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Short Communication

First Report of *Curvularia lunata* Causing Postharvest Fruit Rot of Banana in Pakistan

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Abstract

Banana (*Musa acuminata* Colla) fruits infected with rot disease were collected from the local market of Lahore, Pakistan. The observed symptoms were dry, decayed, sunken lesions with dark grey to greenish in color, damaging quality of the fruit. The pathogen was isolated on malt extract agar plates and observed under light microscope and scanning electron microscope for morphological characterization. The young fungal colony was greenish grey in color which upon maturation turned into downy dark grey to black. The conidia were single or in chains, attached with brown geniculate conidiophores distinguish by septation with dark brown scars. The mature conidia were curved at subterminal ends, dark brown to reddish brown in color with 3 oblique septa. On the basis of these morphological characters, the fungal isolate was identified as *Curvularia lunata* (Wakker) Boedijn. Pathogenicity test was performed by inoculating the pathogen artificially on asymptomatic banana and the re-isolated fungus had the same characters as that of originally isolated one. For more accuracy, the molecular studies of the isolated pathogen *C. lunata* were performed by sequencing its rDNA with two distinct markers ITS and GAPDH. The PCR products of ITS and GAPDH were then submitted to GenBank with MN752153 and MN787829 accession numbers, respectively. To the best of our knowledge, *C. lunata* infecting the banana fruit has not already been reported from Pakistan. Hence, this is the first report of *C. lunata* responsible for post-harvest banana rot in Pakistan. © 2020 Friends Science Publishers

Keywords: *Curvularia lunata*; Fruit rot; *Musa acuminata*; Pakistan; Ribosomal DNA

Introduction

Banana is a renowned and the most consumed staple fruit on the planet, especially in Pakistan. It is grown in more than 90 countries mainly in Brazil, Ecuador, North-America, Japan, Philippines, Colombia, China, India and Costa Rica (Varma and Bebber 2019). In Pakistan, the area under its cultivation is 352 thousand ha with an average yield of 31.4 MT ha^{-1} (Rehman *et al*. 2018). With an increase in urbanization, it is becoming an important cash crop serving as the sole income source to the poor farmers to eliminate the poverty (Memon *et al*. 2016). The fruit is usually curved with a soft flesh rich in starch covered with an enhanced variation of rind which may be brown, green, purple, red, or yellow when ripe (Fu *et al*. 2018). It is a rich source of nutritional antioxidants including potassium, manganese and vitamin B and C. Banana consumption plays a major role on human health in reducing the risk of colorectal cancer, asthma, diabetes, leukemia, high blood pressure and cardiovascular diseases (Ghag and Ganapathi 2018).

Being a perishable fruit, its commercial production is severely affected by post-harvest losses up to 40–45% due to improper handling, inadequate harvest, lack of proper packing skills, insufficient storage conditions and many other uncontrolled factors (Selvaraj *et al*. 2019). Banana plant is attacked by a number of fungal pathogens namely *Fusarium roseum*, *Botryodiplodia theobromae*, *Fusarium semitectium*, *Fusarium moniliformae*, *Colletotrichum musae*, *Lasiodiplodiat heobromae*, *Verticillium theobromae* and *Trachysphaera fructigena* causing blossom end rot, cigar-end rot, Fusarium wilt, crown rot and anthracnose disease (Kuyu and Tola 2018; Shen *et al.* 2018; Vilaplana *et al.* 2018). The genus *Curvularia* is comprised of more than 75 species, most of them are facultative plant pathogens responsible for pre-harvest and post-harvest yield loses in economically important crop plants (Bengyella *et al*. 2019). *Curvularia lunata* is one of the most destructive ubiquitous pathogens responsible for stem blight, leaf spot, leaf blight, root rot and necrotic rot in rice, spinach, strawberry and switchgrass (Gupta *et al*. 2017; Bisht *et al*. 2018; Liu *et al.* 2019). The accurate identification of *C. lunata* has been very confusing because of morphological similarities among the culture isolates depending upon the growing conditions making it doubtful, incorrect or remains unresolved (Kusai *et al.* 2016). Recent studies have shown the importance of molecular identification as these do not correlate with morphological identification. Molecular studies by using the polymerase chain reaction and DNA probes are gaining

importance worldwide for the rapid and specific detection of *C. lunata* for ensuring the quality of fruits, vegetables and cereals (Santos *et al*. 2018; Lu *et al*. 2019). The objective of the present study was to identify the post-harvest pathogen of banana rot in Pakistan through morphological and molecular characters.

Materials and Methods

Sample collection and identification

Banana with greenish grey to dark green, dry, sunken and decayed lesions of rot were collected from the local market of Lahore, Pakistan. Diseased portions were surface sterilized in sodium hypochlorite (3%) for 1 min with three subsequent washings with distilled water and placed on malt extract agar (MEA) containing plates for 5 days. After that, pure culture was obtained by placing the young fungal mycelia, obtained from the colony margins, and incubated at 28°C for 7 days. The mature fungal colony was identified on the basis of its macroscopic features (colony size, shape, texture, exudates and color) and then examined through microscopic characteristics (conidia and conidiophores) under a light microscope at 4, 10, 40 and 100X magnifications.

Scanning electron microscopic (SEM) analysis

Seven-day-old *C. lunata* culture was processed according to Jinfeng *et al*. (2017) with some modifications before SEM analysis. The sample was cut into 1 cm^3 pieces and placed in vials containing 4% glutaric dialdehyde followed by three washings in 0.1 *M* cacodylic sodium trihydrate buffer. Next, the samples were fixed in osmium tetraoxide (1%) for 1 h and washed again with the sodium trihydrate buffer with a subsequent dehydration process in pure ethanol and then passed through acetone. The sample was then placed on a stub for further analysis under electron microscope (Jeol JSM-6480 LV).

Molecular characterization

The genomic DNA of *C. lunata* was isolated by using CTAB method (Doyle and Doyle 1990) and run PCR with ITS and GAPDH primers given in Table 1. Amplified products were subjected to MiSeq Illumina sequencing, USA and submitted to NCBI (National Center for Biotechnology Information) database for BLAST search tool to perform the homology comparisons with other isolated sequences of *C. lunata* by using Clustal W software (Thompson *et al.* 1994). On the basis of aligned sequence data, a neighbour-joining tree was constructed by using MEGA X software (Tamura *et al.* 2012).

Pathogenicity test

For the confirmation of *C. lunata* attack on banana fruit, a pathogenicity test was performed on five surface sterilized banana fruits. The fruits were inoculated by using an inoculation needle carrying fresh mycelia of *C. lunata* grown on MEA. Fruits in control treatment were inoculated with sterile MEA and placed in autoclaved beakers for 7 days. After the confirmation of pathogen establishment, the *C. lunata* conidia were re-isolated from the lesions developed on banana and their morphological characters were studied.

Results

Morphological identification revealed that the fungal colonies were fast growing on malt extract agar with an average diameter of 8 cm in 7 days of inoculation at 28°C. The young colony was greenish grey in color which upon maturation turned into downy dark grey to black with a blackish grey reverse on MEA (Fig. 1). The microscopic examination of the fungal growth revealed that the conidia were single or in the form of chains attached with brown geniculate conidiophores distinguishes by septation with dark brown scars. The apical part of conidiophores was flexuous to straight, unbranched and septate. The mature conidia were 19.8 to 27.3 × 7.3 to 11.8 μm in size, distinctly curved at subterminal ends, ellipsoidal with rounded ends, dark brown to reddish brown in color with smooth conidial walls having 3 oblique septa.

All the inoculated banana fruits showed rot symptoms after 7 days of inoculation (Fig. 2). Initially, small sized dark green sunken lesions appeared on the banana surface which later on expanded by joining together. The fungal pathogen was re-isolated from the infected banana fruits on MEA plates producing the same characteristic features.

The genomic DNA of *C. lunata* was amplified by using two sets of primer pairs *viz.*, ITS and GAPDH generated single fragment PCR products shown in Fig. 3, with MN752153 and MN787829 accession numbers, respectively, resulted in 100% identity match. The obtained sequences were aligned by constructing a phylogenetic tree with each of the individual primer ITS (Fig. 4A) and GAPDH (Fig. 4B) by using a neighbor-joining method in MEGA X software.

Discussion

Major fungi associated with rot decay are *Curvularia lunata*, *C. verruculosa*, *C. tuberculate*, *C. brachyspora*, *C. clavata*, *C. trifolii*, *C. coicis*, *C. inaequalis* and *C. spicifera* (Pei *et al*. 2018; Pornsuriya *et al.* 2018; Wang *et al*. 2019; Balamurugan *et al*. 2020). Among them, *C. lunata*, the dematiaceous mold is considered as the most virulent strain responsible for fruit rot in *Carica papaya*, *Ziziphus mauritiana*, *Solanum lycopersicum*, *Fragaria ananassa*, *Hylocereus polyrhizus*, *Malus pumelo*, *Mangifera indica*, *Phoenix dactylifera* and *Citrus sinensis* on a large scale (Bussaban *et al*. 2017; Bisht *et al*. 2018; Helal *et al*. 2018; Majumdar and Mandal 2019). Similarly, in the present study, the pathogenicity test with *C. lunata* revealed that it is

Table 1: List of oligonucleotide primers used for the characterization of *C. lunata* at molecular level

Primer name	5´ to 3´ sequence	Amplicon size (bp)	Annealing temperature
ITS 1 Forward	TCCGTAGGTGAACCTGCGG	~637	60°C
ITS 4 Reverse	TCCTCCGCTTATTGATATGC		
GAPDH Forward	CAACGGCTTCGGTCGCATTG	~561	60°C
GAPDH Reverse	GCCAAGCAGTTGGTTGTG		

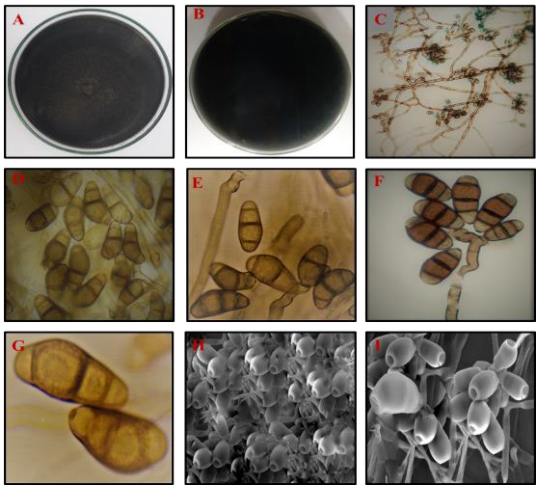

Fig. 1: (A)- Mature colony of *Curvularia lunata* on MEA, **(B)-** Colony reverse on MEA, **(C)-** Conidia at 4X, **(D)-** Conidia at 10X, **(E & F)-** Conidia at 40X, **(G)-** Conidia at 100X, **(H & I)-** Conidia under scanning electron microscope showing conidiophores bearing conidia separately and/or in chains

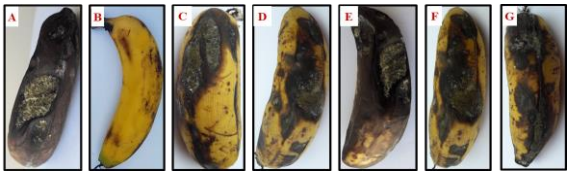

Fig. 2: Pathogenicity test. **(A**)- Symptoms of rot in naturally diseased banana, **(B)-** Control banana (no inoculation) which is symptomless, **(C-G)-** Typical symptoms of *Curvularia lunata* after inoculation on banana

the most pathogenic isolate also capable of inducing rot on banana fruit.

According to Santos *et al.* (2018) pathogen accuracy on the basis of morphological and microscopic characteristics alone poses a diagnostic dilemma because of the absence or infrequent morphological patterns of conidia and conidiophores. In such critical situations, molecular methods provide a great assistance towards their accuracy (Edgar 2018). There are many efforts in this regard to develop such molecular tools for the accurate identification of the pathogens. Wurzbacher *et al.* (2019) reported that ribosomal DNA of *C. lunata*is composed of internal transcribed spacer (ITS) region with major 18S, 5.8S and 28S transcripts. The ITS region is the best DNA marker with high amplification success rate widely used in molecular taxonomy because of elevated variations to differentiate the fungal isolates at inter or intraspecies level

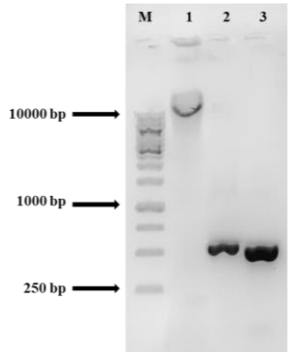

Fig. 3: Agarose gel electrophoresis, **(M):** 1 kb DNA standard marker, **(1):** Genomic DNA of *Curvularia lunata*, **(2):** ITS1/ITS4 amplified PCR product, **(3):** $GAPDH_f$/$GAPDH_r$ amplified PCR product

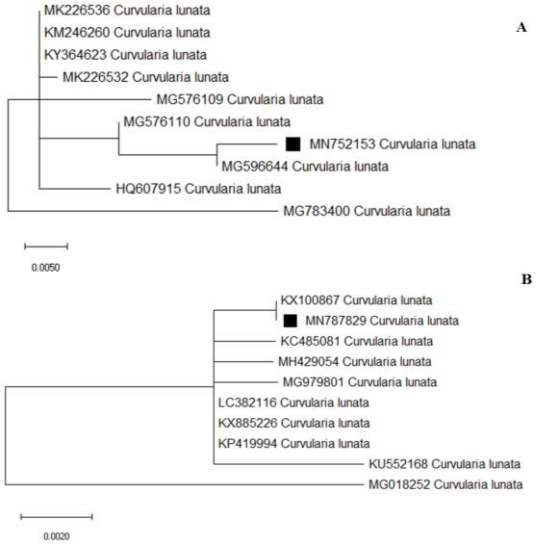

Fig. 4: The ITS (**A**) and GAPDH (**B**) gene sequences of the *C. lunata* isolate from this study was aligned with *C. lunata* sequence isolates from GenBank using Clustal W© program. The phylogenetic trees were constructed using the neighbor-joining method in MEGA X version 10.1 (Tamura *et al.* 2012)

(Badotti *et al.* 2017). In addition, the use of some other secondary DNA markers such as GAPDH is essential for better speciation process within the genus *Curvularia* (Kiss *et al.* 2020). The reason for selecting glyceraldehyde-3-phosphate dehydrogenase region specifically was that it is highly constitutively expressed in *Curvularia* species making it a successful heterogenous gene for the best screening experiments (Silva *et al.* 2017). In previous studies, molecular test with ITS and GAPDH primers were generally used for the confirmation of *C. lunata* (Zhang *et al.* 2017; Xu *et al.* 2018; Liu *et al.* 2019). These markers have also been used for identification of species in many other genera including *Alternaria*, *Aspergillus*, *Fusarium*, *Wallemia*, *Colletotrichum*, *Pythium*, *Botrytis*, *Cochliobolus* and *Ramularia* (Moslemi *et al.* 2017; Garfinkel and Chastagner 2019; Janbozorgi *et al.* 2019; Raza *et al.* 2019).

Conclusion

On the basis of rDNA sequence of *C. lunata* with the corresponding amplified DNA products, morphological, microscopic and molecular phylogenetic results, we believe that *C. lunata* is the first report of banana rot in Pakistan.

Author Contributions

IHK did experimental work and wrote part of paper. AJ supervised the work and also wrote a part of the paper.

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