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RESEARCH ARTICLE

DETECTION OF *FUSARIUM* SPECIES THAT PRODUCE FUMONISIN B1 IN MAIZE KERNELS USING MOLECULAR METHODS

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ABSTRACT

Eighty eight Maize grains samples were collected between November 2013 and May 2014 from local markets and silo of Baghdad city. The aim of study was to identification of *Fusarium* species which associated with maize kernels and their ability to produce FB1 by using traditional and molecular methods. The results revealed that eight fungal genera found in maize samples as follow: *Aspergillus* (58.6 %) was predominant fungus followed by *Fusarium* (17.5%) and then *Rhizopus* (6.8 %), *Alternaria* (6.1 %), *Mucor and Penicillium* (3.9 %), *Bipolaris* (1.9 %), and *Trichothecium* (1.2%). The predominant species among *Fusarium* genera was *F. verticillioides* (70.64%) followed by *F. proliferatum* (8.26%). Specie specific PCR reactions revealed that out of seventy seven isolates only thirteen isolates belong to *F. verticillioides* and out of nine isolates only one isolate belong to *F. proliferatum.* All the thirteen *F. verticillioides* isolates possess *fum1* gene, while absent in *F. proliferatum*.

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INTRODUCTION

The genus *Fusarium* is common plant pathogens occurring worldwide, and produces over one hundred secondary metabolites, which can affect human and animal health. The most important *Fusarium* toxins that can occur at significant concentrations in cereals are: fumonisins, zearalenone and trichothecenes (nivalenol, deoxynivalenol and T-2 toxin) (Sopterean and Puia, 2012). *Fusarium* genus considered taxonomically complex due to contain at least seventy species, because their identification are difficult, molecular methods are required to confirm identification (Healy *et al.,* 2005). The medically important *Fusarium* species that have discovered by molecular methods include: *F. solani, F. oxysporum, F. moniliforme, F. incarnatum, F. chlamydosporum*, and *F. dimerum* (O'Donnell *et al.,* 2004; Migheli *et al.,* 2010). Fumonisins are secondary toxic metabolites produced by the genus *Fusarium*; mainly the species *Fusarium verticillioides* (Bezuidenhout *et al.,*1988), the most abundant and toxic of the fumonisins is Fumonisin B1 (FB1), which representing about 70% of the total food and feed contamination (Dupuy *et al.,* 1993).

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Ingestion of FB1 causes species-specific target-organ toxicity such as: neurotoxic, nephrotoxic, hepatotoxic, teratogenic and pulmonary effects (Bondy *et al*., 1997). FB1 has been related with high rates of human esophageal cancer worldwide and with increased incidences of neural tube defects in infants of mothers consuming maize-based products contaminated with this toxin, also increased levels of fumonisins in moldy maize kernels have been previously linked to the high incidence of human esophageal cancer in several region in the South of Africa (Marasas et al., 1981 and2004). Aim of this study; isolation and identification of *Fusarium spp*. produce FB1 from maize seeds using traditional methods and confirming diagnosis of toxigenic *Fusarium spp.* by molecular methods using species-specific PCR.

MATERIALS AND METHODS

Samples collection

Eighty eight Maize grains samples were collected between November 2013 and May 2014 from local markets and silo of Baghdad city (thirty five from silo and fifty three from markets). Two kilogram of each sample was placed in a plastic bag and stored at 4°C until analysis.

Isolation of Fungi

Fifty grains from each maize sample were surface-sterilized by immersion in 2% sodium hypochlorite (NaOCl) solution in 250 ml conical flask for 1 minute, and washed three times with sterilized distilled water, the grains were dried with sterilized filter paper in a laminar flow hood and placed on Malachite Green Agar (MGA 2.5), as selective media for isolation of *Fusarium* spp., and Potato Dextrose Agar (PDA), as enrichment media for isolation of other genera of fungi, both media contain chloramphenicol (125 mg/L) (5-10 grains / each plate). After incubation for seven days at 25°C, the fungi were isolated and sub-cultured to obtain pure culture. All fungi were identified by morphological characteristics on PDA and microscopic characteristics by scotch tape preparation, according to Barnett and Barry (1998) and Domsch (2007).

Identification of *Fusarium species*

All *Fusarium* isolates were identified to the species level on the basis of colony morphology on PDA, Spezieller Nährstoffarmer Agar (SNA) and Carnation Leaf Agar (CLA). Microscopic characteristics using scotch tape preparation, according to Booth (1977) and Leslie and Summerell (2006). The occurrence and frequency of fungal genera was measured according to Choi *et al*., 1999.

DNA extraction and Species Specific PCR assay

Fusarium isolates were grown on PDA plates for 7 days and mycelia were harvested and ground in liquid nitrogen (Rahjoo *et al.,* 2008). Total DNA was extracted from ground mycelium of each isolate using Geneaid genomic DNA kit according to the manufacturer's instructions.

Primers and PCR conditions were selected according to previously published studies (Mule *et al.,* 2004a; El yazeed *et al.*, 2011). These primers used for diagnosis of *F. verticillioides, F. proliferatum* and FB1 as shown in Table (1& 2). All primers were supplied by Alpha DNA Company, Canada. PCR Master Mix (Promega company/ USA) ready to load (Green) had been used in this work. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel stained with ethidium bromide.

Statistical analysis

All analysis was performed using the statistical package (SPSS) version thirteen; the data were expressed as mean, standard deviation SD, percentage. ANOVA was used to analyze repeated measurement. Results were determined as very high significant at ($P \leq 0.001$), high significant ($P<0.01$) and significant at ($P< 0.05$) and non significant at ($P> 0.05$).

RESULTS AND DISCUSSION

Isolation and Identification of Maize Mycoflora

The results revealed that eight fungal genera were found in maize samples of present study; *Aspergillus* (58.6%) was the most predominant fungus followed by *Fusarium* (17.5 %) as shown in Table (3) There is high significant differences between the fungal genera ($p< 0.001$). these results similar with previous studies (Saleem *et al.*, 2012; Mahmoud *et al.,* 2013; Hassan *et al.,* 2014). *Aspergillus* and *Fusarium* are the major mycotoxigenic fungi and common component of the mycoflora belong to some species associated with many crops especially maize (Palumbo *et al.*, 2008). These two genera are able to produce various types of toxins that have been reported to be; carcinogenic, teratogenic, hepatotoxic, nephrotoxic, neurotoxic, mutagenic, immunosuppressive, tremorgenic, hemorrhagic, and potentially increased susceptibility to HIV (Anonymous, 1993; Hashem *et al.,* 2012).

Isolation and Identification of *Fusarium* spp.

One hundred and nine isolates of *Fusarium* spp. were isolated from different maize samples cultured on MGA and then sub-cultured on PDA (Fig. 1)

MGA medium is a more selective culture medium for *Fusarium* spp. than other media, because it does not allow the development of colonies belonging to other fungal genera. MGA is simple to prepare and less hazardous than other *Fusarium* selective media containing pentachloronitrobenzene (PCNB), also it suitable for isolation and enumeration of *Fusarium* spp. (Castell'a *et al.*, 1997).

All *Fusarium* colonies were transferred onto PDA, CLA and SNA media to identify them to the species level, depending on macroscopic appearance (colony morphology and color) and microscopic appearance (conidia and phialides types), using fungal keys and manuals of Booth (1977) and Leslie and Summerell (2006). The advantage of using CLA and SNA for identification of *Fusarium* isolates to the species level, because conidia produced in PDA are not consistent in size and/or in shape as those produced on CLA and SNA. In addition, these two media (CLA and SNA) produce more and longer microconidia chains, also, chains are easier to see due to less moisture on the agar surface and then fewer droplets in the aerial mycelium.

Table (4) shows the frequency of *Fusarium* spp. in maize seeds were, *F. verticillioides* (70.64%), *F. proliferatum* (8.26%), *F. oxysporum* (7.34%), *F. graminearum, F. solani* and *Fusarium* spp. (4.59%). The high frequent species was *F. verticillioides* followed by *F. proliferatum*. These findings agree with Miller (2001) and Stumpf *et al.* (2013). *F. verticillioides* and *F. proliferatum* are main producer to FB1toxin (Tančić *et al.*, 2012).

Species specific PCR

Seventy seven isolates of *F. verticillioides* and nine isolates of *F. proliferatum* were subjected to molecular analysis using species – specific PCR, as follow; *ver* gene to identify *F. verticillioides* isolates, *pro* gene to identify *F. proliferatum* and *fum1* gene for detection FB1 toxin. Table (5) revealed that gene *ver* appeared in thirteen out of seventy seven isolates for *F. verticillioides*, while gene *pro* appeared in one out of nine isolates for *F. proliferatum,* as shown in Figures (2) and (3) respectively.

Table 1. Primers Used in This Study

Primer Name	Primer Sequence (5' → 3')	Product Size (Bp)	Species-Specificity
ver-F	CTTCCTGCGATGTTTCTCC	578	*F. verticillioides*[a]
ver-R	AATTGGCCATTGGTATTATATATCTA		
pro-F	CTTTCCGCCAAGTTTCTTC	585	*F. proliferatum*[a]
pro-R	TGTCAGTAACTCGACGTTGTTG		
fum-F	CCATCACAGTGGGACACAGT	183	Fumonisin B1[b]
fum-R	CGTATCGTCAGCATGATGTAGC		

[a]Mulé *et al.,* 2004;[b] El yazeed, *et al.,* 2011

Table 2. PCR Condition for *ver, pro* and *fum1* genes

Gene	Initial Denaturation	No. of cycles	Denaturation	Annealing	Extension	Final extension
ver & pro	95 °C/ 5min.	35	94 °C/ 50 sec.	56 °C/ 50 sec.	72 °C/ 1min.	72 °C/ 7min.
fum1	94 °C / 5min.	35	94 °C/1min.	58 °C/ 1min.	72 °C/ 1min.	72 °C/ 10min

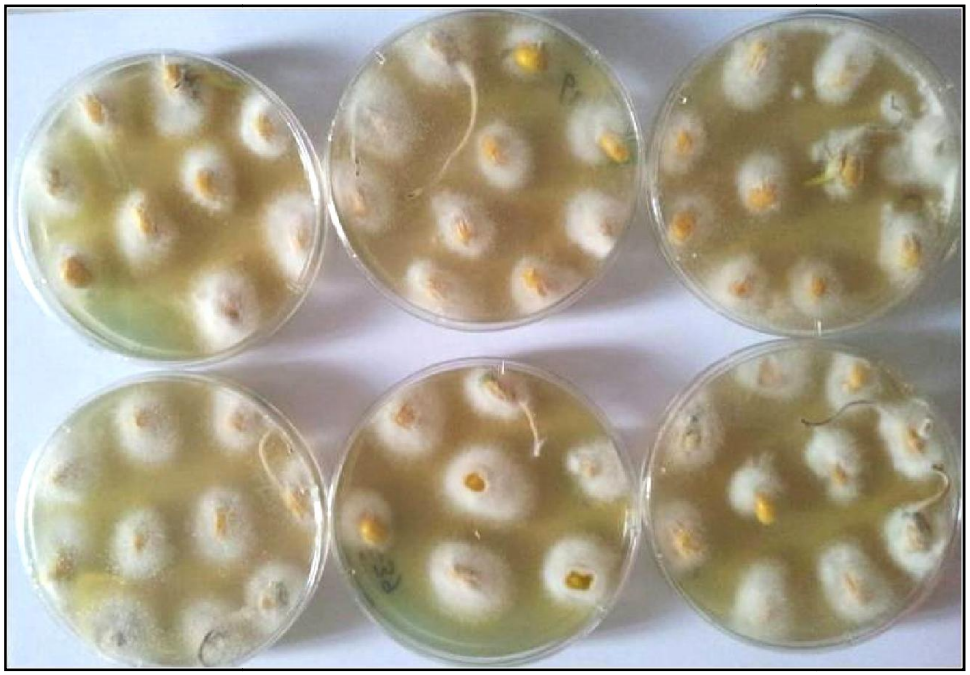

Figure 1. Predominant of *Fusarium spp*. in maize samples on MGA medium

Table 3. Occurrence and Frequency of Fungal Genera in Maize Kernels

Genera	No. of isolates	Occurrence %	Frequency %
Aspergillus	364	32	58.6
Fusarium	109	25	17.5
Rhizopus	42	12.2	6.8
Alternaria	38	9.6	6.1
Mucor	24	10.3	3.9
Penicillum	24	8.3	3.9
Bipolaris	12	1.3	1.9
Trichothecium	8	1.3	1.2
Total	621	100	100

**Significant Differences p<0.001*

Table 4. Frequency of *Fusarium spp.* in Maize Seeds

Isolates	No of isolates	Frequency %
F. verticillioides	77	70.64
F. proliferatum	9	8.26
F. oxysporum	8	7.34
F. graminearum	5	4.59
F. solani	5	4.59
Fusarium spp.	5	4.59
Total	109	100

**Significant Differences p<0.0001*

Table 5. Identification of *Fusarium verticillioides* and *Fusarium proliferatum* by species-specific PCR

Species	No. of isolates	PCR primer results		
		ver	*pro*	*fum1*
F. verticillioides	77	13	-	13
F. proliferatum	9	-	1	-

**Significant Differences p<0.001*

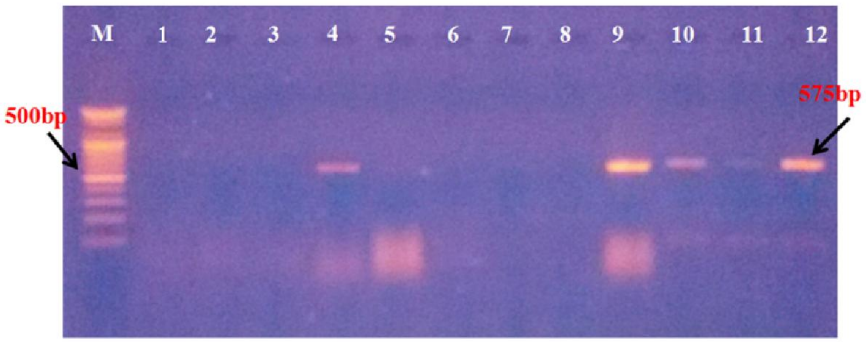

Figure 2. Gel Electrophoresis of *ver* gene (575bp), using 100bp DNA ladder (M=DNA ladder; Lanes (4, 9-12)=positive *ver. F. verticillioides;* Lanes (1-3,5-8) =negative)

Figure 3. Gel Electrophoresis of *pro* gene (585bp), using 100bp DNA ladder. (M=DNA ladder; Lane (7)=positive *pro. F. proliferatum;* Lanes (1-6 and 8) =negative)

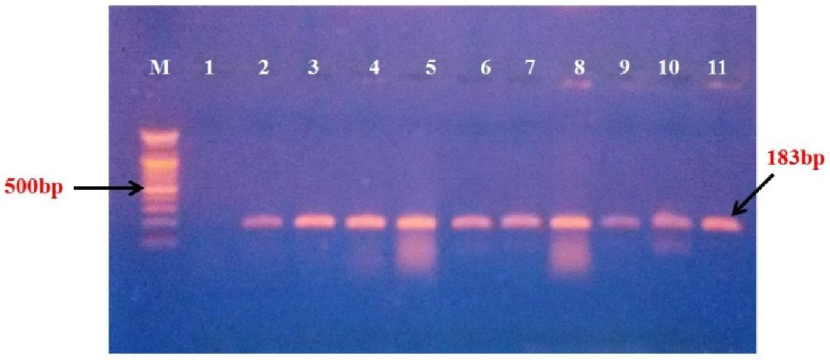

Figure 4. Gel Electrophoresis of *fum1* gene (183bp), using 100bp DNA ladder. (M= DNA ladder; Lane (1)= negative *fum1 F. proliferatum;* Lanes (2-11)= positive *fum1 F. verticillioides*)

Whereas *fum1* gene appeared in all thirteen isolates belong to *F. verticillioides,* and not appeared in single isolate belong to *F. proliferatum,* as shown in Figure (4). All the negative PCR results that use for *ver* gene were subjected to *pro* gene and vice versa, to verify the morphological diagnosis, no positive amplification was obtained with these primers. So, these isolates, with no PCR product to *ver* and *pro* gene, might belong to another *Fusarium* species, have similar micro- and macro-appearance to *F. verticillioides* and *F. proliferatum,* but different genetically. Due to morphological similarity between *F. verticillioides* and *F. proliferatum,* mistake between these two species can be occur, and the only accepted morphology that distinguish between these species is the presence and / or absence of polyphialides (Visentin *et al.,* 2009). In addition,

their diagnosis are time consuming and required trained personal to recognize between them, while species specific PCR are rapid and sensitive tools that give accurate diagnosis to *Fusarium* species (El yazeed *et al.,* 2011). Another important advantage of PCR techniques is that only small quantities of DNA are required to confirm the presence of a pathogen on a host tissue that might be not detected (Chandra *et al.*, 2008). In this study, the results show that all *F. verticillioides* isolates, that possess *fum1* gene, are predominant FB1 producer than *F. proliferatum*, since it is not posses *fum1* gene. In contrast with Sreenivasa *et al.* (2008) and El Yazeed *et al.* (2011), both found that the *fum1* gene was amplified in all *Fusarium* isolates used in their studies (*F. verticillioides*, *F. proliferatum* and *F. anthophilum*). The genus *Fusarium* are complex, because of large number and closely related species, which have been completely classified on microscopic characteristics. As *F. verticillioides* and other fumonisin producing fungi cause latent or asymptomatic infections in maize and the plant tissues, determining their occurrence and frequency on host tissue requires systematic sampling (Grimm and Geisen, 1988). Many studies have been used molecular methods to detect *Fusarium* species contamination in cereals (Abd-Elsalam *et al.,* 2003; Patino *et al.,* 2004; Mule *et al.,* 2004a, b). There are several genes involved in fumonisin biosynthetic pathway which have been used as target for polymerase chain reaction assays to detect the fumonisin producing fungi (Bluhm *et al.*, 2004; Patino *et al.,* 2004; Wang *et al.,* 2010; Ramana *et al.,* 2011 & 2012). FUM1 gene is polyketide synthase (PKA) gene, previously called FUM5, responsible for fumonisins production in fungi, therefore, species specific primers were designed depending on the sequence data from PKA gene (Proctor *et al.,* 1999; Baird *et al.,* 2008). Numerous studies found that the intergenic spacer regions (IGS) have high levels of sequence variability among the species of the same genus, and allow differentiation of genetically related species, therefore, IGS is usually used for identification purposes in taxonomic studies (Edel *et al.,* 2000; Kim *et al.,* 2001; Konietzny and Greiner 2003; González-Jaén *et al.,* 2004; Maheshwar and Janardhana, 2010).

Conclusion

Contamination of maize samples (silo and local market) with toxigenic *F. verticillioides* and *F. proliferatum.* The identification of *F. verticillioides, F. proliferatum* and FB1 gene by the species specific PCR are a practical, short, reliable and more accurate method when compared to the traditional methods. All isolates of *F. verticillioides* possess *fum1* gene have the ability to produce FB1 in different concentrations, in contrast *fum1* gene absent in *F. proliferatum.*

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