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

Fatima A. Karim, Lecturer at The Department of Clinical Laboratories, College of Applied Medical Sciences, University of Kerbala, Iraq.

Ohoud Salman Jawad, Assistant Lecturer at The Department of Clinical Laboratories, College of Applied Medical Sciences, University of Kerbala, Iraq.

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 belongs 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 the main consumables for the population, suggests a potentially reduce in yields and the easy spread throughout the globalized food system. This pinpoints serious health concerns that require more public awareness.

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 infects 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 worldwide and been associated with a variety of diseases in important economical plants, including corn and bananas (8,9). More significantly, it found to cause a disseminated infection in immunocompromised patients (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 that undergo surgical intervention such as corneal transplantation, removal of eye contents, enucleation, or suffer vision deterioration suffered from keratitis(15-18) Furthermore, *F. proliferatum* was mentioned in 2007 as an agent that can cause *Fusarium* keratitis, a condition that causes serious damage to vision (19,20). The fungus was isolated from the contact lens preservation solution, the clinical features and outcomes associated with keratitis from *F. proliferatum*-infection(21) In this study we have shed 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

1. Methods

1.1. 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 weighed 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.

1.2. 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 was 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 cm² of PDA media then incubated at 25±2 °C for 3-7 days in the dark. Afterward, the colonies of different shape and colours were sub-cultured on Potato Dextrose Agar incubated at room temperature 28 °C for 5 to 7 days, and pure culture of each colony was maintained on the slanted PDA medium as discs and stored at 4 °C.

1.1.Total Genomic DNA Extraction

purified fungal isolates was obtained from fungal isolates that showed the typical characteristics of *Fusarium* spp. They were further subcultured on PDA media and fungal DNA was extracted according to the method described by [23] with modification. 20 ml of malt extract broth (MEB, Oxoid) was used to inoculate the fungi isolates then incubated at 28 °C for 5 days. After that, the spores suspension was obtained and transferred using sterile Eppendorf tubes for DNA extraction.

Total genomic DNA of all fungi samples was extracted using DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. The mixture was mixed gently before incubating for 5 min at 80 °C. RNase solution (3 µL) was added to the mixture and incubated at 37 °C for 15 - 60 min. Next, protein precipitation solution (200 µL) was added to the mixture, vortexed continuously and were then incubated in ice for 5 min. Then the cold mixture was centrifuged at 13,000 rpm for 3 minutes. The obtained supernatant was transferred to a clean tube containing 600 µL of isopropanol at room temperature (25 °C) and mixed properly. Second centrifugation for 2 minutes was carried out at 13,000 rpm, and the supernatant was discarded. After that, 600 µL of 70% ethanol was added,

mixed and centrifuged again for 2 min at 13,000 rpm. When the ethanol was aspirated, the pellet was air-dried for 10- 15 minute. lastly, 100 μ L of rehydration solution is used to rehydrate the DNA pellet for 1 h at 65°C or overnight at 4°C. And Reverse primer (ITS 4) (5' GCGAGACCGCCACTAGATTT -3') . 50 μ L reaction mix was used containing Complete 10x PCR-Buffer (5 μ L), 1 μ L dNTP mix (800 μ M), forward primer (0.5 μ M), reverse primer (0.5 μ M) in 2.5 μ L each , 0.5 μ L Taq DNA polymerase. The volume was completed by 36.5 μ L of sterile distilled water (Bioron, Germany). The negative PCR non-template control (water only) was used. The amplification of PCR products was done by using T100 Thermal Cycler (BioRad, Singapore). Five μ L of 3 kb ladder (Norgen High Ranger, Canada) was loaded into the well of agarose gel and used as a standard. 7 μ L of PCR products were loaded together with 3 μ L of loading dye . PCR products were analysed by electrophoresis at 100 Volt for 45 minute using 1% agarose gel containing Gel Red dye and visualised by gel electrophoresis system (Uvitec Cambridge, UK).

High pure PCR product purification kit (Roche Diagnostic GmbH, Germany) was used to purify the amplified products according to the manufacturer's instruction. 5:1 volumes of binding buffer and PCR product was mixed well and transferred into filter tube. After centrifugation at 13,400 rpm (Eppendorf mini spin, USA) for 60 sec at 5°C, The DNA binds to the filter . The flow-through was then discarded. Two wash steps were carried out using 500 μ L and 200 μ L 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 μ L 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 ethidium bromide.

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	Occurance Percentage %
Aspergillus spp.	31	62
Rhizopus spp.	8	16

Fusarium spp.	7	14
Penicillium spp.	3	6
Mucor spp.	1	2
Total	50	100

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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.

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 straight. 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.

2. Conclusion

Fusarium species have recently been 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 indicates the possibility of contamination of their well-known mycotoxins in foods and feeds.

According to the Food and Agriculture Organization (FAO), the world's agricultural production is contaminated with mycotoxins by more than 25%, leading to financial losses in the industry of grains (71).

Contaminated materials may be pathogenic for animals and humans. Thus, one of the most effective measures to protect the public health is to establish a reasonable effective method to contain this key pest and eliminate their toxins and prevent them from wide spreading specially in long stored grain.

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