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Noticeable prevalence of Fusarium proliferatum fungus in Corn seeds in the Middle region of Iraqi

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Summary

In this study we have examined maize samples from 10 different locations across Kerbala, Babylon and Al-Najaf provinces which are located in the middle region of Iraq. 10 species belong to five genera including Deuteromycetes, Zygomycetes and Ascomycetes were present. Among those, Fusarium Spp. was isolated from 14% of the samples. Other genera such as Aspergillus appeared more dominantly with 62% then Rhizopus second with 16% while other genera, Penicillium and Mucor, occurrence rate was 6% and 2%, respectively.

Fusarium proliferatum, one type of fungi that can occur on a wide range of economically important vegetable plants, was isolated then identified based on morphological characteristics in which Fungal colonies produced white aerial mycelia, rarely with a violet pigmentation. Macroconidia appeared colourless and with 3-5 septa. the number of microconidia were more than macroconidia. They were colourless, in chains with one or without septa. As a confirmation, molecular identification was carried out using PCR technique to identify the internal transcribed spacer region (ITS) from the ribosomal DNA (rDNA).

The study reveals for the first time the widespread of F. proliferatum fungus which has been known to cause root rot in number of important crop plants causing major threat to both animal and human health. The isolation and identification of the fungus from maize seeds, which counts as one of main consumable for the population, suggests a potentially reduce in yields and the easy spread throughout the globalized food system. This pinpoint serious health concerns that require more public awareness.

الانتشار الملحوظ لفطر Fusarium proliferatum في بذور الذرة في المنطقة الوسطى من العراق

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الخلاصة

في هذه الدراسة قمنا بفحص عينات ذرة من 10 مواقع مختلفة في محافظات كربلاء وبابل والنجف والتي تقع في المنطقة الوسطى من العراق. 10 أنواع تنتمي إلى خمسة أجناس Deuteromycetes و Zygomycetes و Ascomycetes. بما في ذلك من Fusarium Spp تم عزله من 14٪ من العينات. أجناس أخرى مثل الرشاشيات ظهرت بشكل أكبر بنسبة 62٪ ثم Rhizopus الثاني بنسبة 16٪ بينما كانت الأجناس الأخرى مثل Penicillium و Mucor 6٪ و 2٪ على التوالي.

تم عزل Fusarium proliferatum ، وهو نوع من الفطريات التي يمكن أن تصيب مجموعة واسعة من النباتات المهمة اقتصاديًا ، ثم تم تحديدها بناءً على الخصائص المورفولوجية التي أنتجت فيها المستعمرات الفطريات الهوائية البيضاء ، والتي نادرًا ما كانت مصحوبة بتصبغ بنفسجي. ظهر Macroconidia عديم اللون وبه 3-5 حواجز.حيث كان عدد microconidia أكثر من macroconidia.ةالتي ضهرت عديمة اللون ومقيدة بالسلاسل بحاجز واحد أو بدون حواجز. للتاكد من التشخيص ، تم إجراء الاختبار الجزيئي باستخدام تقنية PCR لتحديد منطقة المباعد الداخلية المكتوبة (ITS) من الحمض النووي الريبوزومي (rDNA).

كشفت الدراسة لأول مرة عن انتشار فطر F. proliferatum المعروف بتسببه في تعفن الجذور في عدد من نباتات المحاصيل الهامة مما يتسبب في تهديد كبير لصحة الإنسان والحيوان. إن عزل وتعريف الفطر من بذور الذرة ، والذي يعتبر من المواد الاستهلاكية الرئيسية للسكان ، يشير إلى احتمال حدوث انخفاض في الغلال وسهولة انتشاره في جميع أنحاء النظام الغذائي المعولم. هذا يحدد المخاوف الصحية الخطيرة التي تتطلب المزيد من الوعي العام.

1. Introduction

Fungal diseases are considered to be a major threat to the most important crops of which humanity depends (1,2). They could appear as a major epidemic causing severely reduced yields (3). Its effects would spread throughout the food system globally (4).

Although, Fusarium proliferatum is a type of fungi that infect a wide range of vegetable plants economically important. However, its role in disease is not always well established (5). In Serbia between the year 2000 and 2001, forty-one samples of

wilting onion and garlic plants were examined, F. proliferatum found to be the predominant fungal species mainly isolated (6,7).

the fungus is distributed a worldwide in important economical plants, including corn and bananas (8,9). More significantly, it found to cause a disseminated infection specially for those patients with weakened immune system (10, 11). As well as body abscesses when trauma is caused by a plant (12)

Recently, both F. proliferatum and F. verticillioides are increasingly blamed especially for invasive and disseminated infections in haemato-oncological patients. (13,14). In the United States, Europe and Asia, 15–27% of the patients suffered from keratitis in particularly those that undergo surgical intervention (15-18).

Furthermore, F. proliferatum was identified as an agent that can cause keratitis, a condition that causes serious damage to vision in 2007 (19,20). The fungus was isolated from the preservation solution of the contact lens. All the clinical features and outcomes associated with keratitis from F. proliferatum-infection (21)

In this study we have shade some light on the distribution of this dangerous fungus among one of the most consumable crops in the country such as maize seeds to investigate potential health risk for populations and animals

2. Methods

2.1. Sample Collection

10 samples of corn seeds were collected from local market across different stores in Karbala and Babylon provinces between the period of 1/11/2018-1/12/2018. Each of which weighted 0.5 kilogram and kept in separate plastic bags which was

labeled with the Sample Number, Date and time of collection and the location of the store from which the sample was taken.

2.2. Culturing of the samples for fungal growth

The fungus was grown from the collected samples via culturing using Potato Dextrose Agar. The PDA (Himedia) media was prepared according to the instructions listed by the manufacturing company, was sterilized in the Autoclave at 121 ° C and pressure 15 psi for 20 minutes. After cooling, 125ml/ L of Chloramphenicol antibiotic was added to the media which were then poured into plates. 5 seeds from each sample were selected randomly and sterilized by 2% of sodium hypo-chlorates. The seeds then washed twice by distal water and dried out using sterilized filter paper. Selected seeds were placed in plates containing each 20 cm^2 of PDA media then incubated at 25±2 0c for 3-7 days in the dark. Afterward, the colonies of different shape and colours were sub-cultured on Potato Dextrose Agar(PDA). The plates were incubated at 28 ^{o} C for 5 to 7 days. Pure culture of each colony was maintained on the slanted PDA medium as discs and stored at 4° C.

2.3. Genomic DNA Extraction

Fungal isolates that were identified as the typical characteristics of Fusarium spp. were selected. further subcultured of the selected isolate on PDA media and fungal. Twenty millilitres of malt extract broth (MEB, Oxoid) were used fungal isolates inoculation and incubated for 5 days at 28° C. Using sterile Eppendorf tubes, the spore’s suspension was collected for DNA extraction.

Total genomic DNA of all fungi samples was extracted using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions. the

spore's suspension was mixed gently before incubating for 5 min at 80°C. The enzyme solution of RNase (3 µL) was then added to the solution and incubated at 37°C for 15 - 60 min. 200 µL of protein precipitation solution was added to the mixture, vortexed continuously and were then incubated on ice for 5 min. The cold mixture was centrifuged at 13,000 rpm for 3 minutes. The obtained supernatant was transferred to 600 µL of isopropanol in a clean tube and kept at room temperature after proper mixing. Identical second centrifugation was carried out yet for 2 minutes in which the supernatant was discarded. The centrifugation step was repeated again after 600 µL of 70% ethanol was added. the generated pellet was air-dried for 10- 15 minute once the ethanol was aspirated. lastly,100 µL of rehydration solution is used to rehydrate the DNA pellet for one hour at 65°C.

2.4. PCR Amplification

The internal transcribed spacer (ITS) gene in the Fusarium samples was amplified using PCR technique. Two primers based on the conserved region of ITS1 and ITS4 primers were employed. The forward primer (ITS1) had a sequence of (5´ GCCTGTTCGAGCGTCATTTC-3´) while the reverse primer (ITS 4) was (5´ GCGAGACCGCCACTAGATTT -3´). 50 µL reaction mix was used. The mix was consisting of 5µL of 10x PCR-Buffer, 1 µL of dNTP mix of 800 µM concentration, two primers of which 2.5 µL from forward and reverse primers of 0.5 µM concentration both and lastly Taq DNA polymerase was employed. 36.5 µL of sterile distilled water (Bioron, Germany) was used to complete the 50 µL of the reaction mix. The reaction mix was run at the same time with the negative control which consist of only water. T100 Thermal Cycler amplifier (BioRad, Singapore) was employed to generate the PCR product.

For agarose gel electrophoresis experiment, 1% agarose gel containing Gel Red dye was used. the gel was loaded with 5µL of 3 kb ladder (Norgen High Ranger, Canada). 7 µL of PCR products were mixed with 3 µL of loading dye then loaded into the wells

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of the gel. PCR products were analysed by gel electrophoresis system (Uvitec Cambridge, UK) at 100 Volt for 45 minutes.

The amplified product was purified using purification kit (Roche Diagnostic GmbH, Germany). According to the manufacturer's instruction, the ratio of 5:1 volume of binding buffer and PCR product was applied. They were mixed well and transferred into filter tube in which centrifugation at 13,400 rpm (Eppendorf mini spin, USA) for 60 sec at 5°C was conducted. The DNA binds to the filter and all flow-through was then discarded. Two wash steps were carried out using 500 µL and 200 µL of wash buffer then centrifuged at 13,400 rpm for 1 min. The flow-through was also discarded every time. Finally, DNA was eluted by adding 50 µL of elution buffer (10 mM Tris-HCl) into a clean 1.5 ml microcentrifuge tube and centrifuged at 13,400 rpm for 1 min. Then, the DNA fragment was run on a 1% agarose gel in Tris-base, acetic acid and EDTA (TAE) buffer, and stained with Gel Red dye.

3.Results and Discussion

3.1 Sample Collection

No of Sample	Date of collection	Time of collection	Location of store
1	1/11/2018	17:00	Karbala Al Amel Quarter
2	3/11/2018	09:00	Karbala/ Dahhan Market
3	4/11/2018	16:00	Najaf/ Kufa Quarter
4	5/11/2018	18:00	Najaf/ Al muskaf Market
5	12/11/2018	10:00	Babylon/ Old market
6	13/11/2018	20:00	Babylon/ Arbaeen Street
7	25/11/2018	17:00	Karbala/ Hindia Market
8	25/11/2018	17:30	Babylon/ Abo Gharaq Market
9	26/11/2018	11:00	Najaf/ Al-Amer Quarter
10	1/12/2018	18:00	Karbala/ Al Zhara Quater

Table 1: shows the location, date and time of the collected samples

Each of the samples weighted 0.5 kilogram and kept in separate plastic bags which was labeled with the Sample Number, Date and time of collection and the location of the store from which the sample was taken.

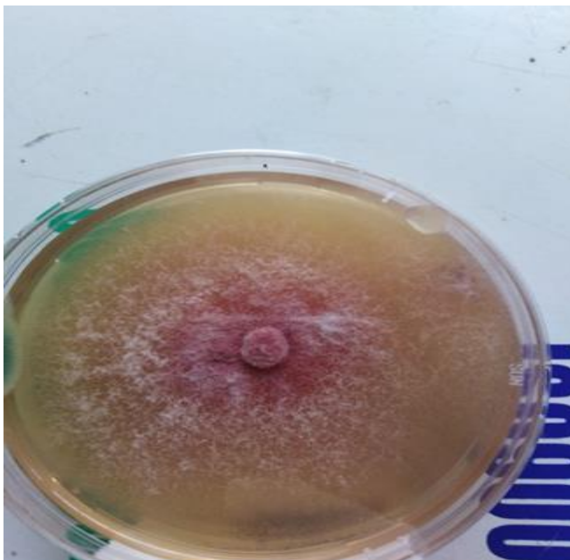
3.2. Isolation of fungi

Type of Fungus	Frequency	Ocurrance Precentage %
Aspergillus spp.	31	62
Rhizopus spp.	8	16
Fusarium spp.	7	14
Penicillium spp.	3	6
Mucor spp.	1	2
Total	50	100

Table 2: shows the frequency and the percentage of the identified isolates. Five genera including Deuteromycetes, Zygomycetes and Ascomycetes were present. Among those, Fusarium Spp.

Fusarium Spp. was isolated from 14% of the samples. Other genera such as Aspergillus appeared more dominantly with 62% then Rhizopus second with 16% while other genera, Penicillium and Mucor, occurrence rate was 6% and 2%, respectively.

3.2 Identification of Fusarium proliferatum



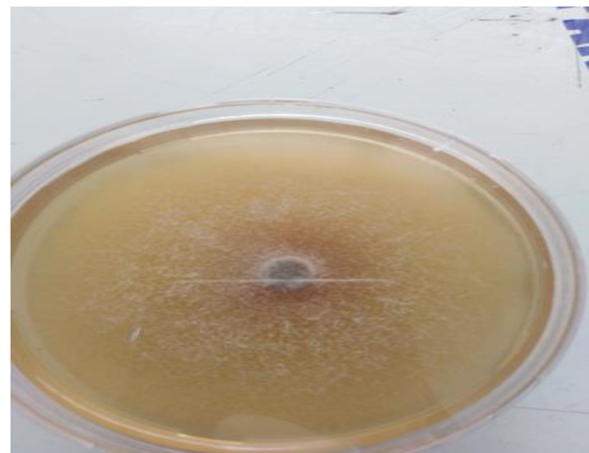


Figure 1: morphological characteristics in which Fungal colonies produced white aerial mycelia, rarely with a violet pigmentation.

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Fusarium proliferatum growth on Potato Dextrose Agar media. The Fusarium fungus grew rapidly on the media giving an overall appearance of colonies appeared initially heavy white aerial mycelium that become tinged with reddish violet to purple as they produce dyes. Some of the isolates did not produce the pigments

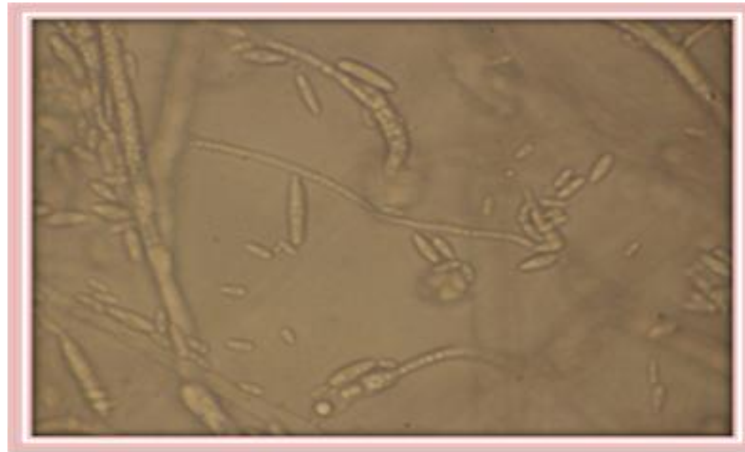
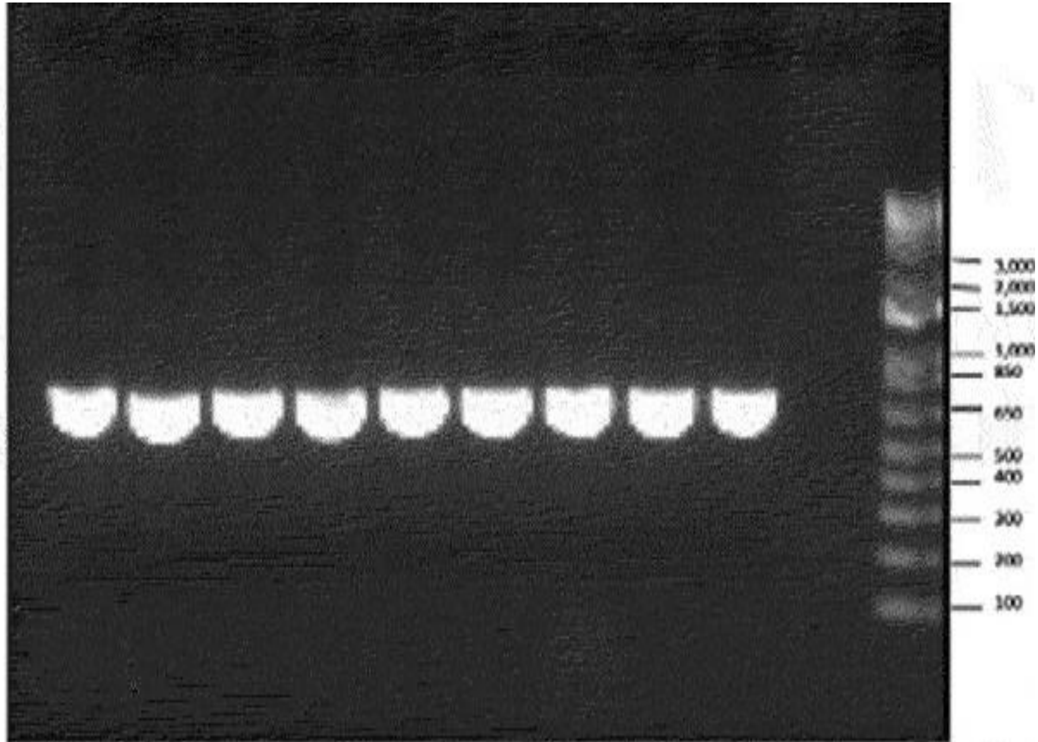


Figure 2 When the fungus was examined microscopically, the fungus contained a set of thread spice-shaped with spores. The fungus Hyphae were septate and slightly curved to almost stright. Conidiophores were medium length either simple or branched. Conidiogenous cells were polyphialides . Microconidia are abundant, single-celled and clavate. Macroconidia appeared colourless and with 3-5 septa. the number of microconidia were more than macroconidia. They were colourless, in chains with one or without septa



PCR of internal transcribed spacer (ITS1 and ITS4) OF Ribosomal DNA for Fusarium samples on 1% agarose gel electrophoresis. 3 kb ladder on the right, then negative control, then PCR products for the fusarium isolates The PCR amplification of the ITS regions resulted in ~650 -bp of fragment.

4. Conclusion

Fusarium species have recently deemed as fundamental fungi causing degradation to certain parts of the plants such as palm groves (22) as well as stem and root rot in others including onion and lucky bamboo in Iraq (23). In this study we have proved that they could cause further damage and sustained their survival by contaminating other important crops such as maize seeds.

The identification of Fusarium proliferatum from most of our isolates suggested the widespread distribution of the fungi in the middle region of Iraqi soil and environment. This inevitably indicate the possibility of contamination of their well-known mycotoxins in foods. The world's agricultural production is contaminated with mycotoxins by more than 25% according to the Food and Agriculture Organization (FAO). Thus leading to financial losses in the industry of grains (71). Contaminated materials may be pathogenic for animals and humans. Hence the urge to establish reasonable effective

method to contain this key pest and eliminate their toxins is very necessary to protect the public health and prevent them from wide spreading specially in long stored grain.

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