Plant Archives Volume 20 No. 2, 2020 pp. 8793-8797 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

EFFICACY OF *LACTOBACILLUS ACIDOPHILUS* IN REDUCING TOXICITY OF *PENICILLIUM GRANULATUM* IN APPLE *MALUS DOMESTICA* FRUITS

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Abstract

This study aimed to isolate and diagnose the fungi associated with imported apple fruits sold in the local markets of Najaf Governorate. results of isolating fungi showed the presence of two species of *Penicillium*, two of *Aspergillus*, in addition to the presence of *Rhizopus* sp. and *Alternaria* sp. *Penicillium* had the highest occurrence ranged from 95 to 100% and a frequency from 0.5 58% and 65.2% in all apple varieties (yellow, red and green), followed by *Aspergillus* with occurrence ranging from 33% to 45% and a frequency between 23.2% and 28.5%, while *Alternaria* sp. had lowest percentage of occurrence ranged from 9% to 14%, with a frequency from 2.3% to 5.2%. *Penicillium* isolates were also diagnosed using PCR technique, where a new isolate was detected on apple fruits in Iraq after being screened in available NCBI data and it was recorded in the name Alhlali and sequences No. MT645651. The TLC test showed that among five *Penicillium* isolates, one isolate *P. granulatum* had an ability to produce the fungal toxin, Patulin. The study showed the efficacy of *Lactobacillus acidophilus* in inhibiting fungal radial growth of *P. granulatum* by 80.87% in PDA compared to the control treatment.

Key words: *Penicillium*, identification, mycotoxin, patulin, PCR

Introduction

Apple *Malus domstica* is one of the most important types of deciduous fruit trees that are widely cultivated in temperate regions of the world (Haris *et al.*, 2002). Apples can be infected during handling and storage period with different types of fungi due to favorable temperature and humidity conditions. These fungi cause great losses and affect the marketing value of the fruits (Du *et al.*, 2001; Naiz and Dawar, 2009). There are many fungi of the genera *Pencillium*, *Aspergillus* and *Fusarium*, which are considered dangerous food contaminants and are known for their ability to produce mycotoxins such as Aflatoxin, Fumonisin and Patulin and for their carcinogenic diseases for humans and animals (Mabett, 2004). Mycotoxins are among the strongest toxins known to have low molecular weights and are also non-antigenic compounds that do not have antibodies, in addition to their resistance to high temperature (Wahba and Al-Nisr, 2010).

Accurate diagnosis of pathogens is important in determining methods of controlling plant diseases of all kinds. Numerous studies have stated that the diagnosis of fungi based on phenotypic characteristics alone is imprecise and requires high experience in classifying fungi morphologically close (O'Donnell *et al.*, 2008). Molecular diagnostics has been relied upon to avoid the problems associated with the phenotypic diagnosis, in particular the diagnosis of economically important fungi (Schroeder *et al.*, 2013). PCR technique is one of the most successful and accurate molecular application methods in diagnosing many organisms based on the pattern of arranging the nitrogenous bases of a DNA strand (Giantsis *et al.*, 2017).

Due to the large losses due to infection of stored food and fruits with fungi, there was a need to reduce or replace chemical pesticides with means that have a rapid effect, economical, easy to apply and with low harm to humans and the environment (Levetin *et al.*, 2001). Tawook, (2005) explained the use of microorganisms to protect fruits and processed foods from infection with fungi during storage by using *P. fluorescens*. El-Nezami, (2002) showed the role of *Lactococcus* bacteria in reducing mycotoxins inside and outside the bodies of living organisms. Therefore, the study aimed to identify mycotoxin producing fungi on infected apples and

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diagnose those fungi using (PCR) technique, and evaluate the efficiency of *Lactobacillus acidophilus* against the mycotoxin producing fungal isolate.

Materials and Methods

A 90 sample of apple fruits (yellow, red and green) were collected from the local markets of Najaf governorate, and pieces (0.5 cm) were taken from the affected part. The pieces were sterilized with 2% sodium hypochlorate for two minutes, after which they were washed with distilled water and then dried with blotting paper. Petri dishes were used as a container on a sterile PDA culture medium with chloramphenicol added 50-250 mg/ml and planted in the infected pieces at a rate of five pieces per plate and with three replications, and incubated at ± 252°C for 48 hours. The growing fungus was purified by transferring a small piece of Haifa tip to the dishes looked new. The phenotypic diagnosis of fungi was carried out by relying on phenotypic features and taxonomic keys (Pitt and Hocking, 1997; Summerell and Leslie, 2011). To confirm the phenotypic diagnosis, the fungus isolates under study were identified using PCR technique.

Ability of *Penicillium* spp. Isolates to produce patulin toxin

Five *Penicillium* isolates were selected based on phenotypic differences of color and the nature of mycelial growth on PDA medium. The five isolates were grown on P.D.A medium and incubated for 10 days under standard conditions, after which the fungal growth for the whole plate for each isolate were taken and cut into small pieces and placed in an electric food processor with 50 ml of distilled water. the mixture was left for 10 minutes, then filtered with a cheesecloth. The filtrate was applied to a separator with an equal volume of auxiliary solution (ethyl acetate + chloroform). Then the samples were ovened at 40°C for 48 hours. Dorner and Sobolev (2002) method was used for detecting the presence of fungal toxin patulin using the thin layer chromatography (TLC) technique. After separation, the samples were examined using UV radiation with a wavelength of 360 nm. Patulin presence was detected by matching the transfer factor (Rf) and fluorescence color of the standard toxin with the migration factor and the color of the fungus isolate extract samples (Al-Jumaili, 2014).

Molecular diagnosis of *P. granulatum* isolates using PCR technique

The molecular diagnosis of the fungus isolates *P. granulatum* secreting toxins that isolated from apple fruit was performed in the virus laboratory 8 Faculty of Agriculture - University of Karbala.

DNA extraction for the toxin-secreting *Penicillium* isolate

The process of extracting DNA for the toxic *Penicillium granulatum* isolate was carried out using the kit (Cat. No: FAPGK100) according to the method described by the manufacture 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 reverse 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 µl (Nuclease-free water).

The DNA of the *Penicillium* isolate 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 (Zou *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-reverse 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.

Effect of *L. acidophilus* on the growth of *P. granulatum*

The isolate of L. acidophilus bacteria was obtained from Toxicology Laboratory/College of Medical and Applied Sciences/University of Karbala. This experiment included the preparation of A. acidophilus suspensions after growing it on Nutrient agar medium by placing 100 ml of this medium in 250 ml glass flasks. the flasks were then inoculating with a one-day-old bacterial colony using an inoculation needle and flasks were incubated at 30°C for 24 hours.

A series of dilutions of bacterial isolate which were grown on nutrient broth medium was prepared at 1 day old to reach final dilution 10^{-10}. A 1 ml of dilution 10^{-6} of the bacterial suspension was taken and placed in a sterile

Petri dish to be mixed with PDA and left until solidified. A tablet of 0.5 cm diameter was taken from the tip of one week old fungus colony and planted in the center of the plate with three replicates. Three plates planted with mushrooms with 1 ml of distilled water without adding bacteria served as a control treatment. The plates were incubated at 25 ± 2°C for 10 days, after which the fungus growth rate and the inhibition ratio were calculated (Montealegre *et al.*, 2003). Also, *L. acidophilus* population density was calculated for the lowest dilution that resulted in inhibition of the pathogenic fungus.

Results and discussion

Regarding to the isolation of fungi infecting apple fruits, the results showed the presence of two species of *Penicillium*, two of *Aspergillus*, in addition to the presence of *Rhizopus* sp. and *Alternaria* sp table 1. *Penicillium* had the highest occurrences ranged from 95 to 100% and a frequency from 0.5 58% and 65.2% in all apple varieties (yellow, red and green), followed by *Aspergillus* with occurrence ranging from 33% to 45%, and a frequency between 23.2% and 28.5%, while *Alternaria* sp. had lowest percentage of occurrence ranged from 9% to 14%, with a frequency from 2.3% to 5.2% table 1. It was reported that the fungus *Penicillium* spp. One of the most important causes that afflict apples after harvesting and cause rotting in many countries of the world (Spadaro *et al.,* 2002). The infection may occur through the entry of the fungus through the lenticel or through wounds or necrotic tissue. The fungus spores remain for long periods inside the contaminated boxes, and the spores are transferred through the air or orchard soil or from rotting to healthy fruits (Scherm *et al.,* 2003).

The ability of *P. granulatum* isolate to produce Patulin toxin

The results of the TLC analysis showed that one isolate of *Penecillium* was able to produce Patulin from among 5 isolates. Patulin toxin was diagnosed for each sample by comparing the Rf value of the stains under test with the stain for the standardized Batulin toxin, which was identical to the tested isolate table 2. This result was consistent with Ali (2011) extracting patulin toxin from *Penecillium* using TLC.

Table 1: The percentage of occurrence and frequency of fungi infecting yellow, red and green apple fruits imported and sold in the local markets.

Isolated fungi	Yellow apple		Red apple		Green apple	
	Occur-rence%	Frequ-ency%	Occur-rence%	Frequ-ency%	Occur rence%	Frequ-ency%
Penicillium spp.	100	65.2	98	61.3	95	58.5
Aspergillus spp.	45	26.3	39	23.2	33	28.5
Rhizopus sp	18	5.5	15	8.5	13	7.3
Alternaria sp	14	2.3	12	6.5	9	5.2

Table 2: Ability of *Penecillium* isolates to produce Patulin toxin.

Isolates *Penecillium*	Isolation source	Ability of producing patulin
*Penecillium*1	Apple	+
*Penecillium*2	Apple	-
Penecillium 3	Apple	-
*Penecillium*4	Apple	-
*Penecillium*5	Apple	-

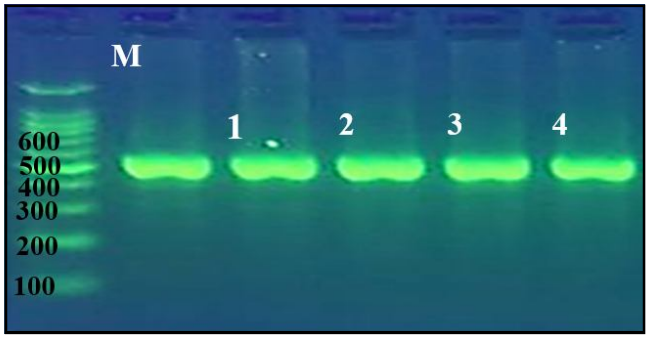

Fig. 1: DNA amplification products by PCR technique for five isolates Penicillium in this study using the primer pair ITS1 and ITS4. M= 1Kbp DNA ladder marker.

The results of the extraction of the fungal toxin, patulin, are in agreement with the findings of Hassen and Jassim (2010) on the extraction and diagnosis of the toxin Patulin using TLC. It also agreed with the results of Frisvad and Filtenborg, (1983) for the extraction of the fungal toxin patulin from *P. granulatum*.

Molecular diagnostics of *Penicillium* spp. using the PCR technique

The results of extracting DNA from *Penecillium* spp. and using PCR showed the possibility of multiplying the DNA product (PCR-amplified product) at the expected size (about 500 nitrogenous bases) by using the front and back primers (ITS1 and ITS4) Fig. 1. the results of amplification of fungal isolates of *Penecillium* spp. were similar to those found by Conrad *et al.,* (2012) using the forward and reverse initial pair (ITS1 and ITS4), which gave amplification results that ranged between (450-870) base pair and considered as being within the sizes of these fungal species.

Through the results of the study and its comparison with the available data (NCBI) using the BLAS program, it was found that *P. granulatum* isolate is genetically different from the isolates that were previously recorded in

(NCBI), so a new isolate was recorded with the number MT645651 and the name Alhlali.

Many previous studies have shown that diagnosing fungi based on (Morphological characters only) is insufficient and sometimes gives imprecise results. Emphasis has been placed on finding advanced classification systems or universal codes such as DNA barcoding, which are considered one of the quick and easy classification methods by using short genetic marker in the genome of organisms. (Chu *et al.*, 2006). As Iwen *et al.*, (2002) mentioned that the current taxonomic studies used ITS Intragenic transcriptional space for the sequential nitrogen bases on the ribosomal DNA strand (rDNA) within the organism's gene, where the difference in ITS regions of fungi was adopted in the diagnosis and classification of many species and strains. Fungal. And the possibility of adopting the ITS region in the diagnosis of many types of *Penecillium* (Seifert *et al.*, 2007).

Effect of L. acidophilus on the growth of *P. granulatum* on the PDA medium

The results showed that the treatments differed significantly in their effect on inhibiting the growth of the fungus compared to the control table 3. The percentage of inhibition increased with increasing the concentration of L. acidophilus bacteria, so that the percentage of inhibition of the fungus at 15% was 80.87%. The reason for the effect of the bacteria is due to its ability to produce some antibiotics, including nisin, acidophilin, lactolin and lactocidin, which have an antagonistic capacity towards pathogens in addition to their production of substances that inhibit the growth of pathogens such as bacteriocins, probionic acids, lactic acid and acetic acid, as well as producing many compounds Influencing the physiological activities of pathogens such as protein formation, cell division, and DNA and RNA biosynthesis. (Ouwehand, 1998; Chaurasia *et al.*, 2005). The results of the study were close to the findings of Saladino *et al.*, (2016) in the use of lactic acid bacteria to inhibit a group of fungi, with an inhibition ratio of 99%, in addition to reducing mycotoxins.

Table 3: Effect of different *Lactobacillus acidophilus* concentrations on fungal radial growth of *P. granulatum* on PDA medium.

L. acidophilus 10^{-6}	% inhibition of *P. granulatum*
0 %	0.00
5 %	68.30
10%	71.04
15%	80.87
Average	55.05
L.S.D. $_{(P\leq 0.05)}$	2.093

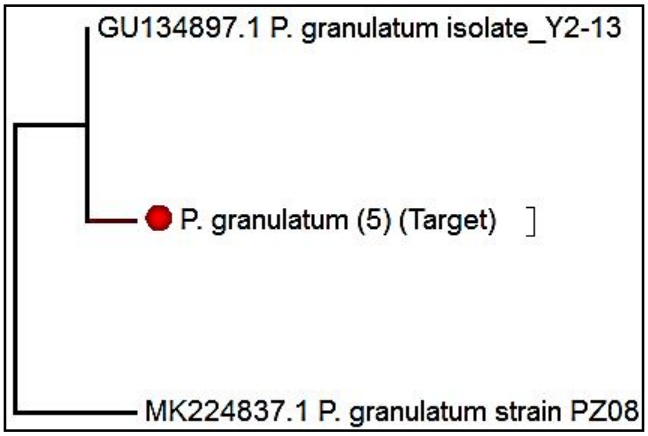

Fig. 3: The Neighbor-joining tree shows the genetic relationship of *P. granulatum* isolated in this study and other isolates of the same fungus previously registered in the NCBI.

Isolate 27
***Penicillium granulatum* (NCBI registered)**
Target (new isolate) …. 90%

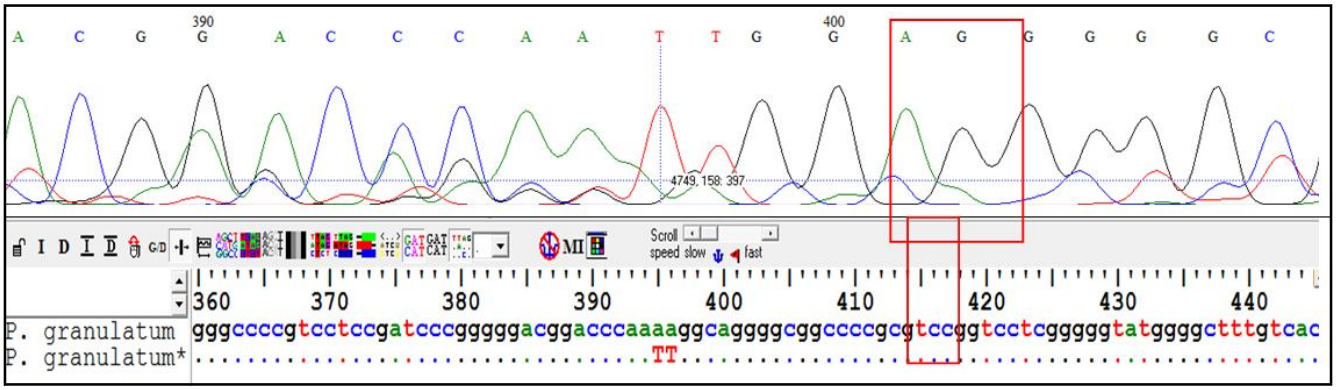

Fig. 2: Sequence variation of some nitrogenous bases of the DNA amplified by PCR from P. granulatum (*lower) isolated in this study and the closest isolate (upper) of same fungus (Y2-13) recorded in NCBI under entry number GU13897.1.

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