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Cultural, morphological, pathogenic and molecular characterization of *Alternaria mali* associated with necrotic leaf spot of loquat

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Abstract

In Pakistan, loquat (*Eriobotrya japonica*) in an emerging fruit and provides well economic return to growers. Percentage disease incidence of necrotic leaf spot (*Alternaria mali*) ranged from 25 to 46.16% in Taxila, Wahcant, Khanpur, Kalar Kahar, Choa Saiden Shan, Tret, Chatar and Murree. 48 isolates were characterized for cultural, morphological and pathogenic variations. Light to dark olivacious colonies were appraised, cottony to velvety with regular to irregular margins and variations in size and shape of conidia were observed between isolates. Length of conidiophores was ranging from 24.6 to 68.5 µm and nine was the highest septation in isolate ALT_7WT_1. Nucleotide sequences of inter transcribed spacer region (ITS1, 5.8S, ITS2) from 4 highly virulent isolates were submitted in the database of NCBI under accession numbersKR232489, KR232490, KT154010 and KT154011 respectively. Evolutionary history was computed with available sequences of *A. mali*, *A. lternata, A. zinnae* and *A. tenuissima* which were reported from Pakistan, USA, Sweden, Newzeland and India. Evolutionary tree showed4 different sub-trees and submitted sequences were observed in *A. mali* sub-tree. Amino acid sequences were exhibiting 100 to 99% genetic homology with *A. mali* isolate (FCBP1343) reported form Pakistan. Amino acid sequences were compared with FCBP1343 and differences at position number 18 (valine was replaced with glutamate), 57 (glutamine with leucine), 84 (alanine with glycine), 163 (glutamine instead of histidine) and 171 (serine was replaced with phenylalanine) were observed. Cultural, morphological, pathogenic and molecular characterization of *A. mali* associated with necrotic leaf spot of loquat was recorded first time in Pakistan.

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Introduction

The Rosaceae family has achieved significant economic value due to its fruit trees and important members of this family are quince (*Cydonia oblonga*), pears (*Pyrus communis*), loquat (*Eriobotrya japonica*) and apples (*Malus communis*) (Ascherson and Schweinfurth, 1887). Loquat is a small tree/shrub of flowering plant commonly cultivated in Japan, China, Florida and Gulf states. In world, loquat growing areas are situated in the latitudes20° north and35°south and Pakistan (latitudes 24°and 37°) falls in this range (Durgac *et al*., 2006). Punjab and Khyber Pakhtunkhwa are two famous loquat producing provinces of Pakistan (Hussain *et al*., 2009). Loquat production provides well economic return to farmer as there is no the fresh fruit available in the market during March/April to compete with it. Recently, a detail study of loquat genotype was provided (Hussain *et al*., 2011) but a very little information about fungal disease is available in the country (Khan, 2003).

Loquat is attacked by several fungal and bacterial pathogens which are reducing the quality and quantity of this emerging fruit. *Alternaria mali* was first described in 1924 in the United States by 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 and loquat producing regions where susceptible cultivars/strains of Delicious are grown (Filajdic and Sutton, 1991). *A. mali* has attained the status of economically important disease in many Asian countries including Japan and India (Jones and Aldwinckle, 1990; Sutton, 1991). Morphological and physiological characters are important aspects to determine the biology of pathogens but variability in sporulation, growth, spore size and shape and pathogenicity has been reported in genus *Alternaria* (Shahzad, 2003). 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.

Morphological identification is confusing and experts are required for proper identification. To overcome this problem, Polymerase Chain Reaction (PCR) assay and sequencing of Internal Transcribed Spacers (ITS1, 5.8S and ITS2)region has been developed (Baffi *et al*., 2012).In Pakistan, loquat is an emerging fruit and its area is increasing with the passage of time but its production is continually decreasing (GOP, 2013). Biotic and abiotic factors may involve reducing the production but no information about these factors is available in the country. No studies on pathogen variability have been conducted in Pakistan and because of the non-availability of host differentials; the present study was conducted with the objective to determine the geographical distribution and morpho-molecular identification of *A. mali* infecting loquat in Pakistan.

Material and methods

Geographical distribution

During the year 2014-15,necrotic leaf spots were collected from34 (5 from Taxila, 5 from Wahcantt, 3 from Khanpur, 5 from Kalar Kahar, 5 from Choa Saiden Shah, 3 from Tret, 3 from Chatar and 5 from Murree) loquat orchards (Fig 1) and prevalence and incidence were determined with the help of formula.

$$\text{Prevalence \%} = \frac{\text{Location exibiting } A.\,mali}{\text{Total locations examined}} \times 100$$

$$\text{Incidence \%} = \frac{\text{Number of infected plants}}{\text{Total number of plants examined}} \times 100$$

Morphological Identification

Sterile blades were used to cut small bits of disease leaf area and bits were sterilized with 10% chlorox. Sterile distilled water was used to remove the traces of chlorox and bits were dried on three layers of sterilized filter papers. The bits were shifted on potato dextrose agar (PDA) and incubated for 7 days at 25°C.Theisolates were purified with single spore suspension method, transferred to PDA plates and morphological characteristics such as color and type of colonies, septation and size of conidia and conidiophore were recorded after 10 days.

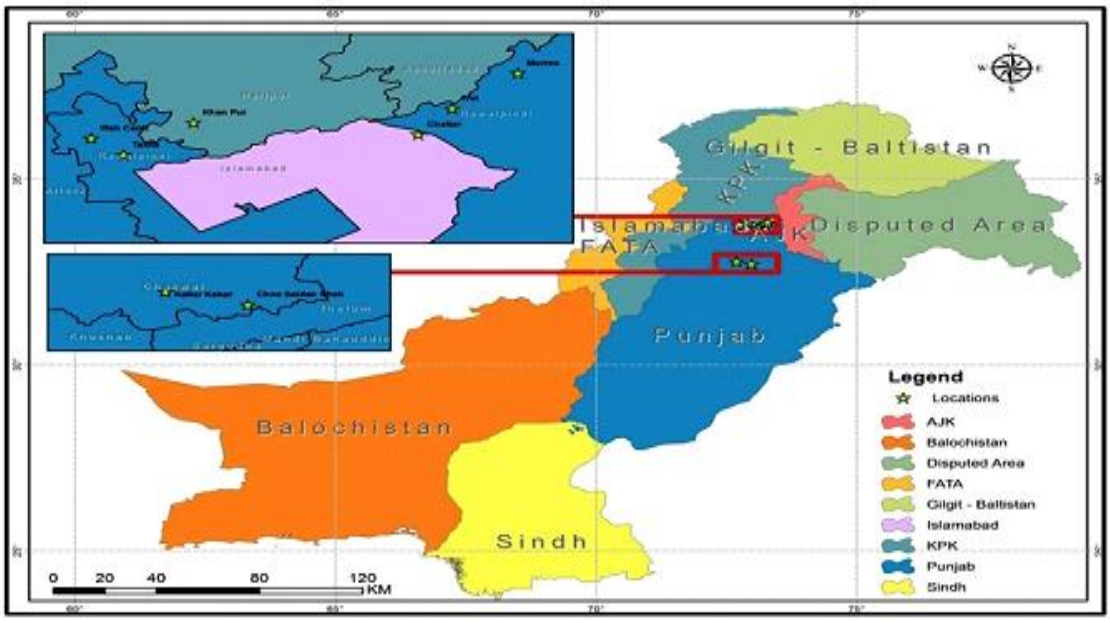

Fig. 1. Geographical distribution of *A. mali* in Loquat orchards of Pakistan.

Pathogenicity tests

For pathogenicity tests, spore suspension (4×10^5spores/ml) was prepared with Haemocyto meter and sterilized healthy detached leaves of loquat were inoculated with suspension. The control leaves were inoculated with sterile distilled water. Sterile petriplates were lined with a wet blotter paper and inoculated leaves were transferred to such petriplates. Petriplates were incubated at 25°C.Re-isolation of pathogen was carried out and compared with original inoculum to satisfy Koch's postulates(Johnston and Booth, 1983).

Molecular characterization

Total genomic DNA of highly virulent 4 isolates was extracted by phenol extraction method (Raeder and Broda, 1985). Molecular diversity was studied through PCR technique and rDNA (ITS1, 5.8S and ITS2) region was amplified with universal ITS1 and ITS4 primers (White *et al.*, 1990). The PCR assay was carried out in 50µl reaction containing Ix PCR buffer, 0.2 mM ofdNTPs, 5unit of *Taq* DNA *polymerase*, 20 ng of DNA template, 10pmols of each primer and 1.5 mM of $MgCl_2$. Amplifications were performed at initial denaturation (94°C for 5 min) followed by 25 cycles of denature (94°C), annealing (59°C) and extension (72°C) for 2 minand a final extension was carried out at 72°C for 5 min. 2% (w/v) agarose gel was used to visualize PCR products.

Sequencing and Sequence Submission

The PCR products were further purified with standard protocol of Gene JET PCR purification kit (Thermo Fisher Scientific) andpurified products were sequenced form Macrogen Korea. The sequences were manipulated with Bio-Edit and final sequences were submitted in the GenBankpublic database.The available ITS sequences of four *Alternaria* species from six different countrieswere downloaded from the public Gen-Bank database (Table 3).Minimum evolution method and maximum composite likelihood method were usedto compute evolutionary distances with the help of Molecular Evolutionary for Genetic Analysis (MEGA) version 6(Saitou and Nei, 1987; Tamura *et al.*, 2004).

Result

Geographical distribution

Necrotic leaf spots were observed in all 34 loquat orchards and prevalence was 100%. Maximum 46.16% disease incidence was recorded in Khan Pur (orchard 3) followed by 44.5% in Tret (orchard 3), 33.5% in Taxila (orchard 1),33.4% in Chatar (orchard 1), 33.34% in Choa Saiden Shah (orchard 1), 29.42% in Wahcant (orchard 3),30.8% in Kalar Kahar (orchard 1) and25% in Murree (orchard 3) (Fig 2).

Morphological identification

Total number of 48 isolates of *A. mali* were obtained from eight locations. Light to dark olivacious with greenish or brownish tinge colonies had velvety or cottony mycelial growth with regular to irregular margins (Table 1).

Table 1. Culture characteristic of *Alternaria* isolates obtained from loquat growing areas of Pakistan.

Sl. No		Colony			Color on the underside of plant
	Isolate	Type	Color	Margin	
1	ALT_1TX_2	Cottony	Lightly Olivaceous green	Regular brownish with white rim	Brown with light grey margin
2	$ALT_{20}TX_2$	Velvety, appressed	Olivaceous	Dark Olivaceous green	Light grey with brown center
3	ALT_2TX_3	Velvety, appressed	Dark Olivaceous green	Regular brownish with white rim	Smoky grey
4	ALT_7TX_3	Velvety, appressed	Olivaceous green	Regular brownish with white rim	Light brown with light grey margin and dark brown center
5	ALT_9TX_3	Velvety, appressed	Greenish with grayish surface	Regular, appressed, green with white rim	Light grey with dark grey center
6	$ALT_{20}TX_3$	Velvety, appressed	Olivaceous	Brownish margin	Smoky grey
7	ALT_4WT_1	Cottony	Dark Olivaceous with dark center	Slightly irregular Olivaceous green	Brown with light grey margin
8	ALT_7WT_1	Velvety, appressed	Greenish with grayish surface	Appressed and green with white rim	Light to dark grey
9	ALT_1WT_2	Velvety, appressed, cottony center	Dark Olivaceous green	Irregular light Olivaceous green with white rim	Dark grey with light grey margin
10	ALT_4WT_2	Cottony	Lightly Olivaceous green	Regular brownish with white rim	Brown with light grey margin
11	$ALT_{12}WT_2$	Velvety, cottony center growth	Olivaceous green	Slightly irregular Olivaceous green	Smoky grey
12	$ALT_{11}KP_3$	Velvety, appressed	Greenish with grayish surface	Regular, appressed, green with white rim	Light grey with dark grey center
13	$ALT_{13}KP_3$	Velvety, appressed	Olivaceous	Brownish margin	Smoky grey
14	$ALT_{14}KP_3$	Cottony	Dark Olivaceous with dark center	Slightly irregular Olivaceous green	Brown with light grey margin
15	$ALT_{19}KP_3$	Cottony	Dark Olivaceous green	Brownish margin	Smoky grey
16	$ALT_{20}KP_3$	Cottony	Lightly Olivaceous green	Regular brownish with white rim	Brown with light grey margin
17	ALT_1TR_1	Velvety, cottony center growth	Olivaceous green	Slightly irregular Olivaceous green	Smoky grey
18	ALT_3TR_3	Velvety, cottony center growth	Olivaceous green	Slightly irregular Olivaceous green	Smoky grey
19	ALT_6TR_3	Velvety, appressed	Dark Olivaceous green	Regular brownish with white rim	Smoky grey
20	$ALT_{13}TR_3$	Velvety, appressed	Olivaceous green	Regular brownish with white rim	Light brown with light grey margin and dark brown center
21	ALT_3CR_1	Velvety, appressed	Greenish with grayish surface	Regular, appressed, green with white rim	Light grey with dark grey center
22	ALT_9CR_1	Velvety, appressed	Olivaceous	Brownish margin	Smoky grey
23	$ALT_{10}CR_1$	Cottony	Dark Olivaceous with dark center	Slightly irregular Olivaceous green	Brown with light grey margin
24	ALT_9CR_2	Velvety, appressed	Greenish with grayish surface	Appressed and green with white rim	Light to dark grey
25	$ALT_{12}CR_2$	Velvety, appressed	Dark Olivaceous green	Irregular light Olivaceous green with white rim	Dark grey with light grey margin

26	$ALT_{14}MR_3$	Cottony	Olivaceous	Irregular dirty white margin	Smoky grey
25	$ALT_{15}MR_3$	Velvety, appressed	Olivaceous	Dark Olivaceous green	Light grey with brown center
28	$ALT_{16}MR_3$	Velvety, appressed	Dark Olivaceous green	Regular brownish with white rim	Smoky grey
29	ALT_6MR_4	Cottony	Dark Olivaceous with dark center	Slightly irregular Olivaceous green	Brown with light grey margin
30	$ALT_{19}MR_4$	Cottony, slightly furrow with appressed center	Greenish with grayish surface	Appressed and green with white rim	Light to dark grey
31	$ALT_{18}KK_4$	Velvety, appressed	Greenish with grayish surface	Appressed and green with white rim	Light to dark grey
32	$ALT_{20}KK_4$	Velvety, appressed cottony center	Dark Olivaceous green	Irregular light Olivaceous green with white rim	Dark grey with light grey margin
33	$ALT_{14}KK_5$	Velvety, appressed cottony center	Dark Olivaceous green	Irregular light Olivaceous green with white rim	Dark grey with light grey margin
34	ALT_4CS_1	Velvety, appressed	Greenish with grayish surface	Appressed and green with white rim	Light to dark grey
35	$ALT_{17}CS_4$	Cottony	Lightly Olivaceous green	Regular brownish with white rim	Brown with light grey margin
46	ALT_4CS_5	Cottony	Olivaceous	Irregular dirty white margin	Smoky grey
47	ALT_7CS_5				
48	$ALT_{12}CS_5$	Velvety, appressed cottony center	Dark Olivaceous green	Irregular light Olivaceous green with white rim	Dark grey with light grey margin

Yellow, brown, black, greenish black and brownish black pigmentation was observed in *Alternaria* isolates. Septate to aseptate conidiophores were exhibiting maximum length of 68.5 µmin isolate $ALT_{12}CR_2$ and minimum 24.60 µm in isolate ALT_9TX_3. Least breadth 2.0 µm was recorded in isolateALT_1WT_2 and maximum 5.6 µm in isolate ALT_1TR_1.9 was the highest septation in isolate ALT_7WT_1 (Table 2) and conidia were exhibiting significant differences in transverse (1-6) and longitudinal (0-4) septation. Maximum 39.8 µm conidial length was observed in isolate ALT_2TX_3while isolate ALT_1TR_1 was exhibiting minimum 9.4 µm. 4 isolates ($ALT_{12}WT_2$, $ALT_{14}KP_3$, $ALT_{12}CR_2$ and $ALT_{14}KK_5$) were exhibiting highly pathogenic response in detached leaves assay.

Sequencing analysis

Final sequences of $ALT_{12}WT_2$, $ALT_{14}KP_3$, $ALT_{12}CR_2$ and $ALT_{14}KK_5$ were submitted in the Gen-Bank public database under the accession numbers KR232489 (PAK1), KR232490 (PAK2), KT154010 (PAK24) and KT154011 (PAK25) respectively. For the reliable confirmation at species level, sequences of *A. mali*, A. *alternata*, A. *zinnae* and A. *tenuissima* reported from USA, Sweden, UK, Newzeland, Pakistan and India were compared with submitted isolates (Table 3).

Evolutionary methods computed the evolutionary tree of alternaria species which clearly indicates four different sub-trees (Fig 3).

The sub-trees were names as *Alternaria mali*, *Alternaria alternata*, *Alternaria tenuissima* and *Alternaria zinnae* because each sub-tree was comprised of same specie. All submitted isolates were recorded in *Alternaria mali* sub-tree with previously reported *A. mali* isolates from USA and Pakistan. Amino acid sequences of PAK1, PAK2, PAK24 and PAK25 were exhibiting maximum 99-100% genetic homology with FCBP1343 isolate. FCBP1343 was used as a reference to compared with amino acid sequences of PAK1, PAK2, PAK24 and PAK25. PAK1 reveals 100% genetic homology with reference isolate because no difference was observed at amino acid composition. Two changes at position number 18 (valine was replaced with glutamate) and 171 (serine was replaced with phenylalanine) were recorded in PAK2 while PAK25 was different at position 57 (glutamine with leucine) and 84 (alanine with glycine).

Table 2. Variability in conidia of *Alternaria* isolates of loquat.

Sl.	Isolate	Conidia					Conidiophore			Pathogenicity Test
		Length (µm)	Breadth (µm)	Septation (No)	Transvers septa (No.)	Longitudinal Septa (No)	Length (µm)	Breadth (µm)	Septation (No)	
1	ALT_1TX_2	11.1-26.8	7.5-12.3	0-7	1-4	0-3	26.4-45.3	3.5-4.5	0-7	+
2	$ALT_{20}TX_2$	11.2-25.2	6.2-14.2	1-7	1-5	0-3	27.5-44.3	3.4-4.3	0-5	+
3	ALT_2TX_3	19.4-39.8	9.9-20.8	1-9	1-6	0-4	28.6-45.5	3.6-4.7	0-6	++
4	ALT_7TX_3	09.7-22.4	5.4-14.2	0-6	1-5	0-3	26.7-47.6	2.8-4.5	0-9	+
5	ALT_9TX_3	18.7-22.4	9.0-20.9	1-4	1-5	0-3	24.6-45.7	3.6-5.0	0-8	+
6	$ALT_{20}TX_3$	12.4-26.4	6.0-15.2	1-7	1-4	0-3	35.5-53.6	4.3-4.3	0-5	++
7	ALT_4WT_1	17.1-31-3	9.1-14.6	0-6	1-4	0-3	27.6-44.4	3.5-4.5	0-6	++
8	ALT_7WT_1	14.4-24.5	9.4-14.3	1-5	1-4	0-3	29.5-46.5	3.8-4.7	2-9	+
9	ALT_1WT_2	14.1-23.3	5.1-14.3	1-6	1-5	0-3	28.7-47.7	2.0-4.9	0-5	++
10	ALT_4WT_2	15.2-24.4	7.2-13.4	1-7	1-4	0-3	27.8-49.8	2.8-3.6	0-4	+
11	$ALT_{12}WT_2$	16.3-25.3	5.3-14.5	1-5	1-5	0-4	33.9-50.0	3.6-4.3	0-6	+++
12	$ALT_{11}KP_3$	16.6-25.7	6.6-16.6	1-5	1-5	0-3	27.8-48.8	2.4-3.5	0-7	+
13	$ALT_{13}KP_3$	09.5-24.8	5.5-14.3	1-5	1-5	0-3	32.6-56.7	3.7-4.8	0-6	+
14	$ALT_{14}KP_3$	15.4-23.9	7.4-14.6	1-6	1-5	0-3	38.5-54.5	3.9-3.5	0-4	+++
15	$ALT_{19}KP_3$	13.7-24.8	5.5-15.7	0-7	1-5	0-3	29.4-43.4	2.6-3.2	0-8	++
16	$ALT_{20}KP_3$	14.6-25.7	6.6-16.8	1-8	1-4	0-4	39.3-55.5	3.9-4.4	1-6	+
17	ALT_1TR_1	09.4-26.6	8.7-17.7	1-9	1-4	0-3	28.3-57.6	2.2-4.8	1-8	++
18	ALT_3TR_3	16.5-25.4	5.3-15.7	1-9	1-5	0-3	44.4-64.8	4.2-5.6	0-6	+
19	ALT_6TR_3	15.6-24.5	6.5-16.8	1-8	1-5	0-3	49.5-68.3	4.7-5.5	0-5	+
20	$ALT_{13}TR_3$	14.7-23.6	7.7-17.6	1-9	1-4	0-3	33.6-55.0	3.5-4.1	0-6	++
21	ALT_3CR_1	13.8-24.7	6.8-16.4	1-8	1-5	0-4	27.7-43.4	3.7-4.4	0-4	++
22	ALT_9CR_1	09.9-25.6	5.9-15.3	1-8	1-4	0-3	33.4-54.5	2.9-4.6	0-9	+
23	$ALT_{10}CR_1$	18.8-26.4	6.6-15.3	1-7	1-5	0-4	27.8-46.6	3.6-4.9	0-4	++
24	ALT_9CR_2	15.5-29.7	7.4-16.3	1-8	1-5	0-4	34.6-58.8	2.4-4.7	0-6	+
25	$ALT_{12}CR_2$	13.4-28.6	8.5-15.4	1-9	1-4	0-4	44.4-68.5	4.2-4.4	0-7	+++
26	$ALT_{14}MR_3$	17.3-29.6	8.5-12.6	1-8	1-4	0-3	33.5-57.3	3.5-4.2	0-4	+
25	$ALT_{15}MR_3$	15.2-28.7	7.6-13.4	1-7	1-5	0-3	45.7-66.5	4.1-5.4	0-5	++
28	$ALT_{16}MR_3$	14.3-27.6	6.7-14.3	1-8	1-5	0-4	47.8-68.2	4.6-5.5	0-7	++
29	ALT_6MR_4	13.4-27.5	7.8-15.5	1-9	1-4	0-3	29.9-34.8	2.9-4.7	0-6	+
30	$ALT_{19}MR_4$	12.5-26.4	8.9-16.3	1-8	1-4	0-4	34.7-55.4	3.7-4.4	0-5	+
31	$ALT_{18}KK_4$	13.7-27.3	6.6-14.6	1-7	1-4	0-3	27.5-37.6	2.4-4.2	0-8	++
32	$ALT_{20}KK_4$	14.5-27.5	5.5-15.7	1-8	1-4	0-3	38.2-48.8	3.6-4.4	0-6	+
33	$ALT_{14}KK_5$	15.4-26.6	6.4-14.5	1-7	1-5	0-3	32.3-59.9	3.9-4.6	0-4	+++
34	ALT_4CS_1	16.5-26.7	5.3-13.4	1-6	1-5	0-4	43.4-63.5	4.3-5.1	0-5	+
35	$ALT_{17}CS_4$	12.7-20.6	5.8-15.6	0-7	1-4	0-4	35.5-54.3	3.5-4.8	0-7	++
46	ALT_4CS_5	13.8-29.6	6.9-14.7	1-8	1-5	0-3	28.6-46.5	2.8-3.5	0-6	+
47	ALT_7CS_5	14.9-28.7	5.8-15.8	1-9	1-4	0-4	33.7-57.7	3.0-4.3	0-5	+
48	$ALT_{12}CS_5$	15.8-26.8	6.7-14.6	1-8	1-4	0-4	29.3-34.9	2.6-3.5	0-7	++

+ = low pathogenic ++ = moderate pathogenic, +++ highly pathogenic.

Maximum 3 changes at position number 18 (glutamine replaced with valine), 163 (glutamine instead of histidine) and 171 (serine replaced with phenylalanine) were observed in PAK24 (Table 4).

Discussion

During the year 1965, Tanaka variety of loquat was introduced in Pakistan and loquat is flourishing in well-drained irrigated areas of the country where the temperature ranges 38 to 25°C in summer and 20 to 3°C in winter(Khan, 2003). Clay loam and sandy loam soils (pH 5 to 8.5) were recorded in these areas (Khan, 2003).

The loquat is not pruned in the country and it is affected by different fungal and bacterial diseases. Previously, none of the government research stations have ever undertaken serious research on loquat diseases to find out the cause of low yield (Khan, 2003).

The current study was conducted to find out the geographical distribution of necrotic leaf spot of loquat in Pakistan. *Alternaria* ranked 10thasleaf, fruit and stem pathogen of more than 4000 plant belonging to ornamental, fruits, cereals crops and vegetables (Farr *et al.*, 1989).

The prevalence was recorded as 100% because not even a single orchard was free from this disease. Disease incidence was 25 to 46.16% in 34 loquat orchards.

The highest disease incidence (46.16%) in Khan purmight be attributed to the source of irrigation i.e. sewerage water and poor sanitary conditions.

Table 3. Available sequences of *Alternaria* species in public data base of NCBI.

Pathogen	Isolate Name	Accession Number	Country	Host	Sources/Reference
A. mali	FCBP1343	KP861906	Pakistan	*Malusdomestica*	http://www.ncbi.nlm.nih.gov
A. mali	RGR 98.0382	HQ238270	USA	*M. domestica*	http://www.ncbi.nlm.nih.gov
A. mali	PAK1	KR232489	Pakistan	*Eriobotry japonica*	http://www.ncbi.nlm.nih.gov
A. mali	PAK2	KR232490	Pakistan	*E. japonica*	http://www.ncbi.nlm.nih.gov
A. mali	PAK24	KT154010	Pakistan	*E. japonica*	http://www.ncbi.nlm.nih.gov
A. mali	PAK25	KT154011	Pakistan	*E. japonica*	http://www.ncbi.nlm.nih.gov
A alternata	Olrim874	AY354228	Sweden	*Betula pendula*	http://www.ncbi.nlm.nih.gov
A alternata	8	HQ846574	USA	*Panicum virgatum*	http://www.ncbi.nlm.nih.gov
A. zinnae	CBS 300.79	KJ718269	UK	*Zinnia elegans*	http://www.ncbi.nlm.nih.gov
A. zinnae	CBS 117223	KJ718270	Newzeland	*Z. elegans*	http://www.ncbi.nlm.nih.gov
A. tenuissima	CHIT-20	KF193437	India	*Sesamum indicum*	http://www.ncbi.nlm.nih.gov
A. tenuissima	CHIT-4	KF193497	India	*S. indicum*	http://www.ncbi.nlm.nih.gov

The infected plant debris viz. fallen leaves, rotten fruits and dead twigs may perhaps the source of inoculum multiplication in all loquat orchards located in the area.Secondly, no literature about fungal disease of loquat is available in the country and farmer is unaware about the proper sanitary measures.

Table 4. Amino acid comparison of PAK1, PAK2, PAK24 and PAK25 with reference isolates of *A. mali* (FCBP1343).

Amino acid positions	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A.mali-(FCBP1343)	S	V	G	E	P	A	E	G	S	L	H	K	Y	E	G	G	L	V	P	L	G	V	T	A	L	L	N	Y	S	P
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amino acid positions	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
A.mali(FCBP1343)	L	S	F	A	Y	F	L	F	P	W	W	V	R	P	P	L	G	Q	T	*	T	F	C	N	C	N	Q	R	Q	*
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK25)	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.	.
Amino acid positions	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
A.mali-(FCBP1343)	Q	I	N	N	Y	N	F	Q	Q	R	I	S	W	F	W	H	R	*	R	T	Q	R	N	A	I	S	S	V	N	C
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.
Amino acid positions	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
A.mali-(FCBP1343)	R	I	Q	*	I	I	E	S	L	N	A	H	C	A	L	W	Y	S	K	G	H	A	C	S	S	V	I	C	T	L
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amino acid positions	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	
A.mali-(FCBP1343)	K	L	C	L	V	L	G	V	L	S	L	A	L	L	E	T	R	L	K	V	I	G	S	R	P	T	G	F	G	A	
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amino acid positions	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	
A.mali-(FCBP1343)	Q	H	K	S	H	S	L	S	A	K	V	*	H	P	L	S	L	F	F	N	F	*	P	R	I	R	*	G	Y	P	
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Amino acid positions	181	182	183	184	185	186	187	188	189	190	191																				
A.mali-(FCBP1343)	L	N	L	S	I	S	I	K	R	R	X																				
A.mali-(PAK1)	.	.	.	.	.	.	.	.	.	.	.																				
A.mali-(PAK2)	.	.	.	.	.	.	.	.	.	.	.																				
A.mali-(PAK24)	.	.	.	.	.	.	.	.	.	.	.																				
A.mali-(PAK25)	.	.	.	.	.	.	.	.	.	.	.																				

The lowest disease incidence (26%) was however, recorded in Murree due to proper sanitary conditions. *Alternaria* has been reported as post-harvest pathogen on various fruits (loquat, apple, tomatoes, blueberries, oranges and lemons) which produce more than 30 different mycotoxins (Stidnson *et al*., 1981; Serdani *et al*., 2002).

More than 40 percent incidence of *Alternaria* on loquat has been reported from Iran (Mirhosseini and Babaeizad, 2015) and it has also been reported as major pathogen of loquat in Florida, Japan, Mexico, Taiwan, Venezuela, Palestine and Greece (Batta, 2005; Ko *et al*., 2010; Tziros, 2013).

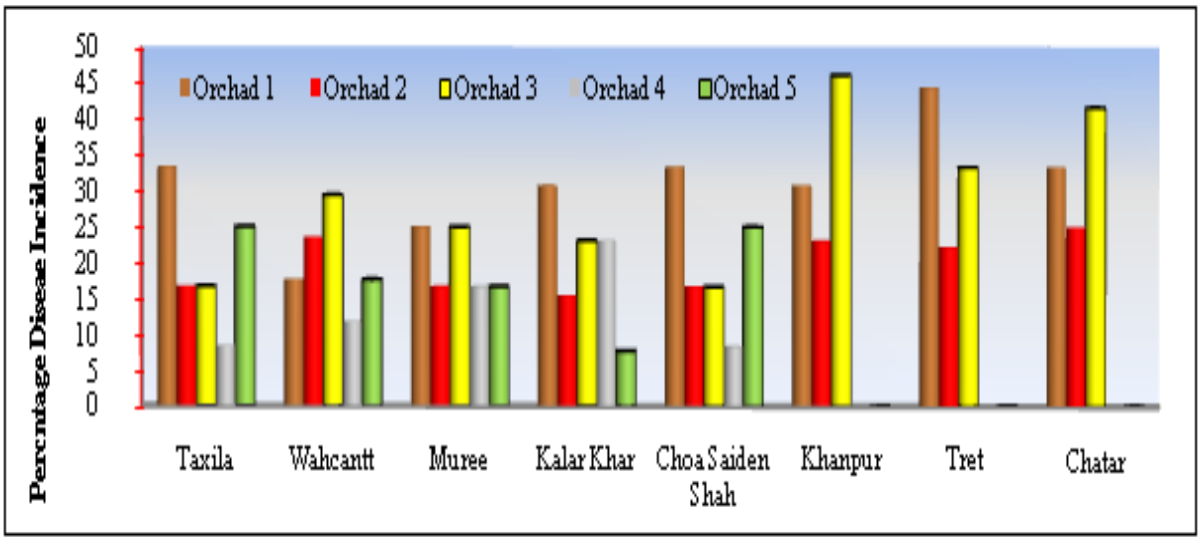

Fig. 2. Percentage disease incidence of *A. mali* in loquat orchards of Pakistan.

However, the isolates were exhibiting light to dark olivacious with greenish or brownish tinge colonies with regular to irregular margins (Table 1) and these are typical colonies characters of *A. mali*. In this study variation in morphological characterization was recorded due to different geographical distribution of obtained isolates.

Previously morphological characterization was used to confirm *A. mali*and morphological identification of 48 isolates were closely resembled to those described byRoberts, 1924; Rotem, 1966; Ramegowda and Naik, 2008; Pusz, 2009; Hubballi *et al*., 2011.

Detach leaf assay technique is more reliable for *Alternaria* species to confirm its pathogenicity (Rotem, 1994; Kumar, 2004; Quayyum *et al.*, 2005; Ozgonen and Karaca, 2006; Soleimani and Esmailzadeh, 2007) and $ALT_{12}WT_2$, $ALT_{14}KP_3$, $ALT_{12}CR_2$ and $ALT_{14}KK_5$ were found to be highly virulent.

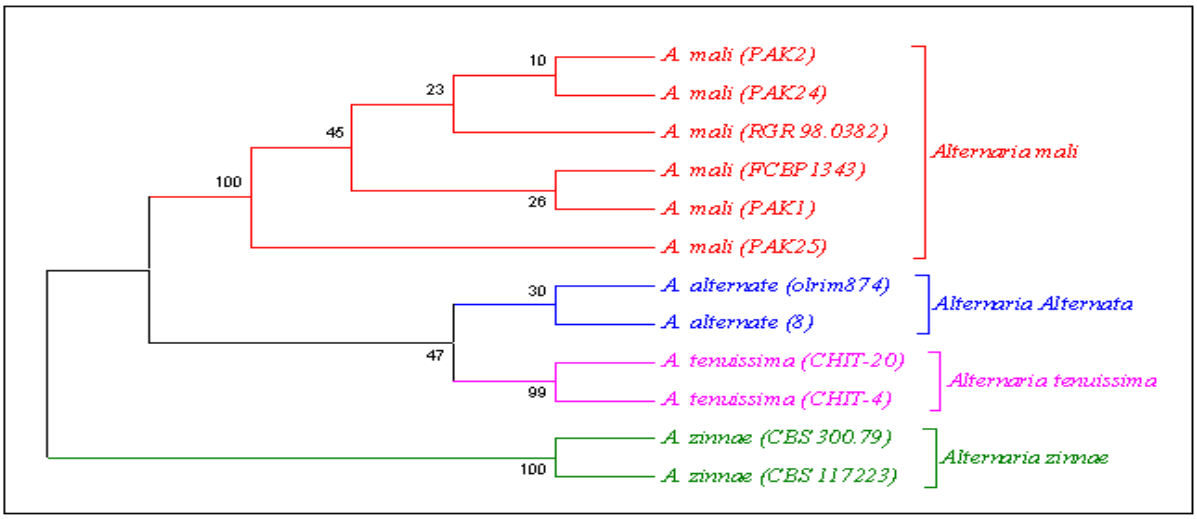

Fig. 3. Evolutionary tree of *Alternaria* species with four sub-trees.

The ITS sequence of each species shared genetic diversity in amino acid sequences of ITS regions and it is one of the main reasons that there are four different sub-trees. Due to maximum genetic homology within the species, each sub-tree comprised of different isolates reported form different countries (Fig. 3). PAK1, PAK2, PAK24 and PAK25 were observed in *Alternaria mali* sub-tree because they are exhibiting maximum 99 to 100% genetic homology with previously reported *A. mali* isolates. The percentage genetic homology of PAK1 was 100% similar while two changes were observed in PAK2 and PAK25 and three differences in amino acid sequences of PAK24 were also recorded.

The variations in amino acids sequences may be attributed to the diverse geographical distribution of these isolates. In the present study, the confirmation of *Alternaria mali* was carried out through morpho-molecular characterization and pathogenicity tests. These tools were also employed for the confirmation of *A. mali* in Yugoslavia, Quebec, Manitoba, North Carolina, Indiana, Florida, Iran and Turkey (Jones and Aldwinckle, 1990; Bulajic *et al.*, 1996; Ozgonen and Karaca, 2006; Soleimani and Esmailzadeh, 2007).

Conclusion

Morpho-molecular identification and pathogenicity tests are reliable tools for the confirmation of *A. mali* infecting loquat in Pakistan.

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