Mycopath (2018) 16(2): 117-121 **Research Article**

Morpho-molecular identification of *Rhizopus stolonifer* causing postharvest soft rot of Loquat (*Eriobotrya japonica*)

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Abstract

Loquat is a fruit of subtropical areas grown in various countries of the world. Owing to its high nutritional value, it is prone to various postharvest fungal diseases deteriorating fruit quality and heavy economic losses. The study aimed to identify *Rhizopus stolonifer* causing soft rot of loquat fruit. During a survey in the local fruit markets of districts Rawalpindi, Attock, Chakwal and capital territory Islamabad during the months of April and May 2017, symptomatic samples of rotted loquat fruits were collected for identification of the causal organism. The pathogen was isolated on potato dextrose agar (PDA), purified on Czapek dox agar (CDA) and was confirmed as *Rhizopus stolonifer* based on morphological characteristics including mycelial growth pattern, size, color and shape of sporangia and sporangiophores. Sporangiophores were globose, ellipsoidal and angular in shape with erected hyphae. The size of sporangia ranged from 120-190 µm. The diameter of sporangiophores ranged from 14~18 µm whereas length varied from 460~2800 µm. The sporangiospores were ranged from 12~14 × 245 µm in size while the size of columella was up to 120 µm. The results of the morphological identifications were further confirmed by sequence analysis of the internal transcribed spacer region (ITS) using a set of universal primers ITS1 and ITS4. Koch's postulates were fulfilled by re-isolation of the pathogen from artificially inoculated loquat fruits. To the best of our knowledge, this is the first report of *Rhizopus stolonifer* causing postharvest soft rot of loquat fruit in Pakistan. The results of the present investigations will be helpful in devising control strategies to manage this disease.

Introduction

Loquat (Eriobotrya japonica) is a subtropical fruit tree belongs to family Rosaceae. It is native to China and cultivated in various other parts of the world including Japan, India, Spain, Brazil, America, Australia and Pakistan. It is consumed both as fresh and processed (Pareek *et al.*, 2014). The fruit starts ripening from late spring to early summer favored by rainy-hot weather (Cai *et al.,* 2006). After harvesting, loquat fruit rapidly turns into brown as fruit has very short postharvest life at ambient temperatures and are sensitive to mechanical and physical damages, moisture and nutrient losses, and postharvest decay (Cao *et al.*, 2014).

Postharvest fungal deterioration is one of the main reasons for fruit decay causing serious problems that reduce the storage and transportation of fresh fruit produce and ultimately lead to severe economic loss (Michailides *et al.,* 2004). Fungal pathogens can tolerate a wide range of temperatures and can cause contamination in postharvest storage conditions of fruits and vegetables (Medveďova *et al*., 2008). *Rhizopus stolonifer* is one of the major postharvest decay causing pathogens, causal of rot in numerous fruits and vegetables (Bautista-Baños *et al.,* 2014). It typically causes soft rot, Rhizopus rot or watery rot, and is a very fast-growing fungus developing on a wide range of temperatures and relative humidities (Nishijima *et al*., 1990). *R. stolonifer* is among the most pathogenic in mycoflora associated with the storage of fruits and vegetables (Shehu and Muhammad, 2011). However, other *Rhizopus* species including *R. oryzae* and *R. artocarpi* are also reported to cause rots in fruits such as papaya, banana, mulberry roots and jackfruit (McMillan, 1986; Yoshisda *et al.,* 2003; Kwon *et al.,* 2012) and considered among the most devastating fungi during storage period of several horticultural commodities. This makes the fungal identification a high priority concern to make them under control and prevent their damage. In consideration of postharvest fungal decay, the present study aimed to identify and characterize the *R. stolonifer* causing postharvest soft rot in loquat fruit.

Materials and Methods

Sampling and pathogen isolation

A survey was conducted in the major fruit markets of three districts of Punjab *viz.* Attock, Chakwal Rawalpindi, and Islamabad Capital Territory, Pakistan during the months of April and May in 2017. During the survey, symptomatic loquat fruits infected with fungal rotting showing dark brown water-soaked lesions in the irregular pattern

were collected in paper bags and were brought to the Fungal Plant Pathology Lab. Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi for further pathogenic detection. Diseased portion of the infected fruits were cut into small pieces of approximately 5 mm, including some fruit flesh parts from the deeper layers and were transferred aseptically into the Czapek dox agar (CDA) medium in sterilized Petri plates. Parafilm sealed inoculated plates were incubated at 25°C for 4 to 5 days. The emerging fungal pathogen was further purified on another fresh Petri plates containing CDA media for cultural and morphological studies.

Morphological Studies

Morphological characterization was done on seven days old pure culture of fungus. Macroscopic features and microscopic characters of the fungus were examined including colony color, growth pattern, types of mycelium, shape, and size of sporangia. Under the biological microscope (Nikon YS100) using 10X, 40X, 100X lens, shape and size of the sporangia were studied and recorded.

Genetic analysis

Results of the morphological identifications were further confirmed by amplification of the Internal Transcribed Spacer (ITS) region of the fungus. Genomic DNA of the fungal pathogen was extracted using the standard protocol of Omni PrepMan™ for Fungi DNA Extraction Kit (G-Bioscience) (Cat. # 786-399). By using an aliquot of genomic fungal DNA as template, ITS region of the fungal genome was amplified using a set of universal primers ITS1 (5′-TCCGTAGGTGAACCTGGGG-3′) and ITS4 (3′-TCCTCCGCTTATTGATATGC-5′) (White *et al.*, 1990). The amplified gene product was sent for nucleotide sequencing and obtained DNA sequences were further manipulated in sense and antisense directions using phylogenetic BioEdit software. The final sequences were deposited to GenBank of NCBI (National Center for Biotechnological Information) to obtain the accession numbers. The percentage nucleotides base homology of ITS regions (ITS1 and ITS2) were determined using the Basic Local Alignment Standard Tool (BLAST) from NCBI.

Pathogenicity test

Non-symptomatic loquat fruits were washed in sterilized distilled water and blot dried were surface sterilized with 1% sodium hypochlorite solution for 60–90 seconds followed by rinsing twice in sterile distilled water. Fruits were then inoculated by creating wounds on the fruit samples and mycelial plugs from 7 days old fungal culture of approximately 2–3 mm were placed onto each wound with the help of a sterile needle. A set of wounded healthy fruits were used as a control. Inoculated and mock-inoculated fruit sets were placed in the separated sterile glass containers incubated at 25 °C for 5 days and rotting were observed regularly. Three independent trials using different isolates were run for conforming to the pathogenicity.

Results and Discussion

A total of 17 fruit market locations were visited and 95 fruit samples were collected. Symptomatic fruits exhibited rotting with soft brown water-soaked irregular lesions which enlarged and turned dark brown later. At least 39 number of fungal isolates were recovered which were further used for their morphological and molecular studies.

Morphological characteristics

Based on morphological keys for identification of *Rhizopus stolonifer* described by Schipper (1984), fungal culture showed aerial erected hyphae forming whitish growth which eventually turned to brownish to dark black spots which were sporangiophores (Fig.1 a & b).Fungal mycelia showed rapid growth which were characterized by abundant stolons connecting groups of unbranched sporangiophores which were hyaline to slight dark in color (Fig. 1 c). Sporangiophores terminated in 82 to 187 × 93 to 197 µm wide columella, shaped cylindrical (Fig. 1 d). Sporangiophores contain single spherical sporangium which were round or conical shaped cylindrical, greyish to brownish, about 245µm in diameter that bears several angular, sub-globose and ellipsoidal with marked ridges. The sporangiospores were ranged from 12 to 14 µm in length with a diameter ranging of 120-250 µm (Fig. 1 e). Rhizoids were found at the nodal position adjacent to the sporangiophore. These morphological descriptions were similar to those described by (Schripper *et al.*, 1984) as *R. stolonifer*. A brief morphological description of the recovered isolates is presented in table 1.

Molecular identification

ITS1-5.8S and ITS2-28S region of fungal genome were amplified using primers pair ITS1 & ITS4 and single compact amplified DNA bands of 650–700 bp sized were observed by running on 1% agarose gel (Fig. 2). The nucleotide sequence of the amplified PCR product was BLAST on NCBI using ITS nucleotide as query which showed 99–100% homology toward the already reported strains of *Rhizopus stolonifer* (accessions: FN401529 and AM933545). The nucleotide products were submitted to NCBI GenBank for accession numbers MH348275 and MH348276.

Confirmation of Kotch's postulates

After the incubation of 5 days, all the fungal inoculated fruits were seen rotten showing the same disease symptoms as observed on the infected fruit

samples collected from the markets. Mock-inoculated fruits remained symptomless. *R. stolonifer* causing soft rot was re-isolated from these infected fruits confirming its pathogenicity towards loquat fruits.

R. stolonifer is among the most destructive fungal species causing different rots in fruits and vegetables. It is one of the most common and fast-growing fungus species from the phylum Zygomycota. It requires mechanical wounds, injuries or cracks to cause infection in fruits in postharvest conditions (Vicente *et al.*, 2005). Often grow within a few days and cause the whole fruit decay considerably cause spoilage in a wide range of fruits and vegetables. Phytopathogenic fungi are commonly identified by taking the consideration of morphological criteria and keys. The most important characteristics for morphological identification of fungi are spores (shape, size, and color) and fruiting bodies and to lesser extinct mycelia; so, it is highly recommendable to follow the keys to genera for an accurate identification (Agrios, 2001).

For identification of *R. stolonifer*, (Farr *et al*., 1989) recommended the generic description published by (Schripper, 1984). It is important to identify and enumerate the conceivable source of variability in *R. stolonifer*. Fungal spores were angular, sub-globose and ellipsoidal with marked ridges (Swingle, 1903). However, globose type shape of spores confirmed as a *Rhizopus stolonifer* by Hernández-Lauzardo *et al.* (2005) which can vary in size, area, and diameter according to the isolate. Rhizoids were found at the nodal position adjacent to the sporangiophore. These characteristics were also described by Lin *et al*. (2016).

The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang *et al*., 2016). Different other species of Rhizopus including *R. oryzea* and *R. sexualis* shared similar characters described by Schripper (1984). However, Conventional techniques of macroscopic and microscopic studies are less sensitive and often proved inadequate for species identification and may give inaccurate results depending on the environmental conditions they are exposed to (Larone, 2002; Watanabe, 2002). Molecular methods of fungal identifications have been accepted as an effective tool (McDonald, 1997). The combination of both morphological and modern molecular techniques is now essential to allow accurate identifications of fungal pathogens. DNA barcodes like ITS, IGR, 28S rDNA sequences are already proved to be the very useful markers in the identification and to differentiate the species and verities for well studied fungal groups. Furthermore, molecular studies and DNA barcodes reveal the morphologically identical species and molecular data are often preferred over morphological data for the confirmation of fungal species (Liou *et al.*, 20017). A molecular amplification of fungal species is a rapid procedure for the assessment of fungal species (Wiemers and Fiedler, 2007). The Internal Transcribed Spacer (ITS) regions consist of ITS1 and ITS2 regions which are highly conserved for the fungal kingdom and by using universal primers set (ITS1 and ITS4) in PCR, can be amplified (White *et al.*, 1990).

Conclusion

To the best of our knowledge, this is the first report of *Rhizopus stolonifer* causing soft rot of loquat fruit from Pakistan.

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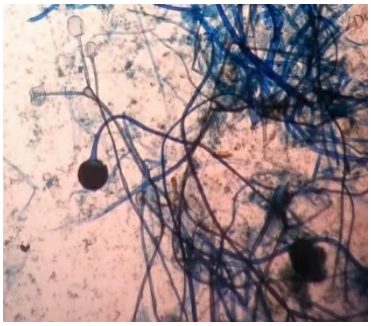
Table 1: Comparison of morphological characteristics of pathogenic *Rhizopus stolonifer* isolated from soft rot of Loquat fruits.

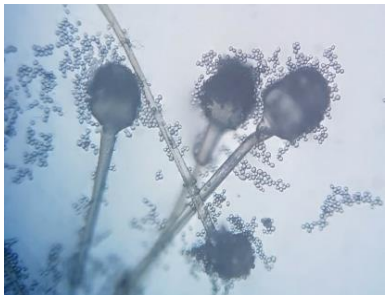
Characters		This Study	*R. stolonifer*
Colony	Color	White cottony to brownish-black	White colony to brownish-black
Sporangia	Shape	subglobuse	Globuse, subglobuse
	Size	120-190 µm	Upto 250 µm
Sporangiophores	Color	Brown	Brown
	Size	460~2800 × 14~18 µm	600~3800 × 10~25 µm
Sporangiospores	Shape	Irregular round or oval	Irregular round or oval
	Size	12~14 × 245 µm	6~15 × 100~275 µm
Columella	Shape	Cylindrical	Conical-cylindrical
	Size	120 µm	Upto 140 µm

a) aerial erected Hyphae with sporangiophores

b) Sporangiophores with Hyphae



c) Fungal mycelia with unbranched sporangiophores



d) Sporangiophores showing cylindrical columella

e) Sporangiospore

Fig. 1: Morphological characters of R. stolonifer under microscope.

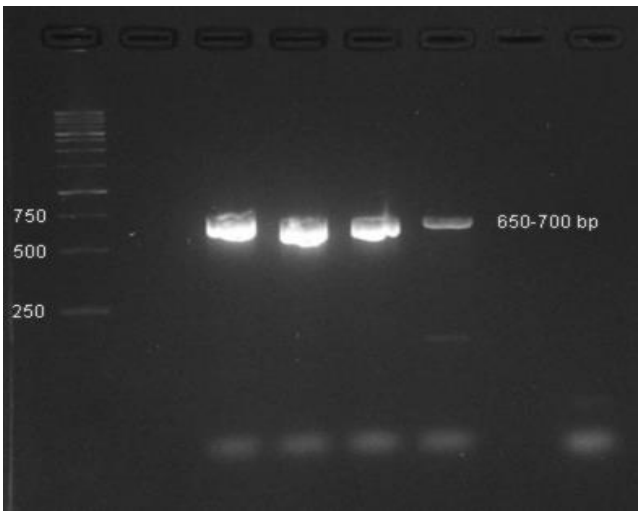


Fig. 2: Agarose gel image showing bands (650-700 bp).

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