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NEW RECORD OF A NON-PATHOGENIC ISOLATE OF THE FUNGUS *PENICILLIUM OXALICUM* ISOLATED FROM CITRUS FRUITS

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Abstract

Orange, lemon and mandarin fruits infected with fruit rot were collected from different markets in the city of Karbala in order to isolate the pathogenic fungi from the rotting areas on the infected fruits. The phenotypic diagnosis of the fungi associated with the infection showed the presence of 12 different isolates, all of which belong to the genus *Penicillium*. The pathogenicity test of *Penicillium* isolates under study was performed on intact oranges, lemons and mandarin. Two isolates were selected: the most pathological isolation and the least pathogenic isolation and they were molecularly diagnosed. The molecular diagnosis of *Penicilium* isolates was carried out in the Plant Virus Laboratory of the Faculty of Agriculture - University of Karbala. The two isolates were diagnosed using the Polymerase chain reaction PCR technique and the Nucleotide sequence of the amplified DNA was determined using ITS1 and ITS4. The results of PCR products using the BLAST program indicated that the diagnosed non-pathogenic isolate was related to *P.oxalicum*. The nucleotide sequence analysis of this isolate was compared with isolates of the same species registered in the NCBI database, an d it was found that there was a genetic difference between the recorded isolates and the isolate under study which was registered as a new isolate under an entry number MT742238.

Key words: *Penicillium*, PCR, fungal isolate, diagnosis, citrus

Introduction

Citrus fruits are one of the most important fruit trees and occupy the second place after grapes in international trade and global consumption, as they are rich in vitamin C in addition to sugars, organic acids and some important mineral nutrients, including potassium Salvatava (2010). The world citrus product is estimated at 140 million tons, according to the Food and Agriculture Organization of the United Nations (2016). Green mold disease and blue rot caused by pathogenic fungi, *Penicillium digitatium* and *P.italicum* are the most economically important causes of citrus, which lead to significant post-harvest losses of up to 30-80%, respectively (Otmani-EL *et al.,* 2011).

The genus *Penicillium* includes more than 400 species and thus it is difficult to distinguish its different types on the basis of phenotypic properties. In order to overcome this difficulty, it is preferable to use molecular methods for diagnosis, especially for the diagnosis of economically important fungi (Schroeder *et al.,* 2013). PCR is one of the most important molecular methods by relying on amplification of a specific region of the DNA of the organism and identifying the differences in the sequence of the nitrogenous bases of the DNA of this region and thus knowing the similarity and difference between living organisms. This technique is characterized by high accuracy in identifying genetic differences and avoiding diagnostic errors by traditional methods. PCR technology was used to diagnose many microorganisms, including fungi such as *Penicillium*, *Fusarium* and *R. solani* (Arif *et al.,* 2012; AL-Fadhal *et al.*, 2018). It is important to accurately diagnose the fungi to determine the pathogen, especially those which are very virulent or cause damage to fruits and separators of toxins in different crops or food products (ALhussaini *et al.*, 2016). Therefore, this study aimed to isolate the fungi that cause fruit rot (orange, lanky and lemon), to test the pathogenicity of these isolates on healthy citrus fruits and to use PCR technology to diagnose the most pathogenic isolation.

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Materials and methods

Fungal isolates of *Penicillium* spp.

Orange, mandarin and lemon fruits showing

symptoms of rot characterized by the presence of white areas surrounded by water areas on the crust of the fruit were collected were from the local markets of the city of Karbala - Iraq. The samples were brought to the Plant Pathology Laboratory in the Faculty of Agriculture - University of Kufa in order to isolate fungi accompanying the infection. Fruits were thoroughly washed with running water, cut into 2 cm small pieces and sterilized with NaOCL solution for 3 minutes. Fruit peels were washed with sterile distilled water several times to remove sterilization residues, dried on sterile filter paper and planted in 9 cm Petri dishes containing PDA with antibiotic Chloramphenicol at 200 mg/L. The dishes were incubated at 25 ± 2 ± for three Days, after which the fungal isolates were purified on the same nutritional medium (PDA) by the haifa tip method (source). The isolates were diagnosed morphologically depending on the colony's characteristics including the colony's color, nature and speed of the growth and the pigments produced into the medium, and microscopically based on shape of the conidia and conidiophores according to the taxonomic keys of *Penicillium* (Pitt, 1988).

Molecular diagnosis of *Penicillium* isolates using PCR technology

A molecular diagnosis was performed to confirm the phenotypic and microscopic diagnosis of *Penicillium* isolates under study on citrus fruits in the Plant Virus Laboratory of the Faculty of Agriculture - University of Karbala. DNA was extracted from two *Penicillium* isolates (the most and the least pathogenic isolates), in this study according to the working method attached to the extract kit obtained from Favorgen Company, Taiwan - China.

The polymerase chain reaction was performed using the (Maxime PCR PreMix (i-Taq), Cat. No. 25026) kit that obtained from the South Korean company iNtRoN. A serial polymerase reaction was carried out with a total volume of 20µl which contains 1 µl of each forward initiator (TCCGTAGGTGAACCTGCGG: ITS1) and revers TCCTCCGCTTATTGATATGC: TS4 (White *et al.*, 1990) as well as 1µl of extracted DNA (DNA). All of the above components were placed in the tube supplied by the manufacturer and the volume was supplemented to 20 امايكرو (Nuclease-free water).

The DNA of the two *Penicillium* isolates was amplified in PCR reaction in conditions and steps involved Initial denaturation of DNA for 5 min at 98 C followed by 35 cycles of Final denaturation for 40 s at 94 C°, Primer annealing for 40 s at 55 C°, then Initial elongation of the PCR-amplified product for 1 minute at 72 C° and completion of the reaction with a Final elongation step at 72 C° (Zhang *et al.,* 2012).

For the diagnosis, PCR amplicons for *Penicillium* isolates with ITS1 and ITS4 were sent to the Korean company Macrogen to determine the Nucleotide sequence of amplified forward-revers DNA products. The Basic Local Alignment Search Tool (BLAST) was used to analyze sequences Nitrogenous bases and the results were compared with the globally diagnosed mushroom data available from the American National Center for Biotechnology Information, NCBI.

Pathogenicity of isolated fungi

Healthy oranges, lemons and mandarin fruits were selected, free from scratches, and homogeneous in terms of ripeness, color and size as much as possible. The fruits were washed with a regular washing solution, washed with running water and sterilized with 70% ethyl alcohol for two minutes then dried on sterile filter paper. Each fruits were wounded by making two 5 cm crossing lines (+) slit using flame sterilized blades after each cut. The wounded fruits were inoculated with a strip (2 x 6 mm) from the fungus culture edge grown on PDA, with three replications for each type of fruit and fungal isolate. Fruits inoculated only with strips of sterile PDA served as control. The fruits were placed disposable cork containers and incubated for five days in laboratory conditions at 25 ± 2 C.

The fruits were examined every two days and the

***Penicillium oxalicum* (NCBI registered), Target (new isolate) …. 90%**

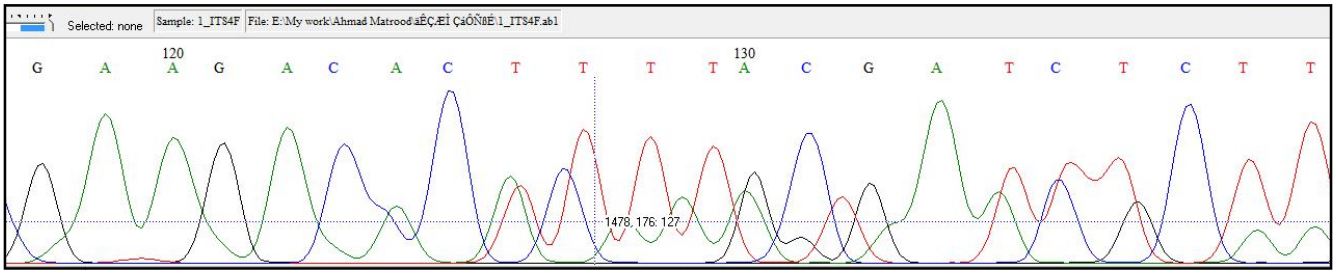

Fig. 1: Sequence variation of some nitrogenous bases of the DNA amplified by PCR from P. oxalicum (* lower) isolated in this study and the closest isolate (upper) of same fungus (CMV010G4) recorded in NCBI under entry number MT742238.

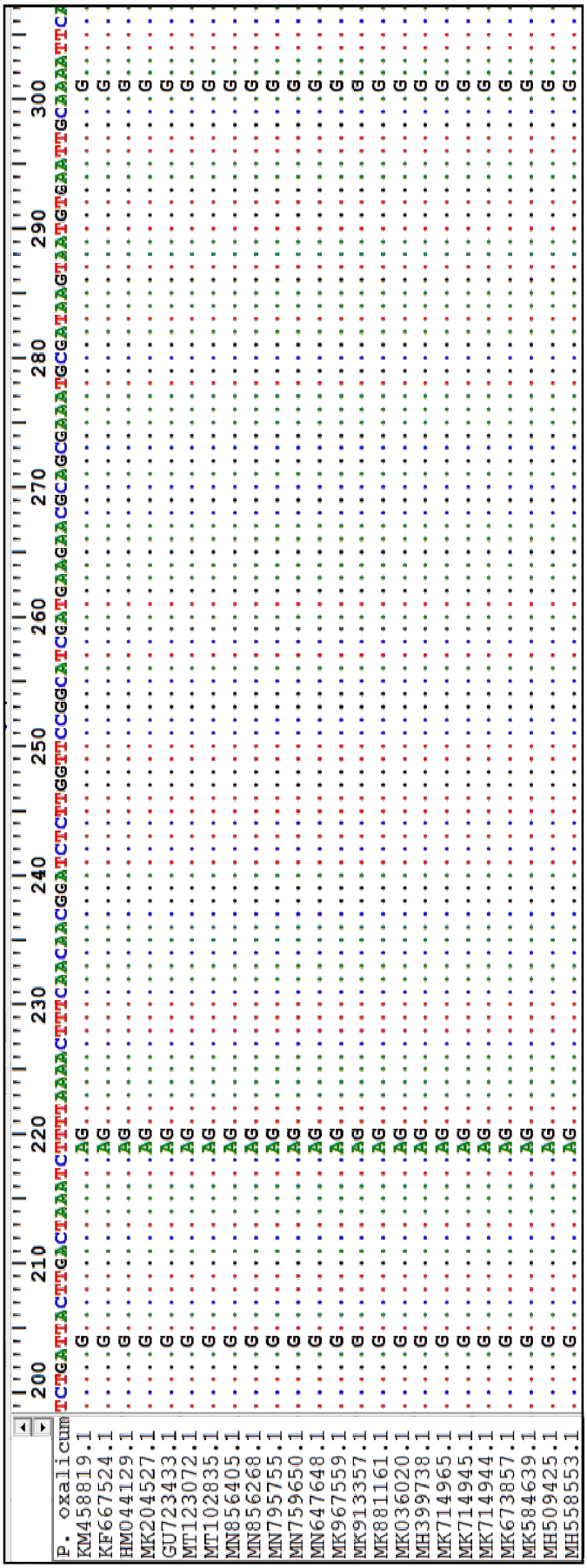

Fig. 2: Nitrogenous base sequences of the PCR-amplified products from *P. oxalicum* isolated in this study which differed in some site locations from the other isolates of the same fungus recorded in NCBI.

development of infection was calculated by measuring two perpendicular diameters of the fungal colony developing on the fruit.

Results and Discussion

Isolation and Diagnosis of *Penicillium* Isolates

Twelve isolates were morphologically identified in the fungus penicllium grown on PDA. 4 isolates PdA1, PdA2, PdA3 and PdA4 from orange fruit, PdB1, PdB2, PdB3 and PdB4 from lemon fruits, and PdC1, PdC2, PdC3 and PdC4 isolated from mandarin fruits. It was found that the isolates pdA1, PdA2, pdA4, PdB1, PdB3, PdC2, pdC3 and PdC4 belong to *Penicillium digitatum* whose colonies are distinguished by the gray-green color and the white septate mycilium, topically brnched erected conidiophores carrying the conidia spores of elongated shape, which are arranged in chains resembling a broom, and the reverse colony's color was brown.

As for the isolates, PdB4, PdB2 and PdC1, they belonged to the *Penicllium italicum*, whose colonies are characterized by bluish green color and white setate fungal mycelium bearing vertically standing conidiophores with apical several branches, each is bearing a chain of conidia. The colony's revers color is also brown. the isolate PdA3 was found to belong to *Penicillium oxalicum* according to the taxonomic key.

Pathogenicity tests

The results of the pathogenicity test table 1 indicated that the pdC4 isolated from infected mandarin fruits was the most pathogenic among the isolates under study. The highest infection development rate was 4.5 cm with the pdC4 isolate after 192 hours, followed by the pdB4 isolate of 3.5 cm while no infection development was recorded for the pdA3 isolate after the same period of incubation. The results also showed that the highest rate of infection was in mandarin with an infection development rate of 2.51 cm, followed by lemons then oranges where the infection development rates were 2.14 cm and 2.43 cm, respectively. The results showed that infection development increased with increasing incubation period regardless of isolation and fruit type, it increased from 1 cm after 48 hours of inoculation to 3.7 cm after 192 hours.

It was observed from the results that orange

Table 1: Pathogenicity of *Penicillium* isolates on orange, lemon and mandarin fruits after inoculation at different incubation period.

Citrus type	Period post inoculation Hour	Diameter of affected area (cm) caused by *Penicillium* isolates													Average of citrus type	Average of incubation period
		Cont.	pd A1	pd A2	pd A3	pd A4	pd B1	pd B2	pd B3	pd B4	pd C1	pd C2	pd C3	pd C4		
Orange	48	0	0.9	1.1	0	0.7	0.7	1.9	0	1.7	1	0	0	2.1	2.14	48h=1
	96	0	2.6	1.8	0	1.6	1.8	2.3	1.7	2.3	2.1	1.3	0.5	3.4		
	144	0	3.3	2.5	0	2.5	2.6	4.6	2.7	4	3.8	1.5	1.2	5.3		
	192	0	4.2	4.8	0	3.1	3.9	5.3	3.2	6	4.9	1.9	1.6	7.1		
Lemon	48	0	1.2	1.2	0	1.2	1.1	2.3	0.9	1.2	1.2	0	1.2	2.4	2.43	96h=1.89
	96	0	3.1	2.3	0	2.1	2.6	2.6	2	3.1	2.4	1.5	1.6	3.7		
	144	0	4	2.6	0	2.6	3	3.6	2.5	5.6	4.1	2	2.1	4.9		
	192	0	5	5.3	0	3.3	4.3	4.3	3.3	6.1	5.5	2.2	2.7	6.4		
Mandarin	48	0	1.4	1.3	0	1.4	1.7	1.9	1.3	1.6	1.1	0	1	2.6	2.15	144h=2.85
	96	0	1.6	1.7	0	1.8	2.5	3	2.2	2.2	2.5	1.7	1.9	4.2		
	144	0	3.1	4.1	0	2.2	3.2	4.2	3.8	4.1	4.7	2.2	2.7	5.6		
	192	0	5	4.9	0	3.3	4	5.1	4.5	4.4	6.1	2.5	3.2	6.9		
Average		0	2.9	2.8	0	2.1	2.6	3.4	2.3	3.5	3.2	1.4	1.6	4.5		192h=3.7
L.S.D. $_{(P\leq 0.05)}$			Isolates = 0.087			Citrus = 0.042			Time = 0.048			Interaction=0.30.				

fruits inoculated with isolate pdC4 recorded the highest diameter of the affected area of 7.1 cm after 192 hours of inoculation. PdB4 recorded infection area of 6 and 6.1 cm on orange and lemon respectively while the same isolate resulted in infection area of 4.4 cm after the same incubation period on mandarin. The variation in the severity of infection among the fruits of different citrus types or varieties may be attributed to the difference in their genotypes and to the phenotypic and physiological characteristics of the fruit peels in addition to the nature of the compounds present (Wang et al. 2014).

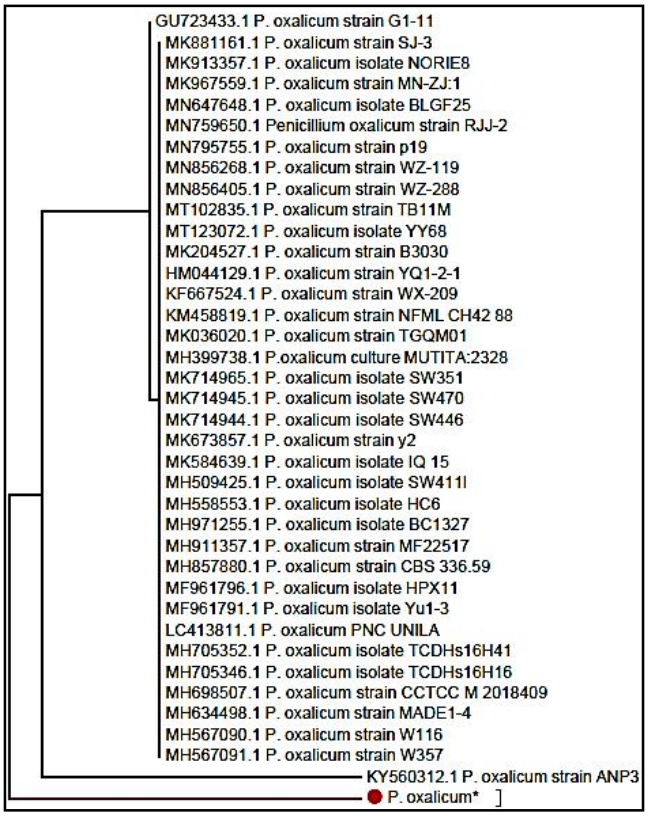

Fig. 3: The Neighbor-joining tree shows the genetic relationship of *P. oxalicum* isolated in this study and other isolates of the same fungus previously registered in the NCBI

Some previous studies have indicated that diagnosis of *Penicillium* species based on their morphological characters alone may give imprecise results for accurate identification (source). Therefore, PCR technique was used in this study in order to diagnose the most and the least pathologic isolates of *Penicillium*.

PCR technology has been used in many studies to diagnose pathogens on plants, due to the high accuracy of this technology in diagnosing many organisms and to avoid problems facing the diagnosis based on phenotypic characteristics. Especially, the phenotypic diagnosis of fungi always requires high experience in this field because some fungal species such as Fusarium types are morphologically very similar (ODonnell *et al.*, 2008).

Zhang *et al.* (2012) indicated that the phenotypic diagnosis is time and efforts consuming, in addition to fungi might be influenced by various environmental factors that may change the colors, sizes and shapes of spores, leading to difficulty in diagnosis. Studying the internal transcribed spacer ITS regions helped in distinguishing the fungal species *Alternaria, Epicoccum, Cladosporium* and *Ulocladium* that attack date palm trees Abass (2016). Many studies indicated that DNA

sequence variations in the ITS region showed high accuracy in diagnosing many fungi of the subspecies *Cladosporium* and *Fusarrium* (Arif *et al.*, 2012; ALhussaini *et al.,* 2016; Al-Fadhal *et al.*, 2018).

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