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Morphological and Molecular Identification of *Penicillium digitatum* Causing Green Mould of Citrus Fruits in Egypt

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Abstract: Nine *Penicillium digitatum* isolates were isolated from citrus fruits and tested for their pathogenicity on Navel orange fruit (*Citrus sinensis* L.). In this regard, the isolates exhibited three different levels of virulence. The isolates were identified morphologically using malt extract agar (MEA) and Czapek yeast extract agar (CYA) for colony characteristics, and MEA for microscopic features. Each one of the isolates has the unique features of *P. digitatum*, especially the largest conidia (6.1-11.9 µm long X 3.2-8 µm width), and phialides (10.1-21.4 µm long X 4-5.1 µm width). In addition, the isolates were identified on a molecular basis using ITS1 and ITS4 primers to confirm morphological identification. Phylogenetic analysis revealed that there was high variance among the isolates, and there were different relations between some of our isolates and other *P. digitatum* registered strains originating from different countries all over the world. The sequences were submitted to respective GenBank nucleotide databases with accession No. OR198852, OR198853, OR198854, OR198855, OR198856, OR198857, OR198858, OR198859 and OR198860. We also isolated a new *P. digitatum* (OB15: OR198859) strain with high virulence and rapid sporulation.

1 Introduction

Citrus fruits have been cultivated for over 4000 years, and they are the most widely grown fruits for human consumption because of their taste, nutritional value, and relatively low price (Albrigo et al 2019). A wide spectrum of products is obtained from citrus essential for humans, becoming used in medicine and other sectors i.e., flavonoids that have anti-inflammatory and anticancer activity (Addi et al 2022). In Egypt, the cultivated area of oranges (*Citrus sinensis* L.) exceeded 129860 hectares in 2021, and the total production of fruits exceeded three million tons in 2021. Meanwhile, Egypt is one of the most important orange exporters in the world by volume (1282563 metric tons) with a value of 714.357 million US$ in 2021, (FAO et al 2023). The contribution of the citrus industry to the Egyptian economy is extensive. The industry generates jobs for more than 500,000 workers through growing, harvesting, handling, storage, transportation, and marketing operations.

Orange fruit is susceptible to infection by pathogenic fungi between harvest and consumption. Wounds that occur on the citrus fruit form passages that help the pathogens penetrate the fruit and consequently cause postharvest wound infection. The most common and economically important disease of citrus fruits after harvest in all production areas with low summer rainfall is green mould caused by *Penicillium digitatum* (Pers.: Fr.) Sacc. (Cheng et al 2020).

By using the traditional identification method that focuses on the texture and color of colonies, the colony rate of growth on selected media, conidiophore morphology inclusive patterns of branching, dimensions as well as embellishments of different parts of the conidiophore and the secretion of certain extrolites, the *Penicillium* species are difficult to differentiate from each other even to expert taxonomists (Pitt 1979, Hawksworth 1990). Also, because these morphological and biochemical characteristics may be influenced or changed by environmental factors, confusion in both identification and taxonomic classification may occur.

Developments in molecular biology during the 1980s resulted in the creation of gene sequences in support of correct differentiation, identification, and classification of species. The fungal molecular identification began when White et al (1990) designed PCR (Polymerase chain reactions) primers for fungal ribosomal genes: 28S or LSU (large subunit); 18s or SSU (small subunit); and the region of ITS (internal transcribed spacer), which includes ITS1 and ITS2, two variable spacers which bracket the conserved 5.8S region. The high conservation of 28S and 18S regions placed them for higher taxonomic declaration. Whereas the ITS1 and ITS2 spacers variability introduced the ITS region as a default marker to study the fungal taxonomy at species–level (Pagano and Rosa 2015). Therefore, molecular methods supplementing morphological ones are very evangelist for the identification of fungal species including *Penicillium* spp. (Khan and Javaid 2021, 2022).

This study aimed to isolate the pathogen of citrus green mould from different regions in Egypt and identify these isolates by morphological methods. In addition, use ITS-rDNA genetic marker to verify species identification and comparison of our ITS sequence to those similar species in the GenBank database.

2 Materials and Methods

2.1 Sample Collection

Samples of citrus fruits with visual symptoms of green mould were collected from orchards and markets located in 9 areas of 5 different governorates in Egypt during the 2018-2019 and 2019-2020 seasons. The samples were placed in separate sterile plastic boxes and transferred as soon as possible to the Postharvest Diseases lab., Plant Pathology Dept., Ain Shams University for the isolation of the pathogen.

2.2 Isolation, Purification, and Maintenance

The infected citrus fruits were surface disinfected with a 2% active chlorine solution for 2 min, washed for 2 min in sterile water for two changes, and dried using sterilized filter papers. Cultures were made by transferring the small pieces of diseased fruit peel surrounded by healthy tissue into Petri dishes containing tap water agar and incubated at 21±1°C. The fungal colonies were microscopically examined after 3 days and were taken separately using the hyphal-tip technique on potato dextrose agar (PDA) slants that were incubated for 5 days at 21±1°C. Serial dilutions of fungal spores for each isolate were completed. On the surface of PDA plates, 0.25 ml of diluted spore suspension was spread equally over the surface of agar then the plates were incubated for 3–4 days at 21±1°C to obtain fungal colonies. The dark yellowish green (olive color) single colonies were considered to be pure isolates (Pitt and Hocking 2009). Pure culture for each isolate was kept on PDA slants until needed at 4± 0.5°C under a phosphate buffer (pH 6.5) (Boeswinkel 1976).

2.3 Inoculum preparation

The pathogen spore suspension (5x 10^5 conidia/ml) of each isolate was prepared as described by Shehata (2015).

2.4 Pathogenicity Test

The fruits of Navel orange were washed, wounded, and inoculated with 25 µl of spore suspension (5x 10^5 conidia/ml) of each isolate to each wound as described by Shehata (2015). Sterile distilled water (SDW) was used to treat wounds of control fruit. Inoculated fruits were placed on glass dishes (7 cm in diameter) in separate sterile boxes lined with well-moistened filter paper on the bottom and covered then the boxes were incubated at 21±1°C and RH ≥ 93% for 7 days. Four replicates for each isolate (treatment), and three fruits were

used in each replicate. Rot development was assessed after the incubation period. The infected area (cm^2) was calculated as $(A/2)^2$ x 3.14, where A= mean of lesion diameter (cm). The fungal isolates were re-isolated as previously described from inoculated Navel orange fruit for identification. Data obtained were subjected to computer statistical software (SPSS Statistics 20) originated by IBM (2011). Data were analyzed using analysis of variance (ANOVA), and mean values were compared using Duncan's multiple range test at a significance level of $P \leq 0.05$.

2.5 Morphological Identification

Nine *Penicillium* isolates obtained from citrus fruits were identified at the species level by the key to *Penicillium* of Pitt and Hocking (2009), and standard parameters of Frisvad and Samson (2004), and Raper and Thom (1949).

2.5.1 Macro-morphology

Colony characteristics were observed on two standard media: MEA and CYA (Pitt and Hocking 2009) at two temperatures (25° and 37°C). Petri dishes (Ø 9 cm) containing MEA and CYA were inoculated with one-week-old culture at three points, equidistant from the center and the edge of the plate, and from each other using a dense conidium suspension (Frisvad and Samson 2004) and incubated in the dark at two temperatures at 25° and 37°C for 7 days. The diameter, color, and texture of the colonies were recorded (Pitt and Hocking 2009).

2.5.2 Micro-morphology

By using the slide culture technique (Taschdjian 1954), microscopic features i.e., stipes, branches, metulae, phialides, conidia measurements, and type of conidiophores of the isolates grown for 7 days on MEA were recorded. After incubation of microscopic slides for 7 days in the dark at 25°C, a drop of 0.1% lactofuchsin stain was added (Carmichael 1955, Pitt and Hocking 2009). Then the slides were examined, and measurement was performed using a compound microscope Leica DM2500 (×400) equipped with a digital camera Leica DFC 320 and software Leica IM 1000.

2.6 Molecular Identification

A culture was developed on PDA for each isolate for 3 days at 25°C before being transferred to a 250 ml Erlenmeyer flask containing 100 ml malt extract broth. The flasks were then incubated at 25°C for 7 days. The grown mycelia were filtrated through sterile Whatman filter paper No. 1. Sufficient quantities of the filtered mycelia were placed into 1.5 ml Eppendorf tubes and stored at -70°C until use (Yin et al 2017).

DNA was extracted by using a modified method of CTAB (cetyltrimethylammonium bromide) as described by Munir et al (2020) and Li et al (2022) as follows: Almost 0.5 g of each isolate mycelium was separated out in liquid nitrogen. The obtained powder was placed into new sterile Eppendorf tubes with adding 1 ml CTAB buffer, then incubated for 30 min in a water bath (65 °C). Later, isoamyl alcohol: chloroform: phenol (5:1:1 respectively, v/v) was added to each tube and centrifuged (12,000 rpm, 10min). Next, the supernatant was transferred into a new sterilized tube followed by adding an isopropanol and sodium acetate mixture (3M) (1:1 v/v) and centrifuged again (12,000 rpm, 10 min). The washing step was carried out by using ethanol (70%) to the resulting pellet. The centrifugation was done once again to dispose of the DNA impurities and the ethanol residues were allowed to be evaporated, then sterile distilled water was added to suspend the pellet of DNA. The obtained DNA samples were kept at -20°C until used later (Munir et al 2020, Li et al 2022). To check the integrity of the DNA, about 5 µl of each isolate was loaded into 1.5 % agarose gel. DNA appears as sharp bands when viewed using the UV transilluminator.

2.6.1 Amplification of ITS region by PCR

Extracted genomic DNAs of the isolates were amplified by using the universal primer pair ITS1(´5TCCGTAGGTGAACCTGCGG-3´) as forward and ITS4 (´5TCCTCCGCTTATTGATATGC-3´) as reverse (White et al 1990). The PCR amplification reaction was performed as described by Li et al (2022). The PCR products were electrophoresed on agarose gel (1.5%). The visualization was conducted under UV light, then photographed, and analyzed by documentation using Syngene Ingenius 3 (Li et al 2022).

2.6.2 Sequence and Phylogenetic Analysis

The ITS fragments were extracted and purified from agarose gel for sequence preparation by using a PCR purification kit (Trans Biotech, Beijing, China). PCR products of targeted bands were sent to the sequence

service (Macrogene, Netherlands) for sequencing. The Sequences were assembled with BioEdit software (Hall 2005) and BLASTn searched for the nearest matches in NCBI (National Centre for Biotechnology Information) GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The GenBank database provided us with the fungal ITS-related sequences for phylogenetic analysis using MEGA11 software (Tamura et al 2021). The phylogenetic tree was constructed to illustrate the relationships that existed among the homologous fungi by using the Maximum Likelihood method and 1000 bootstrap replicates based on the Jukes-Cantor model (Jukes and Cantor 1969). The ITS sequences were aligned using CLUSTAL W. The tree was drawn to scale, with lengths measured in the number of substitutions per site. Twenty-one nucleotide sequences were involved in the analysis.

3 Results

3.1 Sample Collection and Isolation of The Pathogen

Nine isolates of *Penicillium* sp. were isolated from naturally infected citrus fruits (Oranges, and Mandarins) with green mould symptoms that were collected from orchards and markets located in 9 areas of 5 governorates in Egypt, i.e., Cairo, El Beheira, Giza, Menoufia, Qalyubia, during 2018-2020 seasons **Table 1.**

Table 1. Source of *Penicillium* sp. isolates collected from different governorates

Isolate	Host	Location and Governorate	Season
OQ2	Baladi orange	Moshtohor, Qalyubia	2018/2019
OQ3	Navel orange	Sundanhor, Qalyubia	2018/2019
OM5	Navel orange	Quesna, Menoufia	2018/2019
OQ8	Navel orange	Shoubra Elkheima, Qalyubia	2019/2020
MC11	Mandarin	El-Zawya El-Hamraa, Cairo	2019/2020
OG12	Navel orange	H.R. Institute, Giza	2019/2020
OC13	Navel orange	Ain Shams, Cairo	2019/2020
OB15	Navel orange	Nubariya, El Beheira	2019/2020
OQ16	Navel orange	Obour, Qalyubia	2019/2020

3.2 Pathogenicity test

The nine isolates of *Penicillium* sp. were tested as pathogenic agents for causing green mould on the Navel orange fruit. Symptoms were observed 7 days after inoculation. Inoculated Navel orange fruit developed symptoms identical to the green mould, from which the fungal isolates were successfully re-isolated, fulfilling Koch's postulates. Results in **Table 2** indicated that the nine isolates caused complete disease incidence although the differences in disease virulence.

Table 2. Pathogenicity test on Navel orange fruit by obtained *Penicillium* Isolates

Isolate	Lesion diameter (cm)	Area* (cm^2)
OQ2	13.6	144.3 b
OQ3	15.5	189.75 a
OM5	11.8	109.0 b
OQ8	11.6	105.0 b
MC11	12.5	123.0 b
OG12	12.0	113.5 b
OC13	12.3	118.5 b
OB15	16.3	210.3 a
OQ16	9.1	64.5 c

*Area was recorded 7 days after inoculation of wounded fruit with 25 µl $5x10^5$ conidia/ml for each isolate then stored at 21±1°C and RH ≥ 93%.
Means followed by the same letter are not significantly different according to Duncan's multiple range test, (p = 0.05).

According to the statistical analysis of variance, we had 3 levels of significance (a, b, and c). whereas (a) was the most virulent and (c) was the least virulent. Two isolates (OQ3; OB15) were grouped at level a (lesion diameter 15.5-16.3 cm), six isolates (OQ2; OM5; OQ8; MC11; OG12; OC13) were grouped at level b (lesion diameter 11.5-13.5 cm), and only isolate OQ16 was at level c (lesion diameter 9.0 cm). However, it was obvious that isolate OB15 was sporulating rapidly **Fig 1**.

3.3 Morphological identification

3.3.1 Macro-morphology

Morphological investigation revealed that the colonies of all obtained isolates grown on MEA medium for 7 days at 25°C were plane with diameters ranging between 37 and 59 mm, and strictly velutinous

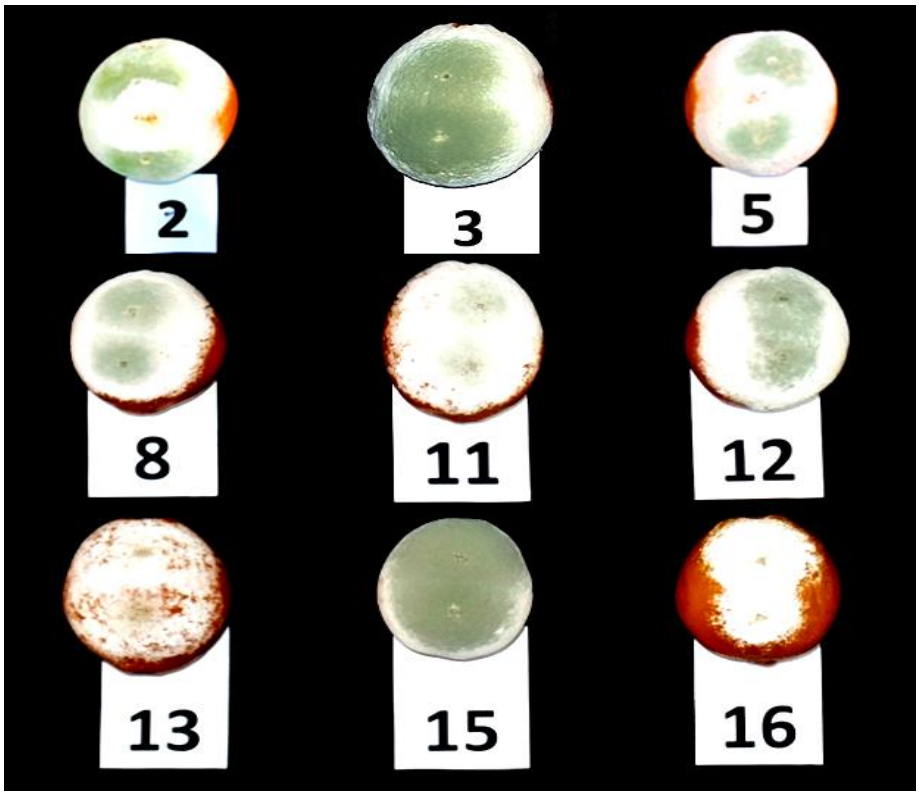

Fig 1. Pathogenicity Test on Navel orange fruit inoculated by a concentration of $5x10^5$ conidia/ml of 9 Isolates of *Penicillium* sp. and stored for 7 days at 21±1 °C

texture with white mycelium. The color of the conidia was greenish olive. While the isolates grown on CYA medium for 7 days at 25°C were plane with diameters ranging between 35 and 45 mm. According to the statistical analysis of the variance of colony diameter, we had 2 levels of significance on MEA and four levels of significance on CYA. No significant difference between isolates no. MC11, OB15, and OQ16 either on MEA or CYA media. **Table 3** and **Fig 2**. However, no mycelial growth was recorded of all obtained isolates either on MEA or CYA media at 37°C.

3.3.2 Micro-morphology

The measurements of conidiophore structures of 9 obtained isolates were recorded. Conidiophores of isolates were borne from the surface or aerial hyphae, stipes length ranged between 61.3-155.3 µm X 4.9 -7.0 µm width, with smooth walls bearing terminal penicilli. Penicilli of isolate MC11 were essentially biverticillate, while the penicilli of the other 8 isolates were predominantly biverticillate and sometimes terverticillate, if terverticillate rami length ranged between 13.4-36.3 µm X 4.5-6.6 µm width. Metulae length ranged between 15.0-26.2 µm X 4.8-6.1 µm width. Phialides were broadly ampulliform 10.1-21.4 µm long X 4-5.1 µm width, and smooth-walled conidia were subglobose to cylindroid 6.1-11.9 µm long X 3.2-8 µm width **Table 4, Fig 3**.

3.4 Molecular identification

3.4.1 Phylogenetic analysis

The PCR products from ITS1 region amplification were approximately 500 bp **Fig 4**. BLASTn searches exposed an extreme similarity (96.57% to 100%) between the ITS sequence of our isolates with those various *Penicillium* species in GenBank. The greatest similarity was with *P. digitatum* (NR_176692) (98.54 % to 100%) **Table 5**.

CLUSTAL W alignment of the ITS region of rDNA nucleotide sequences of nine *P. digitatum* isolates showed the similarity between OQ2, OQ3, OQ16, and OQ8, also few nucleotides variation had been observed in all other isolates **Fig 5**. The sequence data separated the studied isolates into three clear clades and 4 groups/subgroups labeled A, B, C, and D **Fig 6.**

Table 3. Colony diameters (mm) of isolated *Penicillium* sp. grew on MEA and CYA media for 7 days at 25°C

Isolate	MEA		CYA	
	Range	Average*	Range	Average
OQ2	37-41	39.3 b	35-40	36.5 d
OQ3	47-51	48.0 a	41-45	42.8 a
OM5	45-50	47.5 a	35-36	35.5 d
OQ8	37-41	39.0 b	39-43	40.8 ab
MC11	38-45	42.0 b	38-41	39.8 bc
OG12	47-59	50.8 a	35-39	37.0 d
OC13	50-55	52.3 a	37-39	37.8 cd
OB15	38-43	40.3 b	38-41	39.5 bc
OQ16	40-43	42.0 b	37-41	39.8 bc

*Means followed by the same letter at the same column are not significantly different according to Duncan's multiple range test, (p = 0.05)

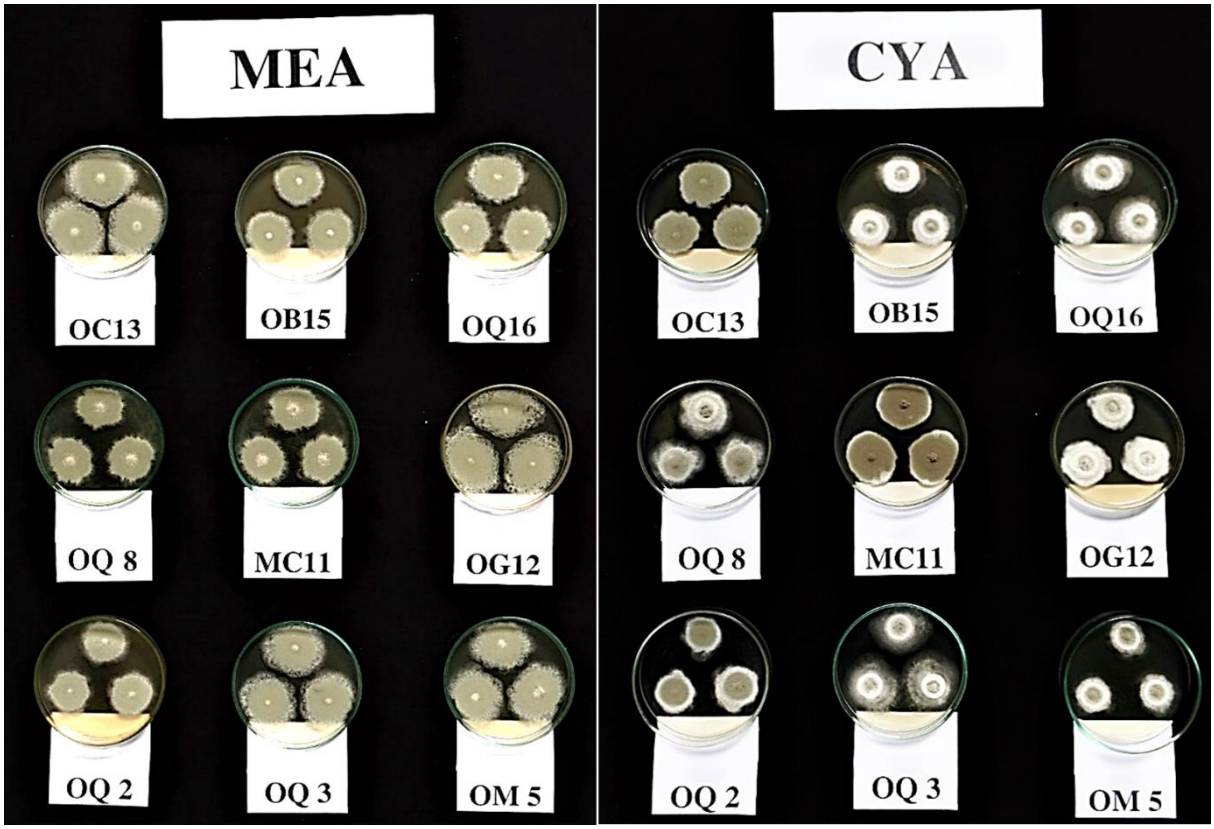

Fig 2. Colony morphology of nine isolated *P. digitatum* grew on MEA and CYA media for 7 days at 25°C

Table 4. The measurements (μ*m*) of conidiophore structures of *Penicillium digitatum* on MEA incubated at 25°C for 7 days

Isolate	Stipe length	Stipe width	Ramus length	Ramus width	Metula length	Metula width	Phialide length	Phialide width	Conidium length	Conidium width
**	60-160	5-7	20-30	5-6	15-25	5-6	10-17	4-5	6-9-14	2.8-6
***	30-100	4-5	-	-	15-30	4-6	15-28	3.5-5	3.5-12	3-8
OQ2	76.5-145.8	5-7	13.4-15	4.5-5.7	15-19.3	5-6	10.1-15.3	4-5.1	6.7-11.2	4.2-5.9
AVG.	95.3	6	14.2	5.1	17.3	5.3	12.9	4.4	8	5.2
OQ3	89.7-154.5	5.1-7	16.3	6	15-21.8	5-6	12-18	4-5	6.5-10.9	4.3-5.7
AVG.	112.6	6.1			18.4	5.3	15.4	4.5	8.2	5
OM5	74.6-155.5	5-6.5	17.5	5.6	15-21.2	5-6.1	13-18.4	4.1-5.1	6.3-9.4	6.1-8
AVG.	99.6	5.8			18.5	5.4	14.9	4.6	8.2	6.8
OQ8	66.9-135.4	5-6.5	36.3	5.8	15-22.9	5-5.5	11.4-21.4	4-5	7.4-10.3	5.7-8
AVG.	98.4	5.6			18.8	5.3	13.9	4.5	8.7	6.8
MC11	70.6-152.7	5.1-7	-	-	16.7-24.8	4.8-5.5	12.8-19.4	4-5	6.4-9.6	4.2-6.2
AVG.	107.8	6.3			20.2	5.1	15.7	4.7	7.3	5.2
OG12	102-155.3	4.9-6.6	20.8	5.1	15-21.8	5-5.5	11-18.7	4-5	6.4-10.1	4.2-5.8
AVG.	126.6	5.8			17.4	5.1	14.0	4.3	7.4	5.0
OC13	64.8-159	5.2-7	16.3-17.6	4.8-6.6	15-20.4	5-6	13.2-18.7	4.2-5	7.2-11.9	4.6-8
AVG.	124.5	6.1	17.0	5.7	17.0	5.6	15.6	4.7	9.1	6.3
OB15	64.9-152.3	5-6.9	15-18.7	4.5-5.3	15.4-26.2	5-5.5	10.2-16.9	4-4.9	6.1-9	3.2-6.1
AVG.	118.5	5.7	16.9	4.9	19.6	5.1	13.2	4.3	7.4	4.5
OQ16	61.3-153.9	5.2-6.7	19.1	6.4	15.2-19.2	5-6	10.9-17	4-5	6.8-11.1	5-6.7
AVG.	105.5	5.8			17.0	5.4	13.2	4.5	8.6	5.9

** Standard parameters of Frisvad and Samson (2004) *** Standard parameters of Raper and Thom (1949)

Table 5. Statistics of the alignment of ITS regions, accession number, total sites, the percentage of the GC content, and percentage of the pairwise identity to *P. digitatum* (NR_176692)

Isolate	Accession No.	Sites	%G~C content	% Pairwise identity
OQ2	OR198852	436	57.6%	100.00%
OQ3	OR198853	434	57.6%	100.00%
OM5	OR198854	455	56.9%	99.76%
OQ8	OR198855	425	58.1%	99.27%
MC11	OR198856	439	57.6%	99.51%
OG12	OR198857	436	58.0%	98.54%
OC13	OR198858	375	57.6%	99.47%
OB15	OR198859	379	58.0%	99.47%
OQ16	OR198860	411	58.2%	99.51%

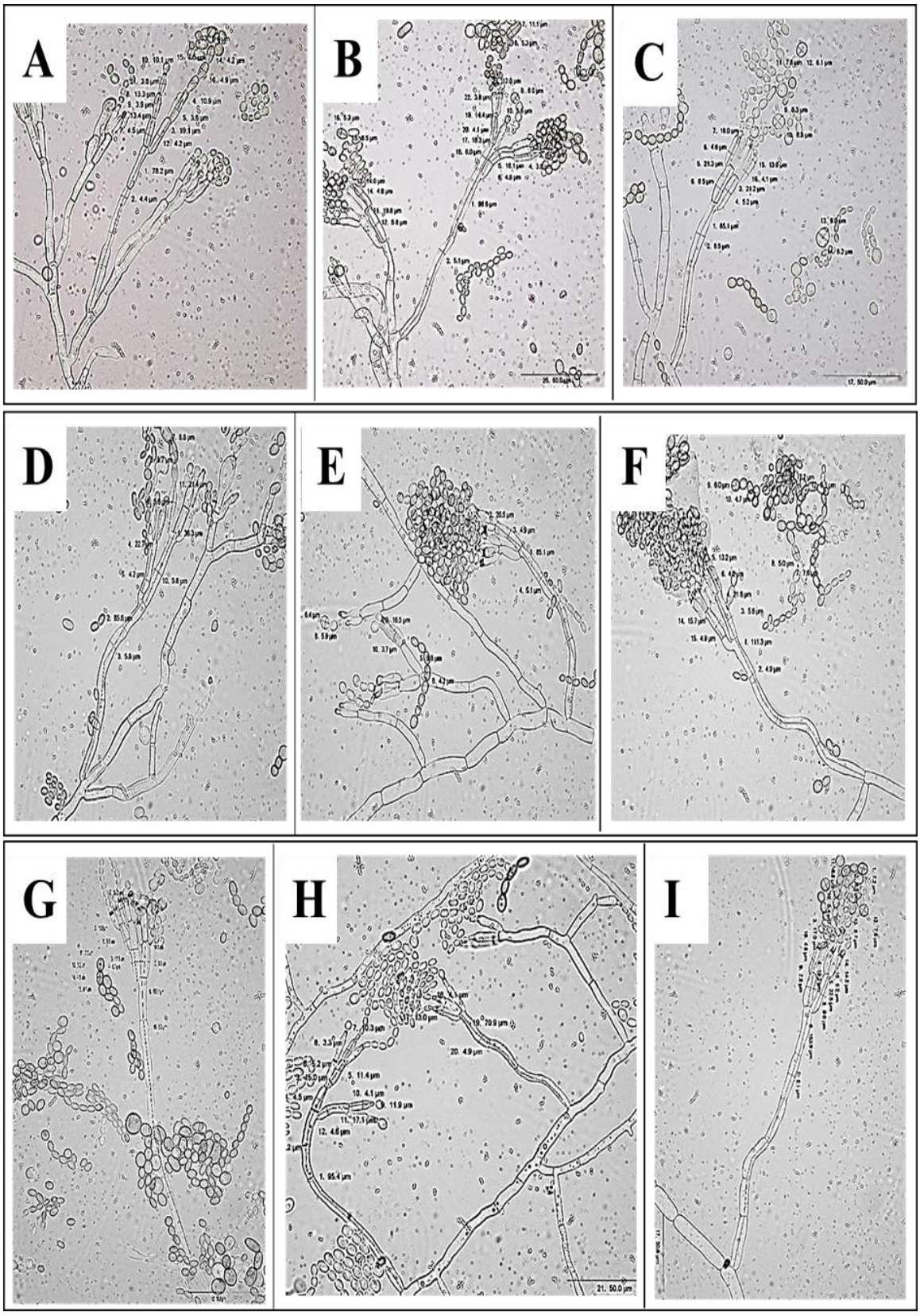

Fig 3. Conidiophores of the *Penicillium* isolates which were grown on MEA and incubated at 25°C for 7 days. (A) Isolate OQ2; (B) Isolate OQ3; (C) Isolate OM5; (D) Isolate OQ8; (E) Isolate MC11; (F) Isolate OG12; (G) Isolate OC13; (H) Isolate OB15; (I) Isolate OQ16

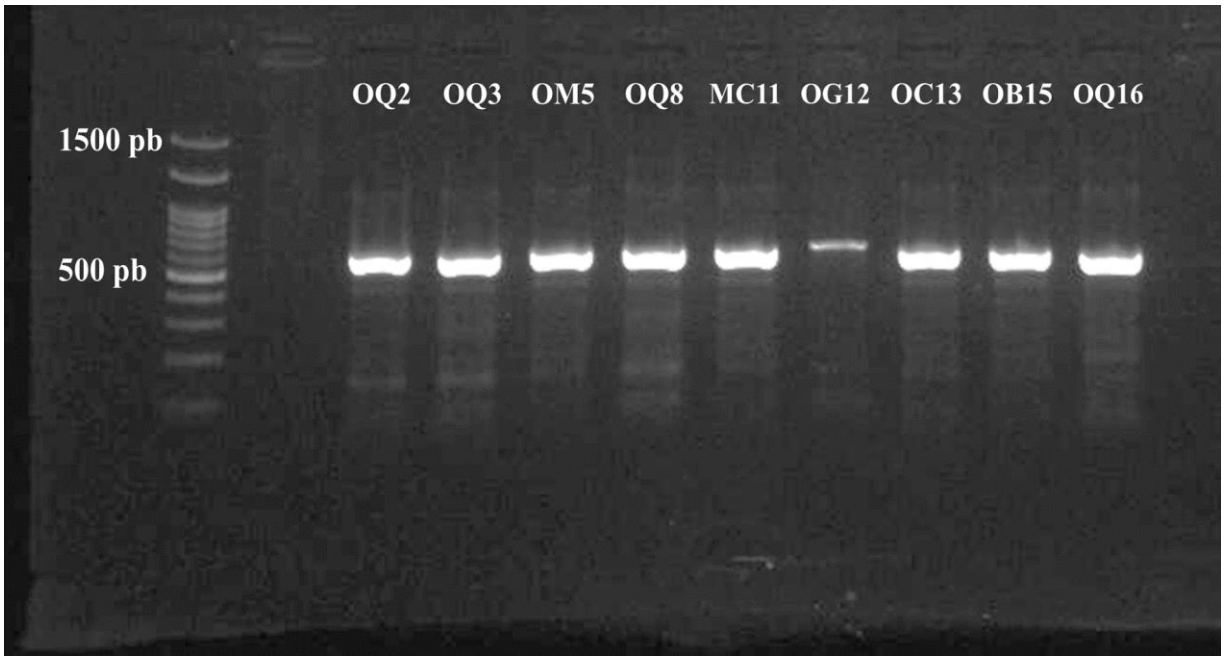

Fig 4. Gel electrophoresis of successfully amplified ITS region of 9 fungal isolates and all bands are presenting in ~500 bp length

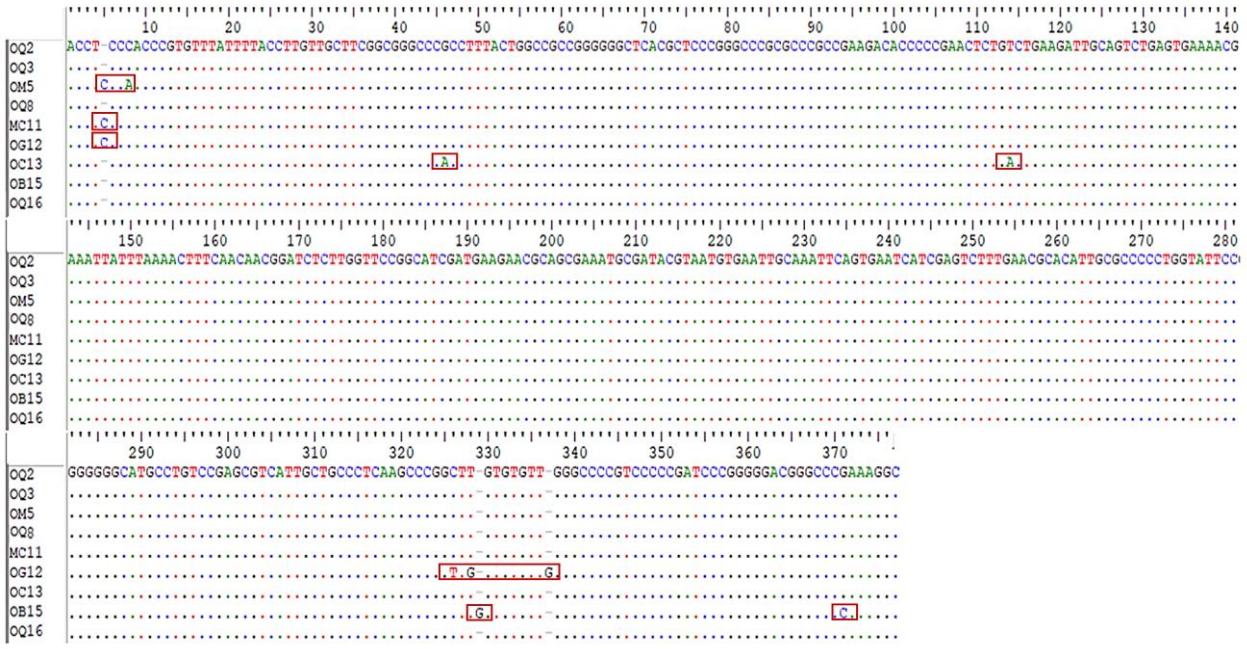

Fig 5. CLUSTAL W alignment of the ITS region of rDNA nucleotide sequences of 9 *Penicillium digitatum* isolates causing green mold disease of citrus fruits

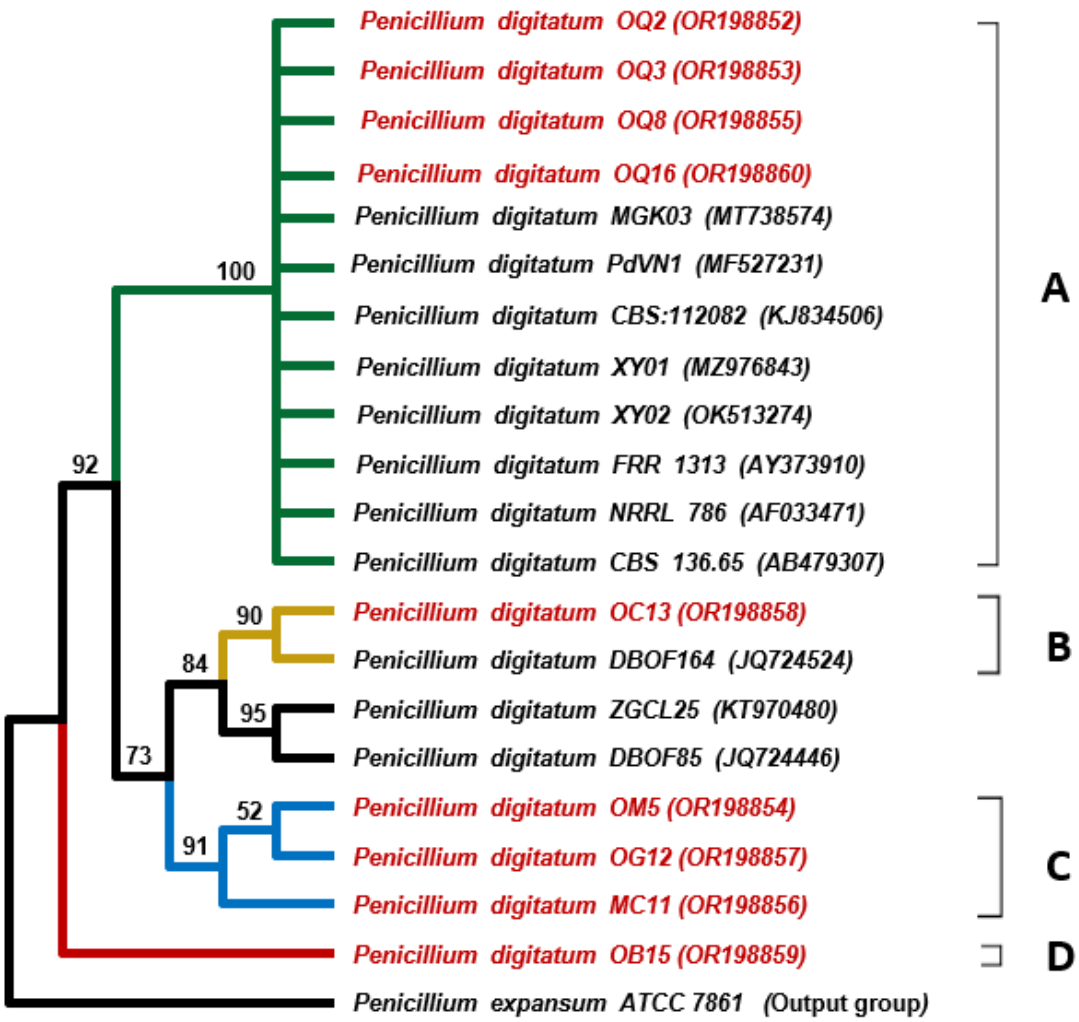

Fig 6. Phylogenetic tree based upon CLUSTAL W alignment of the ITS region of rDNA nucleotide sequences of 20 *Penicillium digitatum* isolates. A maximum likelihood was used, with bootstrap values after 1000 replications of calculated run by using MEGA11 software. The branches numbers indicate bootstrap values. Our own nine sequenced *P. digitatum* isolates are shown in red color. The tree was rooted by *P. expansum* (ATCC 7861)

4 Discussion

Citrus fruits are one of the largest fruit crops which are grown in more than 140 countries around the world. The fruits were severely infected with some *Penicillium* species. Green mould, the serious disease of citrus fruits during shipping and storage caused by the fungus *Penicillium digitatum* leads to huge economic losses worldwide (Cheng et al 2020, Li et al 2022). The nine *Penicillium* isolates identified and examined in the present study caused soft rot of the inoculated healthy citrus fruits, resembling symptoms of green mould, and showed different behaviors when assayed for pathogenicity. There was no significant difference in lesion diameter between the most virulent isolates i.e. OQ3 and OB15 although there was a significant difference between them and all other isolates. Meantime, the isolate OQ16 was the lowest virulent one. However, it was evident that isolate OB15 was the most rapid to sporulate and reveal symptoms.

Although the rate of growth on CYA and MEA media of the two isolates OB15 and OQ16 was very close, their virulence on Navel orange was very far. This result is not in agreement with a previous study by Kumar and Kumar (2017) that reported a strong positive correlation between higher growth rates in media of the fungal bioagent *Metarhizium anisopliae* and its virulence on insects. Also, highly significant correlations were found between the growth rate on the PDA medium of *Verticillium dahliae* and the high disease index of *Verticillium* wilt of cotton (ElSharawy et al 2015), this behavior may be due to differences in the enzyme capability between the two isolates (Armesto et al 2019).

Penicillium identification is a challenging process that has many long and delicate steps. It is based on morphological features incorporating differential media and standardized laboratory conditions. The isolates in this study had colony morphological characters on differential media and microscopic features typical of those described for *P. digitatum* giving us a tentative identification of citrus decay causal agent as *P. digitatum* (Raper and Thom 1949, Frisvad and Samson 2004, Pitt and Hocking 2009).

However, without the aid of molecular technologies, it is frequently challenging to precisely characterize *Penicillium* species because many of the common species share a great deal of macroscopic and microscopic characteristics, and at the same time, there is variability within the species (Hawksworth 1990, Pitt 1979), which is why further identification required other approaches to separate between differing types of *Penicillium* species. Therefore, the precise and reliable identification of closely related *Penicillium* species requires the use of molecular techniques (Akhtar et al 2013, Khan and Javaid 2023).

Phylogenetic investigations by fungal ITS regions of ribosomal DNA are extensively used in genomes because ITS region is an accepted genetic barcode universally, it introduces greater taxonomic determination concerning the different genera of fungi due to the high success amplification and sequencing rate (Javaid et al 2018). This high conservation of the ITS region facilitated the investigation of phylogenetic studies by the utilization of PCR amplification of rDNA (Gardes and Bruns 1993, Li et al 2020).

The results of BLASTn the rDNA-ITS sequence data from Egyptian isolates showed a close similarity to several *Penicillium* species, being the highest similarity with *Penicillium digitatum*. The generated phylogram assured the obtained results from the BLASTn searches. In this study, we used ITS1 and ITS4 primers to differentiate *Penicillium* isolates. Our *Penicillium digitatum* isolates were grouped into 4 groups/subgroups.

Group A included 4 isolates (OQ2, OQ3, OQ16, and OQ8) originating from 4 locations in one governorate (Moshtohor, Sundanhor, Obour, and Shoubra Elkheima in Qalyubia governorate) with a bootstrap of 100 with strains from, Italy (Visagie et al 2014), USA (Haugland et al 2004), South Africa (Tapfuma et al 2022), Japan (Kiyuna et al 2011), and Vietnam (Vu et al 2018).

OC13 (originated from Ain Shams, Cairo governorate was moderately virulent and the lowest at conidia production and symptoms revealing) branched in subgroup B with a strain from India (Velmurugan et al 2013).

Group C included 3 Isolates (OM5, OG12, and MC11) that originated from 3 different locations and governorates precisely (Quesna, Menoufia; H.R. Institute, Giza; and El-Zawya El-Hamraa, Cairo); however, those isolates had the same level of virulence and grouped with a bootstrap of 91 with each other and also bootstrapped by 73 with strains from India (Velmurugan et al 2013) and Pakistan (Kalim and Ali 2016).

Group D included only one unique isolate OB15 originating from Nubariya, El Beheira, which was the heaviest and fastest sporulated isolate with no bootstrapping to any NCBI-submitted *P. digitatum* strains. *In vitro*, this strain may swiftly rot orange fruits and produce green mould.

5 Conclusion

The results show that the variance among *P. digitatum* isolates in their virulence and origins may be an indicator of the variations in molecular features. The isolates were identified morphologically as *P. digitatum* that were recognized by large conidia and phialides. The molecular identification revealed that there is a unique *P. digitatum* Egyptian strain (OB15: OR198859) which would be difficult to explore by morphological identification.

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