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Occurrence of toxigenic *Penicillium polonicum* in retail green table olives from the Saudi Arabia market

Ahmed Mohamed Aly Khalil [a,b], Amr Hosny Hashem [b,*], Amer Morsy Abdelaziz [b]

[a] Biology Department, College of Science, Taibah University, Yanbu, Postal Code 41911, Saudi Arabia
[b] Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, 13759, Egypt

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ABSTRACT

In Saudi Arabia, table olives are widely consumed as appetizers, pickles, and food additives. Many food-borne fungal pathogens such as *Penicillium* specie have ability to survive on prepared and preserved green table olives. *Penicillium polonicum*, an important mycotoxin producer, is much more frequently found in cereals, peanuts, onions, dried meats, citrus fruits and recently in frozen food. The aim of this study was to determine, identify and characterize the occurrence of mycotoxin producing *Penicillium* species from retail green table olives purchased in the Saudi Arabia markets and used for human consumption. Our results revealed that *Penicillium expansum*, *P. citrinum*, *P. verrucosum* and *P. polonicum* were isolated form all samples. *P. polonicum* was the only species that able to grow producing vegetative and reproductive structures in refrigerators. The qualitative determination of citrinin, penicillic acid and cyclopiazonic acid is positive in all investigated samples. This study provides insight into the presence of toxigenic *P. polonicum* not only as a mould but also as a source of toxic metabolites that produced inside food products. Thus, the application of prevention actions should be attained. Furthermore, *P. polonicum* is reported here for first time as both an inhabitant and a pathogen on green table olives.

1. Introduction

Olea europaea L. (Olive) is a cosmopolitan product consumed by human as a part of their diet. More than 90% of olives agriculture distributed in Mediterranean countries which play a vital role not only in an economic contribution but also in an environmental aspects (Cillidag, 2013). Table olives are a term that used to elucidate olives when used as a food rather than being used as a fresh fruits or for oil production. While fresh olives are very bitter in taste, it cannot be used directly without fermentation processes which change it to be more palatable for consumer (Omar, 2010). Many people in Mediterranean countries including Saudi Arabian prefer the scratched or cracked green table olives which produced in loose or packed (canning) form. During fermentation processes of scratched or cracked green olives, the brine and olive fruits can be contaminated by fungal spores which grow during storage in markets resulting in appearance of visible fungal hyphae and spores. Consequently appearance, flavour, taste, and texture of table olives are quickly altered and considered as food spoilage (Franzetti et al., 2011).

Aspergillus and *Penicillium* are the most isolated genera that and cause the fungal spoilage of table olives (Fernández et al., 1997). The growth of these fungi on table olives makes it unaccepted by consumers in addition to their effect on the safety of table olives since mycotoxins may be produced. During preparation and storage of black table olives, *Penicillium verrucosum* and *P. citrinium* were linked to citrinin and ochratoxin A (OTA) production while, aflatoxin B1 (AFB) was linked to *Aspergillus flavus* which also had been isolated during preparation (El Adlouni et al., 2006; Ghitakou et al., 2006; Heperkan et al., 2006, 2009). In addition to OTA and AFB, citrinin, penicillic acid and patulin were also observed in many table olives products (Bavaro et al., 2017). Although, the detected level of toxic metabolites of fungi in table olives was very low to cause a disease, table olives considered as a potential source of fungal toxic metabolites (Medina-Pradas and Arroyo-López, 2015). For this reason, some precautions should be recommended to avoid the presence of fungi and their mycotoxin production along the entire table olive processes including a good handling, storage temperature, salinity and packaging processes (Bavaro et al., 2017).

Penicillium polonicum K. Zaleski is a psychrotolerant xerophilic fungus associated with spoilage of many food and food products such as onions, cereals, dried meats, peanuts, yam tubers and citrus fruits (Frisvad and Samson, 2004; Duduk et al., 2014; Wang et al., 2015). Many harmful metabolites including citrinin, penicillic acid,

* Corresponding author.
E-mail addresses: Amsuliman@taibahu.edu.sa (A.M.A. Khalil), amr.hosny86@azhar.edu.eg (A.H. Hashem).

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nephrotoxic glycopeptides and verucosidin can be produced by this species (Duduk et al., 2014). This study aimed to identify the blue moulds that appear on the pitted and stuffed loose table olives from retail and supermarket in Saudi Arabia. Moreover, study also designed to assess the presence of citrinin, penicillic acid and cyclopiazonic acid in green table olives prepared by Spanish style of Saudi Arabia origin. Pitted and stuffed loose products were selected in particular because the poor hygiene practices are always present.

2. Materials and methods

2.1. Sample collection and isolation

Samples of green table olives were prepared by Spanish-style. Initially, green table olives treated with alkaline solution 2–3.5% sodium hydroxide (w/v) for 4–6 h; then olives were washed repetitively with tap water in order to remove the excess of alkali, finally olives were brined in 8–10% sodium chloride (w/v) where microbial fermentation takes place (Heperkan, 2013). The green table olives were sold in loose with or without symptoms of blue mould purchased from 8 supermarkets in Yanbu city, Saudi Arabia during winter 2016/2017 and kept directly in fridge at 5 - 10 °C for further investigation. Isolation of the fungi was performed by plate diluting or direct transfer technique of blue mould into malt extract agar (MEA) in Petri plates and incubated at room temperature for 7 days. After incubation period, the plates were examined, and the cultures of fungal isolates were transferred to sterile MEA.

2.2. Physicochemical analysis of green table olives and brines

The pH of brines was measured using bench-top pH meter 5500 while the acidity of Brines was determined by titrating brines with a standardized solution of 0.1 M NaOH solution using phenolphthalein as an indicator and expressed as % (w/v) of Lactic Acid. Furthermore, the salt concentration was measured by titrating brines with a standardized solution of $AgNO_3$ (0.1 M) with potassium chromate as an indicator and the results expressed as % (w/v) sodium chloride. Eventually, the residual Sugar Levels were assessed using Fehling method (Kailis and Harris, 2004).

2.3. Macroscopic examination

For studying the morphological features of our isolate, three-point inoculated on media: Czapek yeast extract agar (CYA), malt extract agar (MEA), and potato dextrose agar (PDA) using a dense conidial suspension and incubated at 25 °C (Frisvad and Samson, 2004). Moreover, creatine sucrose agar (CREA) was used to investigate the acid producing by toxigenic *Penicillium* species (Frivsad, 1985). After 7 days the colony diameters were measured. Culture characteristics including colony appearance, surface pigmentation and reverse coloration were evaluated, and three plates per media were inoculated for each isolate.

2.4. Microscopic examination

Microscopic examination was achieved by ripping apart of mycelium in a drop of tween water solution (20/25 ml) or lactophenol blue and examining under bright field microscopy provided with camera. Shape of conidiospores, branching of conidiophores, stipe ornamentation, matula and phialide were examined.

2.5. Scanning electron microscope

Scanning electron microscope was achieved to approve the morphological features of fungal isolate. A small part from the fresh culture of isolate was fixed in 2.5% Glutaraldehyde for 15 min to allow a rapid inter and intra-cellular penetration. The samples were dehydrated by series dilution of ethanol. Acetone was used as intermediate fluid due to it is forming a homogeneous mixture when mixed with carbon dioxide. Critical point drying (CPD) was used to prevent collapse in environmental scanning electron microscopy (ESEM). The specimens were sputter-coated with gold using an Emitech K550X coating unit. The coated samples were then loaded into Environmental Scanning Electron Microscopy FEI (Quanta 200) and detected over a range of magnifications.

2.6. Ehrlich test

According to Lund (1995), the production of cyclopiazonic acid and other alkaloids produced by toxigenic *Penicillium* species was examined by Ehrlich reagent. Preparation of Ehrlich reagent was performed by dissolving 2 g P-dimethylaminobenzaldehyde in 85 ml 96% ethanol, with 15 ml 10 N HCl added. Three disks of agar (8 mm) were cut from the middle of a 7-day old fungal culture medium grown on CYA at 25 °C and a filter paper (~5 × 5 mm) moistened in Ehrlich reagent was positioned on top of the vegetative hyphal side. Subsequently, the reaction was recorded after 5–10 min. The positive results of this reaction means the appearance of violet ring which also means the culture contains cyclopiazonic acid or related alkaloids. Moreover, pink to red or yellow ring means that the fungal species have ability to produce other types of alkaloids. If the positive results of the reaction comes after 10 min it is considered as weak. The experiment was repeated 3 times.

2.7. DNA isolation, PCR amplification, DNA sequencing and phylogenetic analyses

DNA was extracted from agar cultures using Quick-DNA Fungal/Bacterial Microprep Kit (Zymo research; D6007) following the manufacturer's protocol and supported by Sigma Scientific Services Company (Egypt). PCR was performed by using Maxima Hot Start PCR Master Mix (Thermo; K1051). The primers used were Forward ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and Reverse ITS4-R (5′-TCCTCCGCTTATTGATATGC-3′) according to Visagie et al. (2014). The reaction conditions were: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1.5 min; a final extension phase was performed at 72 °C for 10 min. At first, the following components were added for each 50 μl total reaction volume at room temperature: Maxima Hot Start PCR Master Mix (2X) 25 μl, ITS1 Forward primer 1 μl (20 μM), ITS4 Reverse primer 1 μl (20 μM), Template DNA 5 μl, and Water, nuclease-free 18 μl. The obtained PCR product was purified using GeneJET PCR Purification Kit (Thermo K0701) following the manufacturer's protocol and stored at −20 °C. The resulting amplicon was visualized by horizontal electrophoresis on 1% agarose gel against GeneRuler 100 bp Plus DNA Ladder.

Finally, sequencing to the PCR product in GATC Company (Germany) by use ABI 3730xl DNA-sequencer was performed using the same forward and reverse primers mentioned above and by combining Sanger and 454 technologies for DNA sequencing according to manufacturer's instructions. The obtained ITS sequences were aligned by Clustal W (codons) with the required minor manual adjustments. The final sequence was compared with similar sequences retrieved from DNA databases by using the NCBI n-BLAST search program in the National Canter for Biotechnology Information (NCBI). Evolutionary analyses were conducted in Molecular Evolutionary Genetics Analysis MEGA-X (Kumar et al., 2018).

2.8. Citrinin, penicillic acid and cyclopiazonic acid analysis for green table olives

The method of extraction and separation of citrinin, penicillic acid and cyclopiazonic acid which described by (Comerio et al., 1998) was modified. Olive samples (25 g) were mixed with acetonitrile (180 ml), 4% KCl (20 ml) and 20% H_2SO_4 (2 ml), the mixture were blended for 2 min at high speed and filtered through Whatman No 4 filter paper. After filtration, (50 ml) of hexane was added and shake for 15 min in a separating funnel. After separation of layers, (100 ml) from the lower layer were reassigned into a second separating funnel and then extracted with (50 ml) chloroform and (25 ml) distilled water. The extract was evaporated to dryness under nitrogen and used for thin layer chromatography (TLC) analysis.

Silica gel plates were dipped into 10% glycolic acid in ethanol solution for 2 min and dried for 10 min at 110 °C. The plates were developed in a solvent mixture of toluene-ethyl acetate-chloroform-90% formic acid (70:50:50:20, v/v/v/v). The plates were dried and treated with ammonia vapour for 10–15 s (Martins et al., 2002).

For the analysis of citrinin and penicillic acid, the plate were treated with NH_3 vapour (10 mm), followed by heating plate for 5 min at 1100 in oven to give a yellow fluor and blue fluor respectively at 360 nm. While cyclopiazonic acid; plate was spraying with Ehrlich reagent to give daylight violet.

2.9. Statistical analysis

All experiments were achieved in triplicates. Data are presented as mean value ± standard deviation (S.D.).

3. Results

3.1. Symptoms on collected green table olives

Eight samples of green table olives collected from different retails and supermarkets in Yanbu city Saudi Arabia showed presence of some *Penicillium* species namely; *Penicillium expansum*, *P. citrinum*, *P. verrucosum* and *P. polonicum*. Nevertheless, only *P. polonicum* exhibited a bluish grey sporulation extended on the surface and inside pitted table olives after kept in fridge for 21–30 days (Fig. 1).

Physicochemical parameters of the brine and table olives including reducing sugar, lactic acid levels, pH values and brine salt concentrations are the most significant factors that used for preservation and packing the table olives. The pH of the brine should be between 4 and 4.5 while the salt concentration should be up to 7% (Kailis and Harris, 2004). Our results revealed that pH value and salt concentration were 5.5 and 6.2% respectively which considered above the standard levels (Table 1).

3.2. Macroscopic and microscopic characters of *P. polonicum*

Colonies of isolate was velutinous, pale yellow with a white margin on PDA and CYA and bluish grey also with white margin in MEA. Reverse colour on PDA, CYA and MEA was cream to yellowish brown. Clear droplets of exudate were present on CYA. The colony diameter on PDA 28 ± 1 mm; on CYA 29.5 ± 0.5 mm; and on MEA was 30 ± 1 mm. No growth was observed on MEA when the isolate incubated at 35 °C. Microscopic characters were measure on MEA medium. Conidiophores of isolate were terverticillate rising from septate stipes with smooth to finely roughened walls, while the phialides were obviously ampulliform. Conidia were spherical to sub-spherical, smooth-walled, and borne in chains. Conidial diameter were 2.8–3.8 (3.2) × 2.4 to 3.4 (2.9) μm (Figs. 2 and 3; Table 2). Morphological characters in according to the previous describing were for *P. polonicum* K. Zaleski.

Culture characteristics on Creatine Sucrose Agar (CREA) medium showed that the isolates produce acid where the bromochresol purple changed from purple to yellow colour. The positive result in CREA means that the isolate was toxogenic and produce a mycotoxins (Fig. 4).

3.3. Ehrlich test

In reaction with Ehrlich reagent the isolates formed a violet ring in 5–10 min which means isolates produced alkaloids including a cyclopiazonic acid (Fig. 5).

Table 1
Physicochemical characteristic of green table olives and brines.

Reducing sugars in green table olives (%)	pH of brine	Titratable acidity (g of lactic acid/100 ml of brine)	NaCl of brine (%)
3.5 ± 0.2	5.2 ± 0.4	0.31 ± 0.4	6.2 ± 0.2

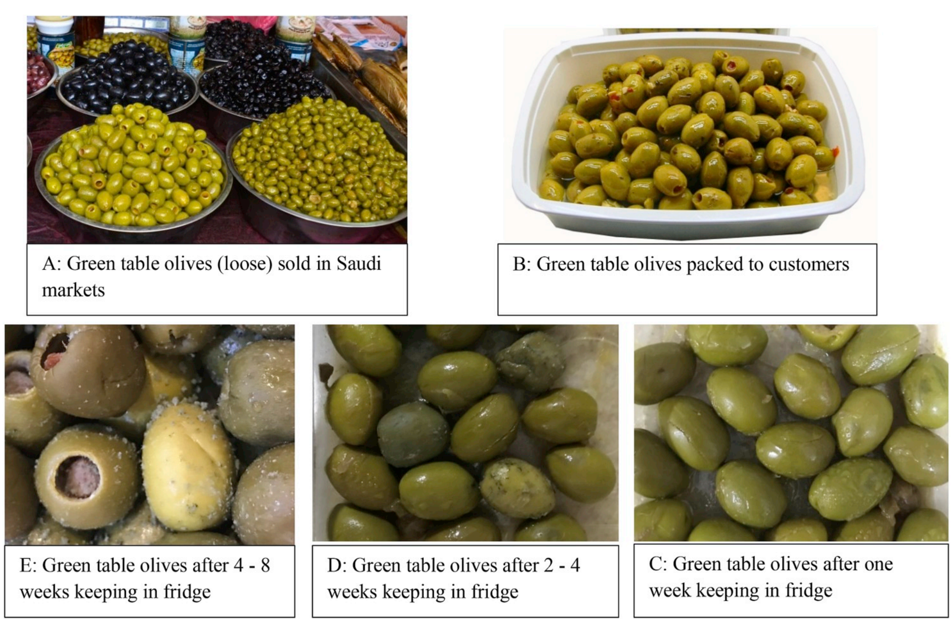

Fig. 1. From market to table, steps of green table olives to become spoilage with blue mould. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

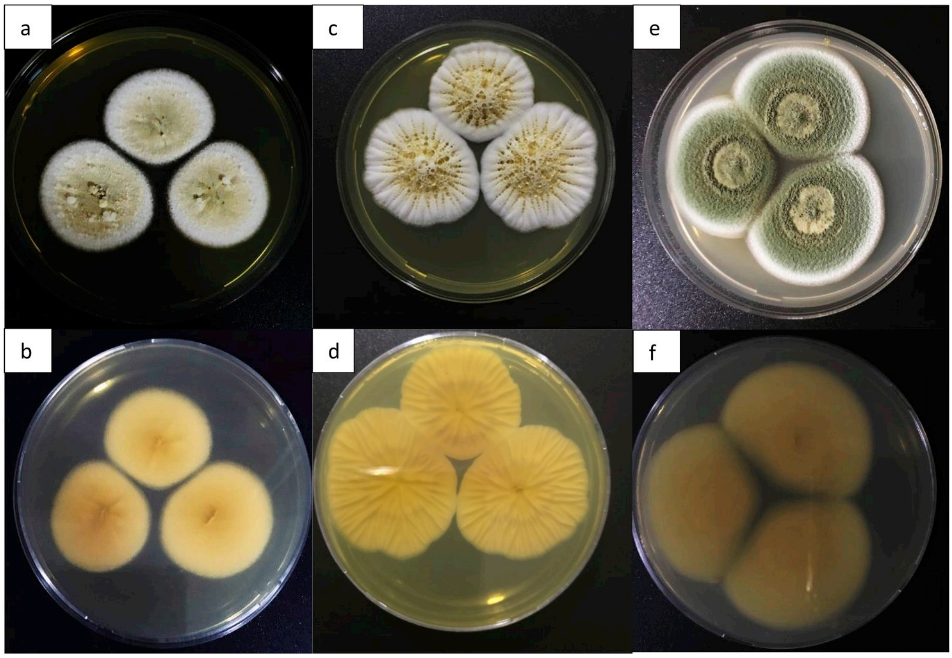

Fig. 2. Colony morphology on different media: a – PDA obverse, b – PDA reverse, c – CYA obverse, d – CYA reverse, e – MEA obverse, f – MEA reverse.

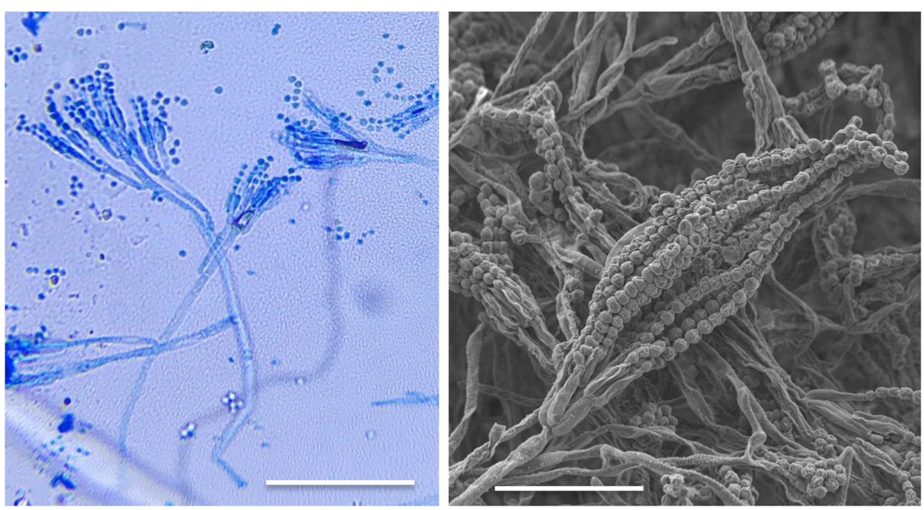

Fig. 3. Bright field and scanning electron micrographs of *Penicillium polonicum* showing terverticillate conidiophores, smooth septate stipe and spherical conidia in chains a– Bar = 250 μm; b – Bar = 25 μm.

Table 2
Culture characteristics of *Penicillium polonicum* in MEA medium.

Characters	Examination on MEA
Growth characteristics	Conidia are blue green with a strong blue element and colonies have a distinct yellow reverse, often with the yellow colour diffusing into the medium
Conidiophore	Conidiophores two-stage branched (terverticillate)
Stipes	Septate and finely rough-walled
Matula and phialides	Ampulliform, 10 μm
Conidia	Conidia smooth and globose to subglobose, 3.2–2.9 μm in diam.

3.4. Molecular identification and evolutionary analyses of ITS rRNA gene of the isolate under study

PCR of ITS rRNA gene produced the expected amplicon size of approximately 600 bp in length (Fig. 6). The obtained ITS assembled sequence was 575 bp in length and deposited in GenBank under accession MK271277.1. This sequence of *Penicillium polonicum* strain KHA, encompassing internal transcribed spacer 1, partial sequence; 5.8 S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. The sequence of KHA strain was closely related to the sequence of *Penicillium polonicum* isolate FZ-17 (GenBank accession, KF848936.1) and showed 99% identity with it. An evolutionary relationship to the nearest 25 *Penicillium polonicum* strains retrieved from GenBank was constructed through MEGA-X (Kumar et al., 2018). The evolutionary relationship was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.01522904 is shown (Fig. 7). The proportions of three repeat trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are revealed next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the matching units as those of the

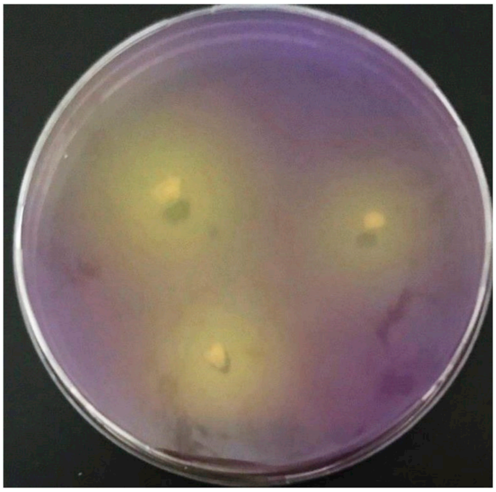

Fig. 4. Colony morphology on creatine sucrose gar medium showing acid production by *Penicillium polonicum.*

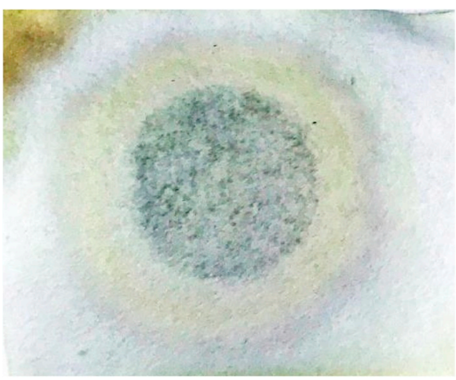

Fig. 5. Violet rings resulting from Ehrlich test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

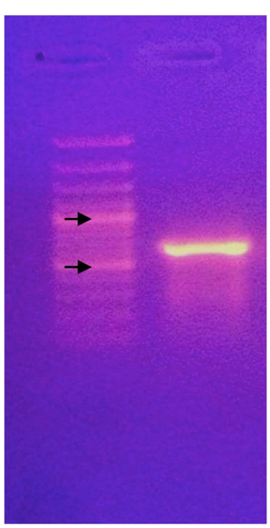

Fig. 6. PCR amplicon (Lane2; 600 bp) of ITS rRNA gene of the isolate under study; strain KHA *Penicillium polonicum*; (Lane1; DNA Ladder, arrowed bands are 1000 & 500 bp).

evolutionary distances used to assume the phylogenetic tree with overall mean distance equals 0.46 (SE 0.01), this is the numbers of base differences per site from averaging overall sequence pairs. The evolutionary distances were calculated using the p-distance method and are in the units of the number of base differences per site (Nei and Kumar, 2000). This analysis of 26 nucleotide sequences was involved. Codon positions 1st+2nd+3rd + Noncoding were included. All positions that have a gaps or/and missing data were excluded. There were overall 513 positions in the final dataset.

3.5. Citrinin, penicillic acid and cyclopiazonic acid analysis for green table olives

Thin layer chromatography (TLC) was used for qualitative detection of citrinin, penicilic acid and cyclopiazonic acid produced by *Penicillium* species in green table olives. All investigated green olive samples which stored at 10–15 °C in home fridge or display refrigerators in retails or supermarkets for 21–30 days exhibited occurrences of some common mycotoxins which produced by *Penicillium* species.

4. Discussion

Moulds that are frequently isolated from table olives include members of the following genera *Penicillium*, *Aspergillus*, *Aureobasidium* and *Geotrichum* (Fernández et al., 1997). The presence of any species belong to these genera in table olives is considered as a contamination which responsible for spoilage (rot, softening of texture, mouldy or bad odour and changing appearance and taste) and may be responsible for toxicity as well (Heperkan, 2013). Moreover, the occurrence of toxigenic moulds in table olives was linked to the presence of mycotoxins. Contamination of table olives with secondary toxic metabolites (mycotoxins) such as Aflatoxin B, citrinin, penicillic acid, patulin and ochratoxin have been approved in cracked and unpacked (loose) table olives (Franzetti et al., 2011).

In the present study, the blue mould appeared on loose and pitted green table olives in markets or in the home fridge (5 - 10 °C) after keeping for short time were isolated and identified. *Penicillium* species are not easy to identify because it is a very large genus including many species that have very similar morphological properties. Therefore, traditional morphological identification combined with biochemical analysis and molecular techniques were used to confirm the identification. The isolates obtained from mouldy green table olives in this study had colony appearance on different culture media; macroscopic and microscopic characteristics typical of those described for *P. polonicum* (Pitt and Hocking, 2009). The same species have been isolated with very rare frequency from table olives in Greece by Bavaro et al. (2017). The psychrophilic and halo-tolerant behaviours of *P. polonicum* are an advantage that helping it to grow and produce blue mould symptoms on green table olives.

The main reason of table olives spoilage is microorganisms, such as bacteria, yeasts and moulds; that introduced from external sources. Washing process before the pickling of table olives through unhygienic procedures may be the most important factor that causes this blue mould in our samples. Furthermore, uncovered table olives with a little amount brine may be permitted the growth of fungi even if stored in refrigerator display case. Monitoring salt concentration, pH of the brine and number of microorganisms is significant during processing, to decrease the risk of infection and avoiding spoilage (Kailis and Harris, 2004).

Creatine sucrose agar was used by Frivsad (1985), as a differential medium for mycotoxin production to divide subgenus *Penicillium* into two equal groups. *P. polonicum* has ability to produce acid and use creatine as a sole nitrogen source and change the pH indicator bromocresol purple to yellow colour after 2–3 days. Moreover, the isolate was tested for producing a cyclopiazonic acid and/or other alkaloid. The reaction with Ehrlich reagent, *P. polonicum* produced a violet ring in less than 6 min which means that it has ability to produce a cyclopiazonic acid

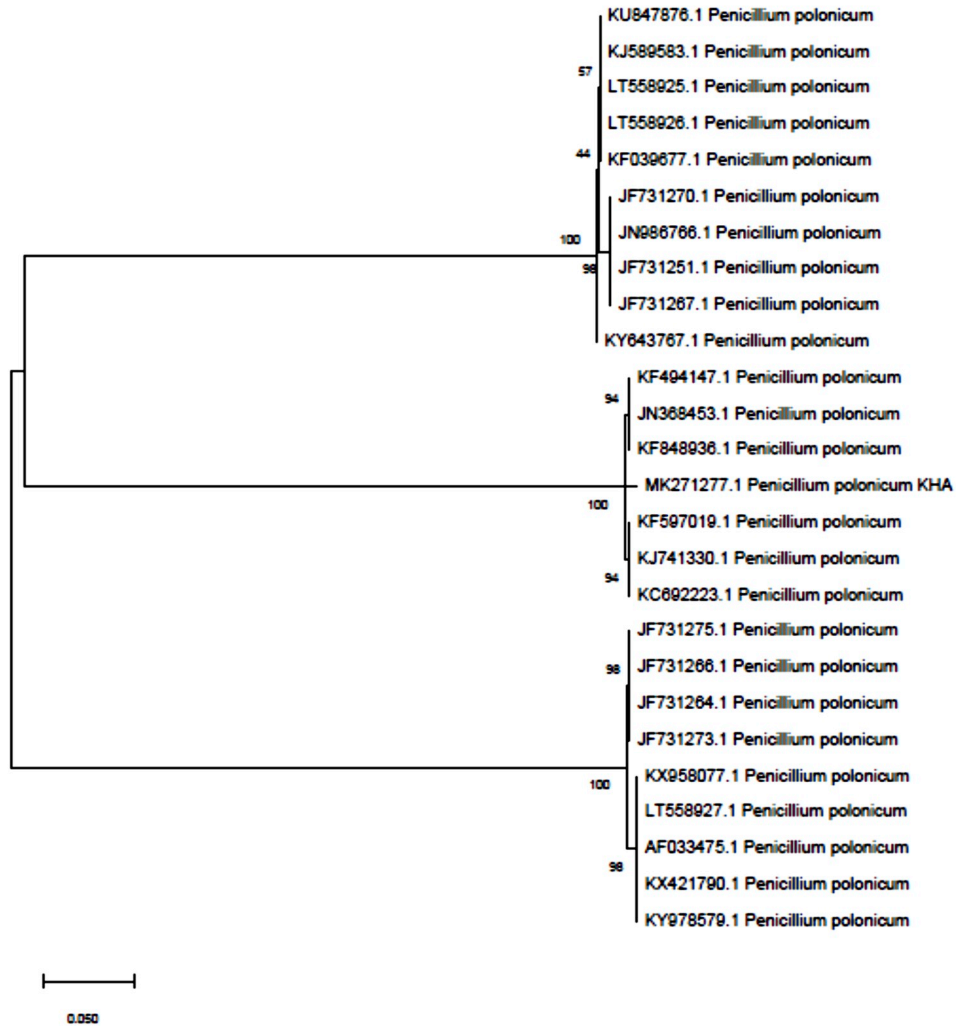

Fig. 7. Neighbor-Joining phylogenetic tree of ITS rRNA gene of the isolate under study; strain KHA (accession no. MK271277) and the nearest 25 *Penicillium polonicum* strains published in GenBank. Numbers represent bootstrap percentage values based on 1000 replicates.

(Lund, 1995). Depending on the previous results, *P. polonicum* is considered as a toxigenic *Penicillium* species (Núñez et al., 1996).

For further identification of our isolates the internal transcribed spacer (ITS) region in the ribosomal RNA (rRNA) operon has been established as the formal fungal barcode. The full ITS region in fungi has an average length of 500 and 600 base pairs (bp) for ascomycetes and basidiomycetes, respectively, and an average length of 600 bp across all fungal ancestries. In our study, the ITS region was sequenced and reached to about 575 bp in 3 length for *Penicillium polonicum* strain KHA. Phenotypic identification of *Penicillium polonicum* strain KHA was assessed by molecular identification of (ITS) region in the (rRNA). Many similar taxonomic studies on *Penicillium* involving polyphasic approach for identification are exist such as those conducted by Anelli et al. (2018).

Pencillium citrinum, *P. expansum*, *P. verecossum* and *P. polonicum* which isolated form investigated olive samples were a well-known citrinin, pinicilic acid and cyclopiazonoic acid producing moulds (El-Banna et al., 1987). However, only *P. polonicum* growth was observed on the surface of pitted and stuffed green table olives. Citrinin, pinicilic acid and cyclopiazonoic acid was detected in all investigated samples after kept in fridge at 10–15 °C for 21–30 days. Although citrinin, penicilic acid and cyclopiazonic acid are considered less toxigenic in compared with aflatoxins, ochratoxin A, patulin, zearalenone and deoxynivalenol (Miller, 1995); there moderately toxic are clearly appeared in humans kidney damage, neurotoxic effects, dilates blood vessels and antidiuretic effects which to be likely a result of prolonged ingestion (Caballero et al., 2003; Shephard, 2008; Bryden, 2012).

5. Conclusion

Our results confirmed that a blue mould on green table olives, collected from markets in Saudi Arabia, is caused by *P. polonicum*. Moreover, *P. polonicum* have ability to produce citrinin, penicillic acid and cyclopiazonic acid in green table olives. The presence of foodborne pathogen such as *P. polonicum* in green table olives is always regarding to the unhygienic practice, storage temperature, selling conditions in addition to the physicochemical properties of table olives and brine. Meanwhile, the effective control strategies against this important toxigenic species should be taken since olives are the main components in the Mediterranean diet. Moreover, since *P. polonicum* colonizes the green table olives, quantitative analysis should be established to assess the amount of mycotoxins on table olives as a substrate.

Conflict of interest

The authors declare that there is no conflict of interest exists in this article. This article does not contain any studies related to human participants or animals.

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