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MOLECULAR CHARACTERIZATION OF *PENICILLIUM EXPANSUM* ASSOCIATED WITH BLUE MOLD DISEASE OF APPLE IN PAKISTAN

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Abstract

The present study was conducted for molecular characterization of the causal agent of blue mold decay in apple (*Malus domestica* Borkh.). *Penicillium expansum* Link is a major post-harvest pathogen responsible for significant yield losses to fruit industry. Specific detection of *P. expansum* is of prime importance for ensuring the safety and quality of food products. The traditional methods for pathogen identification are labor intensive, less reliable and time consuming, therefore, these are being replaced with molecular techniques. The infected apples with pale yellow to brown lesions were collected from the local market of Lahore, Pakistan. The infected samples were cultured on malt extract agar in order to obtain the pure fungal colonies. Pathogenicity of the isolated fungus was confirmed by inoculating the fungal mycelia on asymptomatic apples. After macroscopic characterization, the isolated pathogen was observed under light and scanning electron microscopes and the fungus was identified as *P. expansum*. For molecular characterization, the isolated rDNA of the fungus was tested using ITS, β-tubulin, calmodulin and CF primer pairs and the obtained PCR products were got sequenced and deposited in the GenBank with accession numbers MN752156, MN787826, MN787827 and MN787828, respectively. As per our knowledge, this is the first study regarding molecular characterization of *P. expansum*, the cause of blue mold disease of apple in Pakistan.

Key words: *Penicillium expansum*, Blue mold, rDNA, *Malus domestica*, Pakistan.

Introduction

Apple (*Malus domestica* Borkh.) is the fourth most important deciduous fruit grown widely in Balochistan, Khyber Pakhtunkhwa and northern areas of Pakistan. The total area under its cultivation is 119.8 thousand hectares with 427.9 thousand tons of annual production (Khan *et al.*, 2019). It is a fruit of beautiful shape, attractive color, pleasant flavor, aromatic nature, nutritious properties, crispy in taste and of multiple uses. It contains proteins 0.6 g, carbohydrates 14.3 g, fiber 0.7 g, ash 0.4 g, lipid 0.4 g, starch 0.5 g, sugar 12.3 g, riboflavin 0.05 mg, thiamine 0.03 mg, carotene 0.09 mg, ascorbic acid 5.1 mg 100 g^{-1} and water contents 86.4% (Abid *et al.*, 2019). Moreover, it is well known because of its anticarcinogenic, antiproliferative, therapeutic and anti-inflammatory health benefits against cardiovascular, intestinal as well as viral diseases (Martins *et al.*, 2019). In Pakistan, generally it is consumed fresh or in the form of marmalades, jellies, jams, puddings, snacks, pickles, pie filling, sauces, sweet meat and fermented juices. Because of excessive production, lack of proper transport facilities, insufficient storage conditions and short shelf life, most of the fruit is attacked by several post-harvest diseases caused by fungal pathogens with significant economic losses (Murtaza & Thapa, 2017). *Penicillium expansum*, *Mucor piriformis* and *Botrytis cinerea* are the most important post-harvest pathogens of apple. *P. expansum* causes a psychrotrophic mold known as blue mold on a variety of horticultural products including apple, peach, nectarines and cherries (Kim *et al.*, 2018).

The genus *Penicillium* contains an impressive genetic diversity with a wide range of approximately 350 well-known species that are usually xerophilic, thermophilic, psychrophilic, alkalinophilic or acidophilic (Yadav *et al.,* 2018). Being opportunistic fungal pathogen, it causes substantial economic losses at pre- and post-harvest stages of agricultural products. *Penicillium* species namely *P. expansum, P. crustosum, P. glabrum, P. paneum, P. carneum, P. griseofulvum* and *P. solitum* are known destructive pathogens (Dugan *et al.,* 2017). The accurate identification of *Penicillium* at species level is not easy under light microscope, so there is a strong need to develop advanced and cost-effective techniques for the accurate detection of these pathogens. Moreover, physiological tests are labor intensive, time consuming and often require mycological expertise (Mahmoud *et al.,* 2016). Therefore, molecular identification by using DNA probes and polymerase chain reaction could be of supreme importance for the specific and rapid detection of *P. expansum* and other fungi for ensuring both safety and quality of fruits (Yin *et al.,* 2017; Khan & Javaid, 2020).

In previous studies, *P. expansum* has been isolated from the diseased apple fruits in Pakistan (Ilyas *et al.,* 2007; Khokhar & Bajwa, 2014). However, those studies were confined to identification of pathogen on morphological basis and studies regarding identification and authenticity on molecular basis were lacking. Therefore, the immediate objective of the present research was to identify *P. expansum*, the causal agent of blue mold disease in apple, on molecular basis by using different pairs of primers.

Materials and Methods

Sample collection and identification: Apple fruits with light brown, soft water-soaked lesions were collected from the local market of Lahore. Diseased areas were cut into 4 × 4 mm portions, surface sterilized with 3% sodium hypochlorite solution for 2 min with three subsequent washings each for 2 min in double distilled water, placed on 9-cm diameter malt extract agar (MEA) containing Petri dishes and incubated at 28ºC for one week. Thereafter, pure culture of the isolated fungus was identified to the generic level on the basis of macroscopic (colony shape, size, color, exudates, texture) and microscopic (conidia, phialides, metulae, conidiophores) characteristics at 100X magnification under light microscope (Khokhar *et al.,* 2013).

Scanning electron microscopy (SEM) analysis: The isolated fungal pathogen was grown on MEAand processed before SEM analysis according to Jinfeng *et al*., (2017) with some modifications. The fungal sample was sliced into 1 cm^3 and fixed in 4% glutaric dialdehyde with three subsequent washings with 0.1 M cacodylic sodium trihydrate buffer. Furthermore, the sample was passed through pure ethanol followed by acetone to initiate the dehydration process and placed on a stub for further studies underelectron microscope (Jeol JSM-6480 LV).

Molecular characterization: The genomic DNA of the isolated fungus was isolated as per CTAB method (Doyle & Doyle, 1990) and run PCR with different primer pairs such as ITS, β-tubulin, calmodulin and CF (Table 1). The amplified PCR products were subjected to MiSeq Illumina sequencing, USA and submitted to NCBI (National Center for Biotechnology Information) database to obtain accession numbers. Homology comparison of the selected primer sequences were performed with other isolates of *P. expansum* aligned with our sequences available in GenBank according to Thompson *et al.,* (1994) at different lengths and constructed a neighbor-joining tree by using a software MEGA X (Tamura *et al.,* 2012).

Pathogenicity test: For the confirmation of pathogen attack, a pathogenicity test was performed on five asymptomatic apple fruits after surface sterilization with 1% sodium hypochlorite solution. Next, the fruits were inoculated with 5 mm discs of freshly grown *P. expansum* on MEA and a control was prepared by placing plain MEA discs. Apples were placed in autoclaved beakers for 8 days for the complete establishment of the pathogen and *P. expansum* was re-isolated from the lesions that developed on inoculated apples for the further identification as described earlier in comparison to symptomless control specimen.

Table 1. List of oligonucleotide primers used for the characterization of *P. expansum* at molecular level.

Primer name	5′ to 3′ sequence	Amplicon size (bp)	Annealing temperature	Accession numbers
ITS 1 forward	TCCGTAGGTGAACCTGCGG	~ 582	60 °C	MN752156
ITS 4 reverse	TCCTCCGCTTATTGATATGC			
β-tubulin forward	GGTAACCAAATCGGTGCTGCTTTC	~ 451	62 °C	MN787826
β-tubulin reverse	ACCCTCAGTGTAGTGACCCTTGGC			
CMD5 forward	CCGAGTACAAGGARGCCTTC	~ 567	65 °C	MN787827
CMD6 reverse	CCGATRGAGGTCATRACGTGG			
Cf1forward	GCCGACTCTTTGACYGARGAR	~ 756	57 °C	MN787828
CF4 reverse	TTTYTGCATCATRAGYTGGAC			

Results and Discussion

Morphological investigation revealed that the fungal colonies were circular and fast growing on MEA with an average diameter of 5 cm when incubated at 28°C for 8 days. The young colonies were white in color, which later on turned to dark green with abundant conidia whereas underside of the plate was yellow green (Fig. 1). The conidia were attached with branched conidiophores, elliptical in shape, thin walled, smooth surface with 3.15 to 3.92 × 2.49 to 3.23 µm in size. The examined isolate was identified as *P. expansum* and was morphologically similar to the characteristics recently described by Brito *et al.,* (2020). All the inoculated apple fruits showed similar symptoms of blue mold at 8-day post inoculation (Fig. 2). At initial stages, small sized pale-yellow lesions appeared on the apple surface, which later on increased in size and turned into brown color. The pathogen was re-isolated on MEA by using single spore technique from the infected apples and produced the same characteristic features.

The amplification of the corresponding DNA of tested isolate with specific primers gave distinct products as shown in Fig. 3. Calmodulin and CF like gene were specifically used for *P. expansum* identification in previous studies (Chen *et al.,* 2017). β-tubulin gene also provides accurate identification whereas ITS is not enough to distinguish between all related species. The obtained sequences of *P. expansum* with ITS, β-tubulin, calmodulin and CF showed 99-100% similarities to the corresponding sequences deposited in the GenBank for isolate identification. Accession numbers for sequences of PCR products obtained with ITS, β-tubulin, calmodulin and CF primer pairs were MN752156, MN787826, MN787827 andMN787828, respectively (Table 1). Phylogenetic trees were constructed with each of the individual obtained primer sequences of ITS (Fig. 4A), β-tubulin (Fig. 4B), calmodulin (Fig. 4C) and CF (Fig. 4D). All the sequences were placed within the clade including reference isolates of *P. expansum,* which were carried out by using a neighbor-joining software MEGA X (Tamura *et al.,* 2012). Blue mold decay is caused by many pathogenic *Penicillium* species including *P. expansum, P. crustosum, P. solitum, P. digitatum, P. italicum, P. commune, P. polonicum, P. rugulosum, P. chrysogenum* and *P. verrucosum* (Wang *et al.,* 2019; Zhang *et al.,* 2019). Among these, *P. expansum* is considered as the most virulent toxin producing species responsible for blue mold decay of apple (Abdelhai *et al.,* 2019). The precise characterization of ambiguous *Penicillium* species is often difficult without the use of molecular tools, as many of the common species possess huge similarities within micro and macroscopic features (Sawant *et al.,* 2019). However, the appropriate marker selection is challenging. The internal transcribed spacer (ITS) is the universally accepted genetic barcode, provides a greater taxonomic resolution towards the different fungal genera due to elevated success rate of sequencing and amplification. This is a highly conserved region that can be easily investigated in phylogenetic studies by using PCR

amplification of ribosomal DNA (Li *et al.*, 2020). Unfortunately, ITS sequence has some limitations and alone it is not enough for distinguishing closely related *Penicillium* species. In the present study, for the effective *P. expansum* identification, ITS marker was used in addition to several specific markers such as β-tubulin, calmodulin and CF. In previous studies, these markers exhibited strong identification in many other genera including *Aspergillus, Alternaria, Fusarium, Botrytis, Wallemia* and *Ramularia* (Lee & Yamamoto, 2015; Zarrin *et al.*, 2016; Trabelsi *et al.*, 2019). In agricultural settings, the pathogen accuracy is necessary for its effective toxin control, fungicide resistance and way to alter the proper management strategies.

This study confirms the identification of *P. expansum* on molecular bases, the causal agent of blue mold disease of apple in Pakistan. It is a post-harvest decay pathogen, contaminates food commodities by producing mycotoxins and converts them into inedible products. Further studies regarding its genomic sequence, mechanism and spread are necessary with an emphasis on effective control strategies.

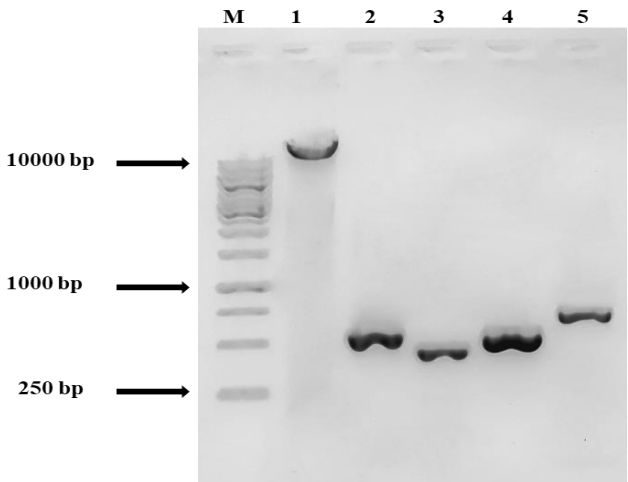

Fig. 3. Agarose gel electrophoresis (M): 1 kb DNA standard marker, (1): Genomic DNA of *Penicillium expansum*, (2): ITS1/ITS4 amplified PCR product, (3): Bt_{2a}/Bt_{2b} amplified PCR product, (4): Calmodulin amplified PCR product, (5): CF amplified PCR product.

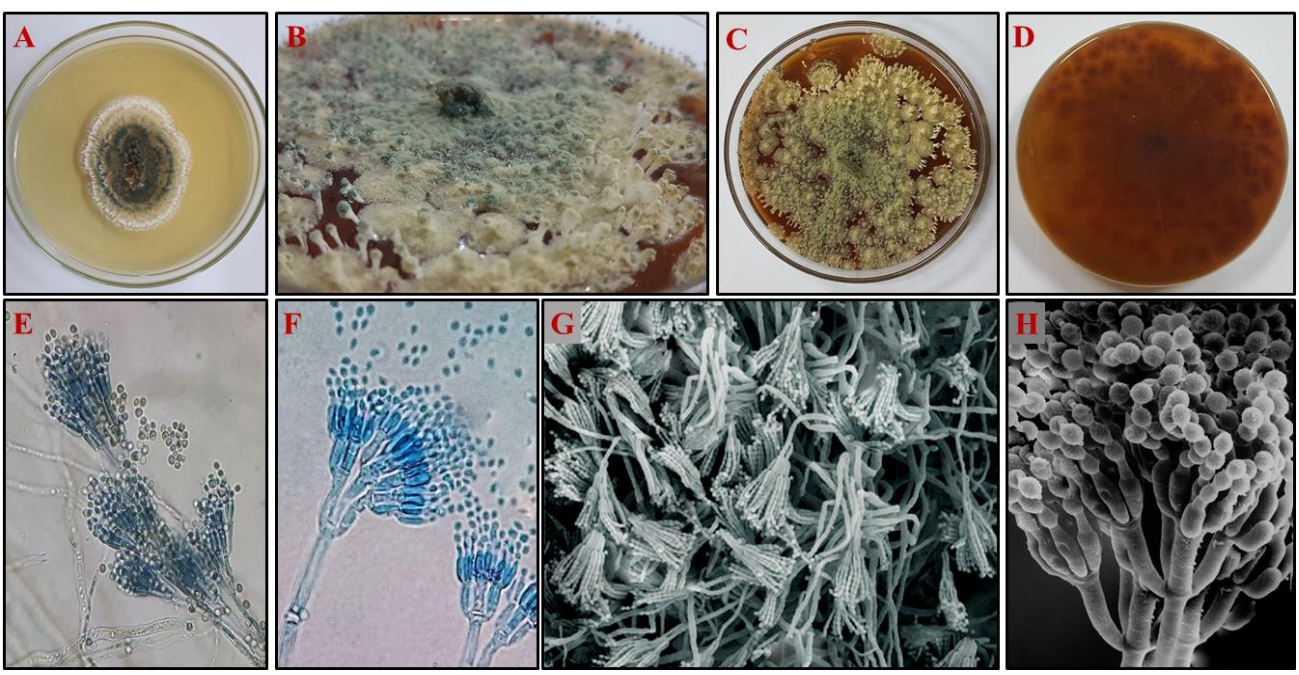

Fig. 1. A)- Immature colony of *Penicillium expansum* on MEA, (B& C)- Mature colony of *Penicillium expansumon* MEA, (D)- Colony reverse on MEA, (E)- Conidia at 40X, (F)- Conidia at 100X (G& H)- Conidia under scanning electron microscope. Conidiophores bearing conidia in chains and attached with sterigma.

Fig. 2. (A)- Apple showing symptoms of blue mold, (B)- Control apple which is symptomless, (C & D)- Typical symptoms of *Penicillium expansum* after inoculation on apples.

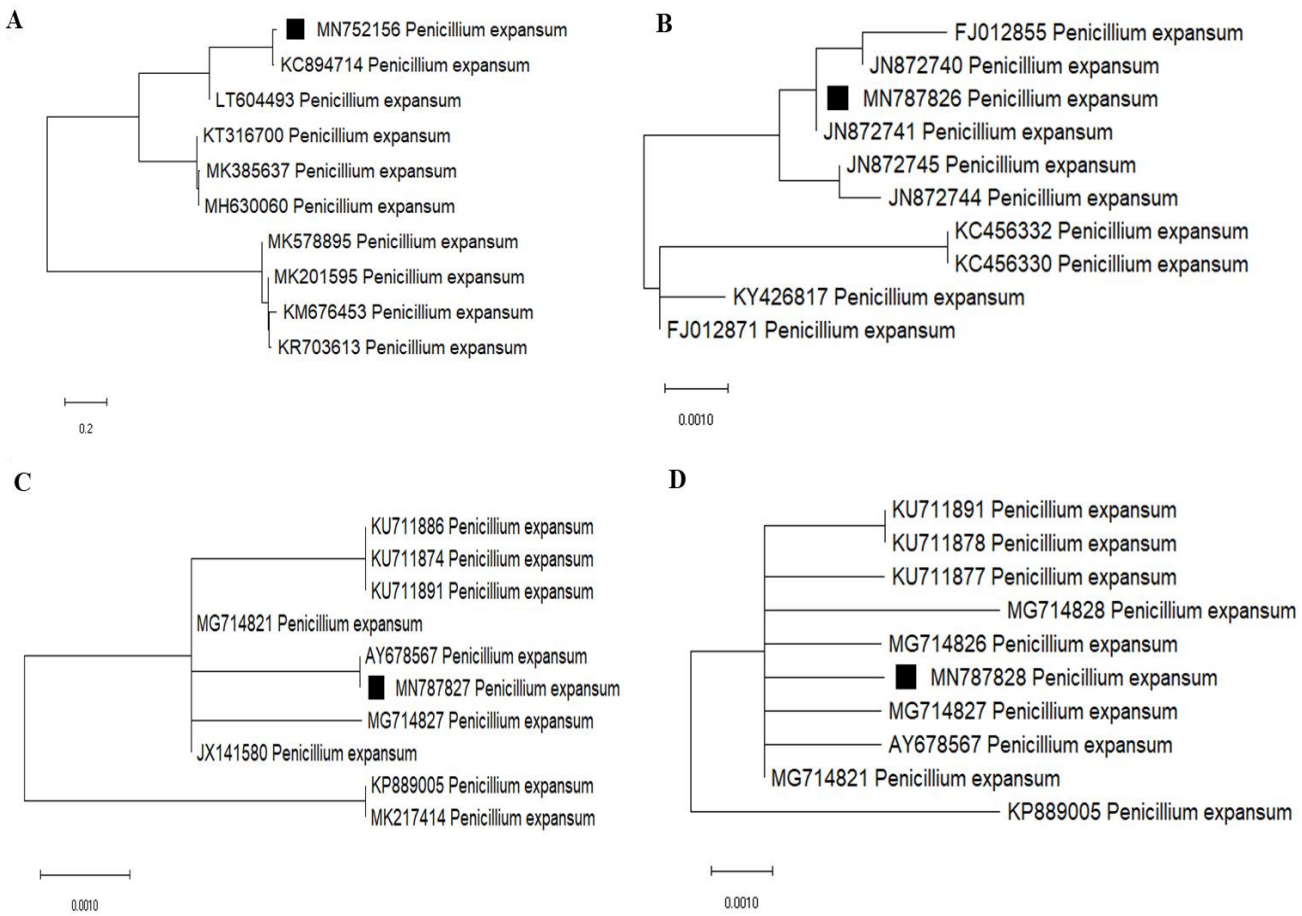

Fig. 4. (A)- ITS1, (B)- β-tubulin, (C)- CMD, (D)- CF gene sequence of the isolate from this study was aligned with reference sequences of *Penicillium expansum* isolates from GenBank using Clustal W© program. The phylogenetic tree was constructed using the neighbor-joining method in MEGA x version 10.1 (Tamura *et al*., 2012).

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