African Journal of Biotechnology Vol. 12(4), pp. 370-381, 23 January, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.2735
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Cultural, morphological, pathogenic and molecular characterization of *Alternaria mali* associated with Alternaria leaf blotch of apple

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Accepted 21 December, 2012

Alternaria blotch (*Alternaria mali*) causes severe foliar damage to apple trees in Kashmir. Twenty one (21) isolates of *A. mali* were collected from different locations and characterized for cultural, morphological, pathogenic and molecular variations. *A. mali* colonies varied in their cultural behaviour ranging from velvety to cottony, mostly appressed, with regular to irregular margins. Colour of colonies ranged between light to dark olivacious. Isolates impregnated media with colour ranging between grey to brown. Growth rate of isolates was between 5.86 to 8.21 mm/day with fast growth in isolate Am-13 and least in Am-5. Morphological variations in size, shape and septation of hyphae, conidiophore and conidia were observed in the isolates with significant variations in conidiophore and conidial septation. Average conidial size ranged from 21.36 to 31.74 x 8.34 to 14.48 µm. Isolates exhibited variations in incubation period, number and size of the lesions were produced. The dendrogram analysis, based on cultural, morphological and pathogenic studies, revealed variation within *A. mali* population. At 67% similarity matrix, all the isolates formed 2 clusters with 12 and nine isolates in cluster I and II, respectively. However, dendrogram on molecular (random amplification of polymorphic DNA, RAPD) basis revealed five clusters at 68% Dice similarity coefficient. There was no congruence between RAPD pattern and cultural, morphological and pathogenic characters. Isolates identical for one spectrum were often dissimilar for other spectrum. The results demonstrate existence of considerable variation in cultural, morphological, pathogenic and molecular characters of *A. mali* isolates prevalent in Kashmir valley.

Key words: Apple, *Alternaria mali*, variability, cultural, morphological, pathogenic, RAPD.

INTRODUCTION

Apple (*Malus domestica* Borkh.) is most ubiquitous temperate fruit cultivated in Europe and Asia from antiquity. Leading apple producing countries in the world are China, USA, Turkey, Italy, India, Poland and France with total world production of 70297212 MT. India ranks 5th in apple production with annual production of 2891000 MT (NHB, 2011). In India, apple is predominantly grown in Western Himalayan states particularly in Jammu and Kashmir (JandK), Himachal Pradesh and Uttaranchal which account for about 90% of total apple production in the country. The apple in JandK State has attained the status of an industry and alone contributes around 65% of total production in the country. Like other horticultural crops, apple is attacked by several pathogens which impair the quality and yield of fruit. However, huge crop losses are incurred by fungal diseases. Amongst these, Alternaria leaf blotch caused by *Alternaria mali,* prevalent

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Abbreviations: RAPD, Random amplification of polymorphic DNA; **UPGMA,** unweighted paired group method of arithmetic average.

in all apple growing areas of the world, is economically important apple disease. *A. mali* was first described in 1924 in the United States by J. W. Roberts and become a problem in the southeastern United States. The disease assumed alarming threat to the crop owing to premature defoliation in North Carolina and has potential of becoming threat especially in those apple producing regions where susceptible cultivars/strains of Delicious are grown (Filajdic and Sutton, 1991). Alternaria blotch has attained the status of economically important disease in many Asian countries including Japan and India (Sawamura, 1990).

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. 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. Shahzad (2003) reported the prevalence of Alternaria leaf blotch in all the districts of Kashmir with variable disease incidence and intensity. He also observed variation in susceptibility amongst different apple cultivars and attributed it to many factors including pathogenic variability.

Understanding pathogen population structure and mechanisms by which variation arises within a population is of paramount importance for devising a successful disease management strategy. This requires continuous monitoring of the development of pathogen variability more so for the breeding programme aimed at developing resistant genotypes to the given set of pathogenic races (Sartorato, 2002). 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. Since the crop and disease are of paramount importance to the JandK state and no studies on pathogen variability have been conducted in India and because of the non availability of host differentials, the present study was conducted with the objective to ascertain prevalence of variability in *A. mali* in Kashmir valley.

MATERIALS AND METHODS

Isolation of *Alternaria mali* isolates

Apple leaves from susceptible cultivar 'Red Delicious' exhibiting typical Alternaria leaf blotch symptoms were collected from twenty one apple orchards across the seven districts of Kashmir valley, namely, Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian. For isolation of fungal pathogen, the diseased leaf area along with some healthy portion was cut into small bits with a sharp sterilized blade. The bits were surface sterilized in 0.1% mercuric chloride for 30 s followed by three washing in distilled water to remove the traces of $HgCl_2$. After blotting dry with sterilized filter papers, the bits were transferred to potato dextrose agar (PDA) medium in sterilized petriplates and incubated for 7 days at 24±1°C. The isolates were purified using single spore isolation technique (Johnston and Booth, 1983).

Pathogenicity

The isolates of *A. mali* were first multiplied on PDA medium and then spore suspension of fungus prepared by flooding the culture plate with sterilized distilled water. The fungal growth on Perti-plate was scrapped with the help of a sterilized razor blade, strained through a double layer of sterile cheese-cloth into a 150 ml flask and spore concentration adjusted to 4×10^5 conidia per millilitre with the help of a haemocytometer. The prepared conidial suspensions were used for pathogenicity. Pathogenicity of fungus was carried out on detached leaves of apple cv. 'Red Delicious'. Healthy leaves were collected, leaf surface rinsed with sterilized water, leaves dried with a laboratory towel and placed in petriplates. Each petriplate was lined with a wet blotter paper on bottom to maintain high humidity. A set of three leaves (injured, uninjured and control) was placed in each petriplate. Before placement on Petri-plates, the injured and uninjured leaves were inoculated with a conidial suspension (4×10^5 spores/ ml) with an atomizer to the whole surface. The control leaves was sprayed with sterilized water. Leaves in petriplates were incubated at 24±1°C with 12 h dark and 12 h light adjustment and monitored for symptom development. Re-isolation of pathogen was carried out and compared with original inoculum to satisfy Koch's postulates.

Cultural variability

Mycelial discs (5 mm) of 7 day old culture of *A. mali* isolates were transferred to the centre of PDA plates and incubated at 24±1°C. Three replications were maintained for each isolate in a completely randomized design. The colony diameter was recorded on 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} day after incubation. Growth per day was calculated by the formula:

$$\frac{\text{Growth observed on a particular day (mm)} - \text{Growth on previous observation (mm)}}{2}$$

The other colony characters *viz.*, type, colour, margin and colour on underside of plate were recorded after 10 days incubation.

Morphological variability

Ten (10) days old cultures of all the isolates were studied for morphological variations. Temporary mounts were studied under compound microscope for hyphae (width), conidiophore and conidia (septation and size). The measurements were taken with the help of software Magnus Pro.MLX Series, New Delhi, India. Fifty recordings per replication were made for the purpose.

Pathogenic variability

Pathogenic variability was carried out on detached leaves of susceptible cultivar 'Red Delicious'. The experiment was carried out by employing the technique similar to that adopted for pathogenicity

test. The time taken for the appearance of first symptoms was recorded while the number and size of lesions were recorded 10 days after inoculation.

Binary data generation from cultural, morphological and pathogenic characters

The data on cultural, morphological and pathogenic variability was digitalized into a two-discrete-character-matrix (0 and 1 for absence and presence of a particular character, respectively). Except cultural variability, the binary data of variability was generated on the basis of critical difference. In case of cultural variability the binary data was generated on the basis of presence or absence of a particular character. The data of all the characters was combined. Binary matrices were analyzed by NTYSYS-PC 2.1 and similarity coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted paired group method of arithmetic average (UPGMA) (Rohlf, 1993).

Molecular variability

The molecular diversity was studied by random amplified polymorphic DNA (RAPD) technique (Williams et al., 1990). The mycelia from freshly grown seven day old cultures were harvested by filtration through a double layered sterilized filter paper, dried between two layers of filter paper under aseptic conditions and stored at -80°C. Total genomic DNA of each isolate was extracted using CTAB method (Murray and Thompson, 1980). RNase was added at 10 µg/ml (MBI Fermentas Life Sciences) and emulsion incubated for 2 h at 37°C. DNA was stored at -80°C (Deep Freezer Co. Pvt. Ltd.) till further use. Thirty 10 mer and twelve 20-mer oligonucleotide primers (Operon Technologies Inc., Alameda, CA, Sigma Genosys) (Table 1) were screened with 2 randomly selected isolates for polymorphism. The primers showing consistency in polymorphism among the isolates were selected for RAPD profiling of isolates. The PCR was carried out in 0.2 ml PCR tube with 25 µl reaction volume containing 1x buffer (20 mM Tris-HCl pH 8.0; 50 mM KCl), 1.5 mM $MgCl_2$, 2.0 µl dNTP mix (0.2 mM), 1 U of *Taq* DNA polymerase (5 U/µl), (Fermentas Life Sciences), 2 µl of DNA tempelate (20 ng), 5 pmols of primer and 17.3 µl of sterilized distilled water. Reaction mixture was vortexed and centrifuged in a microfuge (Thermo Scientific, Thermo electron Corporation). Amplifications were performed using thermal cycler (Whatman Biometra, T Gradient, Goettingen, Germany) programmed for initial denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 37/55°C (37°C for decamer and 55°C for twenty-mer) for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min using fastest ramp time between transitions. The amplified PCR products were resolved by electrophoresis using 1.2% (w/v) agarose gel in 1X Tris borate EDTA buffer (0.5 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0). The gels were stained with ethidium bromide (0.5 µg/ml). The gels were run at 80 V for one and a half hour using Consort Power Pack system (Consort EV 215). The gel images were captured using a combination of Ultracam Digital Imaging (A 650 Canon Camera) and Electronic ultraviolet (UV0 Transilluminator (Ultra. Lum. Inc, 1480 N, Claremont Boulevard, Claremont, CA).

Data analysis

Data analysis for cultural, morphological and pathogenic variability was carried out through MINITAB 13.20. Four most polymorphic and reproducible primers *viz.,* P13, U2, U3 and U9 were used for RAPD profiling. The band pattern obtained from agarose gel electrophoresis was digitalized to a binary-matrix (0 and 1 for absence and presence of RAPD-bands, respectively). Bands that could be scored univocally for presence and absence were included in analysis. Binary matrices were analyzed by NTYSYS-PC 2.1, and Dice coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted paired group method of arithmetic average (UPGMA) (Rohlf, 1993).

RESULTS AND DISCUSSION

Twenty one (21) isolates of *A. mali* were obtained during survey of seven districts in Kashmir valley (Figure 1). The cultural and morphological characters of isolated fungus closely resembled to those described by Roberts (1924). The pathogenicity of *A. mali* (Roberts) isolates was established on wounded detached leaves of apple cv. “Red Delicious”. *A. mali* being a weak pathogen is unable to force its entry and hence requires wounds to facilitate its penetration into leaf. Penetration of host plants through wounds has been shown to be a significant component of infection process for a number of *Alternaria* species (Rotem, 1994). Ozgonen and Karaca (2006) and Soleimani and Esmailzadeh (2007) also confirmed the pathogenicity of *A. mali* isolates by inoculation with conidial suspension on detached wounded apple leaves.

Cultural variability

The isolates of *A. mali* varied in type and colour of colony (Table 2). Colony colour varied from light to dark olivacious with greenish or brownish tinge. Mostly, the colonies had velvety or cottony mycelial growth with slight variations and regular to irregular margin. All the isolates impregnated the media with a colour mostly grey to brown with some variations which were clearly visible from the underside of plates. The results are in agreement with Pusz (2009) who found that the colonies of *Alternaria alternata* isolated from *Amaranthus retroflexus* varied from light grey to dark grey. Similarly, Rai and Kumari (2009) observed loose, cottony, compact and dense colonies with light to dark black colour in *A. alternata* infecting Periwinkle. The findings of Hubballi et al. (2011) showed variation in the pigmentation of 15 *A. alternata* isolates producing black, brownish black, greenish black, brown and yellow pigmentation.

All the isolates depicted variation in growth rate (growth/day) (Figure 2). Isolate Am-13 was fastest among the isolates with mean growth rate of 8.21 mm/day while isolate Am-5 was slowest with mean growth rate of 5.86 mm/day. Isolates in the present study depicted periodic changes in their growth rates. All the isolates showed an increasing trend in growth rate from 2 days to 8 days but decreased afterwards. Almost similar observations were recorded by Pusz (2009) on *A. alternata* with colony diameter ranging from 4.8 to 6.8 cm among 26 isolates after seven days of inoculation. Similar observations were also recorded by Thrall et al. (2005) and Rai and Kumari (2009).

Table 1. Nucleotide sequences of primers used in RAPD.

Primer name	Sequence (5' to 3')	Primer name	Sequence (5' to 3')
OPA-01	CAG GCC CTT C	OPC-06	GAA CGG ACT C
OPA-02	TGC CGA GCT G	OPC-07	GTC CCG ACG A
OPA-03	ATG CAG CCA C	OPC-08	TGG ACC GGT G
OPA-04	AAT CGG GCT G	OPC-09	CTC ACC GTC C
OPA-05	AGG GGT CTT G	OPC-11	AAA GCT GCG G
OPA-06	GGT CCC TGA C	OPC-12	TGT CAT CCC C
OPA-07	GAA ACG GGT G	OPC-13	AAG CCT CGT C
OPA-08	GTG ACG TAG G	OPC-14	TGC GTG CTT G
OPA-09	GGG TAA CGC C	OPC-15	GAC GGA TCA G
OPA-10	GTG ATC GCG T	U1	ATC CAA GGT CCG AGA CAA CC
OPA-11	CAA TCG CCG T	U2	CCC AGC AAC TGA TCG CAC AC
OPA-12	TCG GCG ATA G	U3	GTG TGC GAT CAG TTG CTG GG
OPA-13	CAG CAC CCA C	U4	AGG ACT CGA TAA CAG GCT CC
OPA-14	TCT GTG CTG G	U5	GGC AAG CTG GTG GGA GGT AC
OPA-15	TTC CGA ACC C	U6	ATG TGT GCG ATC AGT TGC TG
OPA-16	AGC CAG CGA A	U7	GGT GAA CAG TGA GAT GAA CC
OPA-17	GAC CGC TTG T	U8	TAC ATC GCA AGT GAC ACA GG
OPC-01	TTC GAG CCA G	U9	AAT GTG TGG CAA GCT GGT GG
OPC-03	GGG GGT CTT T	U10	GAT GTG TTC TTG GAG CCT GT
OPC-04	CCG CAT CTA C	U11	GGA CAA GAA GAG GAT GTG GA
OPC-05	GAT GAC CGC C	U12	GGT TGT AGG CCG ATA TTG TC

Figure 1. District map of Kashmir valley showing distribution of isolates.

Table 2. Cultural variability in *Alternaria mali* isolates.

Isolate	Colony			Colour on the underside of Petri-plate
	Type	Colour	Margin	
Am-1	Velvety, appressed, sparse cottony centre	Dark olivaceous green	Irregular, light olivaceous green with white rim	Dark grey with light grey margin
Am-2	Velvety, cottony central growth	Olivaceous	Slightly irregular, olivaceous green	Brown with light grey margin
Am-3	Cottony, slightly furrowed with appressed centre	Dark olivaceous, with dark centre	Regular, cottony brownish margin with whitish rim	Smoky grey to dark grey
Am-4	Velvety, with cottony central growth	Olivaceous green	Slightly irregular, olivaceous green	Dark grey with light grey margin
Am-5	Velvety, appressed	Light olivaceous green	Regular, light brownish margin with white rim	Smoky grey
Am-6	Cottony, with slighty appressed margins	Olivaceous green with grayish surface	Regular, brownish with white rim	Grey with light grey margin
Am-7	Cottony, appressed centre	Light olivaceous green	Regular, cottony with white rim	Bluish brown with light grey margin
Am-8	Velvety, slightly furrowed with appressed centre	Olivaceous with brownish centre and grayish surface	Irregular, appressed, olivaceous green	Dark grey with light grey margin
Am-9	Cottony	Olivaceous green with grayish surface at centre	Regular, brownish with white rim	Smoky grey
Am-10	Cottony	Light olivaceous green	Regular, with concentric rings of white and green, rim white	Smoky grey to dark grey
Am-11	Velvety, appressed	Olivaceous green with white cottony patches	Regular, green with grayish rim	Brown with light grey margin
Am-12	Velvety, Appressed	Olivaceous	Irregular, appressed, Olivaceous with white rim	Light grey with brown centre
Am-13	Cottony, sub-aerial	Dark green with greyish surface	Regular, dirty white margin	Bluish brown with light grey margin
Am-14	Velvety, appressed	Olivaceous green	Slightly irregular, light green with grayish rim	Light grey with brown centre
Am-15	Velvety, appressed	Olivaceous	Slightly irregular, brownish with dirty white rim	Brown with light grey margin
Am-16	Velvety, appressed	Dark olivaceous green	Irregular, light olivaceous with grayish rim	Smoky grey
Am-17	Velvety, appressed	Dark olivaceous green	Slightly irregular, light olivaceous with grayish rim	Light brown with light grey margin and dark brown centre
Am-18	Velvety, appressed	Olivaceous green	Slightly irregular, light green with grayish rim	Light grey with dark grey centre
Am-19	Cottony, slightly furrowed	Olivaceous	Regular, cottony olivaceous with greyish rim	Greyish with light grey margin and brown centre
Am-20	Velvety, appressed	Olivaceous green	Regular, appressed, olivaceous green	Smoky grey
Am-21	Cottony, sub-aerial	Greenish with grayish surface	Regular, appressed green with white rim	Light grey with dark grey centre

Morphological variability

Mean hyphal width of isolates ranged from 3.28 to 4.28 µm with maximum in isolate Am-4 and minimum in Am-21 (Table 3). In present study, the variation in conidiophore size was observed with maximum length of 68.18 µm in isolate Am-9 and minimum in Am-17 (26.99 µm). Similarly, conidiophore breadth varied from 3.30 to 4.70 µm with least breadth in isolate Am-20 and maximum in Am-3. Conidiophores were aseptate to septate. Both septate and aseptate conidiophores were observed in isolates Am-1, Am-5, Am-9, Am-14, Am-19 and Am-21, while the remaining 15 isolates had only septate conidiophores. The

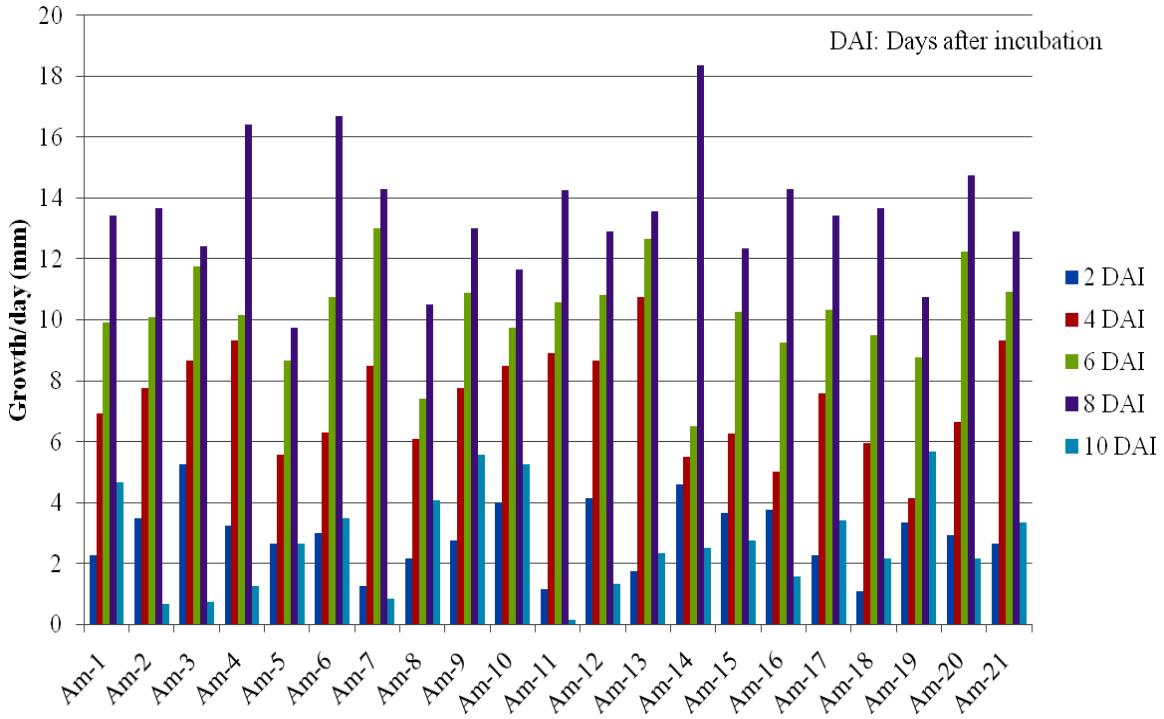

Figure 2. Variability in the growth rate of *Alternaria mali* isolates.

Table 3. Variability in the hyphae and conidiophore of *Alternaria mali* isolates.

Isolate	Hyphal width (µm)		Conidiophore					
			Length (µm)		Breadth (µm)		Septation (No.)	
	Range	Mean*	Range	Mean*	Range	Mean*	Range	Mean*
Am-1	3.75-5.20	4.21	33.54-55.38	47.26	3.48 - 5.09	4.65	0-7	4.44
Am-2	2.94-4.74	4.14	42.35-59.69	55.66	3.85 - 5.47	4.40	1-5	3.08
Am-3	3.81-5.25	4.24	32.44-49.35	42.91	3.35 - 5.53	4.70	1-6	4.43
Am-4	3.80-5.32	4.28	36.57-54.61	47.76	3.47 - 5.40	4.60	1-9	4.54
Am-5	3.92-4.95	4.20	40.28-56.29	50.59	3.36 - 4.95	4.62	0-8	5.90
Am-6	3.25-4.15	3.93	41.32-58.65	53.72	4.06 - 5.62	4.57	1-5	4.06
Am-7	3.45-4.20	3.99	48.25-61.25	57.13	3.81 - 5.47	4.67	1-6	4.35
Am-8	2.55-4.86	3.89	34.55-50.43	43.61	4.27 - 5.05	4.60	1-7	4.78
Am-9	3.65-4.51	4.14	49.27-68.18	62.58	3.38 - 5.15	4.42	0-5	3.46
Am-10	3.35-4.16	4.09	47.49-65.41	60.75	3.67 - 5.24	4.45	1-4	3.69
Am-11	3.71-4.99	4.19	47.31-61.47	55.81	3.36 - 4.95	4.38	1-6	4.74
Am-12	2.74-4.15	3.54	33.29-53.68	42.59	3.49 - 5.08	4.54	1-7	5.14
Am-13	2.94-4.12	3.58	36.29-55.22	45.59	3.65 - 5.24	4.57	1-6	5.23
Am-14	3.31-3.90	3.51	27.28-44.95	33.01	4.20 - 5.34	4.46	0-4	3.28
Am-15	2.78-5.06	4.02	30.25-50.26	38.18	3.68 - 5.06	4.39	2-9	5.64
Am-16	3.31-5.37	4.09	28.01-49.88	37.50	3.36 - 4.78	4.03	1-4	3.09
Am-17	3.45-4.23	3.99	26.99-52.91	41.06	2.19 - 4.70	3.86	2-6	4.27
Am-18	3.55-4.28	4.12	28.71-44.21	34.03	3.21 - 4.68	4.00	1-5	4.19
Am-19	2.91-3.87	3.35	31.32-48.76	39.21	2.55 - 4.63	3.38	0-6	4.21
Am-20	2.83-4.08	3.47	28.21-48.58	36.92	2.79 - 4.41	3.30	1-8	3.94
Am-21	2.19-4.14	3.28	28.15-55.45	39.89	2.35 - 3.59	3.45	0-6	4.32
$CD_{(P = 0.05)}$			0.58	4.44		0.59	0.77	

*Average of 150 observations.

Table 4. Variability in the conidial septation and size of *Alternaria mali* isolates.

Isolate	Transverse septa (No.)		Longitudinal septa (No.)		Length (µm)		Breadth (µm)	
	Range	Mean*	Range	Mean*	Range	Mean*	Range	Mean*
Am-1	1-4	2.26	0-3	1.12	11.32 - 28.17	26.73	8.23 - 16.01	12.01
Am-2	1-4	2.09	0-3	0.36	10.01 - 25.71	23.32	6.22 - 15.82	10.67
Am-3	1-4	2.42	0-4	1.00	11.57 - 31.17	27.17	8.12 - 16.07	11.08
Am-4	1-5	2.45	0-3	1.00	14.16 - 39.06	26.71	6.63 - 13.40	9.97
Am-5	1-6	2.75	0-4	0.91	11.08 - 26.73	23.92	7.66 - 12.24	10.14
Am-6	1-5	2.95	0-3	1.04	10.57 - 24.78	22.74	6.87 - 13.42	9.89
Am-7	1-4	2.30	0-3	0.45	11.10 - 25.22	22.99	6.23 - 14.42	9.41
Am-8	1-4	1.76	0-3	0.33	9.79 - 22.85	22.26	5.57 - 14.28	9.63
Am-9	1-5	2.20	0-4	0.76	18.73 - 34.84	26.66	9.03 - 20.02	14.48
Am-10	1-4	2.19	0-3	0.42	12.35 - 26.89	23.57	7.68 - 13.53	10.42
Am-11	1-6	2.55	0-3	0.92	12.58 - 35.41	26.92	8.04 - 14.49	10.84
Am-12	1-5	2.61	0-4	0.92	9.72 - 32.23	26.49	6.04 - 15.35	11.02
Am-13	1-5	2.92	0-3	0.89	19.10 - 49.12	31.74	7.68 - 14.75	11.66
Am-14	1-4	2.61	0-3	0.22	11.04 - 29.27	24.91	6.93 - 13.87	9.83
Am-15	1-4	2.00	0-3	0.64	10.52 - 24.41	21.91	6.21 - 15.05	10.15
Am-16	1-5	2.30	0-3	0.46	10.46 - 28.38	21.36	6.58 - 12.26	8.34
Am-17	1-4	2.18	0-3	0.76	15.72 - 31.46	28.13	8.57 - 17.39	13.08
Am-18	1-7	2.78	0-3	0.89	9.95 - 27.23	22.44	7.73 - 16.01	11.40
Am-19	1-4	2.29	0-3	0.54	11.06 - 31.00	25.64	7.78 - 15.21	11.03
Am-20	1-4	2.33	0-3	0.51	17.19 - 31.03	26.33	9.19 - 14.67	11.87
Am-21	1-5	2.77	0-3	0.70	12.13 - 35.89	28.05	8.12 - 16.25	12.15
$CD_{(P = 0.05)}$		0.24		0.11		2.52		2.03

*Average of 150 observations.

overall highest septation of 9 was recorded in isolate Am-4 (Table 3). Conidial septation both transverse and longitudinal varied significantly among the isolates. Transverse septa varied from 1 to 7 and longitudinal from none to 4. The highest mean number of transverse septa was recorded in isolate Am-6 (2.95) and lowest in Am-8 (1.76). The highest mean number of longitudinal septa was observed in isolate Am-1 (1.12) and lowest in Am-14 (0.22). In present study, the average conidial size varied from 21.36-31.74 x 8.34-14.48 µm (Table 4). Maximum (31.74 µm) and minimum (21.36 µm) conidial length were noticed in isolate Am-13 and Am-16, respectively, while maximum (14.48 µm) and minimum (8.34 µm) conidial breadth were recorded in isolates Am-9 and Am-16, respectively. In conformity to our observations Ramegowda and Naik (2008) reported that the hyphal width of 9 *A. macrospora* isolates varied from 2.87 to 6.95 µm. Tetarwal et al. (2008) found conidiophore length and breadth among six isolates of *A. alternata* varying from 18.90 to 27.40 and 4.23 to 5.75 µm, respectively. Rotem (1966) found a wide variability in the spore dimensions of 42 isolates of *A. solani*.

Pathogenic variability

A considerable pathogenic variability was observed in incubation period, number of lesions and size of lesions among the isolates (Table 5). The incubation period of isolates varied from 2.0 to 6.3 days with minimum in isolates Am-9, Am-13 and Am-15, and maximum in isolates Am-17. The number of lesions produced by isolates varied from 6.3 to 14.3 with least lesions produced by Am-2 and maximum by Am-16. The mean minimum lesion size of 2.9 mm was observed in Am-17 and maximum of 10.2 mm was seen in Am-1. The findings are in agreement with Thrall et al. (2005) who reported significant variations in the lesion size produced by *Alternaria brassicicola* isolates on wound inoculated *Cakile maritima* plants. Kumar (2004) also reported variation in lesion size and lesion number in *Alternaria triticina* isolates. However, present observations are contradictory to the findings of Quayyum et al. (2005) who did not find any significant variation in the lesions produced by the isolates of *Alternaria panax* on detached leaflets of ginseng.

Cultural, morphological and pathogenic variability in isolates

The dendrogram analysis of isolates on the basis of cultural, morphological and pathogenic studies revealed significant variation within *A. mali* isolates (Figure 3). At

Table 5. Pathogenic variability in *Alternaria mali* isolates.

Isolate	Pathogenic variability		
	Incubation period (days)	No. of lesions*	Mean lesion size (range) (mm)*
Am-1	2.6	12.0	10.2 (7-12)
Am-2	3.6	6.3	9.1 (5-11)
Am-3	4.6	10.0	4.6 (3-6)
Am-4	2.3	10.0	8.5 (4-9)
Am-5	6.0	8.3	6.1 (4-8)
Am-6	5.6	11.0	3.4(2-5)
Am-7	3.0	9.6	5.6 (3-7)
Am-8	2.3	11.6	6.2 (3-8)
Am-9	2.0	11.6	5.8 (3-7)
Am-10	4.0	6.6	6.4 (2-7)
Am-11	3.3	12.3	8.3 (4-10)
Am-12	5.3	9.0	4.5 (2-5)
Am-13	2.0	13.0	5.7 (4-7)
Am-14	3.3	12.0	6.2 (2-7)
Am-15	2.0	12.3	3.1 (2-4)
Am-16	5.0	14.3	4.3 (3-6)
Am-17	6.3	6.0	2.9 (2-4)
Am-18	3.3	8.6	5.6 (4-7)
Am-19	3.0	8.3	5.1 (3-6)
Am-20	4.3	11.3	3.3 (3-5)
Am-21	5.0	12.0	4.6 (2-6)
$CD_{(P = 0.05)}$	0.68	2.41	0.74

*After 10 days of inoculation.

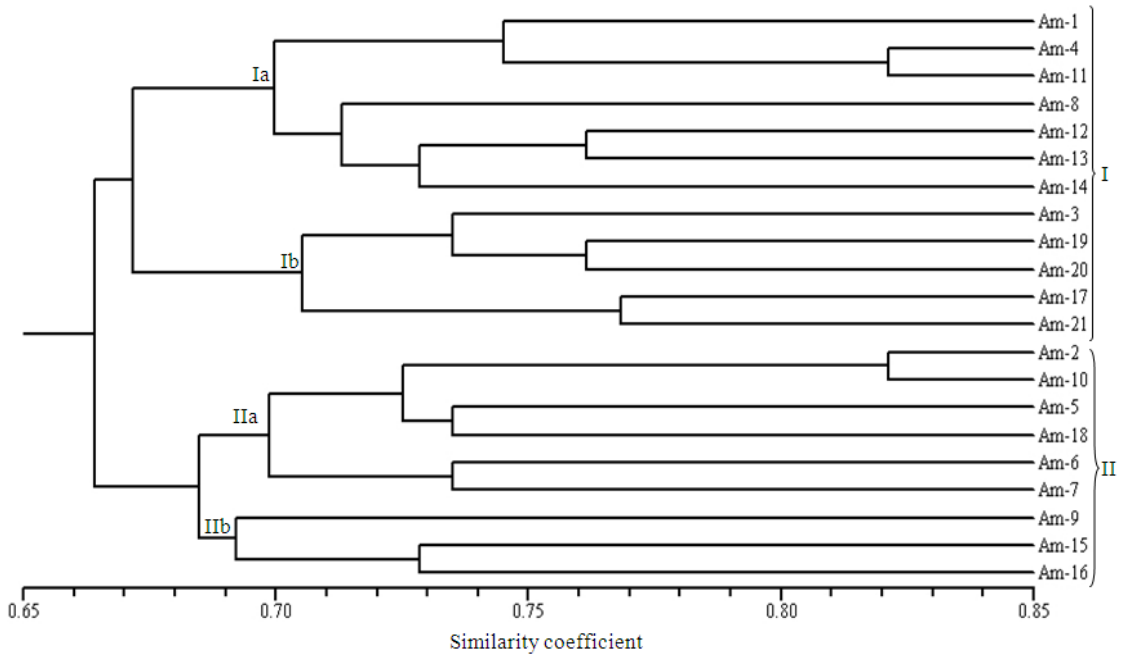

Figure 3. Dendrogram of 21 isolates of *Alternaria mali* generated by unweighted pair group method arithmetic mean (UPGMA) analysis of cultural, morphological and pathogenic characters. Scale at the bottom depicts the similarity values obtained using similarity coefficient.

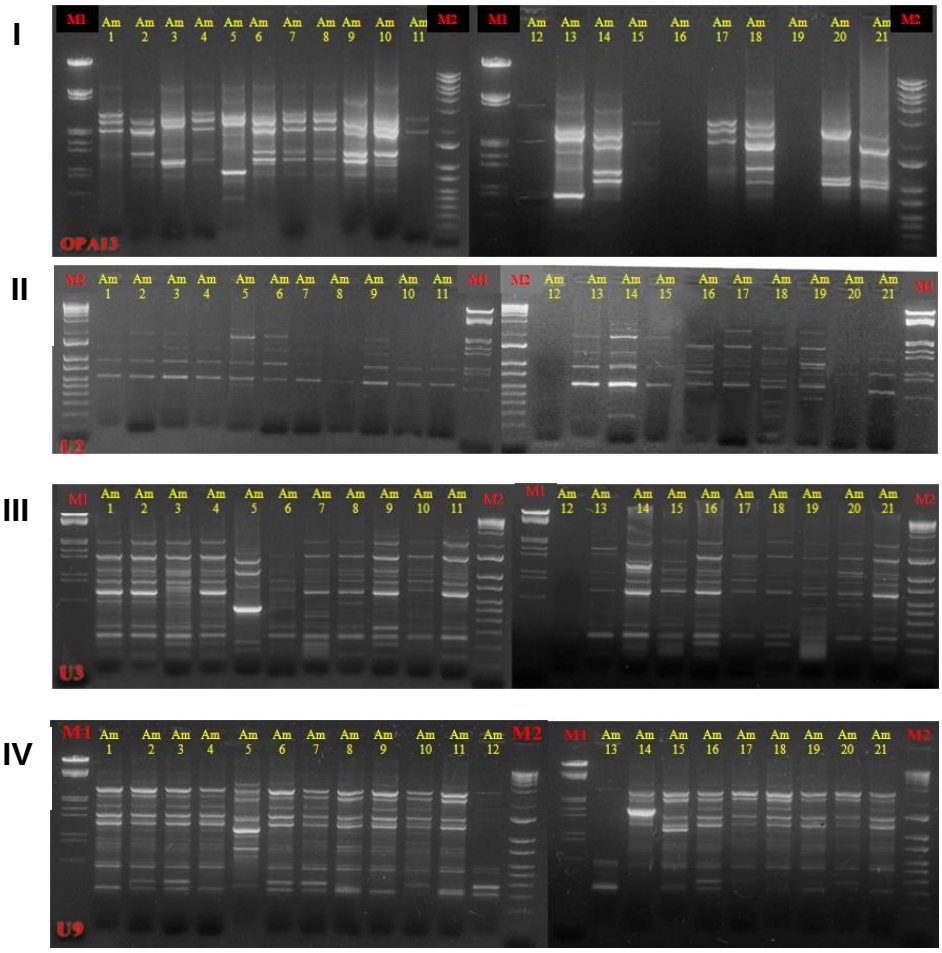

Figure 4. Gel eletrophoresis of the RAPD amplified products from *A. mali* obtained with different primers. Column M1 and M2 shows the band pattern for the fragment size markers (100 bp and Lambda DNA/*EcoR1+Hind III*). I, RAPD profile of 21 *A. mali* isolates with OPA-13 primer; II, RAPD profile of 21 *A. mali* isolates with U-2 primer; III. RAPD profile of 21 *A. mali* isolates with U-3 primer; IV, RAPD profile of 21 *A. mali* isolates with U-9 primer

67% similarity matrix, all the isolates were categorized into 2 clusters (I and II). Cluster I contained of 12 isolates, and cluster II accommodated 9 isolates. Cluster I was further subdivided into Ia and Ib groups accommodating 7 and 5 isolates, respectively. Similarly, cluster II was subdivided into IIa and IIb accommodating 6 and 3 isolates, respectively. Several workers have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. (Singh et al., 2003; Slavov et al., 2004; Tetarwal et al., 2008). It appears that the variation amongst the isolates may be inherent since isolates were collected from different/ distant sites. Phenotypic characters are influenced by environmental conditions so may be responsible for such diversity. Moreover, the isolates in these sites may have acclimatized for many years which may be responsible for this diversity. Many of the phenotypic markers are controlled by many genes most of which have additive effect. The tendency to group isolates in relation to their geographical location was not obtained, as the two major clusters (I and II) and sub-clusters (Ia, Ib and IIa, IIb) contained isolates from different districts having almost similar agro-climatic conditions. Brierley (1920) suggested that variation in fungi imperfecti may be due to mutation or owing to the splitting of originally impure genetic constitution or because of gametic or somatic segregation from heterozygotes. Variants, considered as due to mutation, were found in single spore cultures of *A. mali* (Roberts, 1924).

Molecular variability

Among the 42 primers, one 10-mer (OPA-13) and three 20-mers (U2, U3 and U9) produced consistent polymorphic bands and were selected for fingerprinting of 21 isolates of *A. mali* (Figure 4). This is for the first time that the 20-mer primers were selected for fingerprinting of

Table 6. Number of scorable and polymorphic RAPD bands generated by PCR amplification.

Primer	Scored band	Polymorphic band	Polymorphism (%)
OPA-13	10	10	100
U2	7	6	85.71
U3	14	14	100
U9	11	11	100
Total	42	41	97.61

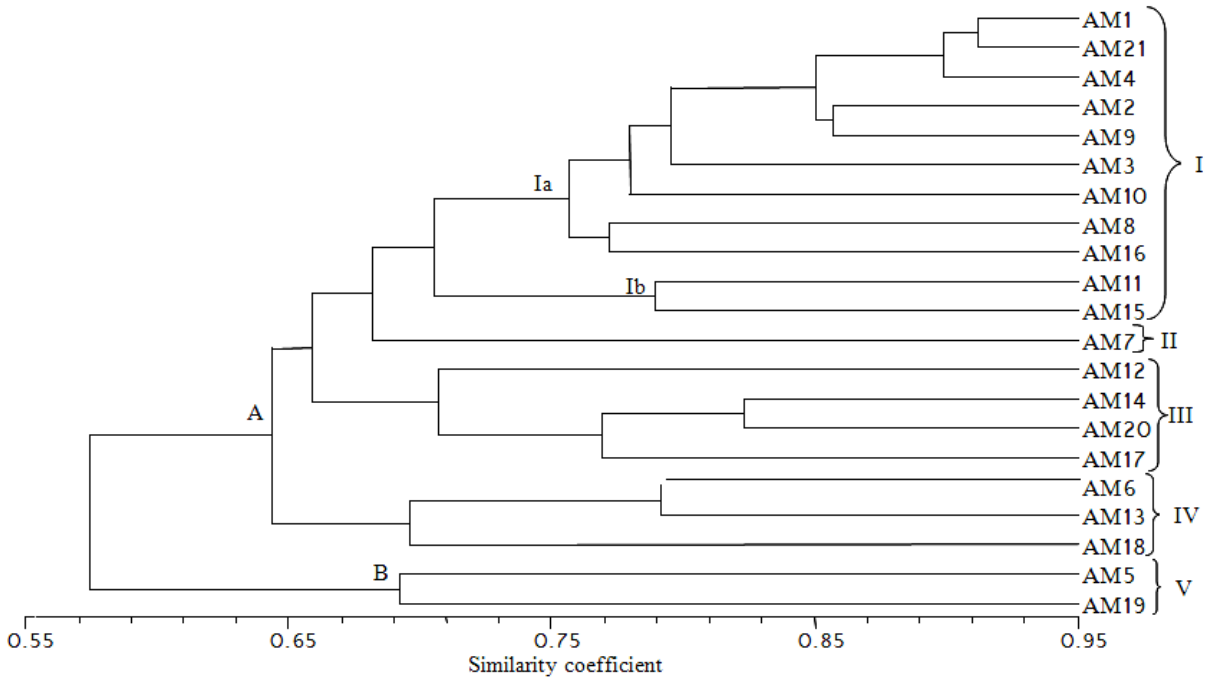

Figure 5. Dendrogram of 21 isolates of *Alternaria mali* generated by UPGMA (Unweighted pair group method arithmetic mean) analysis of RAPD bands obtained with four.

A. mali isolates. Highest scored bands were 14 in U3 showing 100% polymorphism. The least scorable bands were 7 in U2 with 6 polymorphic bands. All the primers showed 100% polymorphism, except U2 with 85.71% polymorphism (Table 6). DNA based molecular markers that are selectively neutral and randomly distributed in a genome are important tools in studies on genetic diversity of pathogen populations (Dini-Andreote et al., 2009). In absence of defined differential set availability, molecular markers offer an appropriate alternative to detect and characterize genetic variability in *A. mali*. RAPD analysis is extremely robust and can separate individuals having intra- and inter-specific variability. It gives more comprehensive information regarding genetic variability in pathogen populations as it is based on the entire genome of an organism (Achenback et al., 1997). Kumar et al. (2008) selected four random primers to study the genetic diversity within isolates of *Alternaria solani* because of their reproducible results of polymorphism between individuals.

The dendrogram analysis of 21 isolates revealed genetic diversity within *A. mali* population in Kashmir valley (Figure 5). At 57.5% Dice similarity coefficient all the isolates formed a single clade, thus showing 42.5% dissimilarity among the isolates. At 68%, all the isolates were categorized into 5 clusters (I to V). Cluster I was further subdivided into Ia and Ib groups accommodating 9 and 2 isolates, respectively. The tendency to group isolates in relation to their location proximity was not obtained, as the 5 clusters (I to V) and sub-clusters (Ia and Ib) contained isolates from different districts. The results presented are in agreement with the observation of Guo et al. (2004) who found high genetic variation within *A. alternata* isolates originating from *Pinus tabulaeformis* and reported that *A. alternata* appears to have the potential for relatively quick evolution which may lead to significant diversification.

The results presented are not in agreement with that of

Pusz (2009) who reported low genetic variability among the isolates of *A. alternata* originating from *Amaranthus;* however, he stated that the low genetic variation may be as a result of high adaptive ability of fungus and due to the close proximity between collection sites and host-plants. Van der Waals et al. (2004) showed high genetic diversity among *A. solani* isolates from South Africa and found no clustering of isolates according to geographical origin. Kumar et al. (2008) also found no effect of origin of isolates, rather two isolates of *A. solani* from two different locations were closer to each other. The RAPD data presented here and other molecular studies (Roberts et al., 2000; Bock et al., 2002; Tigano et al., 2003) confirm high level of variation among isolates of a single species of *Alternaria*. A possible explanation for the high level of genetic diversity found among isolates of *A. mali* could be natural chance mutations, combined with the fact that the fungus can produce abundant numbers of spores in a relatively short period of time as reported in *A. alternata* (Leung et al., 1993).

However, high levels of genetic variations are usually due to recombination, which occur sexually through mating or asexually through the parasexual cycle. It is not known if the parasexual cycle occurs regularly in genus *Alternaria*, and also there is no known sexual cycle for *A. mali*. Morris et al. (2000) suggested an unknown sexual stage as a possible explanation for this diversity as *Alternaria* anamorphs of several *Lewia* spp. have already been described (Simmons, 1986). The diversity in Kashmir valley may be because of mixed cultivars cultivation in our orchard eco-system, diversity in sites and selection pressure due to indiscriminate use of fungicides. Slavov et al. (2004) have argued the opportunities for genetic change through mutation, nuclear migration and anastomosis whereby any mycelium may become heterokaryotic is more likely in *Alternaria*. Obviously, the conidia produced from such mycelium will be genetically different. There was no congruence between the RAPD pattern and cultural, morphological and pathogenic characters. The isolates identical for one spectrum were often dissimilar for other spectrum. The findings are supported by Kumar et al. (2008) who reported that groupings based on RAPD data could not be correlated with the ones based on cultural, morphology and pathogenicity. Peever et al. (2002) also did not find any correlation between RAPD clustering of *Alternaria* isolates and pathogenicity.

The present variability study revealed no grouping of isolates having proximity of the locations. These results indicate the presence of mixed sub-populations which may be due to the distribution of vegetative material over the entire valley (across districts and locations), as the disease is prevalent in apple seedlings as well. Another possible cause for even distribution of genetic variation in *A. mali* may be the dissemination of spores by biotic and abiotic factors. The distribution of genetic variation of *A. mali* gives an indication of the fitness of pathogen to circumvent the effects of natural or artificial stresses on the population, and thus counteract control measures such as fungicide applications. McDonald and Linde (2002) have suggested that in such cases breeding efforts should concentrate on quantitative resistance or on the development of cultivar mixtures that can be used in combination with other control strategies. Understanding the diversity of *A. mali* on apple in Kashmir will thus aid in disease management strategies of Alternaria leaf blotch.

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 imperfecti (Burger, 1921; Boner, 1922; Stevens, 1922). Various other workers have indicated the existence of races of *A. solani* based on morphological, physiological, and virulence differences (Henning and Alexander, 1959; Neergaard, 1945). Stackman et al. (1981) pointed out biological forms as threat in case of stem rust of wheat, similarly biological forms of *A. mali* may pose a serious problem to breeding for resistance to Alternaria leaf blotch of apple.

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