Morphological and Molecular Identification of *Fusarium tricinctum* Causing Fruit Rot of Pumpkin (*Cucurbita pepo*) in Iraq

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Abstract

Thanoon, A.H. 2024. Morphological and Molecular Identification of *Fusarium tricinctum* Causing Fruit Rot of Pumpkin (*Cucurbita pepo*) in Iraq. Arab Journal of Plant Protection, 42(2): 168-173. https://doi.org/10.22268/AJPP-001241

In November 2019, samples of pumpkin fruits with post harvest rotting symptoms were collected from farmers' stores. The causative fungus was isolated from infected pumpkin fruits. Based on morphological traits, Koch's hypotheses and molecular diagnostic tests were employed to confirm the infection and identify the causal agent. Molecular diagnosis using the polymerase chain reaction (PCR) confirmed the identity of the causal fungus. The results of electrophoresis using a 2% agarose gel showed the presence of a 550 bp amplified band. The ITS sequences were found to be homologous. to that of *Fusarium tricinctum* in GenBank database at a similarity level of 99%. GenBank assigned the number MZ166321.1 to the Iraqi isolate. This is the first record of *F. tricinctum* that causes post-harvest pumpkin rot in Iraq.
Keywords: Pumpkin fruit rot, postharvest diseases, *Fusarium tricinctum*.

Introduction

Pumpkin (*Cucurbita pepo* L.) is regarded as an important cultivated cucurbit foods in the world (Jamiołkowska & Thanoon, 2016; Weng & Sun, 2012). America is a native for this crop, and in the sixteenth century it was introduced to Europe and Asia, and nowadays pumpkin is grown all over the world both commercially and in home gardens (Janick & Paris 2006, Paris, 2001). Pumpkin is a crop rich in fiber, and contains necessary substances for human health such as starch, protein, minerals (Na, K, Mg, Fe, Ca, and P), vitamins (B1, B2, A and C), and carotenoids (alpha and beta-carotene, lutein and zeaxanthin) (Lee *et al.*, 2010). Worldwide production of pumpkin in 2016 amounted to 26 million tons. In 2018, China was the most productive country, producing 7 million tons (FAO, 2018). Pumpkins grown in the field are infected with several fungal pathogens (Jamiołkowska *et al.*, 2011, Rahim *et al.*, 2013). Fruit rot in cucurbits is known to be caused by Fusarium species all over the world, including *F. solani*, *F. equiseti*, *F. graminearum*, *F. avenaceum*, *F. culmorum* (Rampersad, 2009; Wyenandt *et al.*, 2010). Pumpkin planted repeatedly in the same field can lead to significant crop loss because of Fusarium fruit rot, a disease which could destroy up to 100% of the crop. The purpose of study was to isolate and diagnose the causative agent of pumpkin fruit rot in Iraq based on morphological characteristics of fungus, molecular analysis of DNA, as well as pathogenicity test.

Materials and Methods

Isolation and diagnosis

Samples of infected pumpkin fruits were collected from farmers' stores, surface disinfected with sodium hypochlorite solution (1%) for 15 seconds, and then washed with distilled sterile water. Around 0.5 cm pieces from the rotted part of the pumpkin fruit were obtained and placed in 8.5 cm Petri dish filled with potato dextrose agar (PDA) and kept at 25±2°C (Jamiołkowska & Thanoon, 2016). The pathogenic fungus was purified by taking portions of the fungus colony edge and transferred to fresh PDA Petri dishes and incubated at 25°C. The isolates' colony type, color, and morphological features, such as the development of conidia, micro and macro spores, and chlamydospores, were all investigated. Isolate can be identified to the species level based on these characteristics, according to Nelson *et al.* (1983) and Burgess *et al.* (1994).

Pathogenicity test of *Fusarium tricinctum*

To demonstrate the pathogenicity of *F. tricinctum*, four ripe pumpkin fruits were surface sterilized with sodium hypochlorite (1%), followed by washings with distilled and sterile water by three times. Using a sterile cork borer, 0.5 cm diameter holes were made in the fungus colony grown in PDA medium and inserted into wounds in pumpkin fruit made by the same sterile cork borer. Disks of sterile PDA media were placed as a control treatment. The fruits were placed in airtight and transparent plastic bags and stored at 30°C to 35°C. 30 days later when rotting symptoms were observed, the causal agent was re-isolated from the infected fruits to prove the pathogenicity of the fungus by applying Koch's postulates (Nuangmek *et al.*, 2023).

Genomic DNA extraction and PCR amplification

The pathogenic fungus *F. tricinctum* was isolated using the single-spore isolation method. It was cultured on a potato dextrose broth (PDB) medium and incubated at a temperature of 25±2°C for two weeks in darkness. The resulting mycelium was collected through filtration using Whatman No.1 filter paper and used directly for DNA extraction. The DNA extraction process was completed

https://doi.org/10.22268/AJPP-001241

using the ZR fungal/bacterial DNA MiniPrep™ Kit (Catalog No. D6005-ZR crop, India) according to the manufacturer's instructions, 50 mg of moist fungal cells was suspended in phosphate buffered saline (PBS) up to a final volume of 200 µl and transferred to a ZR Bashing Bead Lysis tube. In a separate tube, 750 µl of the Lysis solution was added and vigorously vortexed for five minutes. The ZR Bashing Bead Lysis tube was then centrifuged at 10,000 g for 60 seconds. Subsequently, 400 µl of the supernatant was transferred to an Orange Top Zymo Spin IV Spin Filtration tube and centrifuged at 7,000 g for one minute. To the filtrate in the collection tube, binding buffer and 1.2 µl of fungal DNA were added. From this mixture, 800 µl was transferred to a ZymoSpin IIC column 3 collection tube and centrifuged at 10,000 g for one minute. The supernatant was discarded, and the flow from the collection tube was removed by repeating the centrifugation step at 10,000 g for one minute. In a new collection tube, 200 µl of DNA Before wash Buffer was added and centrifuged at 10,000 g for one minute. Next, 500 µl of fungal DNA washing buffer was added to the Zymo-Spin IIC column, followed by centrifugation at 10,000 g for one minute. The contents of the Zymo-Spin IIC Column were transferred to a clean microcentrifuge tube (1.5 ml), and 35 µl of DNA elution buffer was added to the column matrix. To recover the DNA, the preparation was centrifuged at 10,000 g for 30 seconds.

DNA separation by electrophoresis

After extraction, DNA fragments were separated and identified using a horizontal agarose gel electrophoresis unit. According to Sambrook *et al.* (1989), the 1.5% agarose gel was created by mixing 100 ml of TBE buffer with 1.5 g of agarose and heated until boiling, then left to cool and reach 45°-50°C. After adjusting the samples comb, the warm gel was carefully poured into the plate without creating air bubbles, and left to cool down for 30 minutes. The samples comb was then gently removed.

Amplification of ITS region by PCR

ITS gene amplification was performed using a pair of PCR ITS gene detection primers: a reverse primer (ITS4-R: 5′-TCCTCCGCTTATTGATATGC-3′) and forward primer (ITS1-F: 5′-TCCGTAGGTGAACCTGCGG-3′) to amplify an ITS fragment (Integrated DNA Technologies, Canada). Each reaction tube included 5 µl of taq polymerase PCR Premix (Intron, Korea), 1.5 µl of DNA preparation, and 1 µl of each of the four nucleotide bases (10 pmol), and the reaction mixture's total volume was then increased by 25 µl by adding distilled water. Thermal cycling was performed under the following conditions: one denaturation cycle lasting 30 seconds at 94°C, then 35 cycles lasting 45 seconds at 94°C and 60 seconds at 52°C, and finally one extension cycle lasting 3 min. at 72°C.

Alignment of sequences and DNA sequencing

After ethidium or red stain spotting, all PCR products were separated using 2% agarose gel electrophoresis under UV (302nm) light. Using a DNA sequence 3730XL (Applied Biosystem). Gene sequencing was carried out online using the National Instrumentation Center for Environmental Management (NICEM) application, which is available from the National Center for Biotechnology Information (NCBI) online Homology search (NCBI) was conducted using the basic local alignment search tool (BLAST), which is available at the National Environmental Instrumentation Centre.

Antagonistic ability test of *Trichoderma harzianum* T22 against pathogenic fungi

The Dual Culture Technique method was used to investigate the biological agent *T. harzianum* T22's antagonistic capability against the pathogenic fungus. The sterile potato dextrose agar (PDA) media was divided into two equal halves in each 9 cm diameter Petri dish. To inoculate the middle of the first half of the PDA plate, a sterile cork borer was used to remove a 0.5 cm disk from the edge of a one-week-old pathogenic fungal culture. A 0.5 cm diameter disc removed from the edge of a fresh culture of the biological agent was used to inoculate the middle of the second half of the PDA petri plate. The test was conducted with three replicates. The Petri dishes were incubated at 25±2°C for four days, and when the two control colonies touched each other in the Petri dish, the percentage of fungal inhibition was measured, the parasitism level was determined according to (Bell *et al*., 1982):

1= the biological agent covers the whole plate, 2= the biological agent covers 2/3 of the plate, 3= the biological agent and pathogen each cover 1/2 of the plate, 4= 3/2 of the plate is covered in the pathogenic fungus, 5=the whole plate is covered in the pathogenic fungus. The biological agent that produces a parasitism level of 1 or 2 is considered to have a high parasitic ability. The inhibition rate was determined using the following equation:

$$\text{Inhibition rate (\%)} = \frac{\text{Average control diameter - average treatment diameter}}{\text{Average control diameter}} \times 100$$

The antagonistic ability of *Bacillus subtilis* against the pathogenic fungus

A swab of bacteria was taken by a sterile loop, transferred to other plate containing PDA culture medium along the line separating the dish, and incubated at 25°±2°C for 24 hours. *Bacillus subtilis* biological control agent was cultivated on PDA culture medium. By placing a disk of pathogenic fungus *Fusarium tricinctum* that is just beginning to grow, at the age of seven days, 2 cm away from one of the dish's edges, and 3.5 cm away from both ends of a line. Dishes were incubated at a temperature of 25±2°C and three times for each treatment was repeated. According to the same previous equation the inhibition rate was calculated.

Statistical analysis

The experiments were as carried out according to a completely randomized design. The results were analyzed statistically using SAS software and the average were compared according to Dunkin's multiple range test at the probability level of 0.05.

Results and Discussion

Isolation and diagnostic features

The colonies on PDA appeared as white aerial mycelia that changed later to pink color which is typical of *F. tricinctum* (Figure 1-D). Conidia of three different sizes (microconidia, macroconidia, and chlamydospores) were produced by *F. tricinctum*. Other Fusarium species, however, only have macroconidia and chlamydospores. *F. tricinctum* can be identified by the shapes of its macroconidia, microconidia, conidiophores, and chlamydospores (Figures 1-A, 1-B, 1–C). On PDA medium plates, the bottom side is either carmine red or pink. The dimensions of microconidia ranged from 2.0 to 7.1 mm in length and 1.3 to 3.5 mm in width on PDA, and 1.5 to 7.0 mm in length and 2.2 to 4.3 mm in width on SNA. The macroconidia are pyriform, numerous, citriform, napiform, oval or obovate, moderately thin, with 4-7 septa, and measuring 15.3-29.7 mm long by 2.3-4.0 mm wide on PDA and 14.3-25.2 mm long by 3.3-4.2 mm wide on SNA. Chlamydospores have an elliptic or round shape, are intercalary, and are generated in chains with smooth exines in PDA (Leslie & Summerell, 2006).

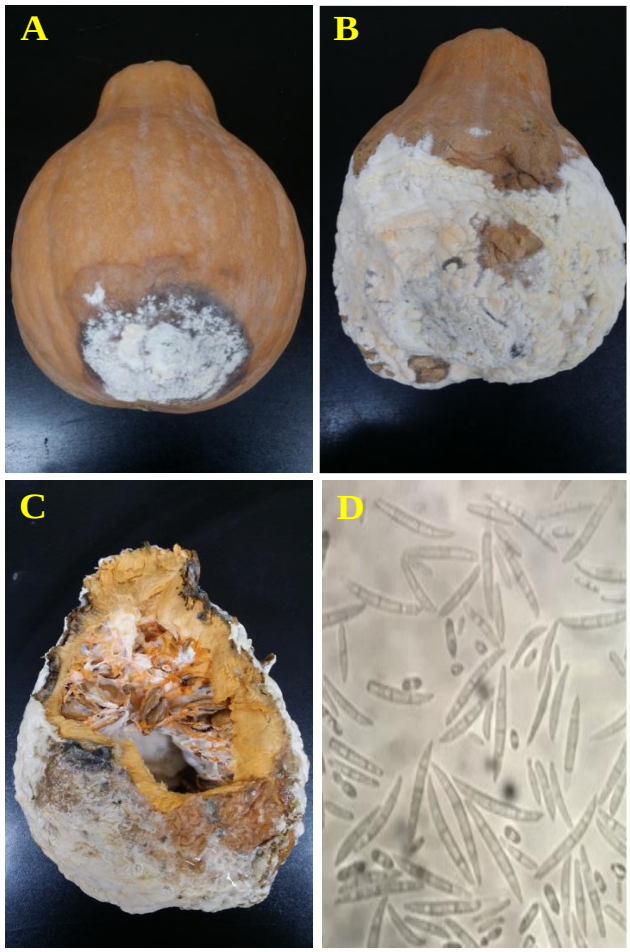

Figure 1. *Fusarium tricinctum*-induced postharvest fruit rot on pumpkin. (A, B) Fruits exhibiting rot symptoms in storage, (C) transverse sections revealed pink mycelia infection in the inside of fruit tissue, (D) microconidia**,** macroconidia and chlamydospores on PDA.

Pathogenicity test

After artificial inoculation, the fruit developed a brown, spongy lesion that expanded over the period of around two weeks from the initial wound location. On the damaged fruit (Figure 1), white mycelia were formed, whereas the control treatment did not produce any symptoms. When the pathogen was reisolated from artificially inoculated fruits, Koch's postulates were achieved. The fungus *F. tricinctum* was identified as the causal pathogen based on morphological characteristics, molecular information, pathogenicity, and symptoms. To our knowledge, this is the first mention of Fusarium fruit rot in pumpkins in Iraq.

Molecular identification of the fungus

The results of the PCR and electrophoresis confirmed the results of the morphological diagnosis through the appearance of a DNA band of 550 bp (Figure 2), which is the expected amplicon for *Fusarium tricinctum* when the mentioned specific primers are used.

Results revealed a tight genetic link between our isolated *Fusarium tricinctum* and those stored in GenBank databases from different regions around the world. When sequences of local isolates of *F. tricinctum* were compared with sequences of *F. tricinctum* strains from the NCBI data base and isolated from various countries, showed 99% similarity with isolate from German which has accession number MW268885.1, 99% similarity with isolate from Iran which has accession number MW268885.1, and 99% similarity with accession numbers EF611094.1, KM880018.1, MH071363.1, KX823405.1, and 99% similarity with accession numbers KM2490831.1, HM068317.1 from the USA, AY188923.1, AB587029.1 from France, and AB587029.1 from Japan, respectively (Figure 3). The GenBank accession number for the ITS nucleotide sequence from the Iraqi isolate is MZ166321.1. This is the first report of *F. tricinctum* producing pumpkin fruit rot in Iraq.

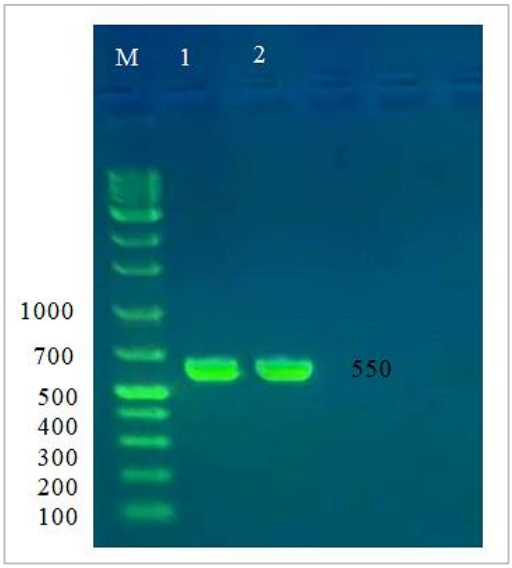

Figure 2. The PCR amplified band size of 550 bp (lanes 1 and 2) after electrophoresis on 2% agarose at 5 volts/cm^2 in TBE buffer for 1.5 hours. M= DNA molecular markers ladder (100 bp).

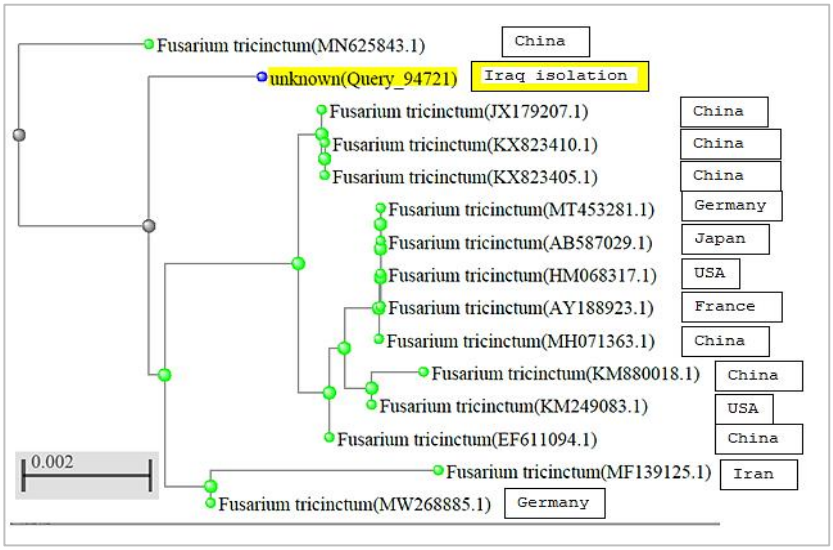

Figure 3. Genetic tree showing the phylogeny and evolution of *Fusarium tricinctum* compared to global isolates in the GenBank.

Antagonistic ability of *T. harzianum* T22 against *F. tricinctum*

As shown in Figures 4 and 5, *T. harzianum* exhibited a high antagonistic efficiency against *F. tricinctum*, with a parasitism level of 1 according to Bell *et al.* (1982) The pathogenic fungal colony's growth was inhibited by *T. harzianum* in this study at an inhibition rate of 45.5%. According to Lo *et al.* (1998), the fungus can produce the hydrolytic enzymes B-1-3 Gluconase, pectinase, chaitinase, protase, and cellulose in addition to the antibiotics Trichodermine, Demadine, Gliotoxin, Acetaldhyde, sesquiterence, and Alkypyrones (Harman, 2000). Trichoderma has the ability to secrete enzymes and antibiotics which widen the scope of its usage against a particular group of plant infections. Their combination also boosted the effectiveness of the fungus Trichoderma against plant pathogens (Howell *et al.*, 2000).

Effect of *B. subtilis* isolate on in vitro inhibition of *F. tricinctum*

The bacterial isolate *B. subtilis* was examined for its ability to inhibit *F. tricinctum* growth. Results obtained indicated that *B. subtilis* inhibition reached 52.5% (Figures 4 and 5). This is expected, due to the fact that *B. subtilis* is known to secrete a large number of antibiotics that are highly effective against plant pathogenic fungi, such as bacilin, sabtiline, subtenoline, fencycin, bacitracinltutin, and bacilomycin. In addition, inhibitory effect can also be due to the bacterial enzymes such chitinase, which dissolve the fungal cell walls, and prevent further growth (Akpa *et al.*, 2001; Athukorala *et al.*, 2009; Bargabus *et al.*, 2002; Oyedele & Ogunbanwo, 2014; Sansinenea & Ortiz, 2011).

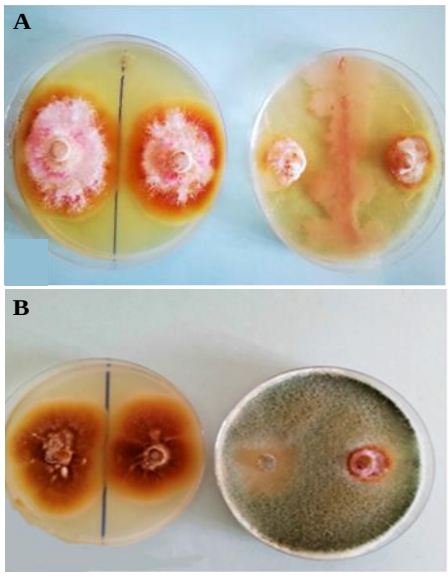

Figure 4. (A) *B. subtilis* antagonism against *Fusarium tricinctum*, (B) *T. harzianum* antagonism against *F. tricinctum*.

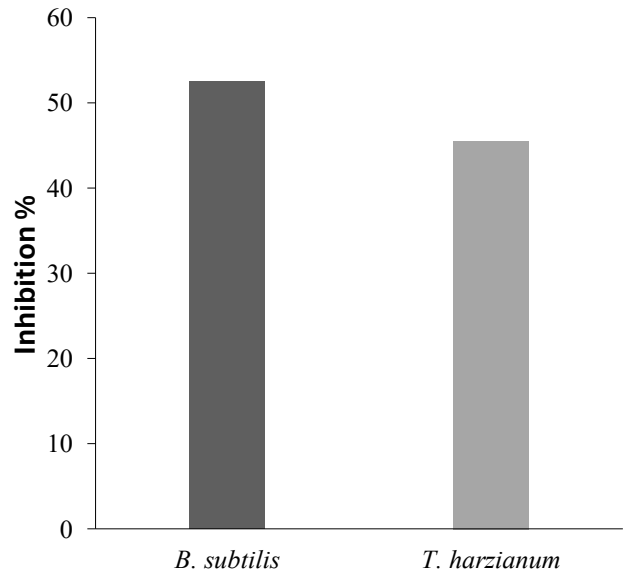

Figure 5. The inhibition rate of *F. tricinctum* fungus by using the bio-control agents *B. subtilis* and *T. harzianum in vitro*.

الملخص

ذنون، علي حمود. 2024. التشخيص المظهري والجزيئي للفطر *Fusarium tricinctum* المسبب لمرض تعفن ثمار اليقطين (*Cucurbita pepo*) في العراق. مجلة وقاية النبات العربية، 42(2): 168–173. https://doi.org/10.22268/AJPP-001241

جمعت عينات من ثمار اليقطين التي ظهرت عليها أعراض التعفن بعد الحصاد من مخازن المزارعين في تشرين الثاني/نوفمبر 2019. تم عزل الفطر المسبب للتعفن على ثمار اليقطين في مختبر وقاية النبات، كلية الزراعة والغابات بجامعة الموصل. تم استخدام فرضيات كوخ/ Koch والاختبارات التشخيصية الجزيئية لتأكيد الإصابة وتحديد ماهية العامل المسبب بناءً على الخصائص الشكلية. وأُكِّدت نتائج التشخيص الجزيئي باستخدام تقانة تفاعل البلمرة المتسلسل (PCR). بيّنت نتائج الترحيل الكهربائي باستعمال هلام الاجاروز 2% ظهور حزمة بحجم 550 زوج قاعدي وذلك من خلال تضخيم فاصل النسخ (ITS) في المنطقة الريبوزومية المحفوظة للحمض النووي، وبالتالي تم تحديد الفطر المسببة للمرض. تمّت مقارنة جميع تسلسلات ITS لعزلات *Fusarium tricinctum* في قاعدة بيانات بنك الجينات مع تسلسل العزلة العراقية وكانت نسبة التشابه 99%. تم إعطاء الرقم MZ166321.1 للعزلة العراقية ، ويعدّ هذا التسجيل الأول للفطر *F. tricinctum* المسبّب لتعفن ثمار اليقطين بعد الحصاد في العراق.

كلمات مفتاحية: يقطين، عفن الثمار، *Fusarium tricinctum*، أمراض ما بعد الحصاد.

عناوين الباحثين: علي حمود ذنون، قسم وقاية النبات، كلية الزراعة والغابات، جامعة الموصل، العراق. البريد الإلكتروني للباحث المراسل: dr.alithanoon@uomosul.edu.iq

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Received: January 18, 2023; Accepted: June 26, 2023

تاريخ الاستلام: 2023/1/18؛ تاريخ الموافقة على النشر: 2023/6/26