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Molecular versus Morphological Identification of *Fusarium spp.* isolated from Egyptian corn.

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ABSTRACT

Fumonisin are important mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*, which are associated with human esophageal cancer. In this study, 56 *Fusarium* isolates - from 31 field maize samples collected from Egypt, were identified using both morphological and molecular methods. The potential production of fumonisin B1 was determined using HPLC and molecular methods. The results of molecular identification showed that all *Fusarium* isolates belonged to *F. verticillioides*, based on *F. verticillioides*-specific primer pair Verpro-F/VERTI-R and primer pair Taqfum- 2f/Vpgen- 3R, which is specific for *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, *F. globosum* and *F. nygamai*, while the morphological and microscopic identification of the 56 isolates showed that 54 isolates (96.4%) belonged to *F. verticillioides*, 1 isolate (1.8%) belonged to *F. proliferatum* and 1 isolate (1.8%) belonged to *F. subglutinans*; showing compatibility of 96.4% with the molecular identification. Although just one corn sample from the 31 contained 9.8 mg kg⁻¹ FB1; which considered higher than the permissible limit (3 mg kg⁻¹). The HPLC analysis confirmed that about 82% of the isolates were able to produce fumonisin B1 in ranges between 1.4 to 555 mgkg⁻¹ medium. Whereas, PCR detection indicated that all isolates were able to produce fumonisin B1. Results of this study revealed that PCR-based technique could be used to differentiate the *Fusarium* species from other genera of fungi as well as to identify fumonisin-producing *Fusarium* species.

Keywords: Fumonisin B1, *Fusarium verticillioides*, *Fusarium proliferatum*, HPLC, PCR.

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INTRODUCTION

Fumonisin (FBs) are a group of mycotoxins produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum* (Marasas, 2001). FBs are produced in maize (Sydenham *et al.*, 1992), wheat and barley (Aziz and Moussa, 2004). Fumonisin B1 (FB1) is a highly toxic FB in vertebrates. FB1 has been found to be teratogenic (Marasas *et al.*, 2004) and carcinogenic (Gelderblom *et al.*, 2001 and Lemmer *et al.*, 1999) and to cause oesophageal cancer (Marasas, 2001) and neural tube disorders in human (Missmer *et al.*, 2006). The International Agency for Research on Cancer (IARC) classified FB1 in Group 2B as “a possible carcinogenic to humans” (IARC, 2002). Therefore, the early detection of grains field’s contamination by *Fusarium* is necessary to reduce food-borne illness.

F. verticillioides and *F. proliferatum*, the most abundant fumonisin producers (Stępień *et al.*, 2011) belong to *Gibberella fujikuroi* species complex. There are limits on the use of morphological characters for the identification of species in the *G. fujikuroi* species complex; as some species have very similar morphological features, *i.e.* *F. proliferatum* and *F. fujikuroi*, *F. sacchari*, *F. subglutinans* and *F. temperatum* as well as *F. verticillioides* and *F. Andiyazi* (Leslie and Summerell, 2006; Rossi *et al.*, 2009; Hsuan *et al.*, 2011 and Scaufflaire *et al.*, 2011). Species-specific PCR reactions are commonly used to clearly identify species inside the complex (Rahjoo *et al.*, 2008 and Hsuan *et al.*, 2011).

Fusarium DNA genomic analysis revealed that the gene *FUM1* encodes a polyketide synthase (PKS) which is necessary for the production of fumonisins and catalyses the initial steps in its biosynthesis (Bojja *et al.*, 2004). Accordingly, it could be used to detect FB1 production by *F. verticillioides* (López-Erassquin *et al.*, 2007). The objectives of the present study were to evaluate the molecular versus morphological methods for the identification of *Fusarium* spp. isolated from field maize samples from different areas in Egypt; as well as to evaluate the chemical versus molecular analysis for the potential production of fumonisin by the isolated strains.

MATERIALS AND METHODS

Samples collection

Thirty one samples of white and yellow dry cob corn were collected from different fields in 3 Governorates (9 Al-Beheira, 9 Al-Daqahlia and 13 Al-Qalyubia). Grains of each Sample were separated manually by hand in aseptic condition. Each sample was divided into two parts, one for microbiological analysis and the other for toxin determination.

Isolation of *Fusarium* spp. from corn using deep Freezing Blotter Method:

To sterilize corn grains, one hundred grains of each sample were surface disinfected with 5% sodium hypochlorite (NaOCl) for 1 min followed by rinsing three times with sterile water. The grains were dried between two layers of sterilized filter paper. The disinfected grains (25 grains/petri dish and four petri dishes/sample) were plated on moisten filter paper by deep freezing blotter method (Limonard, 1966). The plates were first incubated for 24 hrs at 20 °C, kept in freezer at -20 °C for 48hr, and finally incubated at 20 °C for 5 to 7 days. *Fusarium* colonies growing from each sample were recorded.

Morphological identification of the isolated fungi:

Isolated fungi were first identified according to their cultural, morphological and microscopical characters as described by Barnett and Hunter (1972) and Nelson *et al.*, (1983). Identification was further confirmed by Olympus binuclear microscope sc 100 at 40X at the Mycology Research and Survey of Plant Diseases Section, Plant Pathology Research Institute, Agriculture Research Centre, Giza, Egypt.

Chemicals for fumonisin analysis:

Fumonisin B1 (1 mg, 96%; Acros Organics), methanol and acetonitrile (ACN) were purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK). Orthophthalaldehyde OPA, Tween 80 were purchased from Sigma (Sigma-Aldrich, UK). All solvents were HPLC grade.

Standard preparation

Stock standard of FB1 was dissolved in 1ml (ACN: H₂O 1:1). Fifty microliter of stock solution was diluted with the same mixture to 1ml to get 50 µg ml⁻¹. Working standards (0.1, 0.5, 2.5 and 5 µg ml⁻¹ of FB1 in ACN: H₂O) were prepared by appropriate dilution of known volumes of the prepared stock solution (50 µg ml⁻¹) to obtain calibration curves for the chromatographic analysis.

Determination of fumonisin production by *Fusarium* spp.

The medium used in this study was a fumonisin-inducing solid agar medium (Lopez *et al.*, 2007). Fumonisin-inducing solid agar plates were centrally inoculated with 3 µl of *Fusarium* spp. spore suspensions (1x10⁶ spores ml⁻¹). Four replicates of each strain were incubated at 25°C for 10 days. Afterwards, 5 discs of fungal culture were removed from each culture using a sterile cork borer (0.8 cm), transferred to a pre-weighed 2 ml Eppendorf tube and weighed again. FB1 was extracted from each eppendorf by adding 1 ml of (ACN:H₂O, 1:1), shaken 30 minutes well a horizontal shaker and centrifuged. Supernatant was filtered through 0.22µm nylon filter paper. The samples were frozen and stored at -20°C for later fumonisin analysis by HPLC.

Determination of FB1 in corn samples

Fumonisin B1 in samples was extracted and derivatized according to AOAC (2007). The HPLC system used for FB1 analysis was Perkin-Elmer, series 200 system, equipped with quaternary pump series 200, Perkin-Elmer fluorescence detector set series 200 system at 335 nm excitation and 440 nm emission wavelengths. The chromatography column was Phenomenex C18 (150 x 4.6 mm), 5 µm. The mobile phase was (23% 0.1M NaH₂PO₄:77% methanol), adjusted to pH 3.35 using phosphoric acid, with a flow rate of 1 ml min⁻¹.

DNA extraction and PCR

The *Fusarium* isolates were cultured in 0.5 ml of malt extract broth medium (30 g malt extract and 5 g peptone l⁻¹) in Eppendorf tubes at 25°C for three days, after which the mycelium was moved to a new Eppendorf tube. DNA was extracted by octanol/ isopropanol method as described by Paavanen- Huhtala *et al.*, (1999). The quality of DNA was confirmed by using ITS1 and ITS4 (Table 1) primers amplifying *Fusarium* DNA as described by Yli-Mattila *et al.*, (2004). Primers pair, Verprof / VERTI-R which is specific for fumonisin-producing *F. verticillioides* isolates and primer pair Taqfum- f 2/VPgen- R3 which is specific for *F. verticillioides*, *F. proliferatum*, *F. globosum* and *F. nygamai*, were used for the identification and detection of mycotoxins production *Fusarium* isolates as described by Waalwijk *et al.*, 2008 (Table 1). MJ Research thermal cycler (PTC-200) using calculated control and heated lid was used for PCR amplification. Aliquots (5 µL) of each PCR product were analyzed by electrophoresis in a TBE buffer in 1.0% agarose gels and visualized by using Alpha Innotech Corporation Multilmage Light Cabinet with Camera and Filters (Paavanen- Huhtala *et al.*, 1999).

Table 1. Sequences of primers used in PCR: ITS1/ITS4 for confirming the quality of DNA, Taqfum-2F/Vpgen-3R for detecting all fumonisin *Fusarium* producers and Verpro-F/VERTI-R for *F. verticillioides* fumonisin producer's detection.

Primer	5' > 3' sequence	Target sequences and species	Amplicon size(bp)	Authors
ITS1 ITS4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	Fungal Ribosomal DNA	650-700	Yli-Mattila <i>et al.</i> , (2004)
Taqfum-2F Vpgen-3R	ATGCAAGAGGCGAGGCAA GGCTCTCA/ GGAGCTTGCCAT	All fumonisin producers <i>F. verticillioides</i> , <i>F. proliferatum</i> , <i>F. globosum</i> and <i>F. nygamai</i>	180	Waalwijk <i>et al.</i> , (2008)
Verpro-F VERTI-R	GCCATGCGTCACGGCCAC GGAGTAGACAGGGTATTTC	<i>F. verticillioides</i> fumonisin producers	235	Waalwijk <i>et al.</i> , (2008)

RESULTS

Fumonisin B1 level in maize samples and its corresponding producing isolates

The FB1 levels in 31 maize samples from three governorates, Al-Beheira (9 samples), Al-Daqahlia (9 samples) and Al-Qalyubia (13 samples), ranged from 0.015 - 0.076, 0.074- 2.32 and 0.012- 9.8 mgkg⁻¹, respectively. Whereas, the FB1 production in media by its isolates collected from El-Beheira samples (20 isolates), Al-Daqahlia samples (15 isolates) and Al-Qalyubia samples (21 isolates) ranged from 4.4 -380, 3- 360 and 1.4- 555 mg kg⁻¹ media, respectively.

According to the HPLC analysis, the levels of FB1 in 67% of El- Beheira maize samples were undetected. While the rest samples (33%) contained FB1 in range between 0.01 and 0.1mgkg⁻¹. FB1 content in Al-Daqahlia samples divided into 3 equal categories, the first one was undetected, the second one ranged from 0.1 to 1.0 mg kg⁻¹ and the third one ranged from 1.0 to 2.4 mg kg⁻¹. Regarding to Al-Qalyubia samples, FB1 in 69% were undetected, ranged from 0.01 to 1.0 mg kg⁻¹ in 23% of samples and only 1 sample (8%) recorded 9.8 mg kg⁻¹ FB1.

On other hand, the HPLC analysis of FB1 production in culture media referred that 70%, 86.7% and 90.5% of *Fusarium* spp. isolated from El- Beheira, Al- Daqahlia and El- Qalyubia samples respectively were FB1 producer. Most producer isolates were identified as low producers (1.4 to 200 mgkg⁻¹). Just five isolates were characterized as high producer (>200 mg kg⁻¹), 1 isolate from El-Behiera (380 mg kg⁻¹ media), 2 isolate from El-Daqahlia (285, 360 mg kg⁻¹ media) and 2 isolates from Al-Qalyubia samples (260, 555 mg kg⁻¹ media) (Table 2). Surprisingly, all *Fusarium* isolates which had no ability to produce FB1 on fumonisin inducing media were isolated from FB1 free samples with the exception of just two isolates, which were from samples containing FB1. On the other hand, some of FB1-producing isolates were obtained from FB1 free samples.

Identification Fungal species

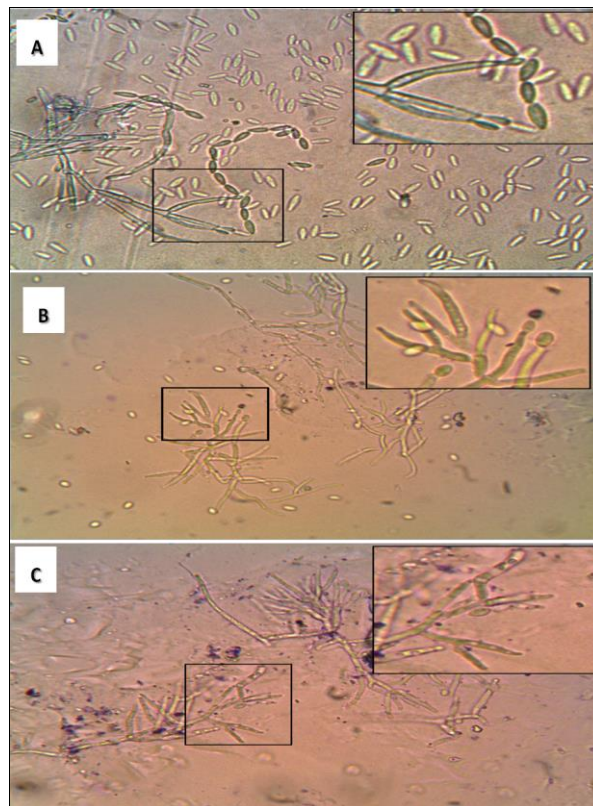


Figure (1). A: *Fusarium verticilloides*– chain of microconidia borne in monopialides, B: *Fusarium proliferatum* microconidia borne in polyphialides and C: *Fusarium subglutinans* microconidia borne in polyphialides but in false head.

Morphological examination of 56 isolates confirmed that all of them belonged to genus *Fusarium* and to the old *Gibberella fujikuroi* species complex. There are limits on the use of morphological characters for identification of species of it as some species have very similar morphology. The results of 56 isolates showed that 54 isolates of *F. verticillioides*, one isolate of *F. subglutinans* and one isolate of *F. proliferatum*. *F. verticillioides* was characterized by mostly zero septum, clavate microconidia with a flat base produced on monophialides in long chains in the aerial mycelium (Fig 1, A). The colonies on PDA were creamish to peach on the observe and pale cream to violet or blue on the reverse. Since, *F. verticilloides* resembles *F. proliferatum* in most of characters except for the formation of polyphialides. The chains of *F. proliferatum* on the polyphialides often appear in the shape of a v shapes in (Fig 1 B). *F. subglutinus* was characterized by oval, single celled but may be 1-3 septa. Microconidia were formed on polyphialides but always in false heads. The colonies on PDA were white mycelium and were sometimes tinged with purple (Fig 1 C).

Table 2. The concentration of FB1 in maize samples and in cultures of *Fusarium* isolates (mgkg⁻¹) by HPLC.

Governorate	Sample				Isolates			
	No.	Positive	% of positive	Range (mg kg ⁻¹)	No	Positive	% of positive	Range (mg kg ⁻¹)
EL-Beheira	9	3	33.3	0.015 - 0.076	20	14	70	4.4 – 380
Al-Daqahlia	9	6	66.7	0.074- 2.32	15	13	87	3- 360
EL-Kalyobia	13	4	30.8	0.012- 9.8	21	19	90	1.4- 555

Molecular identification and detection of fumonisin-producing *Fusarium* isolates

All 56 fungi isolates were subjected to PCR analysis using ITS genus species, Taqfum 2F, Vpgen-3R for fumonisin producers and Verpro-F, Verti-R for just *F. verticillioides* fumonisin producers. All isolates previously identified according to their morphology as 54 isolates *F. verticillioides*, one isolate *F. subglutinans* and one isolate *F. proliferatum* were positive for ITS region (Table 3). On the other hand, all *Fusarium* isolates analyzed by PCR for the ability of fumonisin production using primers Taqfum-2F 2, VPgen-3R were positive for the production (Fig 2). Verpro-F and Verti-R primers specified for producers species of *F. verticillioides* was used to identify the *Fusarium* species. The results showed that all tested *Fusarium* isolates from maize samples belonged to *F. verticillioides* in addition to its ability to produce FB1 (Fig 3).

In general, there was 96.4% compatibility between both morphological and molecular identification. Only 2 isolates were morphologically misidentified as *F. subglutinans* and *F. proliferatum*. Although the data obtained by HPLC revealed that 46 from 56 isolates were able to produce FB1 in media. The findings of PCR detection showed that all isolates were fumonisin producers.

Table 3. The number of *Fusarium* tested and number of *Fusarium* showing positive for ITS, Taqfum 2F, Vpgen-3R and Verpro-F, Verti-R gene as analyzed by PCR.

Fungi (morph. identification)	No. of isolates	positive for ITS region	positive for Taqfum-2f, Vpgen-3R PCR	positive for Verpro-F VERTI-R PCR
<i>Fusarium verticillioides</i>	54	54	56	56
<i>Fusarium porliferatum</i>	1	1	0	0
<i>Fusarium subglutinus</i>	1	1	0	0
<i>Fusarium globosum</i>	0	0	0	0
<i>Fusarium nygamai</i>	0	0	0	0

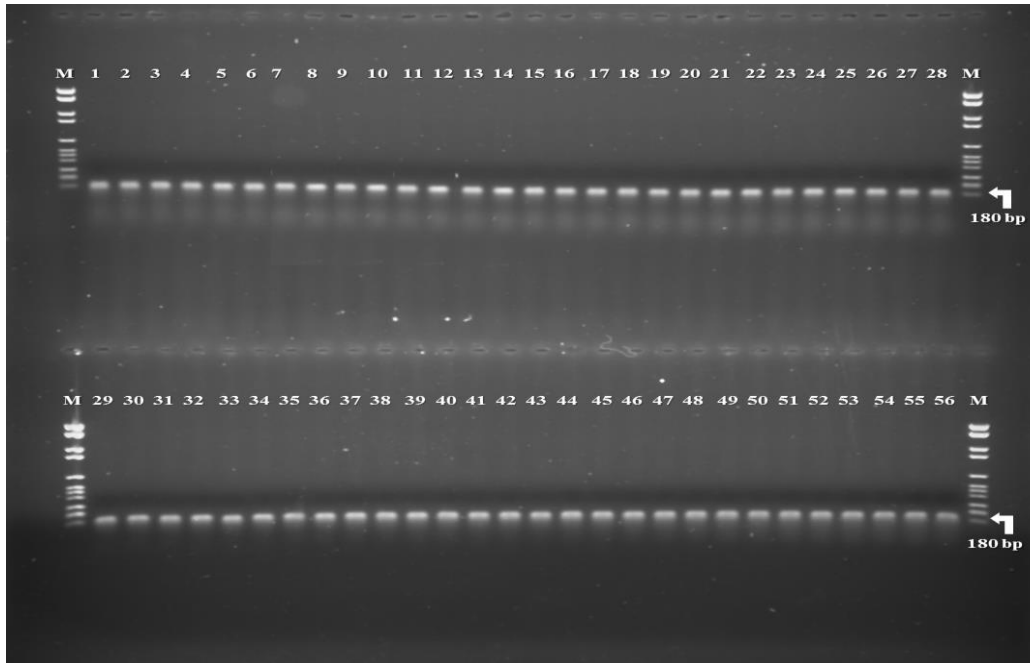


Figure (2). Agarose gel of amplified products using Taqfum-2f, VPgen-3R for identification and detection of fumonisin producing isolates from El- Behira maize samples (lanes 1-20), Al-Daqahlia maize samples (lanes 21-36), Al-Qalyubia maize samples (lanes 37-56). M: 100bp ladder.

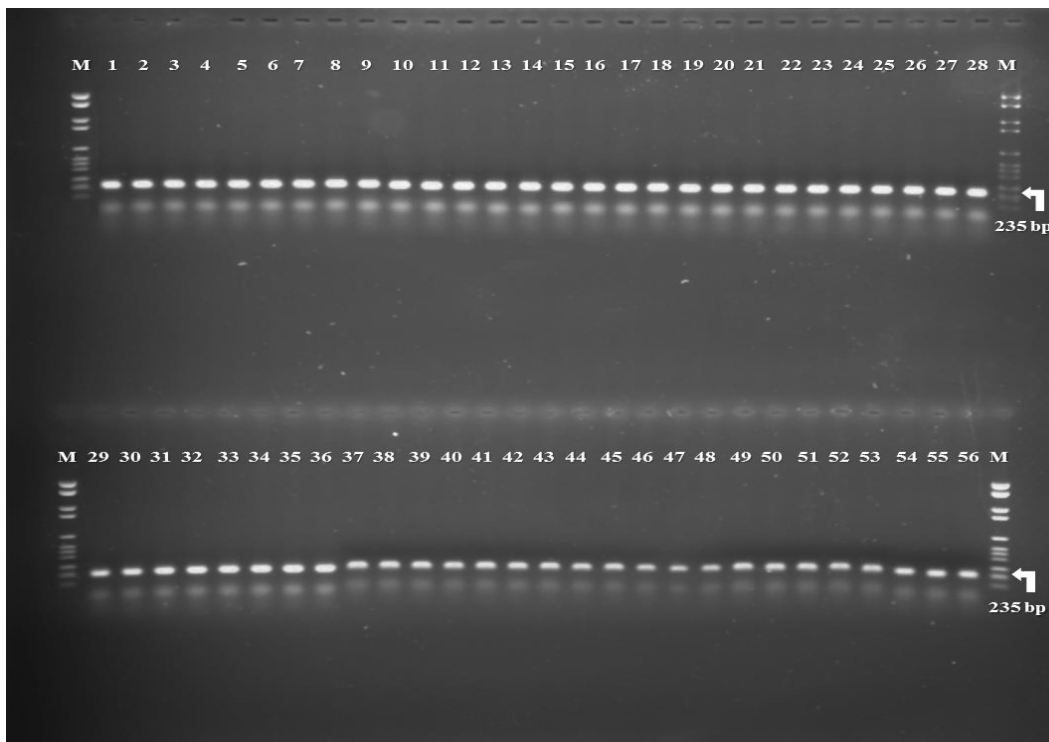


Figure (3). Agarose gel of amplified products of Verpro-f, VERTI-R for detection of fumonisin B1-producing *F. verticilloides* isolates from El- Behira maize samples (lanes 1-20), Al-Daqahlia maize samples (lanes 21-36), Al-Qalyubia maize samples (lanes 37-56). M: 100bp ladder

DISCUSSION

The results of morphological identification of *Fusarium* isolates were in agreement with El-Maghraby *et al.*, (1995) who reported that 45% of *Fusarium* spp belonged to *F. verticilloides* and 9% belonged to *F.*

subglutinans. Also, Fadl Allah (1998) and Abo El Yazeed *et al.*, (2011) found that *F. verticillioideis* isolates from corn grains and animals feed were the dominant species. Likewise, El-Habbaa *et al.*, (2003) collected 68 *Fusarium* isolates from kernels of yellow corn. They found that 40% of these isolates were *F. moniliforme* (*F. verticillioideis*), followed by other species *F. tabacinum*, *F. solani*, *F. semitectum*, *F. tricinctum* and *F. nivale*. Sreenivasa *et al.*, (2006) isolated 36 *Fusarium* species from maize which classified to *Fusarium moniliforme* (33%), *Fusarium proliferatum* (14%), and other *Fusarium* species (42%).

In Egypt, few studies have examined fumonisin production by *Fusarium* species from corn. For example, Fadl Allah (1998) found that 14 from 18 *F. verticillioideis* isolates were producers in range from 69 to 4495 $\mu\text{g g}^{-1}$. Also, Aboul-Nas and Obied-Allah (2013) found that 44 of 58 *F. verticillioideis* isolated from corn samples collected from Sohag Governorate produced FB1 or both FB1 and FB2 (15 and 29 isolates, respectively) on potato–sucrose agar medium. In addition, the results of this manuscript showed a variability of fumonisin production by *Fusarium* isolates which in order represent the biodiversity of fumonisin producing fungi in Egypt. While, Elisabete *et al.*, (2010) reported that a total of 16 *F. verticillioideis* strains isolated from corn feed samples were characterized by fumonisin (FB) production and random amplified polymorphic DNA (RAPD). All the strains produced FB1 and FB2 with levels ranging from 2.41 to 3996.36 $\mu\text{g g}^{-1}$, and from 1.18 to 1209.91 $\mu\text{g g}^{-1}$, respectively. From the 16 *F. verticillioideis* strains, four were identified as low (3.59 to 1289.84 $\mu\text{g g}^{-1}$), eight as intermediate (>1289.84 to 3772.44 $\mu\text{g g}^{-1}$) and four strains as high (>3772.44 $\mu\text{g g}^{-1}$) fumonisin producers. Also, Lorenzo Covarelli *et al.*, (2012) found in Italy that among fumonisin producers, a great variability was observed, with values ranging from 1 to 115 mg kg^{-1} .

In the present study, the levels of FB1 in all tested samples were under the permissible level (3 mg kg^{-1} , FDA, 2001) except one sample which contained 9.8 mg kg^{-1} . Similar results were obtained by AbdAlla El-Sayed *et al.*, (2003) who analyzed 57 corn samples and its-based products. They found that FB1 levels were under the permissible limit ranged from 0.01 to 0.78 mg kg^{-1} . Otherwise, Abo El Yazeed *et al.*, (2011) tested different crops (corn, soybean and barley) for its content of FB1. They reported that all samples contained high levels of FB1 (4.7 to 40 mg kg^{-1}) which was above the maximum limits (3 mg kg^{-1}) except one sample of soybean which was accepted (1.4 mg kg^{-1}).

The molecular detection of fumonisin productivity using Verpro-F/VERTI-R primer pair for *F. verticillioideis* and Taqfum- F2/VPgen- R3 for all fumosinis producers like *F. verticillioideis*, *F. proliferatum* as well *F. globosum* and *F. nygamaias* described by Waalwijk *et al.*, (2008) showed mostly accordance with the result obtained from HPLC analysis of FB1 and classical identification methods of the isolated *Fusarium* species. This means that we can use PCR as a fast and cheap way for screening the contamination of grain samples with fumonisin-producing fungi instead of the time-consuming and expensive ways like HPLC for toxin quantification and agar-plate methods for fungal contamination analysis.

However, there were still some findings, which were not in agreement with traditional identification and this might be refer to the difficulty to identify *Fusarium* species by using morphological methods. Also, there were some differences between the results of HPLC determination of FB1 and PCR results using specific primers for fumonisin production. It might be that the cultivation conditions were not suitable for FB1 production in those isolates that did not produce FB1 and in those grain samples, in which no FB1 was found, although FB1-producing isolates were obtained. This would be in agreement with the results of Rheeder *et al.*, (2002) dealing with the effect of cultivation conditions on toxin production. There might also be a mutation in genes responsible for fumonisin production or secretion. In this regard, further work should be done using different growth conditions to be sure, if all *F. verticillioideis* isolates giving a positive signal with primers specific to fumonisin-producers really are fumonisin-producers and if not what exactly has happened to their fumonisin genes.

The routine analysis of maize and other cereals for toxigenic *Fusarium* species is hindered by difficulties associated with standard methods for isolating and identifying *Fusarium* species. Traditionally *Fusarium* species have been differentiated by morphological characteristics such as presence or absence of micro conidia, shape and size of macroconidia, colony morphology, pigmentation and growth rates. Often morphological analysis is time consuming and requires considerable expertise and skill (Nelson *et al.*, 1983).

PCR-based detection techniques have provided an alternative to microbiological identification of *Fusarium* species. Random Amplified Polymorphic DNA PCR techniques have been used for finding markers for

species-specific PCR assays. Another approach for group- or species-specific detection involves the design of PCR primers for the polymorphic regions of ribosomal DNA. Several researchers have employed polymerase chain reaction (PCR) primers from genes directly involved in fumonisin-biosynthesis, like FUM1 to identify groups of fumonisin-producing *Fusarium* species (Bluhm *et al.*, 2004; Patino *et al.*, 2004 and Abd-Elsalam *et al.*, 2003).

Finally, *Fusarium spp.* was found in all collected maize samples which identified as *F. verticilloides* using sequencing of amplified PCR product of primer Verpro-F, VERTI-R. While, only 96.4% of isolates were *F. verticilloides* when identified by morphological techniques. HPLC determination of fumonisin B1 indicates only 42% and 82% of samples and isolates respectively contain FB1. In contrary, PCR indicate 100% of isolates produce FB1.

CONCLUSION

The aforementioned results led to two main points; the first was that we have to reconsider the culture collection of *Fusarium proliferatum*, *Fusarium subglutinus* as both has the same genetic structure like *Fusarium verticillioides*. The type exchange codes qualify them to be a variety to *Fusarium verticillioides*. The second conclusion is that the molecular approach to detect the toxigenic stain of *Fusarium* is more accurate than the chemical approach by HPLC which did not detect the low level of fumonisin produced by some strains.

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