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Polyphasic approach for detecting toxigenic *Fusarium* species collected from imported grain and seed commodities

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Abstract

The combined approach of bioassay, chemotype, PCR, and LAMP-PCR assays reported in this work allowed a rapid and accurate diagnostic of zearalenone, trichothecene and fumonisin-producing *Fusarium* species isolated from diverse regions. The most frequently isolated *Fusarium* species from grain and seed samples were *F. graminearum* (12.36%), followed by *F. solani* (10.3%), *F. avenaceum* (10.3%), *F. verticillioides* (8.24%), *F. heterosporum* (8.24%), *F. tricinctum* (7.21%) and *F. fujikuroi* (5.15%). Fifty-seven percent of the isolates showed the ability to produce mycotoxins by using *Nicotiana*-based bioassay. Zearalenone (ZEA), trichothecene (TRI), and fumonisins (FUM) toxins were fabricated by tested isolates of *F. graminearum, F. solani*, *F. culmorum, F. equiseti, F. semitectum*, *F. verticillioides*, *F. fujikuroi* and *F. poae.* Tested isolates of *F. acuminatum* produced ZEA at levels ranging from 87–271 µg/ $^{g\ -1}$. The production of fumonisins B1 from *F. equiseti* isolates was observed for the first time. Cluster analysis revealed the occurrence of six groups with similarities ranging from 85–98%. The distribution of some *Fusarium* isolates within the phenogram was not in harmony with chemotype pattern. Three mycotoxin chemotypes were identified by chemical analysis and confirmed by PCR and LAMP-PCR. PCR assays of Zea2, Tri6, and Fum B1 genes were used to forecast whether these isolates could produce ZEA, TRI and FUM, respectively. The presence of Tri5 gene was revealed in 33 of 103 examined isolates (34%), which indicates the potential ability to produce trichothecene mycotoxins by these fungi, while 25 isolates possessed three of the analyzed genes. The greatest number of genes responsible for the production of three mycotoxins was examined in *F. graminearum* and *F. equiseti* while the smallest in *F. avenaceum*.

Key words – cereals – chemotype – *Fusarium* – LAMP-PCR – mycotoxins – PCR

Introduction

A broad range of *Fusarium* mycotoxins occur in cereals and other seeds. Mycotoxins, such as zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FUM) are the most frequent toxins

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produced by *Fusarium* spp. These toxin groups are considered the most risky to animal and human health (Stępień et al. 2014). Twenty-three *Fusarium* species were screened for fumonisin production and only *F. verticillioides*, *F. proliferatum*, and *F. nygamai* produce high levels of fumonisin (Huffman et al. 2010). *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, and *F. poae* isolated from cereals are the main trichothecene producers (Wolny-Koładka et al. 2015). *Gibberella fujikuroi* complex, which includes both *F. verticillioides* and *F. graminearum* (Jeon et al. 2013), can contaminate cereals and many other foods with fumonisins and trichothecenes (Antonissen et al. 2014). Fast screening and detection of toxigenic fungi using biological methods including plants, yeasts, and bacteria were reviewed (Panigra 1993, Rivas-San Vicente et al. 2013). For instance, *Arabidopsis thaliana* was used as a model for observing mycobiota infection and mycotoxin production by *Fusarium* species (Urban et al. 2002). Similar uses of an *A. thaliana*-based bioassay for exploring seed colonization by aflatoxigenic *Aspergilli* (Hammond et al 2007). *Fusarium* species can be identified morphologically, but differences between species are difficult to establish. Traditional toxigenic fungi classification and identification methods are based mainly on morphological characteristics and pathogenicity testing. These approaches require a fully equipped laboratory and usually are expensive, time-consuming and require considerable expertise in taxonomy and physiology of *Fusarium* species (Leslie & Summerell 2006, Gong et al. 2014). To overcome these limitations the development of diagnostic tools for rapid, simple, and cost-effective detection of food-borne fungi is advantageous, especially on-site detection method (Ferrara et al. 2015). The current status and future of research direction is focused on use of multiple genes belonging to different clusters for the detection of toxigenic species. For instance, identify and quantify toxigenic *Fusarium* in cereals was achieved by use of markers targeting the trichodiene synthase encoding gene (tri5 and tri 6) gene in trichothecene-producing *Fusarium*. (Dawidziuk et al. 2014). The FUM cluster genes were used as a novel marker for classification and phylogenetic evaluation of the fumonisin-producing *Fusarium* species (Baird et al. 2008). Real-time PCR tests have been applied to detect food-borne pathogens. Nevertheless, the real time PCR assays need costly fluorescence detection systems (Law et al. 2014). LAMP-PCR was used for detecting fungal contamination in pepper and paprika powder (Zhang et al. 2014). LAMP tests have been developed for rapid detection of *Phytophthora sojae* (Dai et al. 2012), *P. ramorum*, *F. oxysporum* (Lu et al. 2015a), and some *Fusarium* specialized forms and races (Almasi & Li 2013, Peng et al. 2013). Generally, LAMP-PCR was used to detect specific mycotoxin-producing fungi, such as *Aspergillus carbonarius*, *A. niger* and three species of *Aspergillus* section *Flavi* (Storari et al. 2013). Also, LAMP-PCR have been developed for the major FHB and toxigenic *Fusarium* in different plant materials (Niessen & Vogel 2010, Abd-Elsalam 2011, Niessen 2012, Almoammar 2013, Lu et al. 2015b). Several authors have adopted successfully this criterion for selecting mycotoxin biosynthetic genes as LAMP target (Storari et al. 2013). Previous LAMP assays developed for toxigenic fungi were mainly targeted to housekeeping genes (Niessen & Vogel 2010). A target gene involved directly in mycotoxin biosynthesis is preferred, as it is more closely-related to potential toxigenicity. The CYP51C gene species-specific DNA-based assay was used as a phylogenetic marker to identify *Fusarium* species (Wang et al. 2013). The optimized LAMP assay can be used to rapidly identify *F. graminearum* and *F. equiseti* with a lower limit of detection than conventional PCR amplification. The aims of the present research were (1) to describe the construction of a sensitive and inexpensive *Nicotiana*-based assay for fast screening of toxigenic fungi. It would be advantageous to be able to reproduce greenhouse symptoms in sensitive plant could first be confirmed; (2) to characterize and identify the main three toxins production by *Fusarium* isolates associated with cereal and seeds imported from different countries into Saudi Arabia and Egypt; (3) PCR assays of Zea2, Tri6, and Fum6 B1 genes were used to predict whether these isolates could produce zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FUM) respectively; and (4) to evaluate LAMP assay for detection of *F. graminearum* and *F. equiseti* based on the gaoA and CYP51C-genes, respectively,-and demonstrated that the assay is specific and efficient.

Materials & Methods

Fusarium culture

The study was performed using *Fusarium* species isolated from cereal and seeds collected from different countries and imported into Egypt and Saudi Arabia. In order to isolate the tested *Fusarium*, a total of 214 cereal samples were associated and examined. Fungi were isolated by surface-sterilizing 10 seeds per sample in 1% sodium hypochlorite solution in a 50 ml beaker for 1 min and washed with three changes of sterile distilled water. Five seeds each were placed in Petri dishes with potato dextrose agar (PDA) media (Difco). Petri dishes were incubated for 7 days at 24°C. Morphological identification of *Fusarium* spp. was approved according to Leslie & Summerell (2006). The screened isolates were purified and monosporic cultures were maintained on PDA slants at 4°C. The complete list of *Fusarium* isolates with relevant crops, and agro-ecological country is shown in Table 1.

Intact *in vivo* bioassay

The current biological assay is an alternative screening method to assess the phytotoxicity of *Fusarium* mycotoxins. *Nicotiana* seedlings were used to detect the biotoxicity of 103 isolates belonging to 25 *Fusarium* species. *Fusarium* isolates were inoculated on potato dextrose broth (PDB) and incubated for 3 weeks and conidiospores were harvested as previously reported by Hilton et al. (1999). *Nicotiana benthamiana* seedlings were inoculated by placing 10 µl of conidial suspension (containing 10^8 conidia) on each emerging seedling leaf. Three leaves per seedling were inoculated. Control plants were treated with 10 µl of sterile distilled water. Inoculated plants were incubated in a moist chamber for 72 h and kept in a growth chamber at 21°C (day) /18°C (night) temperatures with day/night regime of 16h/8h.

Growth conditions for mycotoxin detection

A total of 103 *Fusarium* isolates (73 from Saudi and 30 from Egypt) collocated from cereals originated from different geographical regions, were screened for their ability to produce three types of mycotoxins. *Fusarium* isolates were cultured in 250 mL Erlenmeyer flasks containing 50 mL of rice culture medium (50 g of rice flour per 500 mL deionized water). The top of each flask was covered with aluminum foil, autoclaved and allowed to cool down in a hood for about 40 min. Mycelia from PDA plates were gently scrapped from the agar, using sterile scalpels. Then, each flask was inoculated by adding the mycelia from the *Fusarium* isolates. The flasks were incubated in a regulated room at 24°C for about 28 days. Mycelia were harvested by filtration through Whatman no. 1 paper. The dry mycelium was ground to fine powder using a coffee blender with ethanol cleaning between samples. The ground samples were stored at 0°C until use.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was used to detect the following mycotoxins: ZEA, TRI and FUM B1. Standards of these toxins were purchased from Sigma Chemical Company (St. Louis, MO, USA) and stored at 4°C in darkness. The procedure was performed as previously described in detail (Kushiro et al. 2015).

DNA isolation

The *Fusarium* isolates were inoculated onto PDA and incubated at 24°C for 7 days. Under sterilized laminar flow workstations, a piece of fresh mycelia and spores was scraped using sterile swabs and dipped in a 0.2 mL Eppendorf tube containing 100 µL 10 × (Tris and EDTA, pH 7.5). The tube was then heated at 95°C for 10 min in a thermblock (Eppendorf) and centrifuged at 3000 r min^{-1} for 1 min. The isolated DNA was stored at 4°C in a refrigerator until used (Jia et al. 2014).

Table 1 *Fusarium* isolates and their crops, agro-ecological country, their mycotoxins bioassay, chemotypes, conventional PCR, and LAMP-PCR specificity used in this study

No.	*Fusarium* species	Crop	Origin country	Mycotoxins[1] Bioassay	Mycotoxins µg/ g -1			PCR			LAMP-PCR	
					ZEA	TRI	FUM-(B1)	ZEA2	TRI6	FUM6	CYP51C	gaoA gene
20	*F. acuminatum*	Wheat	Uzbekistan	■	139	209	ND	□	■	□	□	□
26	*F. acuminatum*	Wheat	France	■	74	87	ND	□	■	□	□	□
53	*F. acuminatum*	Wheat	Uzbekistan	■	410	110	ND	□	■	□	□	□
69	*F. acuminatum*	Wheat	Germany	■	246	271	ND	□	■	□	□	□
1	*F. annulatum*	Barley	Sudan	□	ND	ND	ND	□	□	□	□	□
24	*F. annulatum*	Sesame	Kenya	□	ND	ND	1307	□	□	■	□	□
60	*F. annulatum*	Sesame	Kenya	□	ND	ND	1273	□	□	■	□	□
99	*F. annulatum*	Sesame	Kenya	□	ND	ND	ND	□	□	□	□	□
72	*F. anthophilum*	Wheat	Germany	□	ND	ND	ND	□	□	□	□	□
10	*F. avenaceum*	Wheat	Canada	□	ND	ND	ND	□	□	□	□	□
12	*F. avenaceum*	Wheat	USA	□	ND	ND	ND	□	□	□	□	□
29	*F. avenaceum*	Wheat	USA	□	ND	ND	ND	□	□	□	□	□
44	*F. avenaceum*	Wheat	Uzbekistan	□	ND	ND	ND	□	□	□	□	□
48	*F. avenaceum*	Sorghum	USA	□	ND	ND	ND	□	□	□	□	□
50	*F. avenaceum*	Sesame	Kenya	□	ND	ND	ND	□	□	□	□	□
67	*F. avenaceum*	Wheat	Germany	□	ND	ND	ND	□	□	□	□	□
83	*F. avenaceum*	Wheat	Uzbekistan	□	ND	ND	ND	□	□	□	□	□
87	*F. avenaceum*	Sorghum	USA	□	ND	ND	ND	□	□	□	□	□
89	*F. avenaceum*	Sesame	Kenya	□	ND	ND	ND	□	□	□	□	□
11	*F.brevicatenulatum*	Sorghum	Nigeria	□	ND	ND	ND	□	□	□	□	□
3	*F. camptoceras*	Barley	Kenya	□	ND	ND	ND	□	□	□	□	□
27	*F.chlamydosporum*	Wheat	France	□	ND	97	ND	□	■	□	□	□
42	*F.chlamydosporum*	Soy	Canada	□	ND	213	ND	□	■	□	□	□
103	*F.chlamydosporum*	Soy	Canada	□	ND	86	ND	□	■	□	□	□
13	*F. ciliatum*	Yellow corn	Canada	□	ND	ND	ND	□	□	□	□	□
2	*F. culmorum*	Corn	USA	■	411	212	1366	■	■	■	□	□
25	*F. culmorum*	Wheat	France	■	96	73	1147	■	■	■	□	□
73	*F. culmorum*	Wheat	Germany	■	175	130	1261	■	■	■	□	■
23	*F. decemcellulara*	Barely	Sudan	□	ND	ND	ND	□	□		□	□
22	*F. equiseti*	Barley	Sudan	■	211	407	1.059	■	■	■	■	□
54	*F. equiseti*	Corn	Argentina	■	215	319	ND	■	■	■	■	□

Table 1 Continue

No.	*Fusarium* species	Crop	Origin country	Mycotoxins[1] Bioassay	Mycotoxins µg/ g -1			PCR			LAMP-PCR	
					ZEA	TRI	FUM-(B1)	ZEA2	TRI6	FUM6	CYP51C	gaoA gene
93	*F. equiseti*	Corn	Argentina	□	80	244	0.879	■	■	■	■	□
17	*F. flocciferum*	Wheat	Germany	□	ND	ND	ND	□	□	□	□	□
56	*F. flocciferum*	Wheat	France	□	ND	ND	ND	□	□	□	□	□
95	*F. flocciferum*	Wheat	France	□	ND	ND	ND	□	□	□	□	□
14	*F. fujikuroi*	Wheat	Germany	■	ND	ND	1083	■	□	■	□	□
30	*F. fujikuroi*	Wheat	Australia	■	ND	ND	1374	■	□	■	□	□
40	*F. fujikuroi*	Wheat	Uzbekistan	■	ND	ND	1759	■	□	■	□	□
80	*F. fujikuroi*	Wheat	Uzbekistan	■	ND	ND	987	■	□	■	□	□
6	*F. graminearum*	wheat	Uzbekistan	■	241	615	2020	■	■	■	□	■
7	*F. graminearum*	Wheat	Uzbekistan	■	117	421	1820	■	□	■	□	■
15	*F. graminearum*	Wheat	Germany	■	321	752	2110	■	□	■	□	■
16	*F. graminearum*	Wheat	Germany	■	247	493	1287	□	■	■	□	■
34	*F. graminearum*	Barley	Sudan	■	511	276	1007	■	■	■	□	■
39	*F. graminearum*	Barley	Australia	■	371	813	1243	■	■	■	□	■
41	*F. graminearum*	Barley	Sudan	■	112	147	1055	■	■	■	□	■
55	*F. graminearum*	Wheat	Uzbekistan	■	180	591	1620	■	■	■	□	■
71	*F. graminearum*	Wheat	Germany	■	480	746	1370	■	□	■	□	■
78	*F. graminearum*	Barley	Australia	■	197	837	1711	■	□	■	□	■
79	*F. graminearum*	Barley	Sudan	■	396	134	873	■	■	■	□	■
94	*F. graminearum*	Wheat	Uzbekistan	■	273	219	1110	■	■	■	□	■
35	*F. heterosporium*	Wheat	Uzbekistan	■	147	ND	ND	■	□	□	□	□
63	*F. heterosporium*	Wheat	France	■	233	ND	ND	■	□	□	□	□
92	*F. heterosporium*	Wheat	France	■	176	ND	ND	■	□	□	□	□
58	*F. heterosporum*	Sesame	Sudan	■	420	ND	ND	■	□	□	□	□
64	*F. heterosporum*	Wheat	USA	■	109	ND	ND	■	□	□	□	□
81	*F. heterosporum*	Wheat	USA	■	359	ND	ND	■	□	□	□	□
97	*F. heterosporum*	Sesame	Sudan	□	633	ND	ND	■	□	□	□	□
102	*F. heterosporum*	Wheat	Uzbekistan	■	254	ND	ND	■	□	□	□	□
66	*F. moniliforme*	Wheat	Germany	□	ND	ND	1413	□	□	■	□	□
4	*F. oxysporum*	Wheat	Australia	□	ND	ND	ND	■	□	□	□	□
68	*F. oxysporum* f. sp. *acuformis*	Wheat	Germany	□	ND	ND	ND	□	□	□	□	□
21	*F. poae*	Soy	USA	□	ND	ND	897	□	□	■	□	□

Table 1 Continue

No.	*Fusarium* species	Crop	Origin country	Mycotoxins[1] Bioassay	Mycotoxins µg/ g -1			PCR			LAMP-PCR	
					ZEA	TRI	FUM-(B1)	ZEA2	TRI6	FUM6	CYP51C	gaoA gene
28	*F. poae*	Soy	Canada	□	ND	ND	1000	□	□	■	□	□
46	*F. poae*	Alfa alfa	Canada	□	ND	ND	664	□	□	■	□	□
85	*F. poae*	Alfa alfa	Canada	□	ND	ND	478	□	□	■	□	□
37	*F. proliferatum*	Corn	USA	□	757	ND	1400	□	□	■	□	□
62	*F. proliferatum*	Wheat	Germany	□	430	ND	1211	□	□	■	□	□
76	*F. proliferatum*	Corn	USA	■	819	ND	1545	□	□	■	□	□
101	*F. proliferatum*	Wheat	Germany	■	744	ND	1133	■	□	■	□	□
38	*F. semitectum*	Corn	Kenya	□	211	224	776	■	■	■	□	□
77	*F. semitectum*	Corn	Kenya	□	ND	612	1120	■	■	■	□	□
9	*F. solani*	Soy bean	USA	■	346	**840**	723	■	■	■	□	□
18	*F. solani*	Corn	Nigeria	■	109	259	912	■	■	■	□	□
32	*F. solani*	Yellow corn	Kenya	■	148	476	1140	■	■	■	□	□
45	*F. solani*	Soy	USA	■	228	921	1207	■	■	■	□	□
51	*F. solani*	Sesame	Kenya	■	613	248	934	■	■	■	□	□
57	*F. solani*	Soy	USA	■	412	647	488	■	■	■	□	□
65	*F. solani*	Wheat	Germany	■	387	233	1270	■	■	■	□	□
84	*F. solani*	Soy	USA	■	571	476	1149	■	■	■	□	□
90	*F. solani*	Sesame	Kenya	■	446	623	793	■	■	■	□	□
96	*F. solani*	Soy	USA	■	246	197	918	■	■	■	□	□
33	*F. sporotrichioides*	Yellow corn	Kenya	□	455	ND	ND	■	□	□	□	□
52	*F. sporotrichioides*	Sesame	Nigeria	□	ND	ND	ND	■	□	□	□	□
91	*F. sporotrichioides*	Sesame	Nigeria	□	742	ND	ND	■	□	□	□	□
70	*F. sportichoides*	Wheat	Germany	□	ND	ND	ND	■	□	□	□	□
5	*F. tricinctum*	Wheat	Australia	■	742	ND	ND	■	□	□	□	□
31	*F. tricinctum*	Wheat	Australia	■	389	ND	ND	■	□	□	□	□
43	*F. tricinctum*	Wheat	Canada	■	912	ND	ND	■	□	□	□	□
59	*F. tricinctum*	Barley	Sudan	■	734	ND	ND	■	□	□	□	□
74	*F. tricinctum*	Wheat	Germany	■	841	ND	ND	■	□	□	□	□
82	*F. tricinctum*	Wheat	Canada	■	397	ND	ND	■	□	□	□	□

Table 1 Continue

No.	*Fusarium* species	Crop	Origin country	Mycotoxins[1] Bioassay	Mycotoxins µg/ g -1			PCR			LAMP-PCR	
					ZEA	TRI	FUM-(B1)	ZEA2	TRI6	FUM6	CYP51C	gaoA gene
98	*F. tricinctum*	Barley	Sudan	■	501	ND	ND	■	□	□	□	□
8	*F. verticillioides*	Yellow corn	Canada	□	ND	ND	873	□	□	■	□	□
19	*F. verticillioides*	Wheat	Uzbekistan	□	ND	ND	1394	□	□	■	□	□
36	*F. verticillioides*	Corn	Argentina	■	ND	ND	1190	□	□	■	□	□
47	*F. verticillioides*	Yellow corn	Nigeria	□	ND	ND	1074	□	□	■	□	□
61	*F. verticillioides*	Wheat	France	■	ND	ND	1100	□	□	■	□	□
75	*F. verticillioides*	Corn	Argentina	■	ND	ND	1234	□	□	■	□	□
86	*F. verticillioides*	Yellow corn	Nigeria	□	ND	ND	615	□	□	■	□	□
100	*F. verticillioides*	Wheat	France	■	ND	ND	1220	□	□	■	□	□
88	*Fusarium* spp.	Wheat	Canada	□	ND	ND	ND	■	□	□	□	□
49	*Fusarium* spp.*	Wheat	Canada	□	ND	ND	ND	□	□	□	■	□

Mycotoxins bioassay (sensitivity of *Nicotiana* seedlings to fungal filtration), Mycotoxins were determined by using HPLC.
This isolate was considered unknown. It did not have the ability to form any spores.
ND = not detected. □ negative results; ■ positive results.

PCR assay

PCR amplifications were performed in volumes of 25 mL containing 50 ng of fungal genomic DNA, 0.5 µL 100 mmol l^{-1} of each primer, 12 µL PCR buffer (50 mmol l^{-1} KCl, 1.5 mmol l^{-1} MgCl2, 10 mmol l^{-1} Tris-HCl, pH 8.8, 0.1% Triton X-100), 0.5 µL Taq DNA Polymerase (Jena Bioscience), 2.5 µL of dNTPs (10 mM) and 7 µL PCR water. PCR targeting *zea2*, *tri6* and *fum6* gene was performed as follows. After a 5 min denaturation at 94°C, the PCR mixtures were subjected to 35 cycles of amplification at 94°C for 45 s, 53–56°C for 1 min, and 72°C for 1 min with the final extension of 10 min at 72°C. Amplification was performed by using Biometra T-Personal Thermal Cycler. PCR products were also analysed by gel electrophoresis (5 µL aliquots on 1.5% agarose gel). The DNA molecular weight marker of DNA ladder DL 2000 (TaKaRa, Japan) was used to determine the size of the products. PCR primers used in current research are summarized in Table 2.

Table 2 Primers and their nucleotide sequences and product sizes used in PCR analysis (Dawidziuk et al. 2014).

Primers name	Sequence (5′-3′)	PCR products (base pairs)	Target
ZEA2_dm_fA1	ACM TCA CCA TCM AAR TTC TG	340	Zea2
ZEA2_dm_rA1	GCR TCY CKG TAR TCR CTC AT		Zea2
TRI6_dm_fA2	TAT GAA TCA CCA ACW TTC GA	526	Tri 6
TRI6_dm_rA1	CGC CTR TAR TGA TCY CKC AT		Tri 6
FUM6_dm_fA2	GTY TCR TGT CCK GCA ATG AG	672	Fum6
FUM6_dm_rA1	GGY TCK TTT GAG TGG TGG C		Fum6

Zearalenone polyketide synthase (zea2), Zinc finger transcription factor (tri6), Oxygenase (fum6)

Loop-mediated isothermal amplification.

The LAMP mixture in 25 mL total volume consisted of the following: 1 Thermo buffer (containing 2 mM MgSO4), 6 mM of MgSO4, 0.8 M of betaine (Sigma-Aldrich, St. Louis, MO), 1.4 mM of dNTP, FIP and BIP (1.6 mM each), loop-F and loop-B (0.8 M each), F3 and B3 primers (0.2 mM each), Bst DNA polymerase (8 U) (New England BioLabs), dd H2O and 2 mL of DNA template. The LAMP reaction was carried out at 63°C for 1 hour and terminated at 80°C for 2 minutes in a water bath. The template DNA was omitted in one reaction for negative control. LAMP amplicons were analyzed similarly by naked eye and electrophoresis (Abd-Elsalam et al. 2011). LAMP specificity was evaluated by performing the assay with 103 *Fusarium* DNA template extracted using by simplified protocols. Specificity tests were performed two times. LAMP primers are summarized in Table 3.

Dendrogram analysis for mycotoxin profiles

Dendrogram presents the accurate and defined analysis of the distance in the genetics of *Fusarium* species by groupings the strains based on their mycotoxin producing ability**.** Mycotoxins concentrations per isolate were analyzed using cluster analysis. SPSS 14 software package (www.spss.com) was used for cluster analysis by unweighted pair-group method based on arithmetic mean (UPGMA).

Results

Morphological characterization

The *Fusarium* species most frequently isolated from grain samples were *F. graminearum* (12.36%), followed by *F. solani* (10.3%), *F. avenaceum* (10.3%), *F. verticillioides* (8.24%), *F. heterosporum*

(8.24%), *F. tricinctum* (7.21%) and *F. fujikuroi* (5.15%). In our survey, three species, *F. graminearum, F. solani* and *F. avenaceum*, contributed similarly to around a third of tested isolates. *F. solani, F. avenaceum* (10.3%), *F. sportichoides* and *F. proliferatum, F. poae, F. annulatum, F. acuminatum* (4.12%) were obtained in equal frequency. *F. flocciferum, F. equiseti, F. chlamydosporum, Fusarium* spp., *F. semitectum, F. oxysporum, F. culmorum,* and *F. moniliforme* were found in lower percentages. Agro-ecological variations in *Fusarium* species composition were detected within our sampling (Table 1, Fig. 1).

Table 3 Primers used for loop-mediated isothermal amplification for *F. graminearum* and *F. equiseti*

LAMP- primers for *F. equiseti* (Lu et al. 2015b)			
Primers name	**Sequence (5′-3′)**	**Length**	**Target**
F3 (forward outer)	GCGTACCCGGTACCGAAT	18	CYP51C
B3(backward outer)	GGACTGGTGACAGACTTGTT	20	CYP51C
FIP (forward inner) (F1C + F2)	GGAGGGTCGAGGGAAGAACTCTTAGTGCCTCCGTCCCATAC	41	CYP51C
BIP (backward inner) (B1C + B2)	TGGGATCCTCATCGCTGGGA-CCGAAGCCATAATCCACAGT	40	CYP51C
LF (loop forward)	TGCCAGGAGATGCAAGAAGT	20	CYP51C
LB (loop backward)	CGAGCCTCTTGAGAAGAACGCC	22	CYP51C
LAMP- primers for *F. graminearum* (Abd-Elsalam et al. 2011)			
F3 (forward outer)	AGG GAG TCT TCA GTT CCT GA	20	gaoA gene
B3(backward outer)	GTG AGG GGG CTT TGG ATC	18	gaoA gene
FIP (forward inner) (F1C + F2)	CGCAAGTGACGGCCCAGTTGCTTCGAGCCTCAGCACCTA	39	gaoA gene
BIP (backward inner) (B1C + B2)	TGCAACAAGGCCATTGATGGCCGTTGGCGCCATAGAATGT	40	gaoA gene
LF (loop forward)	GTTGCGAGAAATGGCGCTTCC	21	gaoA gene
LB (loop backward)	ACAAGGATACCTTTTGGCAC	20	gaoA gene

Nicotiana-based bioassay for mycotoxin screening

The filtration of liquid culture media was used to screen 103 *Fusarium* isolates for mycotoxin production. In the positive seedlings, young leaves were tiny and indistinct or chlorotic with asymmetrical margins, spotting or necrotic areas. Leaf tips may yellow and curl downhill. Leaf size was reduced and overall growth will be stunted (Fig. 2). Fifty-seven percent of the total isolates showed the ability to produce simultaneously mycotoxins by using *Nicotiana*-based bioassay (Table 1).

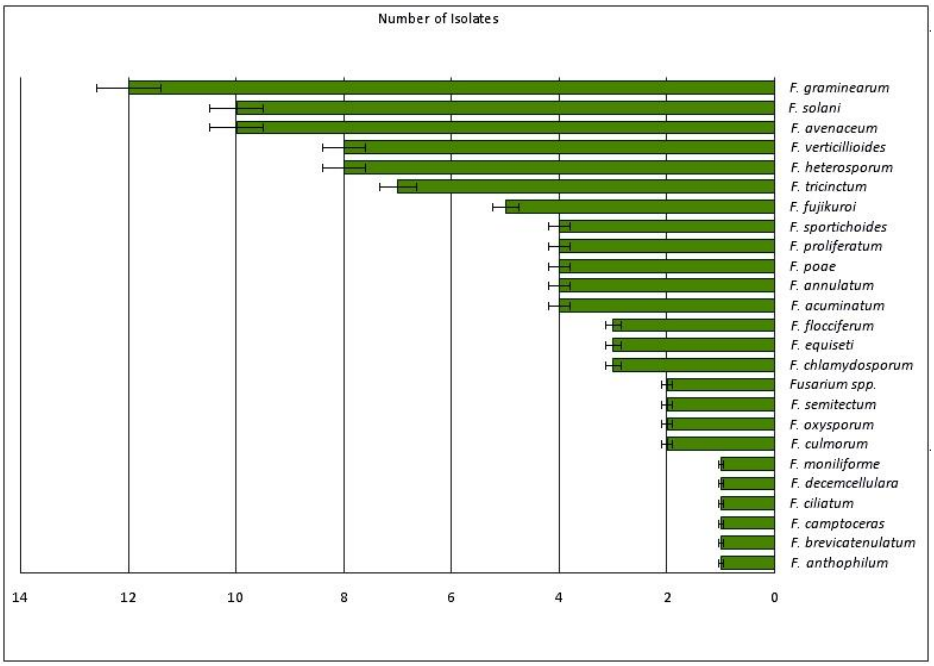

Fig 1 – Differences in relative frequency of isolation were observed among species. Overall, *F. graminearum* had the highest frequency of recovery followed by *F. avenaceum* and *F. solani*, and *F. verticillioides* each ranging from 8 to 12% of the isolates. Other species such as *F. fujikuroi* and *F. tricinctum*, represented 5% to 7% of the total isolates.

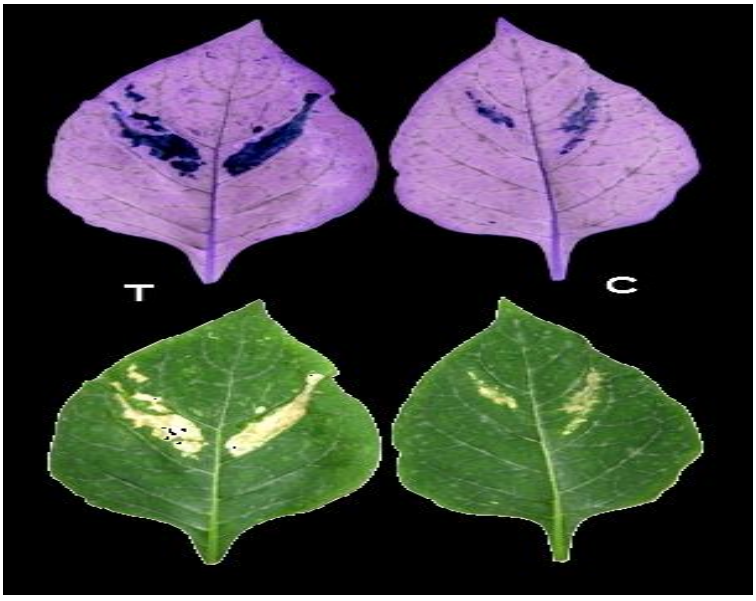

Fig 2 – Mycotoxin-induced lesion formation in *Nicotiana benthamiana* seedlings, the leaves infected with *F. graminearum* isolate 3 have abnormal colours (A). Leaves inoculated with water (C). The dark blue or lime colour indicates cell death.

Chemotype analysis

Results of chemical analyses relevant to the 103 isolates of *Fusarium* species are summarized in Table 1. ZEA, TRI and FUM toxins were produced by tested isolates of *F. graminearum*, *F. solani*, *F. culmorum*, *F. equiseti*, *F. semitectum*. *F. verticillioides*, *F. fujikuroi*, and *F. poae*. All isolates of *F. acuminatum* could produce ZEA at low level ranged from 87–271 µg/ $^{g\ -1}$. *F. verticillioides* is the most important producer of fumonisin B1. Widely variations in fumonisin production were observed among isolates from the same plant host and geographic region. For

example, isolate collected from Nigeria showed total fumonisin production values ranging from 615 (isolate 47) to 1074 µg/ $^{g\ -1}$ (isolate 86) respectively. This is the first report of the production of fumonisins B1 by *F. equiseti* collected from corns and barely at level ranged from 244–407 µg/ $^{g\ -1}$ respectively. A group of 26 isolates was unable to produce detectable levels of mycotoxins screened in the current study. *F. avenaceum, F. flocciferum*, *F. oxysporum, Fusarium* spp. *F. anthophilum, F. brevicatenulatum, F. ciliatum,* and *F. camptoceras* isolates were found to be non-toxigenic. In some cases, no correlations between *Nicotiana*-based bioassay for mycotoxin screening and chemotype analysis, for instance, two isolates of *F. annulatum,* two isolates of *F. chlamydosporum,* and three isolates of *F. verticillioides* showed negative results by using *Nicotiana*-based bioassay, while showed positive by chemical analysis.

Cluster analysis for toxin profiles

A dendrogram based on UPGMA analysis using mycotoxins-producing ability data collected isolated from different grains imported into Saudi and Egypt. One hundred three isolates were assigned into two major clusters, the first cluster comprised 51.4% and the second cluster contains 49.6% of the tested isolates. Cluster analysis revealed the occurrence of six chemotype groups with similarities ranging from 85–98% (Fig. 3). The first major cluster contains three different chemotype ZEA toxin producers *F. heterosporium, F. tricinctum, F. proliferatum,* and *F. sporotrichioides*, TRI toxin producers *F. chlamydosporum*, also, non-toxigenic isolates including *F. avenaceum, F. flocciferum, F. oxysporum, Fusarium* spp. *F. anthophilum, F. brevicatenulatum, F. ciliatum,* and *F. camptoceras* were grouped in the first major cluster together in a single well supported clade in this phylogeny. Whereas the ZEA, TRI, FUM toxin producers (*F. graminearum, F. solani* and *F. culmorum*) and FUM producing species *F. verticillioides, F. fujikuroi, F. poae*, and *F. proliferatum* were grouped within the second major cluster with similarity level ranged from 90–89%. Emphasizing the close evolutionary relatedness among them and agreeing with the type of mycotoxin that these species produce. The average similarities within a cluster were greater than the average similarities between the clusters. No clear-cut relationships between mycotoxin-producing ability and geographic origin of the isolates were evident.

Species-specific PCR assays

The occurrence of Zea, Tri and Fum genes, responsible for the production of three main mycotoxins in *Fusarium* spp., was studied. The specificity of the PCR reaction was tested on a diverse range of *Fusarium* species (25 species) commonly associated with different grains and seeds. Only DNA of *F. graminearum* and *F. culmorum* was amplified with ZEA2_dm_fA1/ZEA2_dm_rA1 and generated a 340 bp PCR product (Fig. 4). No fragment was produced from none ZEA producing species indicating that the described PCR system is specific for ZEA producing species. The specificity of the tri6 primer set TRI6_dm_fA2/ TRI6_dm_rA1 had been tested by the specific amplification of several isolates of *F. graminearum, F. culmorum, F. solani* and *F. chlamydosporum*, whereas no fragment was generated from the none-trichothecene producing *Fusarium* species such as *F. heterosporium* and *F. tricinctum*. None of the *F. avenaceum* isolates resulted in the amplification of the 526-bp tri6 DNA fragment. *F. solani, F. verticillioides, F. proliferatum, F. poae, F. graminearum* and *F. culmorum* species-specific PCR amplification was obtained for all tested isolates with FUM6_dm_fA2/ FUM6_dm_rA1 primer set (Table 1). No amplification was obtained from the other *Fusarium* species. The expected DNA fragment (672 bp) was amplified from FB1 producers (Fig. 5). No PCR product was amplified from the remaining isolates that did not produce FB1. The presence of Tri5 gene was revealed in 33 of 103 examined isolates (34%), which indicates the potential ability to produce trichothecene mycotoxins by these fungi. while 25 isolates possessed three of the analyzed genes. The greatest number of genes responsible for the production of three mycotoxins was observed in *F. graminearum,* and *F. equiseti* while the smallest in *F. avenaceum*.

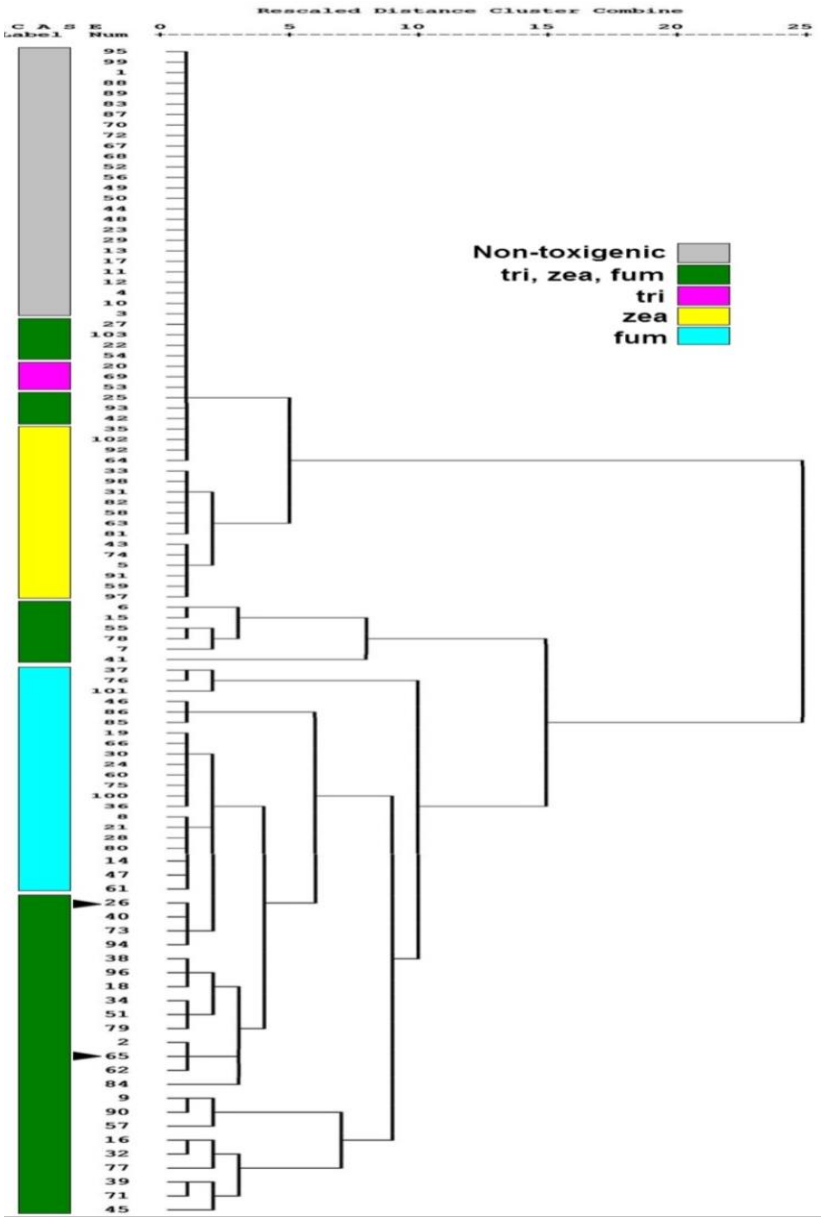

Fig 3 – Dendrogram based on average linkage cluster analysis of chemotype profiles (ZEA, TRI, FUM) obtained from 103 Fusarium isolates.

LAMP-PCR

The gaoA gene LAMP primer was used to detection of *F. graminearum* isolates, the assay was positive only for all *F. graminearum* isolates. There were minor cross reactions only with *F. culmorum* (isolate 73) other *Fusarium* isolates indicating good species specificity of the LAMP-PCR for gaoA gene. No positive DNA products were observed when other toxigenic *Fusarium* (*F. sporotrichioides*, *F. avenaceum*, and *F. proliferatum*) were used as templates (Fig. 6 A). CYP51C gene was used for identification and species-specific detection of different *F. equiseti*, the primers targeting unique regions of the CYP51C was confirmed by testing different *Fusarium* species. The results showed that the LAMP assay was highly specific for the detection of the appropriate species. The three tested *F. equiseti* isolates and *Fusarium* spp. (isolate 49) were produced positive amplification, whereas none of the primer sets amplified the template of any of the closely related species including, *F. graminearum* (tubes 13-15). *F. solani* (tubes 17-19), *F. verticillioides* (tubes 20, 21), and *F. heterosporium* (tubes 22, 23), thereby establishing the specificity of the LAMP assay for *F. equiseti* (Fig. 6 B). Amplification of DNA during the reaction was detected directly in-tube by colour transition of Calcein from orange to light yellow, visible to the naked eye, avoiding

further post amplification analyses. Negative reaction did not show any colour variation and the typical electrophoresis pattern was absent (tubes 8, 16, 24). There was no difference between the LAMP results detected with the naked eye under a UV lamp or agarose gel electrophoresis (data not shown).

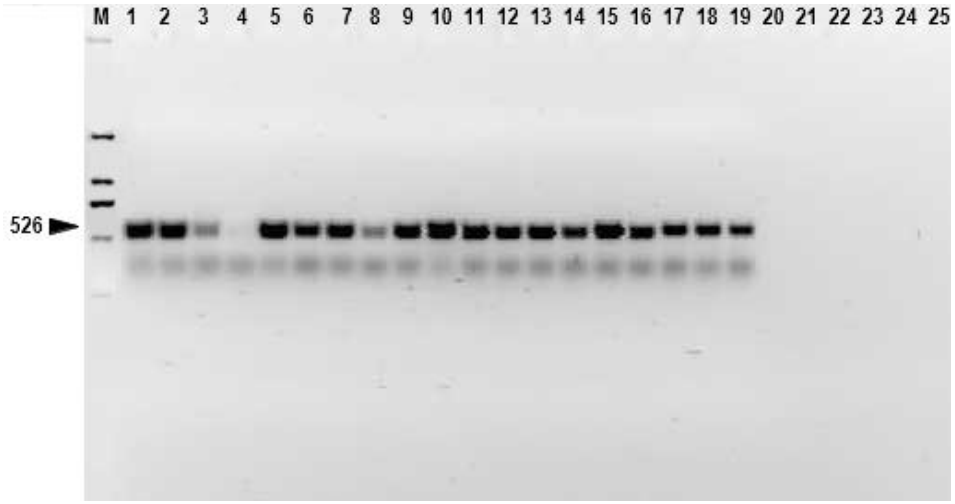

Fig 4 – Results of PCR assay with TRI6_dm_fA2/ TRI6_dm_rA1, trichothecene-specific primers (tri6) and genomic DNA extracted from Fusarium isolates. Lane M, 100 bp DNA ladder; lanes 1-12;*F. graminearum* isolates, lanes 13–15; isolates of *F. culmorum*, lanes 16–17; *F. fujikuroi* isolates, lanes 18–19; *F. sporotrichioides* isolates, lanes 20–21; *F. equiseti* isolates, lanes; 22–23 *F. tricinctum* isolates, lanes 24–25; *F. poae* isolates recovered from grain and seed samples. As size standard, 100 bp DNA ladder DL 2000 (TaKaRa, Japan) was used to determine the size of the products.

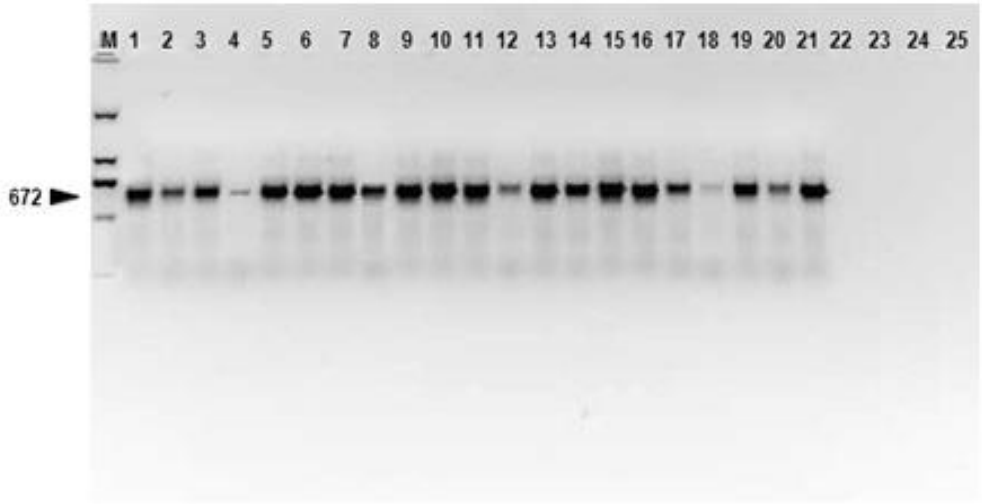

Fig 5 – Results of PCR assay with FUM6_dm_fA2/ FUM6_dm_rA1, fumonisin-specific primers (fum6) and genomic DNA extracted from Fusarium isolates. Lane M, 100 bp DNA ladder; lanes 1-8; *F. verticillioides* isolates; lanes 9–14; *F. proliferatum* isolates of *F. solani*, lanes 15–18; *F. equiseti*, isolates, lanes 19–20; lane 21, *F. moniliforme* isolates, lanes, 22–23; *F. tricinctum* isolates, lanes 24–25; *F. avenaceum* isolates recovered from grain and seed samples. As size standard, 100 bp DNA ladder DL 2000 (TaKaRa, Japan) was used to determine the size of the products.

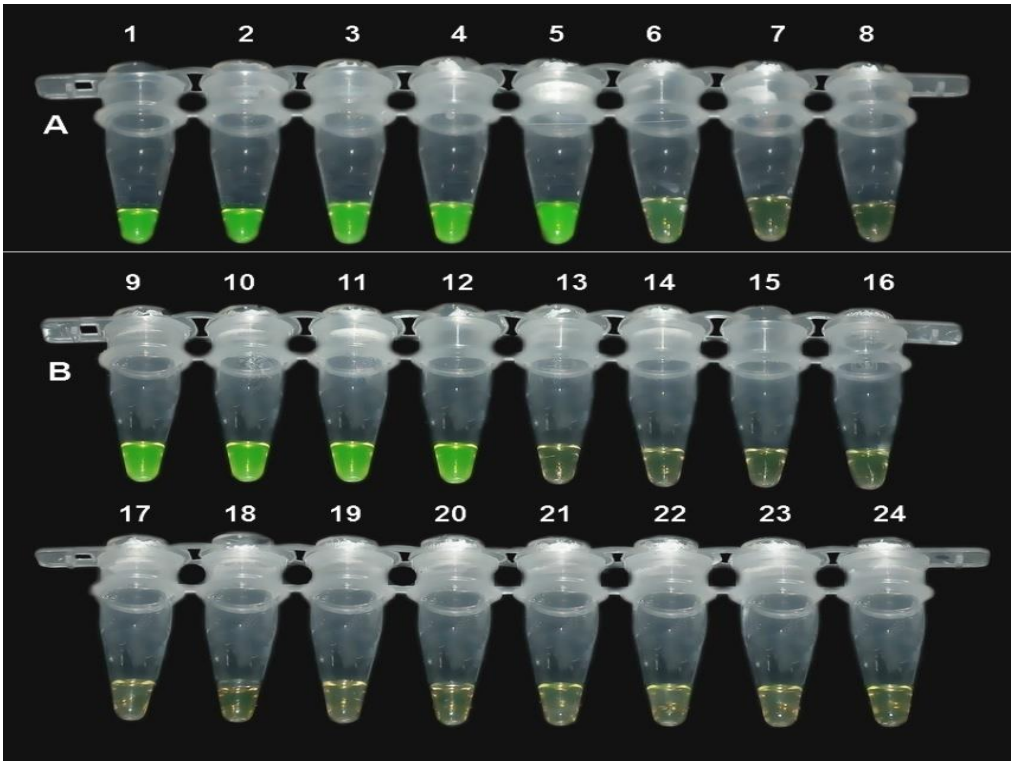

Fig 6 – The detection of loop mediated isothermal amplification (LAMP) products were visualized by fluorescent detection reagent and UV light. (A) LAMP for detection of *F. graminearum* using gaoA gene, *F. graminearum* (tubes 1-4), *F. culmorum* (tubes 5-6), *F. sporotrichioides* (Tube 7), *F. avenaceum* (tubes 20,21), and *F. proliferatum* (tubes 23,23). (B) LAMP detection of the CYP51C gene, *F. equiseti* (tubes 9-11), *Fusarium* spp. (tube 12). *F. graminearum* (tubes 13-15). *F. solani* (tubes 17-19), *F. verticillioides* (tubes 20, 21), and *F. heterosporium* (tubes 22, 23). Positive samples produce a green colour almost immediately (tubes 1, 2, 3, 5, 5, 9, 10, 11 and 12), while negatives remain light yellow. Tubes 8, 16, 24 represent a negative control without target DNA.

Discussion

The genus *Fusarium* is calculated approximately to include at least 300 genealogically exclusive phylogenetic species, although less than half have been formally identified (Aoki et al. 2014). The current research aimed to combine bioassay, chemotype, qualitative and quantitative methods for detecting the major toxigenic *Fusarium*-associated mycotoxins (zearelanone, trichothecenes and fumonisins B1). The tested fungi were isolated from imported grain and seeds imported into Egypt and Saudi Arabia. The analysis was conducted using 103 isolates of *Fusarium* belonging to 25 species. *F. graminearum, F. culmorum, F. poae*, and *F. sporotrichioides* are common in grain and are causative agents of Fusarium head blight disease (FHB), which causes great yield loss worldwide (Leslie & Summerell 2006). *F. verticillioides* was the most widespread *Fusarium* species found on barley rootlets could be correlated with fumonisin B1 contamination (Cavaglieri et al. 2009). It is necessary to redefine the role of bioassay for the field diagnosis of mycotoxicoses, as tests on biological systems designed primarily to confirm the presence of mycotoxins (Panigra 1993, Lachaud et al. 2011). In the present study 57% of the isolates showed the ability to produce three different mycotoxins by using *Nicotiana*-based bioassay. A number of fungal species secrete toxins to kill host cells and promote pathogen growth. The host-selective mycotoxin fumonisin B1 (FB1) is produced by the pathogen. It has been shown that the toxin induces programmed cell death (PCD) in animal and plant cells (Gilchrist et al. 1998). FB1-induced cell death was accompanied with disruption of vacuolar membrane tracked by lesion formation (Kuroyanagi et al. 2005). The higher FB1 dose leads to root growth inhibition and hypocotyl expansion on *N. benthamiana* seedlings (Brandwagt et al. 2001). ZEA, TRI and FUM toxins were

produced by tested isolates of *F. graminearum, F. solani*, *F. culmorum*, *F. equiseti*, *F. semitectum*, *F. verticillioides*, *F. fujikuroi*, and *F. poae*. Our results found that 100% of isolates of *F. graminearum* infested cereals are toxigenic. ZEA is produced by different *Fusarium* species, such as the most important fungal pathogens of maize and wheat, i.e. *F. graminearum F. culmorum* and *F. equiseti*. The main toxins produced by *F. graminearum* are deoxynivalenol (DON) and zearalenone (ZEN) (Bottalico & Perrone et al. 2002). With regard to ZEA and type B trichothecene production, *F. graminearum*, *F. culmorum* and *F. cerealis* are very closely related to one another, both morphologically and chemotaxonomically (Bottalico & Perrone 2002, Nelson et al. 1983). The production of DON by *F. acuminatum* has been previously reported (Marín et al. 2012). In the present study all isolates of *F. acuminatum* could produce ZEA at low level ranged from 87–271 µg/ $^{g\,-1}$. We recommended that this species should not be considered a DON non-producing species, although more isolates and further studies are needed to prove this. The production of fumonisins B1 from *F. equiseti* isolates was reported for the first time. Cluster analysis revealed the occurrence of six groups with similarities ranging from 85–98%. There was no straight relationship between type of mycotoxins and their collected crop or geographic origins. Recently, the probable use of sequence analysis and biosynthetic gene-derived molecular markers was applied to taxonomic and chemotype studies, utilizing both toxic (fumonisins, trichothecenes, zearalenone, fusaric acid, fusarins, enniatins and beauvericin) and nontoxic (bikaverin) metabolites produced by *Fusarium* fungi was described (Stępień et al. 2014). There have been reports of collective detection of trichothecene producing *Fusarium* species with a PCR-based assay based on trichothecene biosynthetic genes (Aoki 2014, Dawidziuk 2014, Stępień et al. 2014) as well as for fumonisin producing *Fusarium* based on genes in the fumonisin biosynthetic cluster (López-Errasquín et al. 2007). Very recently the gene cluster of ZEA biosynthesis has been identified in *F. graminearum* which containing zea2 gene (Dawidziuk et al. 2014). Three chemotypes were identified by chemical analysis and confirmed by PCR and LAMP-PCR. PCR assays of Zea2, Tri6, and Fum B1 genes were used to predict whether these isolates could produce zearalenone (ZEA), deoxynivalenol (DON), and fumonisins (FUM) respectively. The greatest number of genes responsible for the production of three mycotoxins was observed in *F. graminearum*, and *F. equiseti* while the smallest in *F. avenaceum*. PCR amplification of FUM6 gene can be a useful tool for the fast detection of fumonisin B1-producing *F. verticillioides* and *F. proliferatum* isolates. The toxigenic *Fusarium* isolates were detected trichothecenes-, zearalenone-, fumonisins-producing *Fusarium* with the sensitivity ranged from (94–100%) and ranged from specificity (88–95%) (Dawidziuk et al. 2014). There was no correlation between the country of origin and the presence of the marker fragment. PCR assays results were matched quite well with the chemistry for almost of isolates that were shown to produce trichothecenes. PCR assays give structural, rather than functional information, thus reflecting the presence of a sequence or a gene but not necessarily its expression. Numerous developed isothermal DNA-based amplification techniques for food-borne bacterial and fungal contaminants as an alternative to PCR-based assays was reviewed by Niessen (2013). A few number of studies for detection of toxigenic Fusarium using LAMP assays showed that the approach was easier and faster to perform than common PCR assays, as well as being more specific (Niessen & Vogel 2010, Abd-Elsalam 2011, Niessen 2012, Almoammar 2013, Lu et al. 2015b). In this study, we used LAMP primer sets designed from gaoA gene and CYP51C gene for detection of *F. graminearum*, *F. equiseti* isolates, respectively. Our experimental results suggest that LAMP can detect *F. equiseti* and *F. graminearum* faster and with higher sensitivity than the traditional diagnostic method. DNA from more slightly related *Fusarium* spp. except *F. culmorum* was not amplified with the primer set used even if incubation was extended to more than 80 min. Results showed that the LAMP assay for *F. graminearum* is highly specific for that species but also picks up all the genetic lineages of it which were recently erected as species, e.g. *F. graminearum* sensu lato (O'Donnell et al. 2004). These findings are in conformity with earlier studies that the specificity of LAMP method in identification of toxigenic Fusaria such as *F. graminearum* and *F. equiseti* is extremely high (Niessen & Vogel 2010, Dawidziuk et al. 2014). CYP51C is conserved and is common in *F. equiseti* and *F. graminearum*; therefore, the CYP51C gene sequence is a better

for detecting *F. equiseti* and *F. graminearum* than rDNA-ITS or the β-tubulin gene sequence (Deng 2007, Niessen & Vogel et al. 2010). The method uses six primers (FIP, BIP, F3, B3, LF and LB) that recognize eight regions of DNA for the target pathogen and it amplifies DNA within sixty minutes at 65°C in a thermblock, water-bath and thermal cycler, to validate the efficiency and specificity (Notomi 2000, Mori et al. 2004). In addition, great amounts of white magnesium pyrophosphate precipitate were generated in amplified samples, which assisted the identification of *F. equiseti* and *F. graminearum* based on naked-eye examination. Therefore, LAMP method is a widely applied as a diagnostic and detection tool for the most important plant pathogenic fungi (Niessen & Vogel 2010, Abd-Elsalam 2011, Niessen 2012, Almoammar 2013, Lu et al. 2015b).

In conclusion**,** ZEA, TRI and FUM toxins were produced by tested isolates of *F. graminearum, F. solani, F. culmorum, F. equiseti, F. semitectum*. *F. verticillioides*, *F. fujikuroi*, and *F. poae.* The data from the *Nicotiana*-based assay were robust and predictable, and well-correlated with chemical analysis of mycotoxins. The greatest number of genes responsible for the production of three major mycotoxins was detected in *F. graminearum*, and *F. equiseti* while the smallest in *F. avenaceum*. The LAMP assay could serve as a simple and quick technique with potential application for on-site disease detection and field surveys. A diagnostic method which is rapid, accurate, and simple could really help to control of toxigenic fungi and reduction of mycotoxins produced by *Fusarium* species in various agricultural commodities.

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