Mycopath (2018) 16(2): 81-85 **Research Article**

First evidence of Mucor rot infecting loquat (*Eriobotrya japonica* L.) in Pakistan

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Abstract

Loquat is originated from China and it has spread to Pakistan. It is achieving significant value in the country. Present research work is a part of the first ever study on Mucor rot infecting loquat in Pakistan. Prevalence of Mucor rot was 100% in all surveyed locations and disease incidence was recorded as 34%, 40%, 43%, 45%, 49%, 51%, 53% and 56% in Choa Saiden Shah, Kalar Kahar, Murree, Chattar, Tret, Khan pur, Wahcant and Taxila respectively. Morphological characters such as color, texture and spore formation confirmed *Mucor fragilis* as the causal organism of loquat rot. For authentic identification, polymerase chain reaction (PCR) assay was conducted and nucleotide sequences of rDNA region (ITS1, 5.8S and ITS2) of Pakistani isolates (KT312816 and KT312817) were compared with available nucleotide sequences of six phyto-pathogenic Mucor species reported from nine different countries. Both isolates were exhibiting 99.98% genetic homology with *M. fragilis*. Morpho-molecular identification and pathogenicity tests are reliable tools for the confirmation of Mucor rot infection loquat in Pakistan and this information will be helpful for proper management of this disease.

Keyword: Incidence, Loquat, Molecular identification, Mucor rot, Pakistan.

Introduction

Loquat (*Eriobotrya japonica* L.) is originated from China and it is also cultivated in Australia, Brazil, India, Japan, Madagascar, Mauritius Island, Mediterranean countries, Pakistan and United States. Loquat fruits contain sugars, minerals (calcium and phosphorus) with high medicinal value (Wee and Keng, 1992). Seed extract of loquat is very effective for the treatment of liver disorders. In Pakistan, it is an emerging fruit with production of 16,479 tonnes and 98% production achieved from Punjab and Khyber Paktoon Khawa (GOP, 2013). Loquat is well adopted between the latitudes 20° and 45° North. Choa Saiden Shah,Chhattar, Haripur, Hasan Abdal, Kalar Kahar, Kasur, Mardan, Muree, Sargodha, Tret and Wahcant are the famous loquat growing areas of Pakistan (Hussain *et al.*, 2011). Loquat production gives good economic return to farmer. In Pakistan, loquat is propagated through seeds and no standard cultivar is available to the growers (Hussain *et al.*, 2009). Morpho-molecular identification was used for the confirmation of *Diplodia seriata* (Abbas *et al.*, 2018), *Fusarium solani* (Abbas *et al.*, 2017), *Colletotrichum gloeosporioides* (Naz *et al.*, 2017), *Curvularia lunata* (Abbas *et al.*, 2016) and *Alternaria mali* (Abbas *et al*., 2016a) infecting loquat in Pakistan. There are no previous studies on Mucor rot of loquat from Pakistan and present research work is a part of the first ever detailed study on Mucor rot of loquat. Previously fungi were identified through physiological and morphological parameters but these parameters required expertise as they are confusing. Nucleotide sequence analysis of Internal Transcribed Spacers (ITS1, 5.8S and ITS2) were used for identification of associated fungus (Qin *et al.*, 2010; Baffi *et al.*, 2012). It is fast, cost effective and reliable for rapid identification of pathogens at species level. The objectives of the current study were to determine prevalence and incidence of Mucor rot infecting loquat in Pakistan and reliable confirmation by the sequence analysis.

Materials and Methods

During harvesting time, fresh loquat fruit were randomly picked from Taxila, Wahcantt, Khanpur, Kalar Kahar, Choa Saiden Shah, Tret, Chatar and Muree (Fig. 1) and prevalence and incidence of Mucor rot was determined with the help of formula.

$$\text{Prevalence}\,(\%) = \frac{\text{Location exibiting loquat rot}}{\text{Total locations examined}} \times 100$$

$$\text{Incidence}\,(\%) = \frac{\text{Number of infected fruits}}{\text{Total number of fruits examined}} \times 100$$

The infected along with some healthy segments (about 3mm) of loquat fruit was surface sterilized with 0.1% sodium hypochlorite. The traces of

sodium hypochlorite were eliminated by dipping the segments into sterile distilled water for 1 minute. The segments were further shifted to double layer of sterile filter paper. The dry segments were transferred on Czapek Dox Agar medium and incubated at 25±2 °C for seven days. The cultural and microscopic characteristics were used for morphological identification of the pathogen. Healthy loquat fruit were used for pathogenicity tests and compared with un-inoculated fruit (Fig 2B). DNA was extracted with standard protocol of phenol extraction methods as described by Raeder, 1985 and agarose gel was used to visualize DNA on UV transluminator. PCR assay was used to amplify rDNA region. A 50µL PCR reaction was comprised of 10ng of genomic DNA as template, 0.5 mM of universal ITS1 and ITS4 primers, 1U *Taq DNA polymerase*, 2mM $MgCl_2$, 0.1 mM of DNTPs and 1X reaction buffer. PCR reaction was initially denature for 3 min at 95 °C followed by 35 threshold cycles of denature (95 °C) annealing (58 °C) and extension (72°C) for 1 min. each. Final extension was conducted at 72 °C for 7 min. and amplification was confirmed on agarose gels. Un-incorporated nucleotides and primers were eliminated with GeneJET PCR purification kit and sequenced from Macrogen Korea. Obtained sequences were evaluated with BioEdit and compared with previously submitted sequences in public databases using the BLAST program for species identification. Two sequences of rDNA regions were submitted in the GenBank under accession numbers KT312816 and KT312817. Molecular Evolutionary for Genetic Analysis (MEGA) version 6 (Tamura *et al.*, 2013) was used to compute evolutionary history using the Neighbor-Joining method and Maximum Composite Likelihood method was used for evolutionary distances.

Results and Discussion

Mucor rot was observed at the calyx end of loquat fruits (Fig. 2A), infected become juicy and entire fruit rot was recorded after 9 days of artificial inoculation. Prevalence of Mucor rot was 100% in all surveyed locations and disease incidence was recorded as 34%, 40%, 43%, 45%, 49%, 51%, 53% and 56% in Choa Saiden Shah, Kalar Kahar, Murree, Chattar, Tret, Khanpur, Wahcantt and Taxila respectively. Mucor rots have been reported to cause serious decay of pear, strawberries, apple, peach, plum, tomato and potato (Michailides and Spotts, 1990; Li *et al.*, 2014). In Spain, a comprehensive survey of guava fruit was conducted and incidence of *M. fragilis* was reported from rotted guava fruit. Morphological characterization were used for the confirmation of *M. fragilis* (Baffi *et al.*, 2012). In Pakistan, Mucor rot is an important disease of guava (Panhwar, 2005) and citrus (Akhtar *et al.*, 2013). The rot was recorded on artificially inoculated loquat fruit while no symptoms were observed on healthy fruit. For the confirmation of Koch postulates, the pathogen was re-isolated from the artificially inoculated fruit and pathogen was cultured on same nutritional artificial media. The morphological characterizations were similar with the mother culture. A total number of 38 isolates were morphologically identified. At first, white fungal colonies were observed and later turned into grayish. Erect sporangiophores (8.2 to 12.9 µm), brownish black sporangia (14.90 to 89.75 µm), globose to oval columellae (9.87 to 56.46 µm × 9.25 to 51.42 µm) and elliptical to oval sporangiospores (4.33 to 9.87 × 2.19 to 6.58 µm) were observed after 7 days (Fig. 2C). Previously, morphological characterization was used to identification of the pathogen and morphological characteristics of the isolated fungus were similar with finding of Bainier (1884). To verify pathogen identification, molecular tools were used as reliable methods for the identification of pathogens at species level (White *et al.*, 1990) and ITS region is the most common target used for molecular identification of fungi (Sette *et al.*, 2006; Kaushik and Thakur, 2009). 650 bp ITS fragment were amplified through PCR assay. The sequences of FHM-2 and FHM-3 were submitted at National Center for Biotechnology Information (NCBI) under GenBank accession number KT312816 and KT312817 respectively. The nucleotide sequence of both isolates (FHM-2 and FHM-3) were comprised of higher composition of adenine (31%) followed by thymine or uracil (30%), guanine (20%) and cytosine (19%). Average amino acid composition of alanine, cysteine, aspartic acid, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, asparagine, proline, glutamine, arginine, serine, threonine, valine, tryptophan and tyrosine was computed as 2.54, 2.54, 3.56, 4.07, 5.60, 6.11, 2.04, 7.89, 5.60, 11.96, 1.02, 6.11, 4.58, 3.56, 5.34, 9.67, 7.12, 6.36, 1.02 and 3.31, respectively. Available nucleotide sequences of phyto-pathogenic Mucor species such as *M. racemosus*, *M. indicus*, *M. irregularis*, *M. hiemalis*, *M. plumbeus*and *M. fragilis* reported from China, Indonesia, Kenya, Mexico, Poland, Spain, Switzerland, USA and Yunnan were compared with KT312816 and KT312817 (Table 1). Evolutionary tree clearly indicate six different clusters and each species from different countries shared the same cluster (Fig. 2E). Each species is reported from different countries but they are in same cluster due to high genetic homology between rDNA regions. The range of genetic homology of Pakistani isolates was 99.98–99.92%, 97.88–97%. 82%, 97.72–96.36%, 96.97–96.94%, 96.21–96.12% and 95.85–95.73% with *M. fragilis*, *M. indicus*, *M. hiemalis*, *M. plumbeus*, *M. irregularis* and *M. racemosus* clusters, respectively. The rDNA region is not effective for all groups of fungi but a good resolution to the species level may be obtained for Zygomycetes (Balajee *et al.*, 2009). Pakistani

isolates have maximum genetic homology with previously reported *M. fragilis* and it is one of the main reasons that Pakistani isolates shared the same cluster with *M. fragilis* isolates reported from Kenya and Spain. Both isolates (FHM-2 and FHM-3) shared 99.98% genetic homology with isolate FJ499452 of *M. fragilis* and this isolate is the causal organism of guava fruit rot in Spain (Baffi *et al.*, 2012). Recently, 99% homology in nucleotide sequences of rDNA region was used to report *M. fragilis* causing fruit rot (Baffi *et al.*, 2012). For further confirmation, nucleotide evidences of both isolates were compared with FJ499452 and nucleotide differences were recorded at 22 different positions (Fig. 2D).

Conclusion

Morphological identification, pathogenicity tests and nucleotide sequences of rDNA region are reliable tools for the confirmation of Mucor rot infecting loquat in Pakistan.

Table 1: Phyto-pathogenic Mucor species available in the public database of GenBank

Sr #	Species	Country	Accessions #	Host	References
1	*M. racemosus*	China	KC292884	*Stellera chamaejasme*	Jin *et al*. (2013)
2	*M. racemosus*	Poland	AJ878775	*Quercus petraea*	KwaŚNa *et al*. (2006)
3	*M. indicus*	USA	JX045803	*Fragaria ananassa*	Unpublished
4	*M. indicus*	Indonesia	AB113026	Potato	Unpublished
5	*M. irregularis*	Mexico	KR076764	*Carica papaya*	Unpublished
6	*M. irregularis*	China	KM203872	maize	Peng*et al*. (2015)
7	*M. hiemalis*	Spain	KT323343	*Abies alba*	Unpublished
8	*M. hiemalis*	Spain	KT323340	*Pseudotsuga menziesii*	Unpublished
9	*M. hiemalis*	Yunnan	KP686185	*Panax notoginseng*	Unpublished
10	*M. hiemalis*	Switzerland	KM280062	*Ambrosia artemisiifolia*	Unpublished
11	*M. plumbeus*	Poland	KU319075	*Pinus sylvestris*	Unpublished
12	*M. plumbeus*	Switzerland	KP881447	*Platanus x acerifolia*	Unpublished
13	*M. plumbeus*	Switzerland	JX139728	*European horse-chestnut*	Unpublished
14	*M. fragilis*	Kenya	FJ904925	*Grevillea robusta*	Unpublished
15	*M. fragilis*	Spain	FJ499452	*Olea europaea* fruit	Baffi *et al.* (2012)
16	*M. fragilis*	Pakistan	KT312816	*Eriobortica japinica*	In current study
17	*M. fragilis*	Pakistan	KT312817	*Eriobortica japinica*	In current study

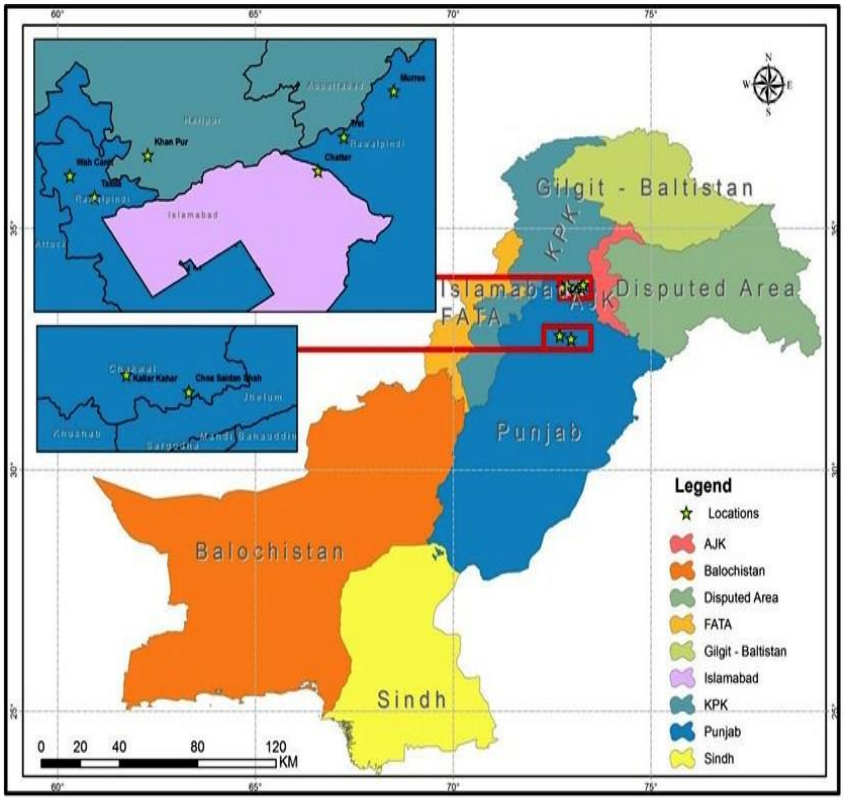

Fig. 1: Survey of major loquat growing areas of Pakistan.

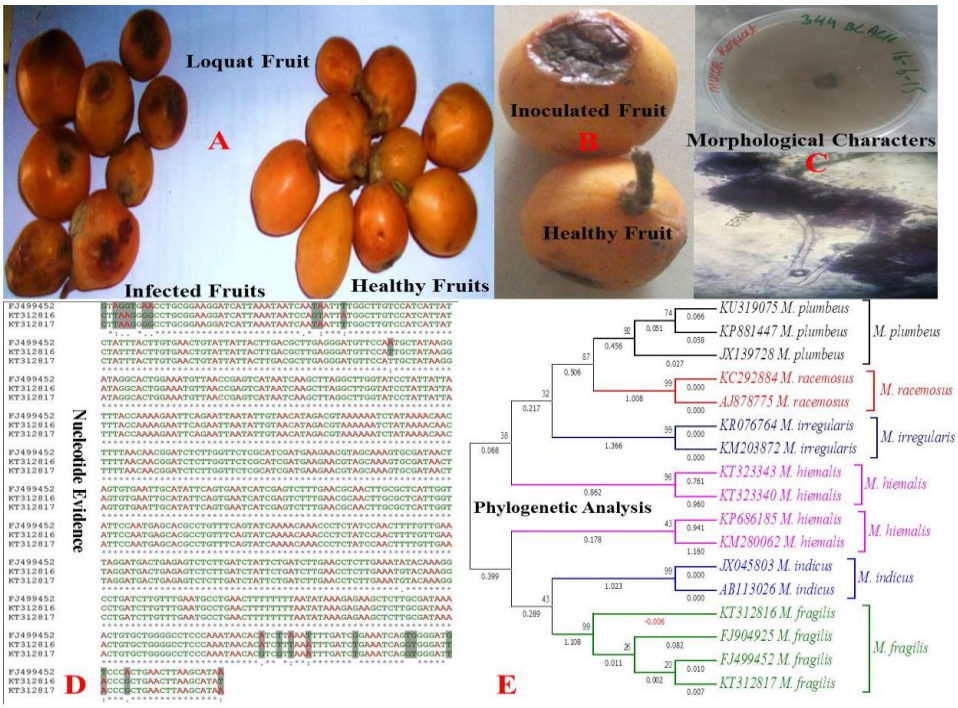

Fig. 2: Rotted loquat fruits (A), pathogenicity tests (B), morphological characterization (C), nucleotide comparison (D) and phylogenetic analysis of Pakistani isolates causing Mucor rot of loquat in Pakistan.

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