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Molecular Identification of the Fusarium Verticillioides Isolated from Corn Grains from Karbala Province

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P A P E R I N F O

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A B S T R A C T

Cereals are an important source of nutrition for both people and their domestic animals. Whether in the field or in storage, these grains are sensitive to fungus infestation . Fusarium verticillioides is the most important one. The results of this study showed that this species was present in all grain samples obtained from diverse locations . By doubling the region of F. verticillioides, the study revealed the molecular diagnosis. The polymerase chain reaction (PCR) technology was used. The results of the molecular analysis showed that this strain was diagnosed for the first time in Iraq, as the results of the comparison of the sequence of the nitrogenous bases of isolate No. 1 showed that it was 99% identical to the isolate registered in Australia under ID: KP132240.1, which belonged to the type F. verticillioides. The results of the alignment also showed that there was a discrepancy at position 390, as the nitrogenous base Guanine was recorded in the sequences of the isolate under study instead of the Adenine nitrogenous base (A\G) and this variation was of the type of Transition (equivalent substitution). Transmutation of the two laws is of the non-equivalent substitution type Transvertion, and that this type was registered under the accession number DI: OP056029.

1. INTRODUCTION

Corn grain is one of the important grain crops in Iraq and the world. Its importance comes through its multiple use as it enters the human diet directly or indirectly, through its use as a basic ingredient in the animal feed (Al-Aswadi, 2002).. Many plant pathogens are spread via seeds, which can result in significant crop losses. Increased examination of grain quality has resulted from recent increases in grain production and sale, with specific focus on worries about contamination with grain-borne diseases such as bacteria, fungus, and viruses that existing on the surface or inside the grain and have the ability to spread. Grains are being harmed, especially with the availability of favorable conditions. [1]

Fungi are a broad group of microorganisms that exist in a variety of environments, including soil, plant parts leaves, roots, fruits, and seeds, water, and food [2,3]. seeds are the main source for the presence of fungi, as the various types of cereals are exposed to fungal infections, whether in the field, during harvesting, or during transportation and storage. The fungal species belonging to the genera Aspergillus , Penicillium and Fusarium are the most common in causing these infections. [4] The safety of food and feed and the freedom from fungi and their toxins is one of the necessary matters that must be focused on , and the danger of many fungi and mycotoxins and their dangerous effects require accurate diagnosis of the fungal species that produce these toxins [5-14].

Mycologists have traditionally used morphological diagnosis based on phenotypic characteristics such as spores formed as a result of mitotic or asexual division or sexual reproduction (meiosis) to identify fungal species [15] .It is still used today as a method of identifying species within fungal groups, despite the use of morphology. It is very important to understand the evolution of phenotypic traits in fungal species, but it requires technical expertise, an experienced specialist, and a long time, especially with less common species [16,17].

In contrast to morphological and biochemical diagnostic tests employed in the laboratory diagnosis of fungi, molecular diagnostics exhibited great accuracy, speed, effort, and specificity in distinguishing between species and sub-species of fungi (Liu et al., 2000). Fungal DNA extraction-based molecular diagnostic tools give a unique barcode for identifying and characterizing various fungal isolates up to the species level [18] .The goal of this study

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was to extract F. verticillioides from seeds and diagnose it using phenotypic, microscopic, and molecular methods.

2. MATERIALS AND METHODS

Fifteen samples have been collected from yellow corn seeds at a rate of 2 kg from markets Karbala Governorate, to isolate the toxin- producing fungi. The samples were transferred to the advanced mycology laboratory / College of Education for Girls / Department of Biology / University of Kerbala. for diagnosis and study. A. Culture media The culture media for Potato Dextrose Agar (PDA) has prepared as recommended by the Manufacturing company and sterilized by Autoclave at 121 C° in 15 minutes for 15pis \ inch2.This method has been used to isolate and grow the fungi B. F. verticillioides is isolated and identified.

The researcher utilized the method used by [19] to isolate Fusarium from seeds, in which 25 seeds were randomly selected from each replicate of each sample and sterilized for two minutes with sodium hypochloride at a concentration of 10 % of the commercial preparation.The seeds were washed three times with distilled water to remove any remaining chlorine from samples , dried on filter paper, and then transferred to 9 cm diameter Petri dishes containing PDA culture medium with five seeds per plate .The plates were incubated in the incubator at a temperature of 25 ± 2 °C for 5 days. Then different fungi were purified, and Fusarium species were planted on the same PDA medium and incubated at 25 °C for a week, after which the fungal isolates were diagnosed using diagnostic keys [20,21]. C.Molecular identification of F. verticillioides isolates.

2.1. DNA was extracted from isolates of the fusarium

Isolates were cultivated on PDA culture medium and then incubated for 5-7 days at 25 ± 2 C°. The DNA was extracted and purified using a Promega Wizard® Genomic DNA Purification Kit and by following the DNA extraction protocols provided in the kit.

2.2. A Gel of agarose was made

According to [22] 1.5 g of agarose was dissolved in 100 ml of the previously prepared TBE solution to make a 1.5 percent agarose gel. To identify DNA fragments, the agarose is heated to a boil and then cooled to 45-50 C°. After cooling, Red Safe dye is add. The gel was poured into the casting plate where the agarose support plate was formed after the comb was fixed to produce holes that would contain the samples. Allow to (30) minutes to cool after pouring the gel slowly to avoid air bubbles. After the comb is carefully raised from the hard agarose, the board is fastened to its holder in the horizontal unit of the electrical relay, which is represented by the tank used for the electrical relay. The gel is coated with a TBE solution , which is put into the tank

.

2.3. Sample Preparation

For electrophoresis, 31 of loading solution (Intron/Korea) was combined with 5 l of DNA extracted as described in section 3-4-6-1. Then, for 1-2 hours, administer a 7 Vc2 electric current until the dye reaches the other side of the gel. A UV source with a 336 nm dimension was used to evaluate the gel.

2.4. The purity and concentration of DNA are determined

A Spectrophotometer (Nanodrope) was employed for this purpose, and the values of DNA concentration and purity were recorded at wavelengths 260 and 280 after calibrating the instrument with a small drop of 0.71 of DNA extract and zeroing it with a corresponding drop of elution buffer solution . Prefixes used in the reaction. The initiators were diluted with distilled water to obtain a concentration of 100 picomoles , the required concentration was prepared by taking 10 liters of the original solution and completing the volume to 100 liters by adding double distilled water to make it ready for use, according to the attached leaflet from the supplying company . The forward ITS-1 starter and the reverse ITS-4 starter, both supplied by IDT (Integrated DNA Technologies, Canada), were utilized in this work as stated in Table 1. [23]

TABLE 1. The purity and concentration of DNA aredetermined

Prim.	Seque.	Tm (°C)	GC (%)	Prod.size
F.	5′-TCCGTAGGTGAACCTGCGG -3′	60.3	50 %	550 bp
R.	5′ TCCTCCGCTTATTGATATGC-3′	57.8	41 %	

2.5. PCR (Polymerase Chain Reaction)

Using the ITS-1 primer with ITS-4, a PCR approach was utilized to amplify the ITS region. A total volume of 25 l was used for PCR amplification, which included 1.5 l of DNA, 5 l of Taq PCR PreMix, and 1 l of each primer (10 pmol). A total of 25 l of distilled water was supplied to the tube.

3. RESULTS AND DISCUSSION

A. DNA extraction from F. verticillioides isolates. The results of the DNA extraction study revealed that the isolate under study was isolated from pure *Fusarium* cultures on PDA medium.presence of DNA in the selected isolates for molecular study was confirmed by electrophoresis on agarose gel at a

concentration of 1.5 percent and at 5 ml/cm for 1 hour; as the bands appeared on the agarose gel, which means the presence of DNA, they reached a purity of 1.9 and a concentration of 76.2 Electrophoresis of PCR replication products

The results of the duplication of ITS1 and ITS4 regions located between rDNA genes using forward and reverse primers of ITS1-ITS4 primers of the species in question. The replication results by the polymerase chain reaction (PCR) technique using specific primers and electrophoresis of the replication products showed the appearance of bands at approximately 550 bp (compared to DNA 1000 plus), which indicates the correlation of the primers and the occurrence of doubling Figure 1.

According to the foregoing results of the molecular analysis using the primers ITS1 and ITS4 to multiply the ITS region using PCR technique, it has been proven that the use of molecular technology has a major role in the accurate diagnosis of fungal species and may be an alternative or complementary to phenotypic diagnosis, and this was confirmed by studies that the use of molecular techniques supports morphological diagnosis through To provide a quick and dependable test for species identification and classification;For plant pathogenic fungi, the first and most difficult step to identify is the determination of morphological and agronomic characteristics, and this is exactly the case with *Fusarium* spp. Because of the difficulty of distinguishing and different between species and their overlapping and their participation in many phenotypic and microscopic characteristics, therefore, molecular technology must be relied upon for accurate diagnosis [24-26]. According to [27] also reported that PCR can be used to identify *Fusarium spp*. , either as an alternative or as a supplement to morphological identification methods. And Molecular diagnostic methods also provide faster and higher accuracy and sensitivity; The correct and rapid diagnosis of the agent being studied is necessary to implement the appropriate treatment, which, if done correctly, helps reduce injuries and the risks of those injuries [28].

The presence of the ITS region in the diagnostic has been the reason for its use. According to [29], the ITS region offers the following benefits: (1). All organisms have several copies of the ribosomal gene, allowing for sensitive PCR detection. (2) The ITS region contains a highly conserved and variable area, which allows for target identification and the construction of PCR primers. It's also because the ITS region is a non-coding area that's extremely polymorphic and contains enough taxonomic units. As a result, the sequences can be separated down to the species level, with results ranging from (450 to 750) base pairs [30].

Figure 1. Electrophoresis of the PCR doubling products using primers ITS1 and ITS4 *F. verticillioides* on a (2)% agarose gel at (5) V for (1.5) hours and the appearance of the bands at ~550 bp compared with the DNA ladder (1000 plus

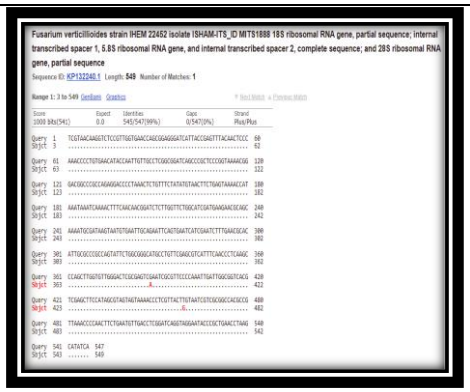

Figure 2. In BLAST, the sequence of nitrogenous bases of isolate No. (4) under investigation and the nearest isolate of *F. verticillioides* found in Iran under accession ID: KP132240.1were aligned. The selection in red highlights the regions of variation, and the proportion of congruence is 99 percent.

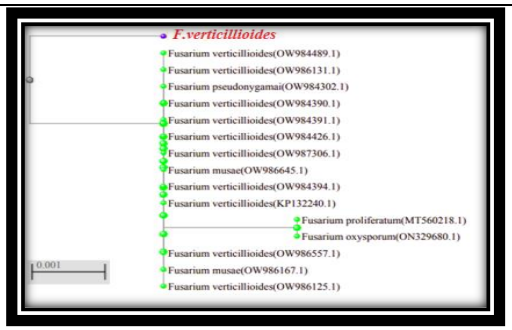

Figure 3. A phylogenetic tree was constructed using the BLAST tool and the Neighbor Joining option for Isolate of *F. verticillioides* under study, and the global isolates registered in the NCBI Gene Bank.

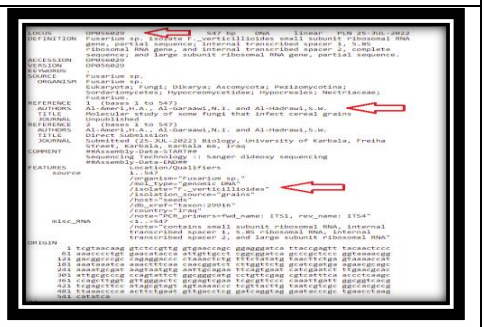

Figure 4. The registration form for the isolate F. verticillioides at the National Center for Biotechnology Information (NCBI) shows the identification number, the name of the fungus, the names of researchers, and the sequence of nitrogenous bases.

4. CONCLUSIONS AND FUTURE

The results showed that fungal infections in yellow corn seeds were caused by fungi, especially the mold studied. The results also showed that molecular diagnosis of fungi is indispensable, as it is a complementary method to the phenotypic diagnosis, and is the means that leads to reassurance of accurate diagnosis.

5. APPLICATIONS

Molecular diagnosis based on DNA barcode extraction of fungi is through which fungal isolates are identified and characterized up to the species level. Through this study, the fungus F. verticillioides is extracted and it is possible to work on biological, chemical or physical control of the fungus by taking appropriate precautions.

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Arabic Abstract

الحبوب هي مصدر مهم للتغذية لكل من الناس وحيواناتهم الأليفة. سواء في الحقل أو في المخزن، هذه الحبوب حساسة للإصابة بالفطريات. ومن أهمها فطر Fusarium verticillioides، وأظهرت نتائج هذه الدراسة وجود هذا النوع في جميع عينات الحبوب التي تم الحصول عليها من مواقع مختلفة. ومن خلال مضاعفة منطقة F. verticillioides، كشفت الدراسة عن استخدام تقنية التشخيص الجزيئي لتفاعل البوليميراز المتسلسل (PCR). وبينت نتائج التحليل الجزيئي انه تم تشخيص هذه السلالة لاول مرة في العراق، كما اظهرت نتائج مقارنة تسلسل القواعد النتروجينية للعزلة رقم 1 مطابقتها للعزلة المسجلة في استراليا بنسبة 99%. تحت المعرف: KP132240.1، الذي ينتمي إلى النوع F. verticillioides. كما أظهرت نتائج المحاذاة وجود تباين عند الموضع 390 حيث تم تسجيل القاعدة الآزوتية Guanine في تتابعات العزلة قيد الدراسة بدلاً من القاعدة الأدينينية (A\G) وكان هذا التباين من نوع الانتقال (استبدال يعادل). تحويل القانونين هو من نوع الإحلال غير المتكافئ Transvertion، وأن هذا النوع تم تسجيله تحت رقم الانضمام DI:OP056029.