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Diversity in Indian Isolates of *Alternaria brassicicola* (Schwein) Wiltshire Causing Black Leaf Spot Disease in Cauliflower

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Abstract: Morphological, cultural, pathogenic and genetic variability in thirty two *Alternaria brassicicola* (Schwein) Wiltshire isolates infecting cauliflower (*Brassicae oleracea* var *botrytis*) from different parts of India were studied here. Dark olivaceous black fungal colonies were observed with small, obpyrifom, septate, brown colored spores forming in chain having no beak. A significant ($p<0.05$) morphological variability was found within the isolates in respect to conidia length, width and number of septa whereas less cultural variability was seen with respect to colony colour and growth. High sporulation intensity was found in most of the isolates. RAPD (42) and ISSR (12) primers generated a total of 337 amplicons out of which 16 were monomorphic, indicating presence of high level of genetic diversity within the pathogenic *A. brassicicola* isolates. No major clusters were formed, rather isolates of mixed geographical regions of collection are found to be interlinked with each other. The internal transcribed spacer region of all the thirty two isolates showed 100% similarity with *A. brassicicola* accessions from NCBI.

Key words: *Alternaria brassicicola* (Schwein) wiltshire, leaf spot, crucifers, diversity analysis

INTRODUCTION

As a second largest producer of vegetables next to China in the world, India accounts for about 15% of the world's production of vegetables. Among vegetables, crucifers were reportedly affected with different fungal diseases specifically Alternaria blight which play a major role in production loss in many parts of the world (Hodgkin and MacDonald, 1986; Dillard *et al*., 1998; Pattanamahakul and Strange, 1999), including India (Meena *et al*., 2012; Sharma *et al*., 2013b). Severe damage of the foliage or seed germination in crucifers occurs due to *Alternaria* blight caused by *Alternaria brassicae* (Berk) (Kolte, 1985; Kolte *et al*., 1987; Tewari, 1991; Verma and Saharan, 1994; Sharma *et al*., 2013a, b) and *A. brassicicola* (Schw) (King, 1994; Jung *et al*., 2002; Sharma *et al*., 2013c). In vegetable brassica seeds, especially white cabbage and cauliflower, *A. brassicicola* is the dominant pathogen which causes more severe disease than *A. brassicae* (Maude and Humpherson-Jones, 1980; Humpherson-Jones, 1985; Maude *et al*., 1984; Kubota *et al*., 2006). Considerable damage occurs to cabbage and cauliflower in transit (Ellis, 2001). These species can survive in seeds for several months at different temperatures and relative humidity (Kumar and Gupta, 1994; Abul-Fazal *et al*., 1994) and the disease is spread during the growing season by wind-blown or rain-splashed spores (Rotem, 1994; MacKinnon *et al*., 1999; Oliver *et al*., 2001). The pathogen attacks most parts of the plant and it is thought to induce its chlorotic effect by release of phytotoxins (Jung *et al*., 2002).

Major taxonomic criteria for delimitation of fungal species including *Alternaria* are provided by morphological characteristics of conidia and conidiophores and sometimes host plant association (David, 1991; Pattanamahakul and Strange, 1999). All commercial cultivars of brassicas are susceptible to *Alternaria brassicicola* and no resistance reported till 1991 (Tewari, 1991). Many reports on the existence of morphological variability within the isolates of different Alternaria species have been reported by earlier workers (Verma and Saharan, 1994; Varma *et al*., 2006). Variability studies were done on the basis of morphology, sporulation, mycelial growth and other cultural characteristics (Ansari *et al*., 1989; Patni *et al*., 2005; Kaur *et al*., 2007). Morphological and cultural variability among the isolates of *A. brassicae* were reported from across the rapeseed-mustard and cauliflower growing region of India (Meena *et al*., 2005;

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Kaur *et al*., 2007; Singh *et al*., 2007; Goyal *et al*., 2011; Sharma *et al*., 2013a, b). However variability study in *A. brassicicola* infecting vegetable crop like cauliflower is still missing.

To understand host-pathogen co-evolution, epidemiology and resistance management, the study of genetic diversity of plant pathogen populations is also a basic need (Milgroom and Fry, 1997). The PCR based marker assisted genotypic identification of phytopathogens have been successfully going on at the species and subspecies level (Benali *et al*., 2011). Characterization of genetic variation in populations of *Alternaria* species pathogenic to crucifers by RAPD and nuclear ribosomal DNA sequences (Sharma and Tewari, 1995, 1998; Roberts *et al*., 2000; Tigano *et al*., 2003; Jasalavich *et al*., 1995) was reported with high level of similarity among the isolates of *A. brassicae* from different origin (Cooke *et al*., 1998; Sharma *et al*., 2013a, b). Occurrence of substantial genetic variability in *A. solani* and *A. alternata* infecting tomato and potato. Weir *et al*. (1998) and Morris *et al*. (2000) suggested diversity can be maintained by mutation, uniform host selection, extensive dispersal or the existence of a cryptic sexual stage. While resolving this, Bock *et al*. (2002, 2005) found moderate levels of genetic diversity in the form of substantial polymorphism between isolates from five populations of *A. brassicicola* attacking *Cakile maritima* along the New South Wales coast of Australia but the inter/intra species level variation among the Indian isolates of *A. brassicicola* (Schwein) Wiltshire infecting vegetable crucifers is still lacking. Therefore, the present study is aimed to study the morphological, cultural, pathogenic and genetic variability among the *Alternaria brassicicola* isolates collected from infected cauliflower (*Brassicae oleracea* L. var *botrytis*) from different part of India.

MATERIALS AND METHODS

Collection of isolates: Thirty two isolates (Rajasthan-6, Delhi-2, Tamil Nadu-3, Haryana-2, Jammu and Kashmir-2, Bihar-1, Uttar Pradesh-3, Himachal Pradesh-1, West Bengal-3, Uttarakhand-1, Maharashtra-1 and Assam-1, Orissa-2, Gujarat-1, Jharkhand-1, Punjab-1, Karnataka-1) of *Alternaria* spp. were obtained from infected leaf samples of cauliflower collected from seventeen different regions of India during 2009-2012. Blighted leaf pieces (2 mm) were surface sterilized with 0.1% Mercuric chloride ($HgCl_2$) for 1 min, 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 and was confirmed to be *A. brassicicola* by microscopic (Carl Zeiss, Germany) observation (Ellis, 2001). Single spore cultures of these isolates were purified on Potato Dextrose Agar (PDA) plates at 25±1°C which were further maintained on PDA slants at 4°C.

For all isolates, morphological characteristics of the colony and sporulation apparatus were determined from single-spore colonies. The nature of mycelia growth (colony colour and radial 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 media on growth and sporulation: Seven types of media were tested to determine their effect on growth and sporulation of the *A. brassicicola* isolates. Seven different growth media were used namely Potato dextrose agar (PDA), Cauliflower Leaf Extract Agar (CLEA) made of Dextrose (20 g), agar (20 g) and Cauliflower leaf infusion from 200 g in 1000 mL water, Carrot Potato Agar (CPA), Oat Meal Agar (OMA), Czapex Dox Agar (CDA), V8 Juice Agar (VJA) and Corn Meal Agar (CMA) from Himedia, India. The experiment was arranged in a completely randomized design with 3 replicates. Measurements on radial colony diameter (in cm) were taken on 7th day after inoculation whereby six plates were sampled each time for each media treatment.

To determine conidial concentration of each isolates, cultures grown on seven different media plates were considered. The 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: Thirty two *A. brassicicola* isolates from cauliflower were tested for symptom production on a susceptible cultivar of cauliflower viz., DC-23000. The seeds of cauliflower were sown in nursery beds during October, 2010-11 and 2011-12 seasons. Cauliflower seedlings were transplanted after two weeks in fields with a spacing of 30×40 cm. After 60 days leaves from both the cauliflower plant were taken for the detached leaf inoculation method (Sharma *et al*., 2004, 2013b). Test leaves were properly washed under running tap water and then surface wiped off with 70% alcohol and 2 μL of 4×10^4 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 greenhouse conditions and were observed for appearance of disease symptoms on 5 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 0.2-0.5 cm ranked as single plus (+), spot with diameter 0.6-1.0 cm ranked as double plus (++) and spot diameter more than 1cm were ranked as three plus sign (+++).

Genomic DNA isolation: The thirty two *A. brassicicola* isolates were grown on Potato Dextrose Broth (PDB) medium for 7 days at 28°C. Mycelial mat 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; Connolly *et al*., 1994; Sharma *et al*., 2013b). Around 300 mg of lyophilized fungal mat 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 and centrifuged (4000 rpm, 10 min). 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. Then pellet were solubilized in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH- 8.0) and treated with RNase A (20 mg mL^{-1}) for 1 h at 37°C. Equal volume of Phenol:Chloroform:Iso-amyl alcohol (25:24:1) was added to the DNA solution and mixed by swirling for 5 min. After centrifuge at 10,000 rpm for 5 min, supernatant was collected in a fresh tube. This was followed by extractions with chloroform: iso-amyl alcohol (24:1). The purified DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5.6) and 2.5 times (v/v) along chilled ethanol (95%). Extra salts were removed by further washing with 70% ethanol and DNA was pelleted and dried under vacuum. The quality and quantity of DNA was analyzed both spectrophotometrically using Nanodrop (Thermo-Scientific Ltd.) and in 1% agarose gel. The pellet was dissolved in a minimum volume of TE (10:1) buffer at room temperature and stored at -20°C.

RAPD analysis: Forty two different 10-mer oligonucleotide primers (Operon Technologies, USA) were tested on a set of 32 *A. brassicicola* isolates to identify polymorphic amplicons. PCR was performed in 15 µL volumes containing 1 µL DNA (50 ng), 1.5 µL primer (10 pmol), 1.5 µL of 10×PCR buffer, 1 µL of 25 mM $MgCl_2$, 0.5 µL *Taq* DNA polymerase (0.5 U), 1.5 µL dNTP mix (2 mM), 8 µL double distilled water. Amplification were performed on a Bioneer-Mygenie 32 thermocycler programmed for 5 min at 95°C (denaturation), 40 cycles each of 1 min at 94°C, 1 min at 32°C (annealing), 1 min at 72°C and final extension of 2 min at 72°C (Sharma *et al*., 2013b). The amplified products were loaded onto 1.5% agarose gels (Agarose T2 Low EEO, Biomatrix) containing 0.2 mg L^{-1} Ethidium bromide (Sigma, USA) and electrophoresed in 1×TAE buffer. All the PCRs were repeated thrice to check the reproducibility of the amplicons.

ISSR analysis: Twelve different ISSR primers obtained from SBS Genetech Co., Ltd. were also tested on the above thirty two isolates of *A. brassicicola* to identify polymorphism. PCR was performed in 15 µL volume reaction mixture same as for RAPD analysis but with different ISSR primers. The PCR amplifications were performed in the same thermocycler for 5 min at 95°C (denaturation), 40 cycles of 30 sec at 94°C, 30 sec at 51°C (annealing), 45 sec at 72°C and final extension of 7 min at 72°C. Again the amplified products were electrophoresed in 1.5% agarose in 1×TAE buffer after adding Ethidium bromide.

Internal Transcribed Spacer (ITS) analysis: Thirty two *A. brassicicola* isolates were analyzed by amplifying the regions of the rDNA repeat from the 3'end of the 18 s and the 5' end of the 28 s gene using PCR conditions with the two universal primers, ITS1-3'TCCGTA GGTGAACCTGCGG5' andITS4-3'TCCTCC GCT TA TT GATATGC5' 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 $MgCl_2$, 2 mM of dNTP mix, 20 mM of each primer with 50 ng μL^{-1} of template and 2.5 U of *Taq* polymerase. These reactions were subjected to an initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 35 sec, primer annealing at 60°C for 1 min 30 sec and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C in the thermal cycler. Aliquots (10 µL) of the amplified products were analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA [pH 8]), stained with ethidium bromide (1 µg mL^{-1}) and electrophoresis was carried out at 70 V 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 gel extraction kit as per instruction manual.

Nucleotide sequencing and in silico analysis: The sequencing of the PCR product was carried out in automated Sequencer at Xcelris Lab., Ahmadabad, 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). The multiple sequence alignment and pairwise alignment were performed using the ClustralW algorithm in Bioedit (Hall, 1999) and Phylogenetic analysis was done using MEGA 5.0 (Tamura *et al*., 2011) software. To assess the possible phylogenetic relationship neighbour-joining tree (Saitou and Nei, 1987) was created using maximum composite likelihood method (Tamura *et al*., 2004) and Parsimony tree was created using Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 0 in which the initial trees were obtained with the random addition of sequences (10 replicates). The sequences were aligned among themselves as well as with other published sequences available in GenBank using BlastN.

Statistical analysis: Averages of colony diameter on each media plates and number of conidia per milliliter 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.

Dendrogram were constructed employing nested un-weighted pair group method using the arithmetic averages (UPGMA) and the sequential, agglomerative, hierarchical, non-overlapping (SAHN) clustering was obtained by NTSYS-PC Version 2.02 h (Rohlf, 1997). Resolving Power (RP) of the RAPD and ISSR primer was calculated as per (Prevost and Wilkinson, 1999). $RP = \Sigma IB$, where IB (Band informativeness) = $1-\{2\times (0.5-P)\}$; P is the proportion of the 32 isolates of *A. brassicicola* analyzed that contains the band.

RESULTS AND DISCUSSION

Single spore culture of thirty two isolates of *Alternaria brassicicola* (Schweinitz, Wiltshire) were isolated from seventeen different cauliflower growing states of India (Table 1). These isolates were further maintained on PDA slants at 4°C for further study.

Colony colour and radial growth were observed among thirty two isolates of *Alternaria brassicicola* infecting cauliflower in India. The colour of *A. brassicicola* colonies on PDA plates varies between olive gray to dark olivaceous black. Mycelia colour of all tested isolates was found to be brown. Conidial characteristics of each isolate were found similar to each other viz., obpyriform conidia having brown colour with smooth surface and short beak (Table 1). A Significant ($p<0.05$) variation was observed in conidia length and breadth (Table 2) of the thirty two single spore cultures of *A. brassicicola*. Average conidia length varied between 32.57 μm in CaAbcP1 from Punjab to 40.08 μm in CaAbcUP2 from Uttar Pradesh. Similarly average conidial breadth varied from 6.23 μm in CaAbcJK1 from Jammu and Kashmir to 9.40 μm in CaAbcWB3 from West Bengal. Average number of horizontal septa varied between 1.5-3. No vertical septum was found in any of the conidia. From the conidia size and shape it was found that all the isolates of *A. brassicicola* are almost similar to each other. Similar results were also showed by Ellis (1971), Pattanamahakul and Strange (1999) and Jung *et al*. (2002). The fungus differs from *A. brassicae* which is also majorly responsible for the black leaf spot in crucifers in India (Sharma *et al*., 2013a; Meena *et al*., 2012; Singh *et al*., 2012) in its beakless and smaller conidia (Cho *et al*., 2001). All the thirty two isolates of *A. brassicicola* showed significant ($p<0.05$) morphological variability in respect of conidia length, conidia width and number of septa.

Seven different nutrient media brought slight variation in the mycelial colony growth of thirty two isolates of *A. brassicicola*. According to average radial growth of different isolates on different media, Cauliflower Leaf Extract Agar (CLEA) and Potato Dextrose Agar (PDA) was found optimum for all isolates. On CLEA most of the isolates attended maximum radial growth (>6 cm) with CaAbcO1 from Orissa having maximum 7.5 cm except nine isolates viz., CaAbcR1, CaAbcR3 (Rajasthan), CaAbcH1 (Haryana), CaAbcJK1 (Jammu and Kashmir), CaAbcWB2 (West Bengal), CaAbcMH1 (Maharashtra), CaAbcO2 (Orissa), CaAbcD1 (Delhi) and CaAbcK1 (Karnataka) with the least in CaAbcWB2 (4.83 cm). On PDA, highest radial growth was obtained in CaAbcUP3 from Uttar Pradesh (6 cm) and least in CaAbcR6 from Rajasthan (4.40 cm). On CDA, PCA, CMA and VJA highest growth were obtained in CaAbcR4 (5.63 cm), CaAbcR5 (5 cm), CaAbcWB2 (5.03 cm) and CaAbcP1 (5.97 cm), respectively whereas least growth was found in

Table 1: Morphological and cultural characteristics of thirty two isolates of *Alternaria brassicicola* collected from Cauliflower growing states of India with their accession numbers

Isolate code[a]	Place of collection state	Accession No. of ITS sequences[b]	Culture appearance on PDA plate	Mean sporulation mL^{-1} ($in\times10^4$)[c]	Mycelium colour	Conidial characteristics				
						Colour	Surface	Shape	Beak	Horizontal septa
CaAbcR1	Jaipur, Rajasthan	KC888991	Olive Gray	24	Brown	Brown	Smooth	Obpyriform	Short	3-4
CaAbcH1	Faridabad, Haryana	JX258817	Olivaceous Black	62	Brown	Brown	Smooth	Obpyriform	Short	1-3
CaAbcJK1	Kathua, Jammu and Kashmir	JX258819	Olivaceous Black	165	Brown	Brown	Smooth	Obpyriform	Short	1-3
CaAbcB1	Samastipur, Bihar	JX258821	Olive Gray	243	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcUP1	Ghaziabad, Uttar Pradesh	JX258822	Light Olive Green	297	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcAs1	Jorhat, Assam	KC888992	Light Olive Green	277	Brown	Brown	Smooth	Obpyriform	Short	3-5
CaAbcO1	Bhubaneswar, Orissa	KC888993	Dark Olive Green	604	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcR2	Kota, Rajasthan	KC888994	Light Olive Green	26	Brown	Brown	Smooth	Obpyriform	Short	1-2
CaAbcHP1	Solan, Himachal Pradesh	JX258824	Dark Olive Green	230	Brown	Brown	Smooth	Obpyriform	Short	1-2
CaAbcG1	Ahemadabad, Gujarat	KC888995	Olivaceous Black	136	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcWB1	Nadia, West Bengal	JX258825	Light Olive Green	732	Brown	Brown	Smooth	Obpyriform	Short	0-2
CaAbcWB2	Kolkata, West Bengal	JX258826	Light Olive Green	256	Brown	Brown	Smooth	Obpyriform	Short	1-3
CaAbcJK2	Jammu, Jammu and Kashmir	JX258820	Dark Olive Green	312	Brown	Brown	Smooth	Obpyriform	Short	0-2
CaAbcUP2	Bulandsahar, Uttar Pradesh	JX258823	Dark Olive Green	313	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcUK1	Dehradun, Uttarakhand	JX258827	Light Olive Green	524	Brown	Brown	Smooth	Obpyriform	Short	2-4
CaAbcH2	Shikohpur, Haryana	JX258818	Dark Olive Green	54	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcJr1	Ranchi, Jharkhand	KC888996	Light Olive Green	88	Brown	Brown	Smooth	Obpyriform	Short	1-2
CaAbcMH1	Pune, Maharashtra	JX258828	Light Olive Green	747	Brown	Brown	Smooth	Obpyriform	Short	1-2
CaAbcWB3	Durgapur, West Bengal	KC888997	Light Olive Green	131	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcP1	Moga, Punjab	KC888998	Dark Olive Green	470	Brown	Brown	Smooth	Obpyriform	Short	2-4
CaAbcO2	Cuttack, Orissa	KC888999	Dark Olive Green	196	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcR3	Jaipur, Rajasthan	KC889000	Light Olive Green	139	Brown	Brown	Smooth	Obpyriform	Short	0-3
CaAbcD1	IARI field, Delhi	KC889001	Light Olive Green	122	Brown	Brown	Smooth	Obpyriform	Short	3-4
CaAbcUP3	Meerut, Uttar Pradesh	KC889002	Olive Gray	84	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcR4	Jaipur, Rajasthan	KC889003	Olive Gray	86	Brown	Brown	Smooth	Obpyriform	Short	0-3
CaAbcR5	Samode, Rajasthan	JN108901	Light Olive Green	295	Brown	Brown	Smooth	Obpyriform	Short	1-3
CaAbcR6	Kota, Rajasthan	JF439451	Light Olive Green	76	Brown	Brown	Smooth	Obpyriform	Short	3-4
CaAbcD7	Yamuna Bank, Delhi	JF439452	Olivaceous Black	49	Brown	Brown	Smooth	Obpyriform	Short	2-3
CaAbcT2	Palani, Tamil Nadu	JF439453	Olivaceous Black	40	Brown	Brown	Smooth	Obpyriform	Short	2-4
CaAbcT3	Dindigul, Tamil Nadu	JF439454	Olivaceous Black	322	Brown	Brown	Smooth	Obpyriform	Short	2-4
CaAbcT4	Kannavadi, Tamil Nadu	JF439455	Olivaceous Black	141	Brown	Brown	Smooth	Obpyriform	Short	0-3
CaAbcK1	Bengaluru, Karnataka	KF515684	Olivaceous Black	229	Brown	Brown	Smooth	Obpyriform	Short	3-4

[a]Isolate coding was done as Ca: Cauliflower, Abc: *Alternaria brassicicola*, Alphabets and numbers in the Isolate code: State of collection and number of sample isolated. [b]Accession numbers of ITS sequences of the *A. brassicicola* isolates deposited to NCBI database. [c]Mean Sporulation mL^{-1} ($in\times10^4$) of *A. brassicicola* isolates on different nutrient media

Table 2: Variation in *A. brassicicola* isolates based on colony growth on different nutrient media, conidial size and pathogenicity response on susceptible cauliflower cultivar (DC-23000)

	A. brassicicola colony diameter (cm)[a]								Average conidia size (µm)[b]		
Isolate code	PDA	CLEA	CDA	PCA	CMA	OMA	VJA	Mean diameter (cm)	Length	Breadth	Pathogenicity response[c]
CaAbcR1	5.00	4.50	3.03	4.60	3.53	3.57	4.97	4.17	34.54	8.47	++
CaAbcH1	4.83	5.17	2.17	4.60	4.53	3.00	5.13	4.20	36.28	7.73	++
CaAbcJK1	4.60	5.20	3.80	4.30	4.67	3.80	2.97	4.19	38.22	6.23	+++
CaAbcB1	5.97	6.23	5.27	4.70	4.67	4.63	3.97	5.06	39.31	6.33	+
CaAbcUP1	5.37	6.37	5.30	4.77	4.57	3.63	2.97	4.71	35.55	6.80	++
CaAbcAs1	5.83	7.43	5.30	4.40	4.47	3.90	5.10	5.20	38.88	7.23	+
CaAbcO1	5.37	7.50	5.27	4.53	3.93	3.57	3.40	4.80	34.47	7.10	+
CaAbcR2	5.60	6.00	5.23	4.60	4.00	3.73	3.43	4.66	38.48	7.67	+
CaAbcHP1	4.50	6.20	4.73	4.83	4.73	3.43	5.77	4.89	35.24	7.90	+++
CaAbcG1	4.97	7.17	4.00	3.37	4.53	3.10	4.23	4.48	34.03	6.60	+++
CaAbcWB1	5.57	6.13	5.00	4.83	4.03	3.60	3.20	4.62	35.13	7.23	+
CaAbcWB2	5.37	4.83	3.97	3.63	5.03	3.43	4.23	4.36	34.55	8.50	+++
CaAbcJK2	5.63	7.30	5.47	4.00	4.47	4.03	5.20	5.16	38.18	8.17	+
CaAbcUP2	5.20	6.80	5.23	3.23	3.97	4.37	3.83	4.66	40.08	8.90	+
CaAbcUK1	5.17	7.07	4.80	3.40	3.90	4.37	4.53	4.75	39.61	7.90	++
CaAbcH2	5.00	7.53	4.83	4.03	4.87	3.23	4.97	4.92	34.98	7.50	+
CaAbcJr1	5.30	7.03	3.67	4.23	4.53	3.57	5.03	4.77	35.77	8.40	++
CaAbcMH1	5.17	5.57	5.57	4.50	5.07	3.23	4.90	4.86	37.75	8.13	+++
CaAbcWB3	5.63	6.03	5.00	4.87	4.67	3.90	4.53	4.95	37.72	9.40	+++
CaAbcP1	5.40	7.23	5.47	4.07	4.10	4.07	5.97	5.19	32.57	9.17	+
CaAbcO2	4.90	5.50	5.23	4.67	4.50	3.50	3.73	4.58	35.69	6.40	+
CaAbcR3	4.80	5.77	4.07	4.23	4.23	3.63	4.27	4.43	34.73	7.20	+
CaAbcD1	5.43	5.40	4.73	4.57	4.67	2.93	4.47	4.60	36.43	7.80	+
CaAbcUP3	6.00	6.03	5.07	4.30	4.63	3.37	5.03	4.92	37.01	8.40	+
CaAbcR4	5.63	7.40	5.63	4.57	4.37	4.10	4.53	5.18	33.49	8.30	+
CaAbcR5	6.00	7.00	4.70	5.00	4.47	4.57	4.00	5.10	34.71	8.33	+++
CaAbcR6	4.40	6.90	4.83	4.00	5.00	4.13	5.60	4.98	34.04	8.80	+
CaAbcD7	5.97	6.17	4.07	4.73	4.50	3.80	4.80	4.86	32.97	8.40	++
CaAbcT2	5.23	7.00	5.47	5.13	4.80	4.13	4.43	5.17	33.56	7.20	+++
CaAbcT3	5.20	7.23	5.00	4.43	4.37	3.67	4.57	4.92	37.71	7.83	+++
CaAbcT4	5.43	6.77	4.83	4.70	4.23	3.57	4.33	4.84	35.74	8.10	++
CaAbcK1	5.17	5.97	5.23	4.53	3.80	3.57	4.20	4.64	36.22	8.60	++
	SEM= 0.58								LSD = 0.72	LSD =0.29	
	CD (p<0.0001) = 1.64								CV(0.05%) =5.64	CV(0.05%) = 10.53	

[a]Mean radial growth (cm) of *A. brassicicola* isolates measured on different nutrient media in six replications. [b]The size of conidia (length and width in µm) was determined using ocular and stage micrometer. [c]Pathogenicity response of *A. brassicicola* isolates on susceptible cauliflower cultivar (DC-23000) where 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 0.2-0.5 cm ranked as single plus (+), spot with diameter 0.6-1.0 cm ranked as double plus (++) and spot diameter more than 1cm were ranked as three plus sign (+++)

CaAbcH1 (2.17 cm), CaAbcUP2 (3.23), CaAbcR1 (3.53 cm) and CaAbcJK1 and CaAbcUP1 (2.97 cm each), respectively. The OMA showed the lowest radial growth ranging from 2.93 cm (in CaAbcD1) to 4.63 cm (in CaAbcB1) (Table 2).

Sporulation pattern of each isolates of *A. brassicicola* on 7th day on seven different nutrient media was similar to each other. The lowest sporulation (20-50×10^4 mL^{-1}) was observed for the isolates from Rajasthan (CaAbcR1, CaAbcR2), Delhi (CaAbcD7), and Tamil Nadu (CaAbcT2). Moderately sporulating (50-100×10^4 mL^{-1}) were found for the isolates from Haryana (CaAbcH2), Jharkhand (CaAbcJR1), Uttar Pradesh (CaAbcUP3) and Rajasthan (CaAbcR4 and CaAbcR6). Rest of the isolates were found highly sporulating (>100×10^4 mL^{-1}), among which the Orissa isolate CaAbcO1 was found to be the highly sporulating with 604×10^4 mL^{-1} followed by CaAbcUK1 (524×10^4 mL^{-1}) from Uttarakhand, CaAbcP1 (470×10^4 mL^{-1}) from Punjab and CaAbcT3 (322×10^4 mL^{-1}) from Tamil Nadu (Table 1).

All the *A. brassicicola* isolates from cauliflower were found pathogenic in nature. Among these nine isolates viz., CaAbcJK1, CaAbcHP1, CaAbcG1, CaAbcWB2, CaAbcWB3, CaAbcMH1, CaAbcR5, CaAbcT2 and CaAbcT3 were highly pathogenic as the spot size produced by them are >1cm in diameter. Eight isolates namely CaAbcR1, CaAbcH1, CaAbcUP1, CaAbcUK1, CaAbcJr1, CaAbcD7, CaAbcT4 and CaAbcK1 were found moderately pathogenic as they producing spot size of 0.6-1 cm and rest fifteen isolates were found lowest pathogenic as the spot size were 0.2-0.5 cm in diameter (Table 2). These results are in agreement with earlier workers on different *Alternaria* species (Awasthi and Kolte, 1989; Varma *et al*., 2006; Meena *et al*., 2005, 2012; Kaur *et al*., 2007; Singh *et al*., 2007, 2012; Reis and Boiteux, 2010; Sofi *et al*., 2013; Sharma *et al*., 2013b; Sun and Zhang, 2008), who observed morphological, cultural, pathogenic variability in different geographical isolates.

Forty two random decamer oligonucleotide primers were utilized for RAPD analysis but two primers did not give satisfactory amplification, so were not considered further. Thirty nine primers resulted in the amplification of distinct and reproducible bands in the present investigation. All the primers produced wide range of fragments/amplicons ranging from 100-1500 bp. The highest number of fragments (199) was amplified by the primer OPA8 and the lowest by the primer OPX13 (7). The number of polymorphic bands produced were maximum with the primers OPA3 and OPA17 (9 each), while minimum (3) with the primers OPA9 and OPC5. In case of monomorphic bands the maximum number of bands (3) was produced by the primer OPA9 followed by OPA6 and OPA13 with two bands each. Among the rest, seven primers viz., OPA4, OPA5, OPA8, OPC7, OPE3, OPE4 and OPE6 yielded each single monomorphic band. The Resolving Power (RP) was highest in case of OPA8 (12.4375), whereas the lowest RP was observed in OPX13 (0.4375). Total 14 monomorphic bands were amplified with all the RAPD primers used in the present study. The details of RAPD banding pattern was presented in Table 3 and Fig. 1a. Jaccard's similarity coefficient was calculated from the RAPD data showed that all the

Table 3: Details of RAPD and ISSR banding pattern in different isolates of *Alternaria brassicicola* of Indian origin

Primer	Nucleotide sequence	Fragment size (bp)	Total No. of bands	Polymorphic bands	Monomorphic bands	Percentage of polymorphic loci (PPB)	Total No. of bands amplified	Resolving power (RP)
OPA1	CAGGCCCTTC	300-600	5	5	0	100.00	36	2.2500
OPA2	TGCCGAGCTG	250-800	8	8	0	100.00	111	6.9375
OPA3	AGTCAGCCAC	250-900	9	9	0	100.00	168	10.5000
OPA4	AATCGGGCTG	250-900	9	8	1	88.89	191	11.9375
OPA5	AGGGGTCTTG	250-700	6	5	1	83.33	129	8.0625
OPA6	GGTCCCTGAC	250-700	6	4	2	66.67	157	9.8125
OPA7	GAAACGGGTG	250-600	6	6	0	100.00	91	5.6875
OPA8	GTGACGTAGG	250-900	8	7	1	87.50	199	12.4375
OPA9	GGGTAACGCC	250-700	6	3	3	50.00	145	9.0625
OPA10	GTGATCGCAG	300-900	7	7	0	100.00	184	11.5000
OPA11	CAATCGCCGT	250-800	7	7	0	100.00	114	7.1250
OPA12	TCGGCGATAG	250-700	6	6	0	100.00	83	5.1875
OPA13	CAGCACCCAC	300-800	6	4	2	66.67	165	10.3125
OPA14	TCTGTGCTGG	400-800	5	5	0	100.00	58	3.6250
OPA15	TTCCGAACCC	300-800	6	6	0	100.00	117	7.3125
OPA16	AGCCAGCGAA	500-800	4	4	0	100.00	42	2.6250
OPA17	GACCGCTTGT	200-1500	9	9	0	100.00	181	11.3125
OPA18	AGGTGACCGT	200-700	6	6	0	100.00	163	10.1875
OPA19	CAAACGTCGG	250-800	6	6	0	100.00	55	3.4375
OPA20	GTTGCGATCC	300-800	6	6	0	100.00	12	0.7500
OPC1	TTCGAGCCAG	400-900	6	6	0	100.00	90	5.6250
OPC2	GTGAGGCGTC	500-900	5	5	0	100.00	92	5.7500
OPC3	GGGGGTCTTT	400-900	6	6	0	100.00	50	3.1250
OPC4	CCGCATCTAC	600-1000	5	5	0	100.00	70	4.3750
OPC5	GATGACCGCC	100-300	3	3	0	100.00	29	1.8125
OPC6	GAACGGACTC	200-800	6	6	0	100.00	110	6.8750
OPC7	GTCCCGACGA	100-800	8	7	1	87.50	152	9.5000
OPC9	CTCACCGTCC	300-800	6	6	0	100.00	69	4.3125
OPC10	TGTCTGGGTG	200-700	6	6	0	100.00	36	2.2500
OPE1	CCCAAGGTCC	200-900	8	8	0	100.00	142	8.8750
OPE2	GGTGCGGGAA	300-800	6	6	0	100.00	116	7.2500
OPE3	CCAGATGCAC	200-900	8	7	1	87.50	176	11.0000
OPE4	GTGACATGCC	200-900	8	7	1	87.50	90	5.6250
OPE5	TCAGGGAGGT	500-900	5	5	0	100.00	77	4.8125
OPE6	AAGACCCCTC	500-900	5	4	1	80.00	68	4.2500
OPE7	AGATGCAGCC	300-1000	7	7	0	100.00	119	7.4375
OPE9	CTTCACCCGA	300-800	6	6	0	100.00	69	4.3125
OPE10	CACCAGGTGA	400-800	5	5	0	100.00	32	2.0000
OPX13	ACGCCAGCAA	600-1000	5	5	0	100.00	7	0.4375
ISSR1	$(GA)_8AC$	400-1500	7	7	0	100.00	127	7.9375
ISSR2	$(AG)_8$ AT	300-1500	9	9	0	100.00	208	13.0000
ISSR3	YCT $(GT)_7$	500-1200	7	7	0	100.00	153	9.5625
ISSR4	$CCCGCATCC(CA)_9$	300-1500	9	9	0	100.00	152	9.5000
ISSR5	$CCCGGATCC(GA)_9$	300-1500	7	7	0	100.00	130	8.1250
ISSR6	$(CA)_8G$	300-1500	6	6	0	100.00	68	4.2500
ISSR7	$(CT)_8AC$	400-1500	7	7	0	100.00	114	7.1250
ISSR8	$(CT)_8TG$	600-1500	5	5	0	100.00	72	4.5000
ISSR9	$(CA)_6AC$	400-1500	7	7	0	100.00	104	6.5000
ISSR10	$(GA)_6GG$	200-3000	9	9	0	100.00	201	12.5625
ISSR11	$(GT)_6CC$	100-3000	9	9	0	100.00	166	10.3750
ISSR12	$(CAC)_3GC$	300-3000	10	8	2	80.00	199	12.4375
Total			337	321	16	95.4 (Avg)	5689	

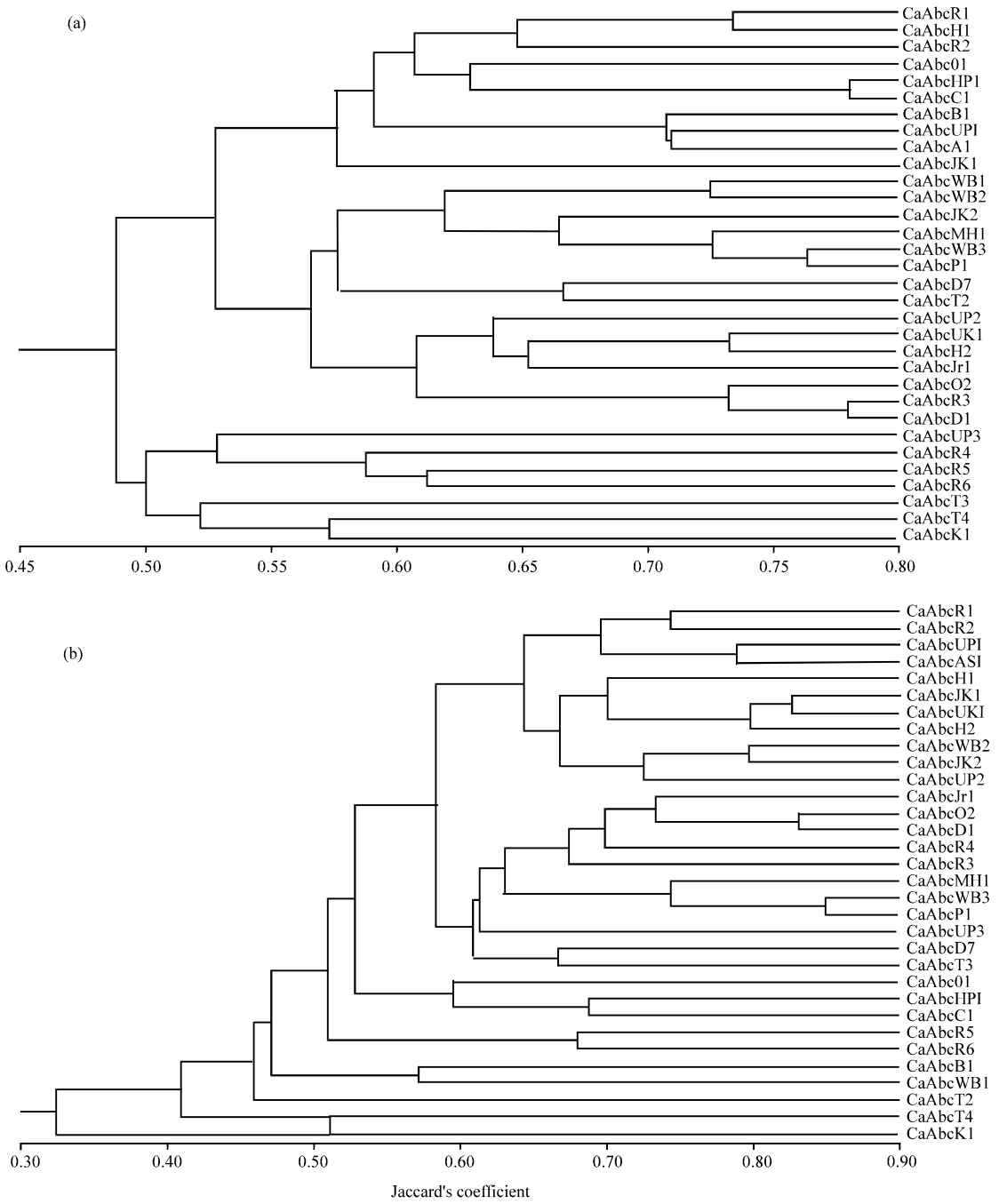

Fig. 1(a-b): Dendrogram generated by the unweighted pair group method with arithmetic means (UPGMA) of Jaccard's similarity coefficients based on the data amplified from the 32 isolates of *Alternaria brassicicola* using (a) RAPD and (b) ISSR primers

isolates were correlated with each other with an average similarity of 0.56. The isolates CaAbcD1 and CaAbcR3 were the most closely related, while CaAbcR4 and CaAbcR1 were widely apart with similarity coefficient 0.78 and 0.35, respectively (Table 1).

Twelve ISSR primers yielded a total of 92 reproducible bands. Among these 92 loci only two were found to be monomorphic, whereas the rest were polymorphic. The Resolving Powers (RP) for ISSR were comparatively higher than the RAPD markers which

proved ISSR a microsatellite based marker can produce extremely high variability and high mapping density. The highest RP was observed for the primer $(AG)_8AT$ (13.00) (Table 3, Fig. 1b). Jaccard's similarity coefficient calculated from the ISSR data showed that all the isolates were correlated with each other with an average similarity of 0.53. The isolates CaAbcP1 and CaAbcWB3 were closely associated with similarity coefficient of 0.85 while the isolates CaAbcK1 and CaAbcH1 were most distantly separated having similarity coefficient of 0.22. In the present study, both RAPD and ISSR could successfully differentiate among the isolates of *A. brassicicola* showing high level of polymorphism. Gherbawy (2005) reported considerable genetic variability among *Alternaria* isolates, even within the same species group. This result was also well endorsed by findings of other species of Alternaria infecting crucifer (Sharma *et al*., 2013b; Meena *et al*., 2012; Sangwan and Mehta, 2007) and other crops (Kale *et al*., 2012). The phylogenetic relationship could not group the isolates based on geographical origin. Rather a high level of genetic variability among the studied *A. brassicicola* isolates was found using RAPD and ISSR markers. This result was similar to the findings of Bock *et al*. (2002) who got genetic differences among a small sample of isolates of *A. brassicicola* using AFLP markers and Bock *et al*. (2005) who detected substantial polymorphism between isolates from five populations of *A. brassicicola* attacking *Cakile maritima* along the New South Wales coast of Australia with a maximum of two genotypes being shared between population pairs. Varma *et al*. (2006) reported similar results while studying genetic diversity among the *Alternaria solani* isolates infecting tomato crop. The RAPD and ISSR analysis proved to be an efficient method for detecting genetic variability amongst different *Alternaria spp*.viz., *Alternaria brassicae*, *A. brassicicola* and *A. raphani* (Sharma and Tewari, 1998), *A. cassiae* and *A. alternata* (Tigano *et al*., 2003; Iram and Ahmad, 2005; Pusz, 2009), *A. alternata, A. arborescens* and *A. tenuissima* (Hong *et al*., 2005, 2006), *A. solani* (Varma *et al*., 2006; Kumar *et al*., 2008; Leiminger *et al*., 2010), *A. radicina* and *A. carotiincultae* (Park *et al*., 2008). Conserved primers ITS1 and ITS4, directed the amplification of an approximately 600 bp ITS rDNA fragments from all isolates of *Alternaria brassicicola*. DNA sequencing revealed that these fragments ranged in size from 584-624 bp. The rDNA revealed presence of partial sequences of 18S and 28S rRNA and complete sequences of ITS1, ITS2 and 5.8S rRNA gene. The GenBank accession numbers for all the amplified ITS sequences determined in this study are provided in Table 1. In addition to the sequences used in this study, the alignment included 15 ITS sequences of different *Alternaria* spp. (including *A. brassicae* (Accession: HQ674659), *A. brassicicola* clone Abra0318 (Accession: AY781078), *A. brassicicola* strain P4838 (Accession: GU983654), *A. alternata* (Accession: AB369904), *A. carotiincultae* (Accession: AF229465), *A. mimicula* (Accession: FJ266477), *A. radicina* (Accession: EU781949), *A. japonica* (Accession: AF229474), *A. dianthi* (Accession: AY154702), *A. compacta* (Accession: AF314573), *A. gaisen* (Accession: EU520078), *A. mali* (Accession: AY154683), *A. citri* (Accession: DQ339104), *A. porri* (Accession: AB026159), *A. solani* (Accession: HQ270462) obtained from GenBank. The sequence alignment revealed all the 32 *A. brassicicola* isolates are closely associated with each other with bootstrap value of 100% identity.

Neighbour-joining analysis of ITS sequences showed minor variations where the optimal tree was found with sum of branch length of 0.40. The neighbour-joining tree (Fig. 2a) was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method, in the units of the number of base substitution per site. The analysis involved 47 nucleotide sequences containing the 32 *A. brassicicola* isolates along with other 15 *Alternaria* sp. from GenBank. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 454 positions in the final dataset. All the 47 *Alternaria* spp. were grouped into one large clade in which 32 *A. brassicicola* isolates of the present study revealed 100% identity with the *A. brassicicola* clone Abra0318 (Accession: AY781078) and *A. brassicicola* strain P4838 (Accession: GU983654) obtained from Genbank. Moreover these isolates showed maximum similarity with the rDNA sequence of *A. mimicula* isolate from NCBI. The *A. brassicicola* isolates showed slight variation from two other species *A. brassicae* and *A. japonica* which were also highly responsible for causing leaf spot in crucifers, whereas the neighbour joining tree revealed *A. japonica* is more closely associated with the *A. brassicicola* rather than *A. brassicae*. Under the large Alternaria clade, distinct small species clades were revealed which include the other *Alternaria* species showing 95-100% identity with each other.

Parsimony analysis of ITS data set revealed 123 most parsimonious trees, out of which one tree (Fig. 2b) having length = 196, the consistency index = 0.642857 (0.595376), the retention index = 0.825871 (0.825871) and

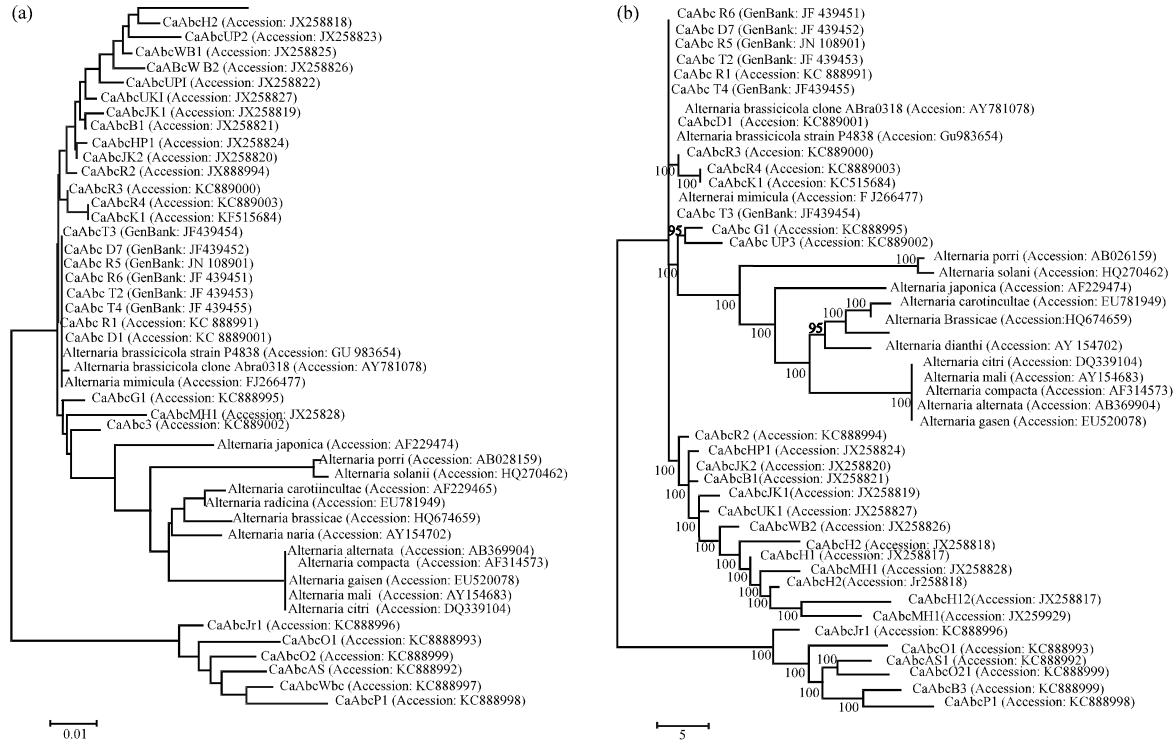

Fig. 2(a-b): Evolutionary relationships among thirty two *Alternaria brassicicola* isolates of India by aligning rDNA sequences using neighbour joining method in MEGA5 software, (a) The phylogenetic tree having sum of branch length 0.40 in the unit of number of base substitutions per site is constructed using maximum composite likelihood method and (b) The phylogenetic tree was obtained using the close neighbour interchange algorithm with search level 0 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis included 15 nucleotide sequences of other *Alternaria* spp. obtained from NCBI GenBank. The name of the isolates and *Alternaria* spp. along with the GenBank accession numbers are indicated on the termini of branches. The scale bar indicates the number of nucleotide substitutions. The consensus values (>50%) are mentioned below the branches

the composite index is 0.530917 (0.491703) for all sites and parsimony-informative sites (in parentheses) had near identical topology to the tree generated with neighbor-joining analysis (Fig. 2a). The large Alternaria clade was again supported by a consensus value of 100% with all 32 *A. brassicicola* isolates having 100% identity with each other and the *A. brassicicola* isolates of NCBI.

The inter transcribed spacer region is widely used in classifying fungi (Chillali *et al*., 1998) due to its variability nature among species (Jung *et al*., 2002). This region is suitable for PCR amplification, restriction analysis and sequencing procedure (Pryor and Gilbertson, 2000). Molecular systematic studies among *Alternaria* spp. based on nuclear ribosomal DNA have been previously analysed (Kusaba and Tsuge, 1994, 1995; Pryor and Gilbertson, 2002; Chou and Wu, 2002; Pryor and Bigelow, 2003). *Alternaria* species pathogenic to crucifer varied from each other by rDNA sequence analysis (Jasalavich *et al*., 1995). In the present study, all the thirty two *A. brassicciola* isolates were found closely associated with each other with bootstrap value of 100% identity. Both the neighbor joining and parsimony tree analysis showed *A. brassicicola* isolates were forming a large group showing 95-100% similarity with other *A. brassicicola* isolates considered from NCBI. They were closely related to the *A. mimicula* and *A. japonica* species under the big *Altermnaria* clade. Pryor and Gilbertson (2000) also found 98% similarity *A. brassicicola* and *A. japonica* species. *A. brassicae* which is also majorly responsible for black leaf spot in cauliflower grouped distantly apart from *A. brassicicola* (Sharma *et al*., 2013b).

CONCLUSION

In conclusion the overall morphological, cultural, pathogenic and genetic variability showed high level of

polymorphism among the *A. brassicicola* isolates infecting cauliflower in India with no clear grouping of isolates on the basis of their geographical origin. Morphological characteristics are correlated with the RAPD and ISSR analysis in showing polymorphism. Both the RAPD and ISSR primers proved to be efficient for detecting genetic variability among the *A. brassicicola* isolates. ITS analysis clearly indicates 99-100% similarity among the *A. brassicicola* isolates grouping them in a large group of species, different from other *Alternaria* spp. under the *Alternaria* genus/clade.

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