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Isolation and Molecular Analysis of Aspergillus spp. Associated with Melon Seeds

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ABSTRACT

Seed-borne fungi are a big menace to agriculture and food safety in that they decrease seed viability, favor spoilage and may even induce mycotoxin into the food chain. The purpose of the research was to isolate and molecularly characterize Aspergillus species related to melons (Cucumis melo) seeds sampled in Penjwen. Isolation was done through the agar plating method on potato dextrose agar (PDA) and morphologically identified. Six Aspergillus species were obtained: A. flavus, A. niger, A. corrugatus, A. fumigatus, A. terreus and A. tamarii, with A. flavus having the highest prevalence (30%). The polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS1/ITS4), large subunit ribosomal RNA (LSU) and b-tubulin (Bt2a/Bt2b) gene segments were used to determine molecular identification. Fragmentary products of anticipated sizes, namely 650 bp (ITS), 1200 bp (LSU) and 495 bp (b-tubulin) were visualized and verified through agarose gel electrophoresis. Species identity and evolutionary relationship of the isolates were further confirmed by sequencing and phylogenetic studies. The prevalence of A. flavus and other species of Aspergillus highlights their ability to adapt to dry seed conditions and possible ability to generate spoilage-related enzymes and secondary metabolites. These results demonstrate the necessity of introducing appropriate seed management and storage to prevent fungal contamination and guarantee the quality of seeds.

***Keywords*:** Aspergillus spp., melon seeds, seed-borne fungi, molecular identification, ITS, LSU, b-tubulin.

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INTRODUCTION

In recent years, one of the most important issues in agriculture and food safety has been fungal contamination of seeds. In addition to serving as the basis for vegetative life, seeds can also serve as the starting point for the spread of pathogens [1]. Additionally, it has been shown that when storage and post-harvest handling circumstances are unfavorable, a significant portion of seed deterioration is caused by fungal diseases. The quality of seeds, the degree of germination, and other factors are impacted by these fungal pollutants, they can spread toxins to the food chain [2]. The commonest genera of fungi that may cause contamination of seeds include *Aspergillus*, *Fusarium*, and *Penicillium* with *Aspergillus* spp. being the most common after being able to survive in dry and hot condition [3]. As a rule, the seed-borne fungi infect the seeds on infected parent plants (systemic infection), or when handling, harvest, storage, and transportation [4]. Fungal infections decrease the viability of seeds and play a significant role in spreading plant diseases. They are also involved in the synthesis of harmful secondary metabolites, like aflatoxins and ochratoxins [5]. Such seeds are often the source of fungal infection in farm lands that could trigger plant disease outbreaks when conditions are favorable for them. The species of Aspergillosis Aspergillus, especially A. flavus, A. niger, A. fumigatus, A. parasiticus, and A. nidulans, are common among cereals, legumes, and oilseed plants [6]. These organisms are well known for their capacity to colonize dry seeds owing to their ability to synthesize certain enzymes such as amylase, protease, and cellulose that break down seeds causing spoilage [7]. Besides, Aspergillus fungi have the capability of producing secondary metabolites, which are essential virulence factors for successful infection of seeds [8]. The Polymerase Chain Reaction (PCR) is one of the most extensively employed molecular identification tools for fungi due to its capacity to amplify certain DNA segments utilizing conserved primer sets for various species [9]. The internal transcribed spacer (ITS) gene has become the universal barcode for fungi taxonomic identification because of its genetic diversity between different species and similarity within the same species [10]. Nevertheless, despite the usefulness of ITS gene amplification in genus differentiation, it may face problems in discriminating

closely related species of Aspergillus. β-Tubulin genes have proven themselves highly effective for detecting seed-borne fungi. Fungal seed infection identification in previous molecular studies was successful in isolating *A. flavus*, *A. parasiticus*, and *A. niger* through ITS, LSU, and β-tubulin primer sets. [11].

Materials and Methods

2.1 Collection of Samples

The experimental study involved the collection of melon seeds from rotted melons in Penjwen through the use of the laboratory in the garden designing unit of Sulaimani Polytechnic University.

2.2 Fungi Isolation

In fungi isolation, the technique used was agar plating. In this process, the contaminated maize grains were sterilized with sodium hypochlorite ($NaClO_2$) for 3 minutes [12].Then the seeds were washed in three phases with sterile distilled water and dried on a sterile blotter paper in two minutes. Each Petri dish contained a maximum of five seeds that were plated on the sterile PDA agar with streptomycin solution in 0.005 mg/ml and incubated at 25+- 2 o C with 7 days of incubation period, which were transplanted to a second set using PDA to purify fungal isolates [13]. Morphological and microscopical properties were used to identify the colonies that emerged as the main identification was done by the use of fungal identification keys [14]. In order to preserve fungal samples, they were cultured in the slant.

2.3. DNA Extraction

Some of the selected fungi isolates were used to extract their genomic DNA using the Favorgen Biotech Corp. Fungi/Yeast Genomic DNA Extraction Mini Kit according to the manufacturer's procedure. This DNA was kept at −20°C for further analysis.

2.4. Ribosomal RNA (rRNA) Gene

PCR Amplification. Polymerase chain reaction (PCR) was done in a total reaction volume of 50 uL to amplify partial ribosomal RNA (rRNA) genes. To 25 uL of 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S, Denmark), 2 uL of ITS1 (50- TCCGTAGGTGAACCTGCGG-30) ITS4 (50- TCCTCCGCTTATTGATATGC -30) LSU LROR (5'- ACCCGCTGAACTTAAGC -30) LR5 5'- CGCCAGTTCTGCTTACC-30) Vigalys Hester PCR amplification was done in PTC-200 Gradient Thermocycler (Bio-Rad). The thermal cycling conditions were 5 min of initial denaturation at 95 C°, then 35 cycles of denaturation at 95C° of 50 s, annealing of primer at primer-specific temperatures of 60 s and extension at 72C° of 1 min. An extension step was finally done at 72C° and lasted 10 min[15]. Agarose Gel Electrophoresis

2.5. Agarose gel Electrophoresis:

was used to analyze PCR products and extracted DNA. Agarose gels (1.0-1.5) were made by dissolving the agarose powder in 1x TBE buffer and heating it in the microwave till it melted. The solution was then left to cool down to a temperature around 45-37 degC and 5-10 uL of ethidium bromide was introduced to it and mixed thoroughly. Agarose was heated in molten form and then poured into a casting tray with combs and allowed to cool at room temperature. The gel was put in an electrophoresis tank which could hold enough 1x TBE buffer to submerge the gel by 2-3 mm. In the case of DNA samples, 5 uL of each extract was combined with 2-3 uL of 6x loading dye and loaded into different wells. A molecular size marker was a DNA ladder (1001500 bp) loaded. The products of PCR were loaded without the use of any loading dye since the master mix was a red tracking dye. The process was continued until satisfactory separation had occurred in electrophoresis at 50-100 V. The DNA fragments were placed under UV (ultraviolet) light and photographed using the UV transilluminator and recorded.

2.6.Visualization of DNA Fragments

Visualization of the DNA fragments was done using ethidium bromide, a DNA intercalating dye which is used to visualize DNA molecules via UV radiation by its binding property to DNA and giving fluorescence. The band sizes and their positions on agarose gel were determined based on the molecular weight marker.

2.7.DNA Sequencing

A partial sequencing was done for PCR products with the help of an Applied Biosystems ABI Prism Terminator Sequencing Kit and was performed at Microgene Center, Korea. Finch TV software was used for the base-calling and error detection in the chromatograms.

2.8. Sequence Alignment and Comparison

The acquired nucleotide sequences were analyzed by comparing them with reference sequences available at the GenBank database using the Basic Local Alignment Search Tool (BLAST) from the NCBI website. The sequence alignment study helped in finding out the degree of sequence homology as well as the closely related taxa.

2.9. Phylogenetic Tree Construction and Homology Searching

The sequences generated were analyzed through homology searching by aligning them with existing sequences present in the NCBI GenBank database to find out which species were similar. After performing the homology searching, a

phylogenetic tree was constructed using the sequences obtained to establish evolutionary relationships between the isolates**.** used the Nucleotide Basic Local Alignment Search Tool (BLASTn) program (Altschul et al., 1990). The bootstrap (100X) analysis of the phylogenetic tree was done in MEGA-11 software [16].

Results and Discussion

Isolation of fungi According to the frequency of occurrence (Table 1), six Aspergillus species were obtained in the melon seeds or seeds including A. flavus, A. niger, A. corrugatus, A. fumigatus, A., terreus and A. tamari the highest frequency of occurrence was registered to A. flavus (30), then A. corrugatus (23.33), A. fumigatus (16.66), and A. niger (13.33), A. terreus (10 Both A. flavus and A. corrugatus had higher frequencies of occurrence than the other species. Frequency Percentage =Number of samples with fungus/Number of samples with fungusx100.

Table (1): Frequency of Fungi isolated on melon seeds.

Fungal Species	Number of Occurrence	Frequency (%)
Aspergillus flavus	9	30
Aspergillus niger	4	13.33
Aspergillus corrugatus	7	23.33
Aspergillus fumigatus	5	16.66
Aspergillus tereus	3	10
Aspergillus tamarii	2	6.66
Total	30	100

Molecular identification

Identification of the Aspergillus strains at the molecular level was done through PCR amplification of multiple genetic markers such as the internal transcribed spacer (ITS) region (ITS1 and ITS4), the large subunit ribosomal RNA (LSU) gene, and the β-tubulin gene (Bt2A and Bt2B). DNA isolation was performed on genomic DNA and used as the PCR template in order to allow specific binding of the primers to the target areas within the Aspergillus genome. The amplification of the ITS region produced a DNA fragment of about 650 bp, whereas the amplification of the LSU gene resulted in an amplicon of about 1,200 bp. The amplification of the β-tubulin gene gave a fragment *of about 495 bp.*Electrophoretic separation of the fragments was done on a 2% agarose gel (Fig. 1A, B, C).

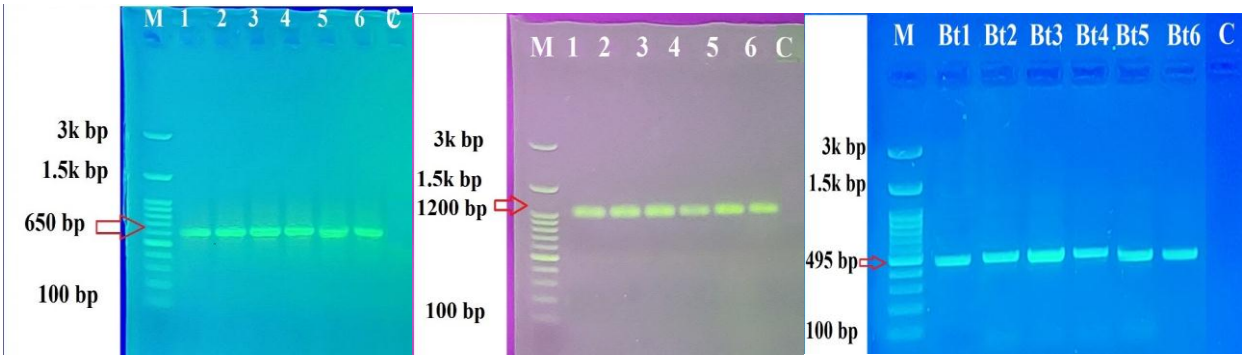

Fig 1 (A) PCR amplification for partial sequence 5.8 S rRNA (ITS) gene of Aspergillus isolates (lanes 1 to 6) was successfully achieved, M indicate to ladder (3K bp-100 bp) and C is negative control, Fig 1 (B) PCR amplification of partial 28S rRNA of LSU gene from Aspergillus samples from lane 1-6 related fungus PCR product, M indicate to ladder (3k bp-100 bp) and C is negative control, Fig 1(C) PCR amplification of partial 28S rRNA of Bt2 gene from Aspergillus samples from lane 1-6 related fungus PCR product, M indicate to ladder (1500 bp-100 bp) and C is negative control

Table (2): Alignment of partial COX subunit I gene sequences with homologous NCBI sequences after database submission.

BLAST Identification	Given Accession No.			Identic Number	Query Cover
	ITS	LSU	Bt2	%	%
Aspergillus flavus	OP554765	OP550093	OQ184733	100%	100%
Aspergillus niger	OP554766	OP550094	OQ184736	100%	100%
Aspergillus corrugatus	OP554769	OP550097	OQ184731	100%	100%
Aspergillus fumigatus	OP554779	OP550107	OQ184738	100%	100%
Aspergillus terreus	OP554767	OP550095	OQ184732	100%	100%
Aspergillus tamarii	OP554768	OP550096	OQ184737	100%	100%

Phylogenetic inferences

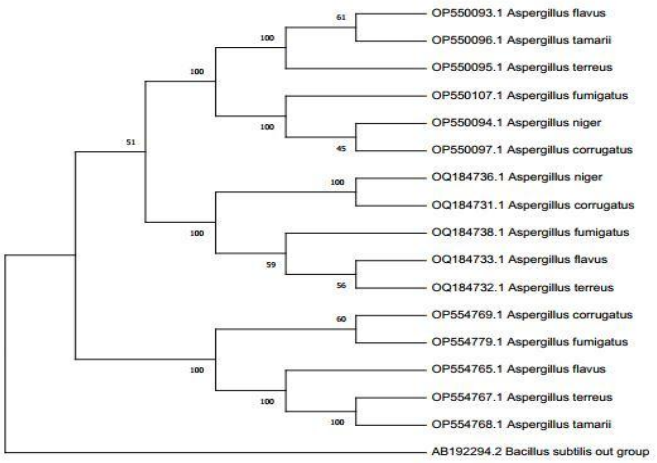

Fig (2) A phylogenetic tree was generated using Neighbor-Joining algorithm based on the concatenated sequences of ITS, LSU, and β-tubulin genes using MEGA11 software.

Discussion

Analysis of frequency distribution for the isolated fungal species showed a predominance of *Aspergillus* spp., although the frequency differed among the isolated species. The most frequently isolated species was Aspergillus flavus, accounting for 30% of the total number of fungi. This result is in line with the findings of earlier studies that indicate the prevalence of this fungus as one of the most common seed-borne pathogens. Being able to grow in hot and humid environments, *A. flavus* can infect seeds both pre-harvest and post-harvest [14]. The high prevalence of *A. flavus* is especially alarming because of the ability of the fungus to produce aflatoxins – one of the most toxic mycotoxins [17].

The second most common species was *Aspergillus corrugatus*, with 23.33% of the isolates belonging to it. Despite being less studied compared to other members of *Aspergillus* genus such as *A. flavus* and *A. niger*, its relative abundance could be explained by adaptation of the fungi to the seed environment. The prevalence of the species might be linked to either storage conditions or substrate specificity as certain *Aspergillus* species prefer some seeds [18].

Aspergillus fumigatus comprised 16.66% of the identified isolates. Thermotolerance of *A. fumigatus* is a well-known feature which helps this species to thrive under unfavorable conditions [19]. In spite of its reputation as an opportunistic human pathogen, the presence of the fungi on the seeds implies their ability to act as saprotrophs decomposing organic material.

The proportion of *A. niger* was 13.33%. This fungus is well known as a frequent contaminant of seeds and agricultural products due to fast growth and abundant conidia formation which enable easy dispersion [20]. Although *A. niger* is regarded as less toxigenic than A. flavus, nevertheless it can negatively influence seed viability and quality of storage conditions.

The proportion of *A. terreus* and *A. tamarii* was lower than the proportion of other isolated fungi – 10% and 6.66%, respectively. The lower proportion of these two species may be caused by some differences in ecological adaptation and growth peculiarities. However, both of these fungi are significant from the point of view of agriculture. *A. terreus* is known as the cause of seed rot and opportunistic infection while *A. tamarii* is usually associated with oilseeds and fermentations [18].

In conclusion, from the current study it can be stated that that the studies carried out indicate that the genera of Aspergillus fungi constitute the most common fungal contaminants of melon seeds. It is believed that the observed variation in their occurrence rates can be due to variations in their ecological fitness, adaptation, and growth behavior.

Conclusion

In this research, it was proven that seeds of melon (Cucumis melo) collected in Penjwen are associated with some species of Aspergillus. A total of six different types of the fungus including *A. flavus*, *A. niger*, *A. corrugatus*, A. fumigatus, *A. terreus,* and *A. tamarii* were isolated and characterized based on their morphological traits and molecular analysis.

The predominant species of *Aspergillus* isolated was *A. flavus*, which demonstrates its good adaptation to the seed environment. The prevalence of this species is very alarming since it is capable of producing aflatoxins, which cause serious harm to seeds' quality, food safety, and people's health. The molecular identification using ITS, LSU, and β-tubulin genes was reliable in terms of fragment amplification and sequence similarity (100%).

The presence of many *Aspergillus* species, especially toxigenic species like *A. flavus*, indicates the dangers associated with them in regard to the quality of the seeds, their ability to germinate, and food safety. Consequently, proper handling, management of the seeds, and regular monitoring of the seeds through morphological and molecular identification is highly important to avoid the problem of fungal infection and mycotoxins production. In addition, further researches are suggested on the basis of aflatoxin determination and control.

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No Supplementary Materials.

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The study was conducted following the protocol authorized by the Head of the Ethics Committee, Sulaimani Polytechnic University(SPU).

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Data available upon request.

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The authors declare no conflict of interest.

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Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

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العزل والتحليل الجزيئي لأنواع Aspergillus المرتبطة ببذور البطيخ

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الخلاصة

تشكل الفطريات المنقولة عن طريق البذور تهديدًا كبيرًا للزراعة وسلامة الغذاء من خلال تقليل قابلية البذور على الإنبات، وتشجيع التلف، وإمكانية إدخال السموم الفطرية (الميكوتوكسينات) إلى سلسلة الغذاء. هدفت هذه الدراسة إلى عزل وتشخيص الأنواع الفطرية من جنس ***Aspergillus*** المرتبطة ببذور البطيخ (***Cucumis melo)*** المجمعة من منطقة بنجوين. تم إجراء العزل الفطري باستخدام طريقة زراعة الأكار على وسط البطاطس-ديكستروز (***PDA)***، تلاها تحديد مورفولوجي للأصناف المعزولة. تم استرجاع ستة أنواع من ***Aspergillus*** وهي: ***A. flavus***، ***A. niger***، ***A. corrugatus***، ***A. fumigatus***، ***A. terreus***، و ***A. tamarii***، حيث أظهر ***A. flavus*** أعلى معدل تكرار بنسبة 30%. تم إجراء التحديد الجزيئي باستخدام تفاعل البوليميراز المتسلسل (***PCR)*** لتضخيم مناطق الجين للموصل الداخلي (***ITS1/ITS4)***، ووحدة كبيرة من الحمض النووي الريبوزومي (***LSU)***، وجين بيتا-توبولين (***Bt2a/Bt2b).*** لوحظت قطع ***DNA*** المضخمة بالأحجام المتوقعة— حوالي 650 زوج قاعدة (***ITS)***، 1200 زوج قاعدة (***LSU)***، و495 زوج قاعدة (—***β-tubulin)*** تم تأكيدها عبر الرحلان الكهربائي على الأكاروز جيل. كما أظهرت عمليات التسلسل والتحليل التطوري تحديد الأنواع وكشفت عن العلاقات التطورية بين المعزولات. يبرز غلبة ***A. flavus*** وأنواع أخرى من ***Aspergillus*** قدرتها على التكيف مع بيئات البذور الجافة وإمكانية إنتاج الإنزيمات المرتبطة بالتلف والمنتجات الثانوية. تسلط هذه النتائج الضوء على أهمية تطبيق ممارسات مناسبة للتعامل مع البذور وتخزينها للحد من التلوث الفطري وضمان جودة البذور.

الكلمات المفتاحية: أنواع الأسبرجيلس (Aspergillus spp.)، بذور البطيخ، الفطريات المنقولة بالبذور، التعرف الجزيئي، ITS، LSU، بيتا-توبولين (β-tubulin)