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Research Article

First Report of *Penicillium digitatum* (Pers. ex Fr.) Sacc. causing a postharvest green mould of Oranges (*Citrus* × *sinensis*) in Pakistan

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Abstract

In this study *Penicillium digitatum* was found to be the cause of postharvest rot of stored Oranges (*Citrus* × *sinensis*) in Pakistan. Infected fruit tissues were cultured on Czapek Dox Agar, 2% Malt extract agar, Czapek Yeast Autolysate Agar and 25% Glycerol Nitrate Agar at 25 ◦C medium. The pathogen was identified as *P. digitatum* on the basis of morphological and molecular characteristics. Pathogenicity tests conducted on healthy fruits under laboratory conditions showed typical rot symptoms after seven to fourteen days. This is the first report of posthavest green mould of Oranges caused by *P. digitatum* in Pakistan.

Keywords: Orange, *Penicillium digitatum*, Green mould, Morphology, Molecular characterization.

Introduction

Pakistan is the sixth largest producer of Kinow (mandarin) and Oranges (*Citrus* × *sinensis*) in the world, with 2.1 million tons. Pakistan world mandarin and oranges market share during the year 1997 was 0.9 percent and 3.6 percent in terms of value and volume respectively. Pakistan is also the largest producer of '*Citrus Reticula*' variety (Kinow), this unique variety of citrus is indigenous to this part of the world. According to an estimate approx. 95 percent of the total Kinow produced all over the world is grown in Pakistan. Citrus fruits such as orange, lemon, and lime, have been cultured widely and processed into juice (Olaniyan, 2010). In tropical developing countries the loss due to post harvest diseases represents a major economical burden and fungal decay is one of the major factors contributing to loss in stored fruits. The deterioration of food by fungi results in economical losses ranging from 5 to 20% of the production in developed countries and can be as high as 50% in regions with a tropical climate (Eckert and Ogawa, 1985; Usall *et al*., 2000; Janisiewicz and Korsten, 2002). It is estimated that up to half of all fruits harvested is lost due to fungal and pests decay worldwide (Burden *et al*., 1989). Postharvest fungal decay may cause significant losses to the citrus industry. Injuries on citrus fruit caused during harvest, provide entries to wound pathogens, including *Penicillium digitatum* Sacc. and *P. italicum* Wehmer, causal agents of green and blue mould, respectively. These pathogens occur in almost all citrus growing regions of the world (Palou *et al*., 2001).

Materials and Methods

Sample collection and fungus isolation

In December 2011 and 2012 during a survey of local vegetable and fruit market in Lahore (Pakistan), samples of decayed oranges have been collected. To clarify the causal agents of those symptoms, orange samples were obtained from a local vegetable and fruit market, kotlakhpat, Lahore and examined at laboratory. From the necrotic areas, a green fungal growth was observed. Temporary slides of diseased

tissues were made and observed under light microscope. Small pieces (3 mm) of rotting tissue from the oranges and surface sterilized with 1 % Na (O) Cl, were placed onto 2% malt extract agar (MEA) and incubated at 25 °C in darkness for 5 days.

DNA sequencing

To confirm the identity of the causal fungus, extraction of total DNA from the mycelia and conidia of the isolates was done by modified 2% CTAB method (Doyle and Doyle 1990). The internal transcribed spacer (ITS) region of rDNA was amplified with primers ITS1/ITS4 (White *et al*., 1990).

Tests for pathogenicity

Pathogenicity of the isolated organism was confirmed on healthy oranges. Conidial suspension (2×10^4 conidia mL^{-1}) from a pure culture of the fungus was directly inoculated by means of a sterile needle into the subcutaneous layer of oranges. Infested oranges were incubated at 25 °C for 7 to 14 days.

Results

Morphological characterization

As a result, a species belonging to the genus *Penicillium* subgenus *Furcatum* was consistently found associated to the described symptoms. According to key (Raper and Thom 1949; Pitt 1979, 1985; Ramírez 1982; Samson *et al*., 1995; Pitt and Hocking 1997) this species is primarily characterized by its relatively slow growth on Czapek-based media and MEA at 25 °C, heavy green sporulation, forming crusts, production of a dark brown pigment on the media, and inability to grow at 5 and 37 °C on both Cz and MEA. Thus, in order to allow the confirmation of the fungus identity obtained in this study the resulting fungal colonies were subcultured on Czapek–solution agar (Cz), 2% MEA, CYA and G25N at 25 °C (Plate: 1).

The description of our fungal specimen is as follows:

MEA, 25 ^{o}C, 7 days

Colonies were variable in size from 35 -70 mm in diameter, plane, relatively sparse, very deep and floccose with an overlay of white mycelium, margins irregular. Mycelium became dull green due to moderate conidiogenesis. Reverse of the colony was pale or brownish. Exudates and soluble pigment absent.

CZ, 25 ^{o}C, 7 days

Colonies were variable in size from 35-45 mm in diameter, plane or centrally lightly crateriform, surface texture velutinous to deeply floccose, mycelium white. Mycelium became greyish green to olive or grey olivaceous due to conidiogenesis. Reverse of the colony was yellow or yellow brown. Exudates and soluble pigment absent.

CYA, 25 ^{o}C, 7 days

Colonies were variable in size from 35-45 mm in diameter, plane or centrally lightly crateriform, surface texture velutinous to deeply floccose, mycelium white. Mycelium became greyish green to olive or grey olivaceous due to conidiogenesis. Reverse of the colony was pale or brown. Exudates and soluble pigment absent.

G25N, 25 ^{o}C, 7 days

Colonies were variable in size from 6-12 mm in diameter, plane, sparse, mucelium when aerial white. Reverse of the colony was pale (Plate 1) or olive. Exudates and soluble pigment absent.

Conidiophores borne from subsurface or surface hyphae, with stipes commonly 70-150 × 5.0-7.0 µm. Stipe was smooth walled, bearing terminal penicillin, when best developed terverticillate but frequently biverticillate or irregular. Rami 20-30 × 5-6 characteristically terminating in well defined verticils of 2-3 metulae, 15-25 × 5.0-6.0 µm. Phialides were ampulliform to cylindroidal, in verticils of 3-5, 10-15(-20) × 4.0-5.0 µm, narrowing abruptly to large cylindroidal collula. Conidia smooth walled, ellipsoidal (Fig. 1) to cylindroidal, 6.0-8.0(-15) × 2.5-5.0-(6.0) µm.

DNA sequencing

A GenBank BLAST search with the present data revealed that the ITS sequences showed 100% similarity with that of *P. digitatum* (JN942856). The resulting approximately 600 bp ITS sequences were

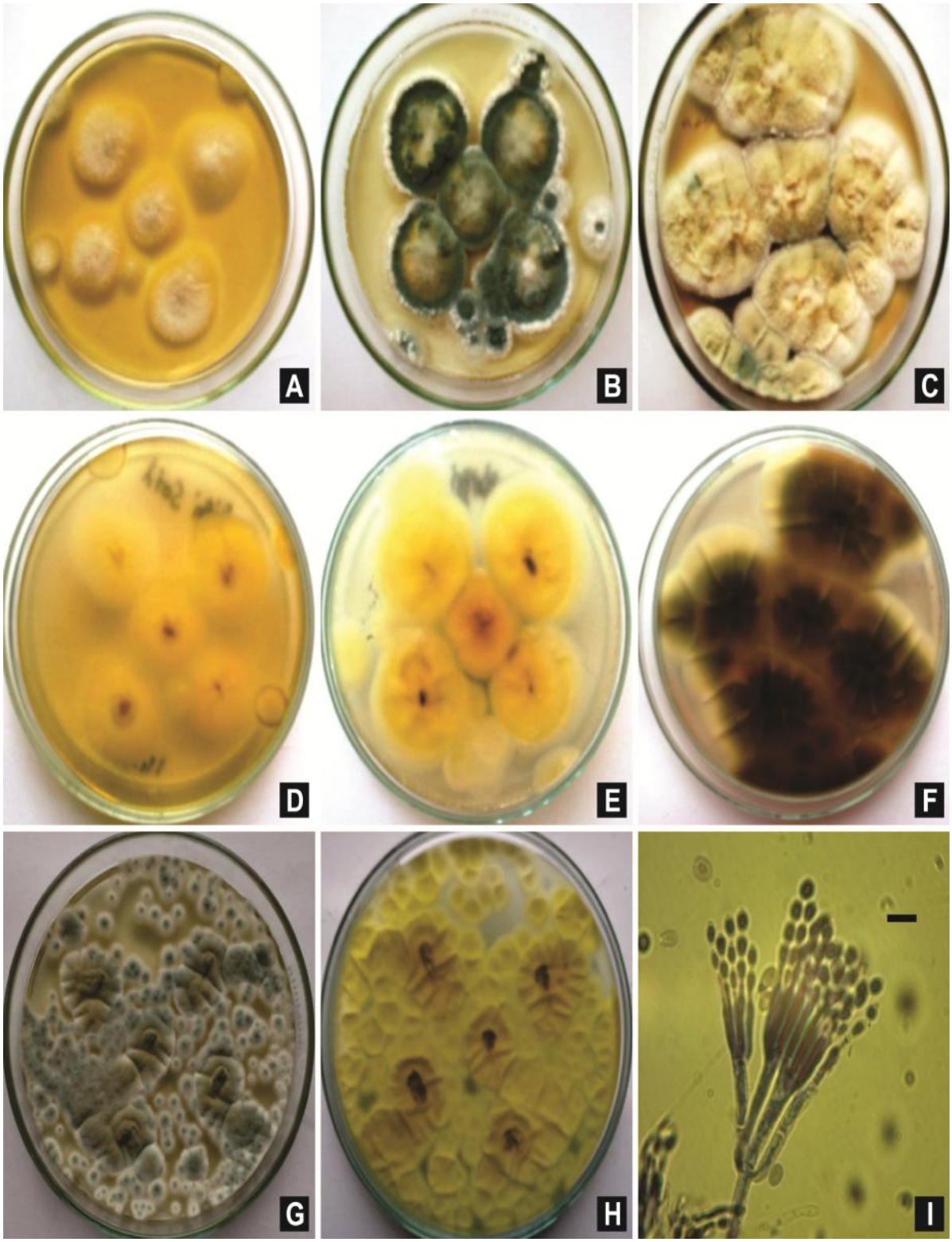

Plate 1: ***Penicillium digitatum***. **A-C & G**, 7-days old Colony at MEA, CZ, CYA and G25N, respectively. **D-F & H**, Reverse on MEA, CZ, CYA and G25N, respectively**. I**, Microphotographs (100 X). (bar = 10µm).

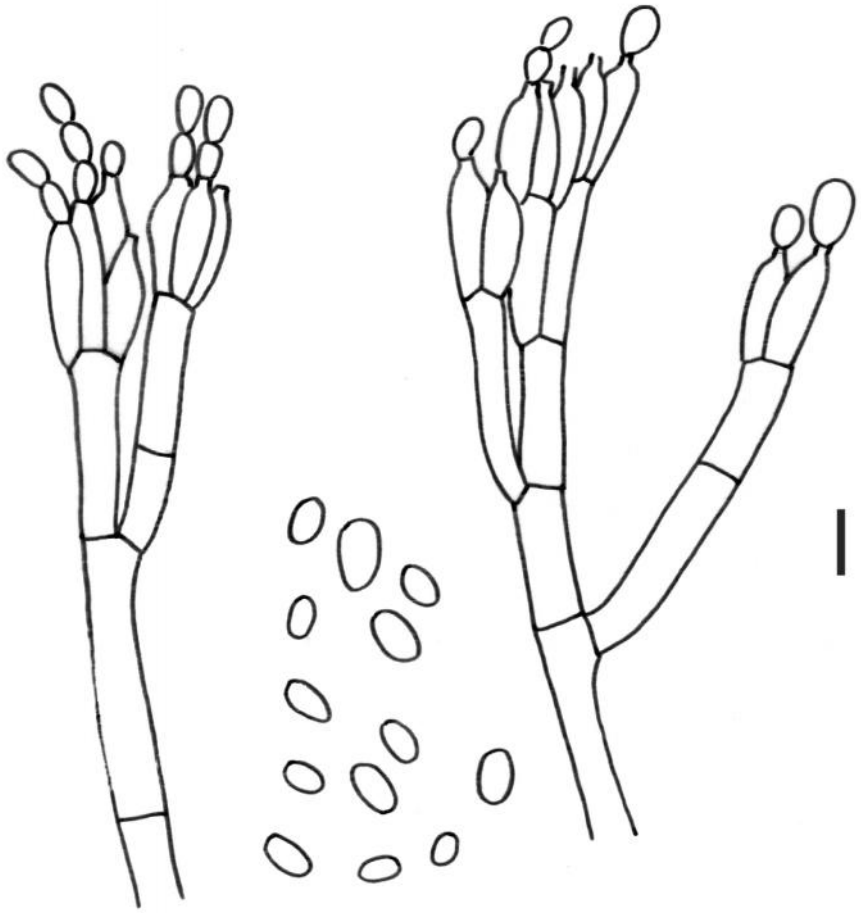

Fig. 1: Conidiophores and conidia of ***Penicillium digitatum***. (bar = 10µm).

deposited in GenBank at NCBI (National centre for biotechnology information) under accession numbers HG326303.

Tests for pathogenicity

Typical symptoms were produced on the inoculated oranges after 7 days. The pathogen from the inoculated oranges was re-isolated on 2 % MEA medium as described above. The morphological and molecular characteristics of the re-isolated organism were compared with the original pathogen. The pathogen was identified from all infected orange samples.

Discussion

Various investigations have also revealed cold tolerance of genus *Penicillium* and demonstrated by the fact that many species grow on food preserved in refrigerators (Pitt and Hocking, 1999). Present findings are coinciding with those reported by other investigators previously with constant presence of *Penicillium* on rotted fruits and vegetables from different areas in the world (Roman *et al*., 2005, Nevarez *et al*., 2008, Katleen *et al*., 2008).

In a survey of Oranges postharvest losses in commercial markets in Lahore, Pakistan, blue mould symptoms were observed on up to 50% of Oranges. After doing pathogenicity test results show that *P. digitatum* is the cause of green mould symptoms on oranges and postharvest losses worldwide (Burden *et al*., 1989). A culture of the fungus has also been deposited at First fungal culture bank of Pakistan (FCBP), Institute of Agricultural Sciences, University of the Punjab Lahore, Pakistan, for further studies.

Results of this research indicated that *Penicillium digitatum* was found to be the cause of postharvest rot of stored oranges (*Citrus* × *sinensis*) in Pakistan. This is the first report of posthavest rot of oranges caused by *P. digitatum* in Pakistan.

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