

Statistical analysis

Averages of colony diameter on each media plates and number of conidia/ ml for each isolates were taken for subsequent data analysis. Analysis of variance and separation of means to determine differences in growth rates on media types was analyzed by performing two-way ANOVA using statistical software PRISM version 3.0 at $p<0.0001$. Similarly the conidial characters were also statistically analyzed at $p<0.05$ by performing one way ANOVA and least significant difference was calculated by student's t-test.

RESULTS

Morphological characterization

Differences in rates of linear growth and colour of the colony were observed among all 32 isolates of *A. brassicae* (Table 1; Figure 1) obtained from the infected Cauliflower and Mustard leaves. The color of the colonies and the conidia grown on PDA showed little variation. The color of the *A. brassicae* isolates varies between light olive gray to olivaceous black. The mycelia colour varies between brown and golden. The conidia characteristics were also similar to each other among the isolates viz., conidia color was golden or brown with mostly smooth surface. Most of the conidia were long obpyriform in shape with long beak.

The 32 single-spore cultures of *A. brassicae* showed significant ($P < 0.05$) morphological variability in respect of conidia length, conidia width and number of septa (Table 2; Figure 1). Average conidial length, which varied from 37.88 to 57.65 µm, was highest in West Bengal cauliflower isolate (CaAbW1) that is, 57.65 µm and lowest in Delhi mustard isolate (MAb2) that is, 37.88 µm. Average conidial width, which varied from 6 to 9.5 µm, was highest in West Bengal cauliflower (CaAbW2) isolates that is, 9.5 µm and lowest in Delhi cauliflower isolate (CaAbD1) that is, 6 µm. The average number of transverse septa, which varied from 2.33 to 6, was highest in Delhi cauliflower (CaAbD4) isolates that is, 6 and lowest in Delhi mustard isolate (MAb6) that is, 2.33.

Finally it was revealed that the smallest size of conidia and lowest number of septa was seen in Delhi isolate (MAb2, CaAbD1 and MAb6 isolates respectively). Microscopic examination of conidia at 40X magnification revealed variability in conidia size and could be categorized into two groups, that is, small (<47 µm) and long (>47 µm) but not according to their geographical origin. The small group included isolates from Delhi (CaAbD1, CaAbD2, CaAbD5 and CaAbD6, MAb2, MAb3, MAb4), Uttar Pradesh (CaAbU2, CaAbU3, CaAbU4, CaAbU5, CaAbU9) and Rajasthan (CaAbR3, CaAbR4) while long group included isolates from West Bengal (CaAbW1, CaAbW2, CaAbW3), Uttar Pradesh (CaAbU6, CaAbU7, CaAbU8,MAb10, MAb11), Delhi (CaAbD3, CaAbD4, MAb1, MAb5, MAb6, MAb7, MAb8), Haryana (CaAbH1), Kerala (CaAbK1) and Tamil Nadu (CaAbT5) states.

Effect of different media on radial growth

Mycelial growth and sporulation varied among the different isolates in different synthetic media. According to the average of radial growth of different isolates on different media, Potato Dextrose Agar and Cauliflower Agar was optimum for all the cultures. Radial growth on the 7th day of 14 isolates [Uttar Pradesh (CaAbU3, CaAbU4, CaAbU7), Delhi (CaAbD1, CaAbD2, CaAbD3, CaAbD4, MAb6), Rajasthan (CaAbR3, CaAbR4), Haryana (CaAbH1), Kerala (CaAbK1), West Bengal (CaAbW1), Tamil Nadu (CaAbT5)] were higher on PDA medium (>8 cm). Among them Uttar Pradesh (CaAbU3) and Delhi (CaAbD3) showed highest growth (8.87 cm) while Uttar Pradesh (CaAbU8) grew the least (4.86 cm). On Cauliflower agar media, Carrot Potato and Oat Meal Agar media highest growth were observed in Haryana (CaAbH1):7.60 cm , Haryana (CaAbH1) and Rajasthan (CaAbR4): 7.47 cm and Delhi (CaAbD4): 7.37 cm respectively whereas least growth was found in Delhi (MaAb5):5.43 cm, Uttar Pradesh (CaAbU2):5.77 and Tamil Nadu (CaAbT5):5.17 cm respectively. Similarly in Czapex-Dox agar media highest growth was found in Delhi (CaAbD1):6.93 cm and least growth in Haryana (CaAbH1): 4.83 cm. Rest two media viz., V8 juice agar and Corn meal agar showed the lowest growth ranging (2.93-5.13 cm) and (2.13-4.43 cm) respectively.

A dendrogram (Figure 2) was constructed based on the data for mycelial growth of *A. brassicae* isolates on the 7th day at different nutrient media at a temperature of 25°C, from the similarity coefficient by using Unweighted Pair Group Method with Average Means (UPGMA). The dendrogram identified two major clusters with 82% similarity. One cluster (group I) comprised of 16 isolates from Uttar Pradesh (CaAbU2, MAb11), Delhi (CaAbD3, CaAbD5, CaAbD6, MAb2, MAb3, MAb4, MAb6, MAb8), Tamil Nadu (CaAbT5), Kerala (CaAbK1), West Bengal (CaAbW1, CaAbW2, CaAbW3) and Rajasthan (CaAbR3) while another cluster (group II) comprised of remaining 16 isolates of which one Delhi isolate (CaAbD4) was separated from the rest fifteen isolates at 86% similarity level. The fifteen isolates of group II shares 100% similarity among the isolates viz., Uttar Pradesh isolates (CaAbU3, CaAbU4, CaAbU5, CaAbU6, CaAbU7, CaAbU8, CaAbU9 and MAb10), Delhi isolates (CaAbD1, CaAbD2, MAb1, MAb5, MAb7), Haryana isolate (CaAbH1) and Rajasthan isolate (CaAbR4).

Effect of different media on sporulation

Sporulation of each *A. brassicae* isolates on the 7th day on different media was almost similar. The lowest sporulation was observed for the isolates of Uttar Pradesh (CaAbU2, CaAbU3), Delhi (CaAbD3) and Haryana (CaAbH1) which was in a range 2.67-6.33 x 10^4/ml. Moderately sporulating within a range of 7-10 x 10^4/ml were

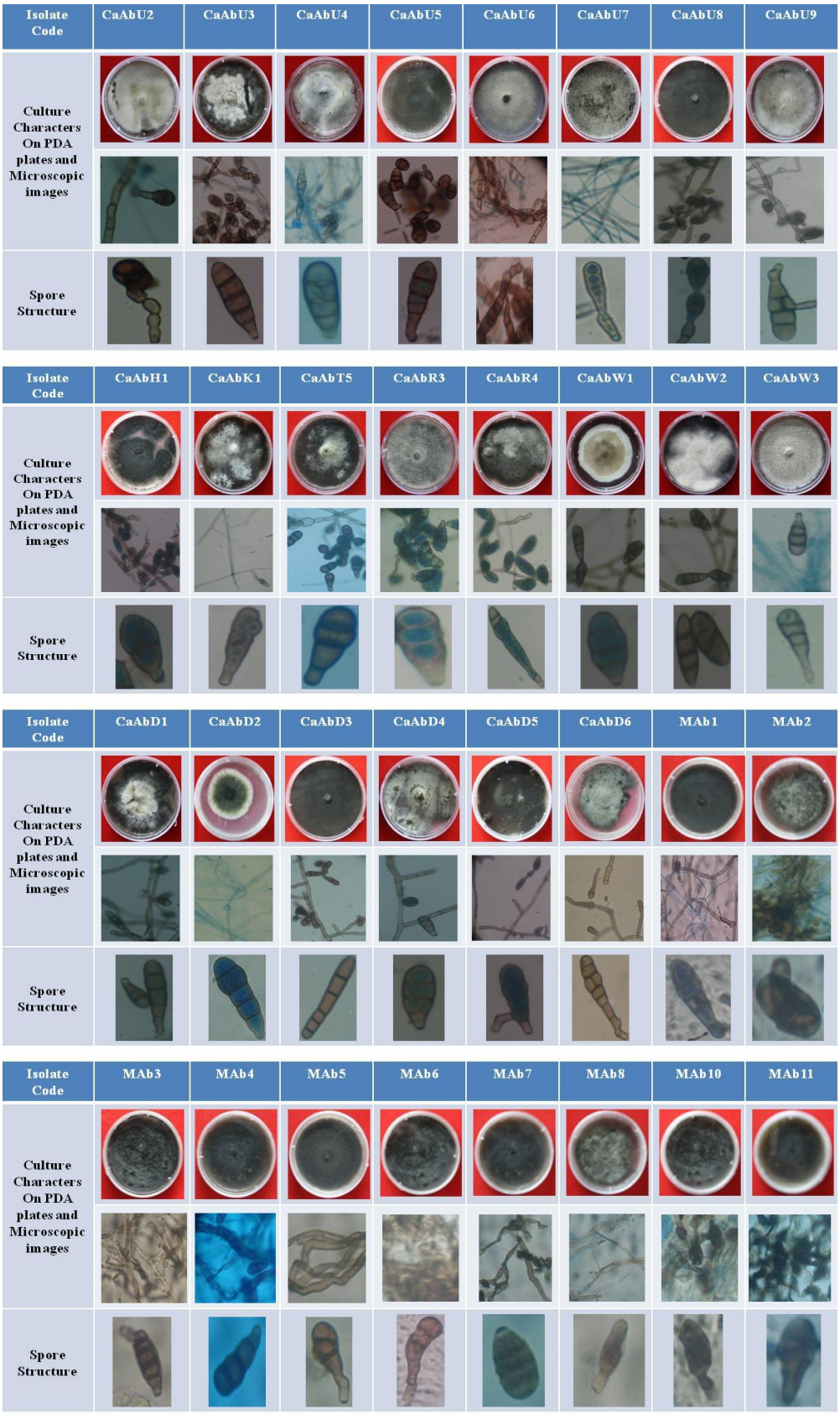

Figure 1. Spore morphology and cultural characters on PDA plates and microscopic images of *Alternaria brassicae* isolates.

Table 2. Conidial size and septation of *Alternaria brassicae* isolates.

Isolate	Average conidial length (µm)	Average conidial breadth (µm)	Average Number of Transverse septa
CaAbU2	40.32	7.50	5.33
CaAbU3	38.43	7.90	5.67
CaAbU4	38.22	6.20	4.00
CaAbU5	45.54	7.20	4.67
CaAbU6	50.43	8.50	5.00
CaAbU7	53.28	8.60	3.67
CaAbU8	49.50	7.40	4.33
CaAbU9	38.48	6.10	4.67
CaAbD1	42.16	6.00	4.33
CaAbD2	44.36	6.80	4.00
CaAbD3	48.64	7.40	5.00
CaAbD4	47.98	7.10	6.00
CaAbD5	38.98	6.40	5.00
CaAbD6	40.08	7.20	3.67
CaAbR3	42.04	7.80	4.33
CaAbR4	46.87	8.40	5.67
CaAbH1	48.76	8.60	3.67
CaAbK1	48.88	8.40	4.00
CaAbW1	57.65	9.40	4.33
CaAbW2	52.11	9.50	4.67
CaAbW3	48.99	8.70	5.00
CaAbT5	54.77	9.60	5.00
MAb1	56.39	8.60	5.00
MAb2	37.88	7.50	2.67
MAb3	43.35	8.40	2.67
MAb4	44.99	8.30	4.67
MAb5	50.48	8.80	2.67
MAb6	52.62	8.40	2.33
MAb7	53.36	8.50	4.00
MAb8	47.56	7.90	2.33
MAb10	48.87	8.10	2.67
MAb11	56.43	8.60	4.67
LSD (0.05)	2.08	0.34	0.36
CV (%)	12.48	12.12	23.65

The size and shape of conidia (length and width) was determined using ocular and stage micrometer. Numbers of septa were also recorded.

seen for the isolates Rajasthan (CaAbR3, CaAbR4), West Bengal (CaAbW1), Kerala (CaAbK1), and Uttar Pradesh (CaAbU6, CaAbU7). Rest of the isolates were highly sporulating within a range of 12.33-50 x 10^4/ml. Tamil Nadu isolate (CaAbT5) was found to be the highest sporulating with 50 x 10^4/ml. Similar observations were found in all nutrient media.

Another dendrogram (Figure 3) was constructed based on the 7th day for sporulation of *A. brassicae* isolates in the same way as for mycelial growth. This dendrogram produced two major clusters with 15% similarity. One cluster (group I) comprised of four isolates from Uttar Pradesh (CaAbU2, CaAbU3), Delhi (CaAbD3) and Haryana (CaAbH1). This group is also the least sporulating group with less than 6 x 10^4/ml sporulation. Another cluster (group II) comprised of remaining twenty eight isolates which was further sub-clustered. Among the sub-clusters one comprised of twenty two isolates which were highly sporulating (>10 x 10^4/ml). This sub cluster include Delhi isolates (CaAbD1, CaAbD2, CaAbD4, CaAbD5, CaAbD6, MAb1, MAb2, MAb3, MAb4, MAb5, MAb6, MAb7, MAb8), Uttar Pradesh isolates (MAb10, MAb11, CaAbU4, CaAbU5, CaAbU8, CaAbU9), Tamil Nadu isolates (CaAbT5), West Bengal (CaAbW2, CaAbW3). Another two sub-clusters comprised of isolates from Rajasthan (CaAbR3, CaAbR4), West Bengal (CaAbW1), Kerala (CaAbK1) and Uttar Pradesh (CaAbU6, CaAbU7).

Cluster analysis also revealed cultural variability among thirty two *A. brassicae* isolates and found a close relationship among Delhi and Uttar Pradesh isolates in respect of

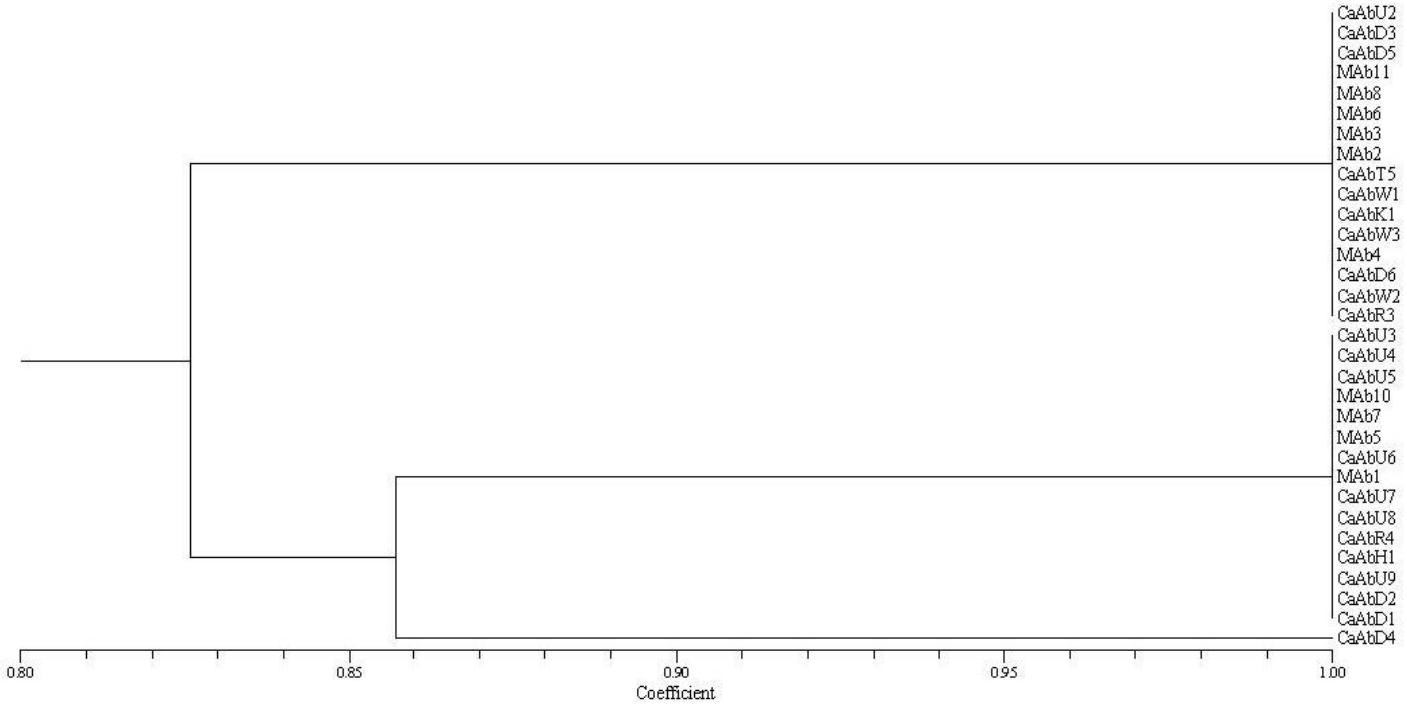

Figure 2. Dendrogram showing cultural variability in mycelial growth of 32 *Alternaria brassicae* isolates at different nutrient media.

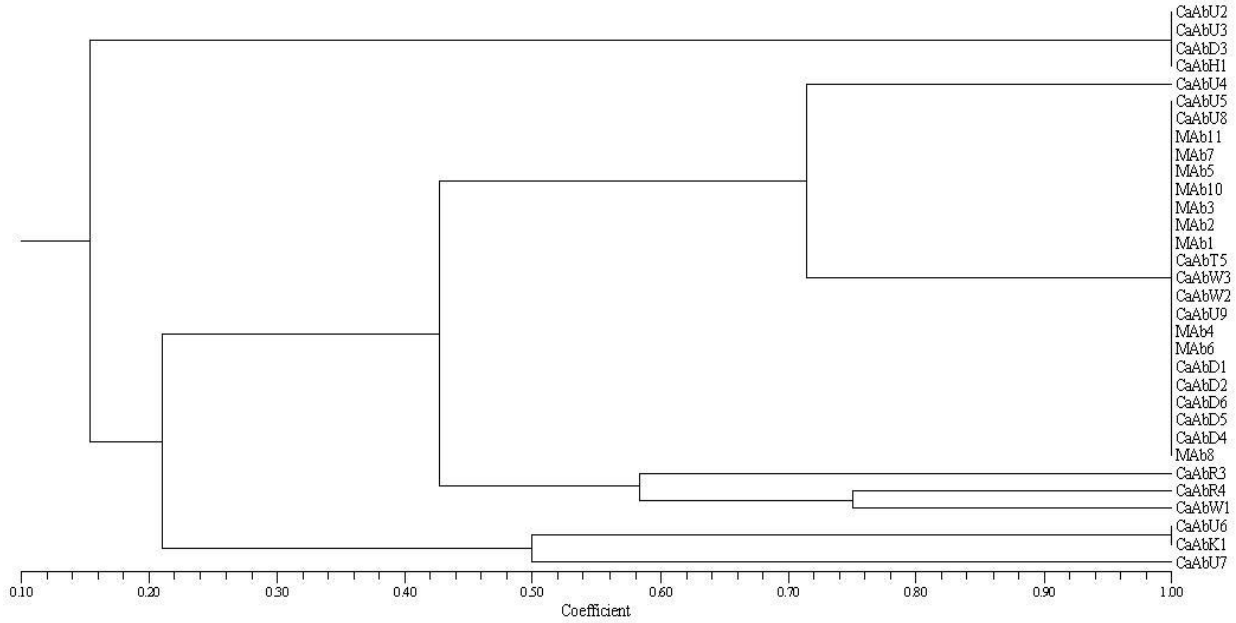

Figure 3. Dendrogram showing cultural variability in sporulation of 32 *Alternaria brassicae* isolates at different nutrient media.

mycelial growth and sporulation on different nutrient media.

Pathogencity

All the *A. brassicae* isolates both from cauliflower and mustard were found to be pathogenic in nature (Table 3). Among the cauliflower isolates CaAbT5 from Tamil Nadu, CaAbD5 from Delhi and CaAbU8 from Uttar Pradesh were found to be highly pathogenic as the spots produced by them were >1cm in diameter. Cauliflower isolates CaAbU2, CaAbU3, CaAbU4, CaAbU6, CaAbU7 (Uttar Pradesh), CaAbD3 (Delhi), CaAbR3, CaAbR4 (Rajasthan), CaAbH1 (Haryana), CaAbK1 (Kerala),

Table 3. Pathogenicity testing of *A. brassicae* isolates on the respective hosts cauliflower and mustard.

Cauliflower *A. brassicae* isolate	Cauliflower cultivar: DC-23000	Cauliflower *A. brassicae* isolates	Cauliflower cultivar: DC-23000	Mustard *A. brassicae* isolates	Mustard cultivar: Pusa Jagganath
CaAbU2	+	CaAbD4	++	MAb1	++
CaAbU3	+	CaAbD5	+++	MAb2	++
CaAbU4	+	CaAbD6	++	MAb3	++
CaAbU5	++	CaAbR3	+	MAb4	++
CaAbU6	+	CaAbR4	+	MAb5	++
CaAbU7	+	CaAbH1	+	MAb6	++
CaAbU8	+++	CaAbK1	+	MAb7	++
CaAbU9	++	CaAbW1	+	MAb8	++
CaAbD1	++	CaAbW2	++	MAb10	++
CaAbD2	++	CaAbW3	++	MAb11	++
CaAbD3	+	CaAbT5	+++		

Symptoms observed were ranked as minus (-) for no symptom and plus (+) for black leaf spot with yellow halos on inoculated leaves. Appearance of symptom again divided into three groups viz., black spot with diameter 0.2-0.5cm ranked as single plus (+), spot with diameter 0.6-1.0cm ranked as double plus (++) and spot diameter more than 1cm were ranked as three plus sign (+++).

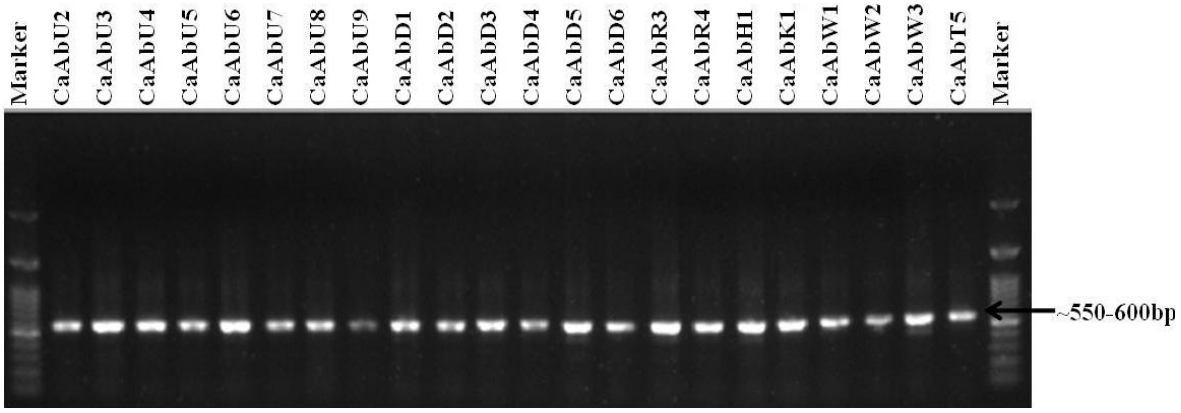

Figure 4. Amplification of the internal transcribed spacer region of the twenty two *A.brassicae* isolates collected from the infected cauliflower host from different part of India with the universal primer ITS1 and ITS4. Lane M: 100 bp ladder (GeneDirex RTU, Biochem Life Science, India).

CaAbW1 (West Bengal) were found least pathogenic as the symptoms of black spot produced were 0.2-0.5cm in diameter. Isolates CaAbU5, CaAbU9, MAb10, MAb11 (Uttar Pradesh), CaAbD1, CaAbD2, CaAbD4, CaAbD6, MAb1, MAb2, MAb3, MAb4, MAb5, MAb6, MAb7, MAb8 (Delhi), CaAbW2, CaAbW3 (West Bengal) were found moderately pathogenic producing dark spots of size 0.6-1 cm.

ITS analysis

DNA was successfully extracted from the fungal isolates using CTAB method and yielded the PCR products of amplicons of ~550 to 600bp. Preliminary fingerprinting of the 32 isolates using internal transcriber spacer primers ITS1- 5'TCC GTA GGT GAA CCT GCG 3' and ITS4-5' TCC TCC GCT TAT TGA TAT GC 3' confirmed that these isolates to be of *Alternaria* species (Figures 4 and 5). Analysis of the ITS regions revealed 90-100% identity among the thirty two isolates in the *A. brassicae* species group. All the ITS sequences were submitted to NCBI database (Table 1). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.20085009 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 37 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 513 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura

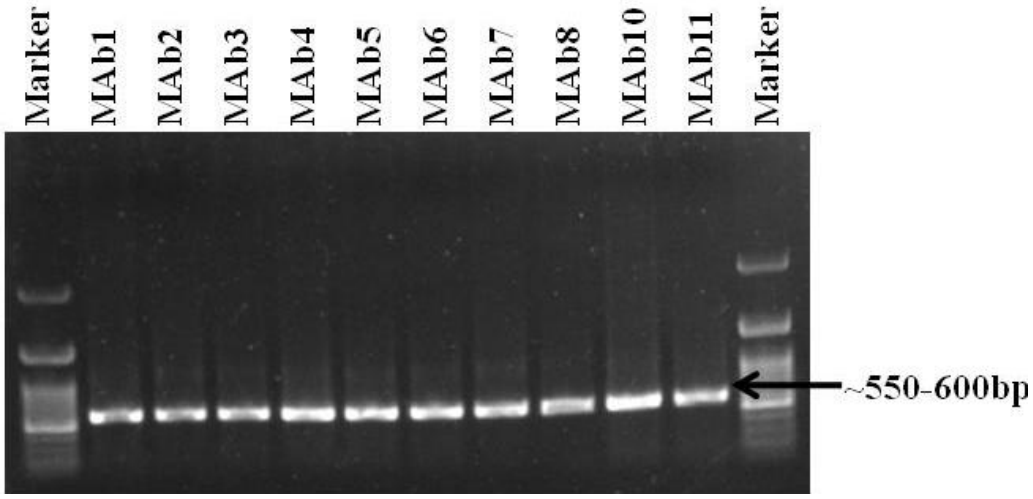

Figure 5. Amplification of the internal transcribed spacer region of the ten *A.brassicae* isolates collected from the infected cauliflower host from different part of India with the universal primer ITS1 and ITS4. Lane M: 100bp ladder (Gene Direx RTU, Biochem Life Science, India).

et al., 2011).

The phylogenetic tree (Figure 6) formed three distinct clusters of the *A. brassicae* isolates but there are not of much difference. First cluster comprises of only one isolate that is CaAbD2, second group was formed constituting only two isolates of Rajasthan origin and cauliflower as source plant of isolation viz., CaAbR3 and CaAbR4 which are 99% similar to each other. The third group containing the rest of all isolates having >56% similarities with each other. When some other accessions of *A. brassicae* were considered from NCBI it showed a close relationship with the isolates in the present study.

DISCUSSION

In a recent report Goyal et al. (2011) reported variation in conidial morphology, mycelial growth, sporulation of thirteen isolates of *A. brassicae* collected from different geographical zones were dependant on temperature and geographical origin. Similarly, variability in the morphological characteristics in *A. brassicae* isolates of different regions of India have been reported (Meena et al., 2005; Kaur et al., 2007; Singh et al., 2007). Some researchers have worked on cultural variability in *Alternaria* species in respect of mycelial growth and sporulation (Ansari et al., 1989; Patni et al., 2005; Kaur et al., 2007). Cauliflower and mustard are winter crops and are affected by *A. brassicae*. This present study was to understand the variability among the isolates infecting both the crops grown in different states of India on basis of morphology, cultural, pathogenic and molecular level. Significant variation in growth, sporulation and conidial morphology of *A. brassicae* isolates were found on different nutrient media irrespective of crop and geographical states. All the isolates were found pathogenic in nature against their respective host.

Due to the systematic and taxonomic usefulness the ITS region has been used in classifying fungi (Chillali et al., 1998) as it is suitable size for PCR amplification, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well (Jung et al., 2002). Molecular relationships amongst *Alternaria* species based on nuclear ribosomal DNA and host-specific toxins (Kusaba and Tsuge, 1994, 1995) or with other related fungi have been analyzed (Pryor and Gilbertson, 2002; Chou and Wu, 2002). Variation in nuclear ribosomal DNA sequences among *Alternaria* species pathogenic to crucifers has been reported from one isolate each of *A. brassicae*, *A*. *brassicicola*, *A*. *raphani* and *A*. *alternata* (Jasalavich et al., 1995). BLAST analysis of the internal transcribed spacer region of all thirty two *A. brassicae* isolates in this study showed high similarity among the isolates with *A. brassicae* of the NCBI database.

All the thirty two isolates of *Alternaria brassicae* though different at cultural and morphological level were found to be pathogenic in nature. Irrespective of the host and geographical origin the *A*. *brassicae* isolates are dispersed in the whole country sharing a single gene pool.

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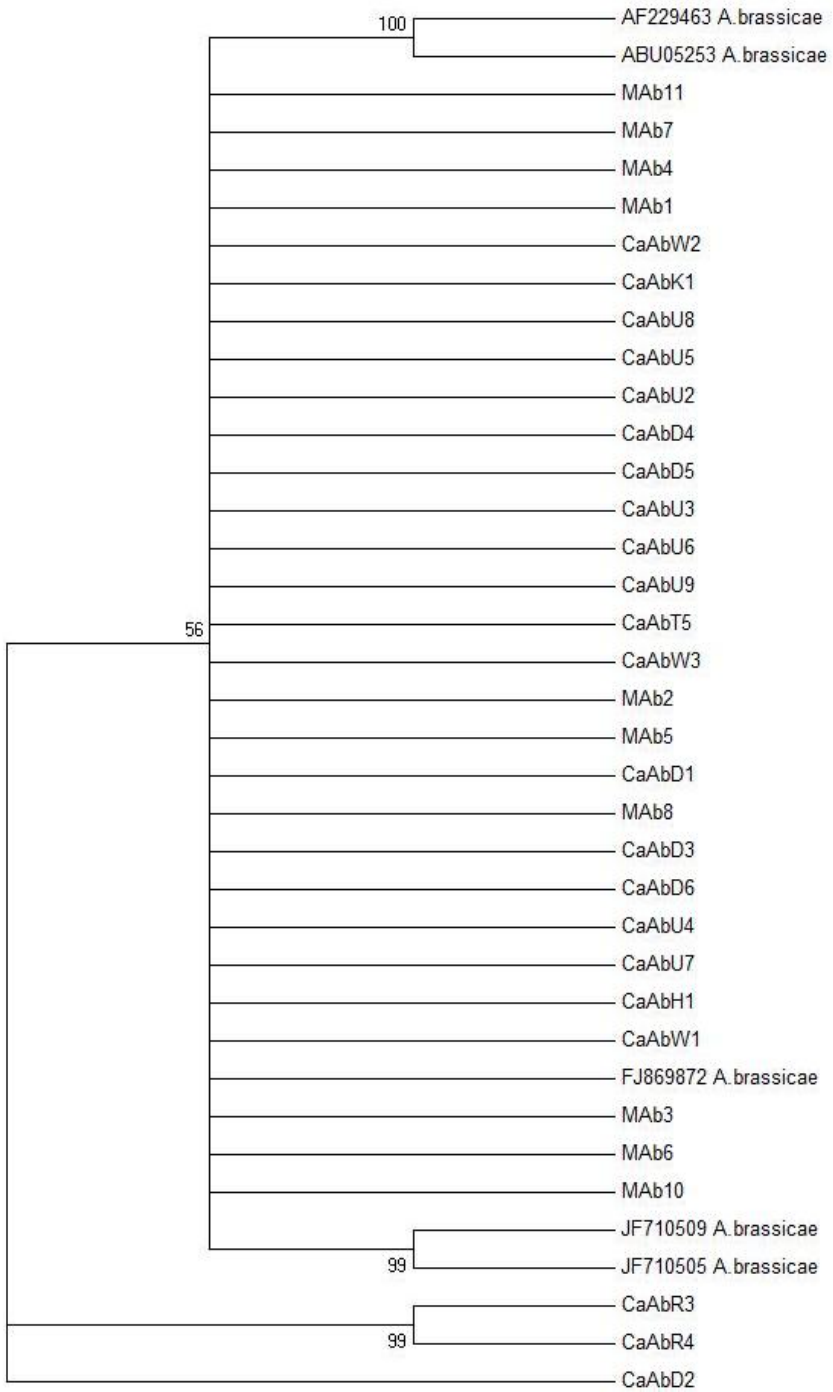

Figure 6. Dendrogram representing Internal Transcribed Spacer region of thirty two *Alternaria brassicae* isolates.

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