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RESEARCH ARTICLE

Occurrence of *Fusarium* species and the potential accumulation of its toxins in Egyptian maize grains

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Abstract

Surveys were carried out in 2011, 2012 and 2014 in three Governorates of Delta (El-Beheira, El-Dakahlya and El- Qalyubia) to evaluate the presence of *fusarium* and *its* toxins in maize grains at harvest. *Fusarium* species were the most abundant species detected in maize kernel using deep freezing blotter method. While, *Aspergillus* section *flavi* and *nigri* were the most dominant species detected in maize kernels on Potato Dextrose Agar, followed by *penicillium* species. *F. verticilloids* was the most prevalent *Fusarium* species, followed by *F. proliferatum* and *F. subglutinans,* representing of 67.2%, 51.7 and 43% in samples from El-Beheira, El-Dakahlya and El- Qalyubia during 2011, respectively. While during 2012, the percentage averages were 37.5 %, 20.2% and 35.1% in samples from El-Beheira, El-Dakahlya and El-Qalyubia, respectively. Fumonisin B1 and zeralenone were the predominant mycotoxin. Fumonisin B1 was detected in 32.3, 36.8 and 93.3% of samples in 2011, 2012 and 2014, respectively. Also, zeralenone were detected in 38.7, 36.8 and 33.3% in 2011, 2012 and 2014, respectively. The maximum value of FB1 (9.8 mg kg^{-1}) was detected in El- Qalyubia in 2012, whereas the highest value of zeralenone was detected in El- Qalyubia in 2014 recording 7.2 mg kg^{-1}. Deoxynivalenol was not detected in any sample.

INTRODUCTION

Maize is the third major field crop grown in the world after rice and wheat, also considered as one of the principal crops in Egypt. It is planted in Egypt on approximately 378,000 Fadden of land, and 50% of its production is used for livestock poultry feed **(FAO 2013)**. In the field as well as during storage period many pests and parasites attack maize. Insects are most often considered as the principal cause of grain losses **(Gwinner *et al.*, 1996).** However, fungi are also important and ranked as the second cause of deterioration and loss of maize (**Ominski *et al.*, 1994**). **Kossou and Aho (1993)** reported that fungi could cause about 50 – 80 % of damage on farmers' maize during the storage period if conditions are favorable for their development. The major genera commonly encountered on maize in tropical regions are *Fusarium, Aspergillus* and *Penicillium* (**Samson 1991; Orsi *et al.*; 2000, El-Shabrawy 2001 and Madbouly *et al.*, 2012**). In Egypt, *Fusarium* has received considerable attention from the pathological viewpoint **(Abdel-Monaim 2012 and Ziedan *et al.* 2012 and Hathout *et al.*, 2014).** Maize contamination by fungi not only renders grains unfit for human consumption by discoloration and reduction of nutritional value, but can also lead to mycotoxin production. Mycotoxins are poisonous secondary metabolites produced by some fungi in staple foods and foodstuffs. They are potentially hazardous to man and domestic animals **(Suleiman *et al.*, 2013)**. Many of them are considered to be important worldwide, but the five most often reported and well documented are deoxynivalenol, zearalenone, ochratoxin, aflatoxins and fumonisins **(Pitt 2000)**. Fumonisins are a group of secondary metabolites mainly produced by *Fusarium verticillioides* and *F. proliferatum*, the principal causal agents of the ''pink ear rot'' of maize (**Bottalico 1998 and Garrido *et al.*, 2012**), during kernel colonization. Among fumonisins, fumonisin B1 (FB1), the diester of propane-1, 2, 3-tricarboxylic acid and 2-amino- 12,

16-dimethyl- 3,5,10,14,15-pentahydroxyeicosane is most prevalent. In addition to its toxicity, due to the capacity of inhibiting the synthesis of sphingolipids in membranes, altering lipid metabolism, it is also carcinogenic, immunotoxic, hepatotoxic, pneumotoxic and nephrotoxic (**IARC 1993**). The concentrations of fumonisins in raw maize are influenced by environmental factors such as temperature, humidity, drought stress and rainfall during pre-harvest and harvest periods. For example, high concentrations of fumonisins are associated with hot and dry weather, followed by periods of high humidity (**Shelby *et al*., 1994 a, b and Mohamed 1999**). Zearalenone is [(3S, 11 E)-14, 16-dihydroxy- 3-methyl-3, 4, 5, 6, 9, 10-hexahydro-1H-2-benzoxacyclotetradecine-1, 7(8H)-dione]. It presents an estrogenic and anabolic action in several animals. In young male swine the toxin causes prepuce oedema, testicle atrophy and increasing of mammary gland (**Edwards *et al.*, 1987**). In relation to the toxic effects in human beings, there are cases in which ZEA was reported because it caused premature puberty in children aged between 7 and 8 years (**Painter 1997**). ZEA has been shown to be immunotoxic and hepatotoxic and nephrotoxic and an enhancer of lipid peroxidation (**Pistol *et al*., 2014**). It is mainly produced by *F. graminearum* and produces estrogenic effects (**Anukul *et al*., 2013**). Deoxynivalenoln (DON), known as vomitoxin, is a type B tricothecene, an epoxysesquiterpenoid, produced by *Fusarium graminearum* and *F. culmorum.* The principle causal agents of the red ear rot of maize (**Garrido *et al*., 2012 and Anukul *et al*., 2013)**. Due to the health hazards of these compounds for humans and animals and the occurrence in foodstuffs for human consumption, so, The United States Food and Drug Administration proposed a guideline to allow a maximum content of 3 ppm in corn and corn products for human consumption (**FDA 2001**). The European Commission has set maximum permitted levels in breakfast cereals for ZEA, 50 μgkg^{-1} in the case of breakfast cereals, excluding maize-based products, and 100 $\mu g\ kg^{-1}$ for maize-based breakfast cereals (**EC 2007)**. The safe limit of DON was set at 200 µgkg-1 for processed cereal-based foods, foods for infants and young children by the Commission of the European Communities **(EC 2005).**

The aim of this study was to evaluate of *Fusarium* species and its toxins contamination in sixty five of Egyptian maize samples collected from fields.

Material and methods

Samples Collection:

Sixty five of dry cob maize samples (about 500 g each) in triplicate were collected after harvest in three seasons (31 samples in 2011, 19 samples in 2012 and 15 samples in 2014) from three governorates of Delta region in Egypt (El-Beheira, El-Dakahlya, and El- Qalyubia). Grains of each sample were separated manually by hand in aseptic condition. Each sample of 2011 and 2012 were divided into two parts, one for microbiological analysis and the other for toxin determination. The last, fifteen samples, 5 samples for each governorate, were collected during September 2014 to confirm the data belonging to contents of *Fusarium* toxins. Samples of microbiology analysis were kept at 4°C, until fungal enumeration within 48 hr. and samples of toxins analysis were kept at - 20°C till later analysis.

1- Determination of *Fusarium* species of maize samples:

A- Planting on Potato Dextrose Agar media (PDA):

One hundred grains of each sample was plated on potato dextrose agar **(ATCC 1984)** containing 15 % sodium chloride to inhibit the growth of *Rhizopus* and *Mucor*. The plates were incubated for 5 to 7 days at 25°C. The colonies of *Fusarium* and other fungi isolated from each sample were recorded. The percentage of infection, total fungal counts (TFC $100gram^{-1}$) were determined.

B) Planting by Deep Freezing Blotter Method:

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 with sterilized filter paper. The sterile grains 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. Total *Fusarium* counts (TFUC $100gram^{-1}$) were determined.

1- 2- Morphological identification of the isolated fungi:

Isolated fungi were first identified according to their cultural, morphological and microscopically characters as described by **Barnett and Hunter (1972) and Nelson *et al.*, (1983)**. Identification was further confirmed by using 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.

2- Quantitative determination of *Fusarium* toxins:

2-1- Determination of fumonisin:

Fumonisin B1 in maize samples was extracted and derivative according to **AOAC (2012)**. 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 NaH_2PO_4:77% methanol), adjusted to pH 3.35 using phosphoric acid, with a flow rate of 1 ml min^{-1}.

2-2- Determination of zeralenone (ZER):

Zeralenone (ZER) was extracted from maize samples according to (**AOAC 2012**). Same HPLC system used for fumonisin B1 was used for determination at 274 nm excitation and 440 nm emission wavelengths. The mobile phase system according to (**Chiraz Zaied *et al.*, 2012**) (acetonitrile: water: methanol 48:50:3 v/v/v) was isocratically used at flow rate 1ml min^{-1}. Data were collected and integrated using Totalchrom Navigator Chromatography Manager Software.

2-3- Determination of deoxynivalenol (DON):

Deoxynivalenol (DON) was extracted from maize grains according to the official method **(AOAC 2012)** and determined using TLC and HPLC methods. Regarding to TLC method, the resulting spots -color were then marked on TLC. The marked areas were determined using CD-60 densitometer (DESAGA- Germany) using wavelength 365 nm in Faculty of pharmacy, Cairo University. The HPLC system used for DON analysis was an Agilent 1100 system equipped with quaternary pump model G1311A, UV detector model G1314A set at 220 nm wavelength, autosampler model G1329A. Agilent Zorbax Eclipse XDB C18 4.6 mm × 150 mm, 5m column was used for deoxynivalenol separation. Data were integrated and recorded using Chemstation Software program.The mobile phase was according to **(Gupta *et al.* 2011)** (Water: Acetonitrile: Methanol 5: 4: 1, v/v/v), with a flow rate of 0.7 ml min^{-1}.

Result:

***Fusarium* profile in maize samples:**

All the collected maize kernels samples didn't show any sign of fungal infection symptoms. The kernels were good and healthy. Planting on specific media was the route to understand the *Fusarium* contamination on the kernels. The average of total fungal count (TFC) by using PDA (cfu $100gram^{-1}$) and total *Fusarium* count (TFUC) by using deep freezing blotter as a specific method for the isolation of *Fusarium* species are illustrated in Table (1). There were no significant differences between the average count of TFC and TFUC in samples for the three governorates. The higher average of total fungal count was recorded in El-Beheira (592.5 cfu $100gram^{-1}$) followed by El-Qalyubia (547.6 cfu $100gram^{-1}$) in 2011, whereas, total *Fusarium* count was higher in El-Qalyubia and El- Dakahlya than that in El-Beheira governorate. In 2012, the total fungal count was high in El- Dakahlya (596.5 cfu $100gram^{-1}$) followed by El-Beheira. The average of total *Fusarium* count in 2012 was higher than those in 2011 in the three governorates.

Table (1). The average of Total fungal count and Total *Fusarium* count (cfu /100gram)

Governorates	2011		2012	
	TFC / 100g	TFUC/100g	TFC / 100g	TFUC/100g
El-Beheira	592.5±41.5 [a]	169.5± 24.1 [a]	586.1±122.1 [a]	309.4± 30.4 [a]
El-Dakahlya	469.9±42.0 [a]	216.4± 18.5 [a]	596.5±57.3 [a]	254.0±15.4 [a]
El-Qalyubia	547.6±65.0 [a]	222.8±24.9 [a]	457.6±52.1 [a]	263.7±20.6 [a]

Means in the same column with the same letter are not significantly different. Average (mean± SE). TFC: total fungal count; TFUC: total *Fusarium* count.

Morphological identification carried out on spores of isolates from maize kernels on PDA media in 2011 and 2012 are shown in Fig. (1). *Aspergillus, penicillium, Eurotium* and *Fusarium* were the most common genera. *An Aspergillus genus was* dominant in all governorates during two seasons followed by *penicillum* and *Eurotium* genera. Whereas, *Fusarium* genus was found with very low percentage ranged from (0 to 2.6%) by planting on PDA media.

In Fig (2) showed that the most prevalent fungi were those belonging to *A. flavus* with incidences of 38.1%, 43.5%and 34.9% in 2011 and 35.1%, 20.6% and 34.5% in 2012, in samples from El-Beheira, El-Dakahlya and El-Qalyubia samples, respectively. Colonies of the *A. niger* were also detected with percentages of 26.2%, 39.5% and 28.3 % in 2011 from El-Beheira, El- Dakahlya and El-Qalyubia, respectively.

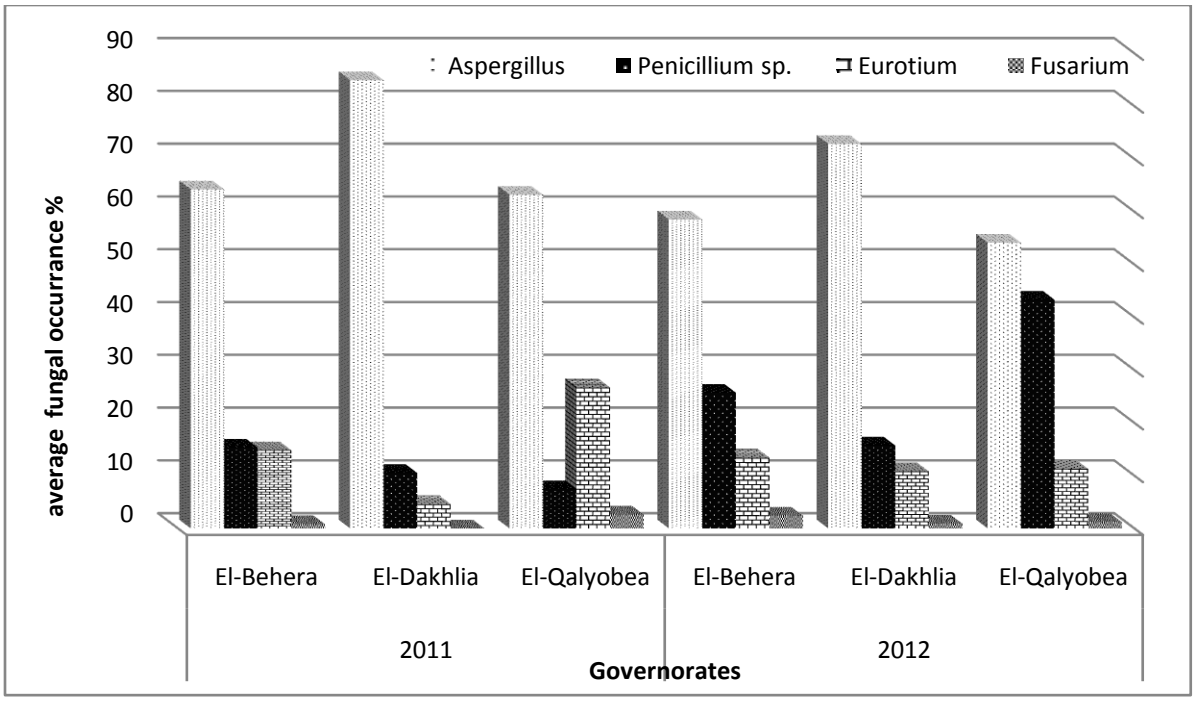

Fig (1) the average percentage of occurrence different genera of fungi on PDA from different governorates

While, in 2012 its percentages were 23.4% 35.5% and 52.2%, respectively. P*enicillum* speices represented 15.3% in El-Beheira, 11.8% in El-Dakahlya and 7.4% in El-Qalyubia in 2011. These percentages increased in 2012, recording 25.7%, 15.2% and 15.7 % in samples from El-Beheira, El- Dakahlya and El-Qalyubia, respectively. *Fusarium* species was found only in El-Beheira 0.8% and El-Qalyubia 1.3% in 2011,whereas, it represented 2.4%, 1.0% and 1.1% in 2012 in El-Beheira, El- Dakahlya and El-Qalyubia samples, respectively.

On specific and selective method, deep freezing blotter was used for isolation of *Fusarium* species from maize. Fig (3) showed that *Fusarium*, *Aspergillus* and *penicillium* were the most common genera but *Eurotium* genus was disappeared. The *Fusarium* genus was recorded a higher occurrence percentage of other genera than that recorded on PDA media. The high percentage of *Fusarium* genus was showed in El- Beheira, followed by, El- Dakahlya in 2011.

Among *Fusarium* species, *F. verticilloids* was the most predominant species in both 2011 and 2012 with incidence of 67.2%, 51.7% and 43% in 2011 and 37.5%, 20.2% and 35.1% in 2012, in El-Beheira, El- Dakahlya and El-Qalyubia, respectively (Fig. 4). The incidence of *F. prolifertum* was varied widely between two years in three governments. *F. subglutinus* was found in 2012, with incidence 20 % and 6.5% in El-Beheira and El- Qalyubia.

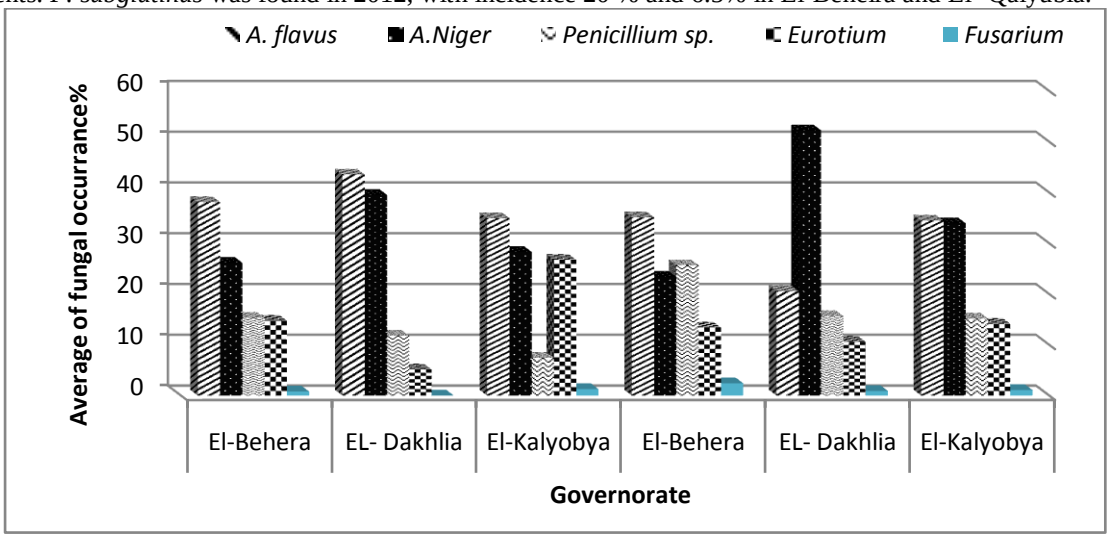

Fig (2) Average percentage of fungal species occurrence on maize grains on PDA media from different governorates

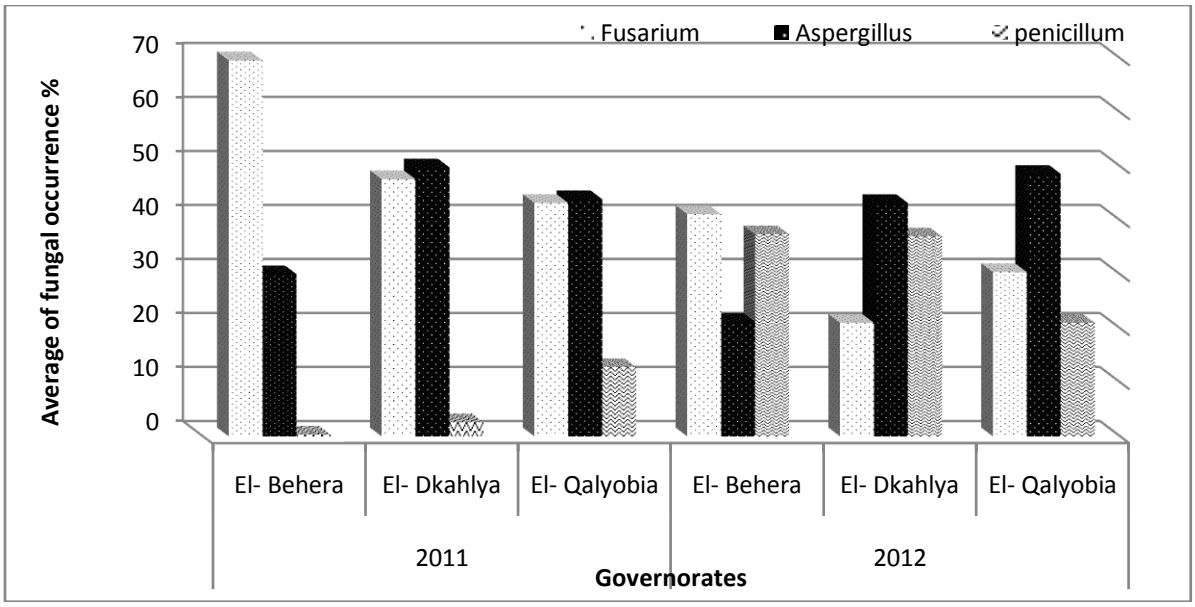

Fig. (3). The average percentage of occurrence different genera of fungi by using deep freezing blotter method from different governorates

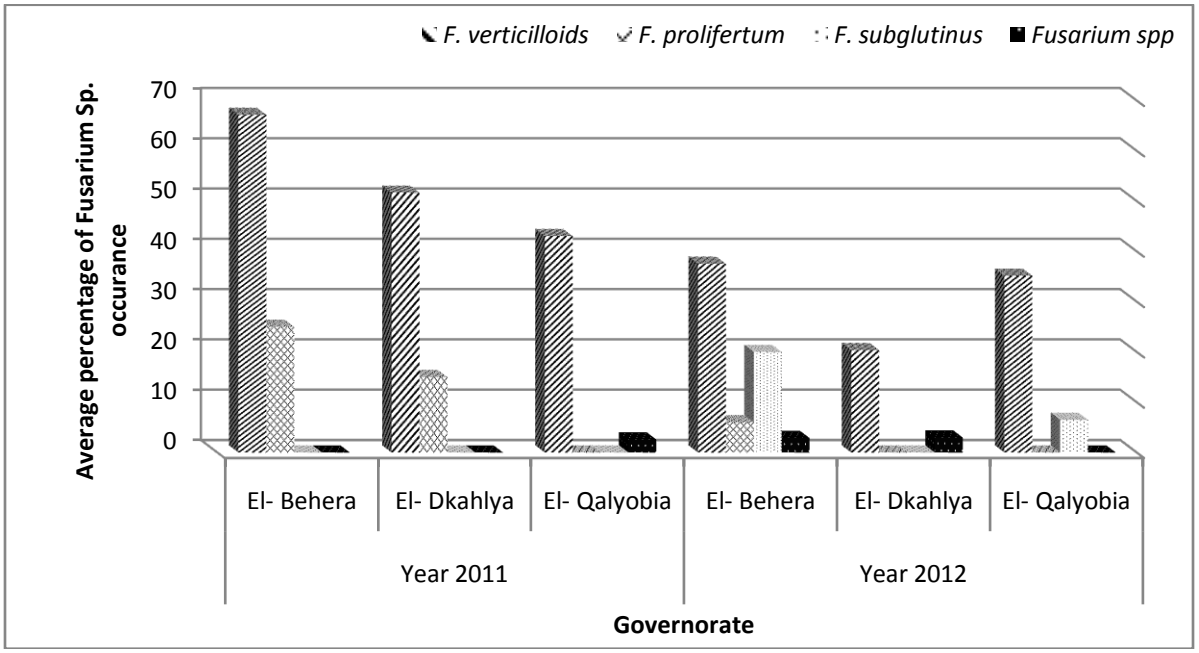

Fig (4) Average percentage of *Fusarium sp*. occurrence in maize samples from different Governorates

Mycotoxin analysis:

The percentage of positive samples ranges and averages of *Fusarium* toxins in maize samples are illustrated in Table (2). In 2011, 32.3% of samples were contaminated with fumonisin B1 in three governorates in range from 0.012 to 1.4 mg kg^{-1}. While, in 2012, 36.8% of the samples were contaminated with fumonisin which ranged from 0.01 to 9.8 mg kg^{-1}. Only one maize sample from 65 samples contained high amount of FB1 (9.8 mg kg^{-1}) which exceeded the permissible limit (3 mg kg^{-1}) (**FDA 2001**). Although, 93.3% of the samples in 2014, contaminated with Fumonisin B1. The average of its content was low (0.2 mg kg^{-1}).

The levels of contamination with zeralenone were generally high contaminations of 0.8, 0.1 and 0.5 mg/kg in 2011, 2012 and 2014 El-Beheira, El-Qalyubia and El-Dakahlya, respectively. Thirteen samples of 24 contaminated samples with zeralenone were higher than permissible limit (0.1 mg kg^{-1}) for maize-based breakfast cereals (**EC 2007)**.

Ranges which varied from 0.04 to 3.0 mg/kg in 2011, from 0.03 to 0.2 mg/kg in 2012 and from 0.02 to 7.2mg/kg in 2014 for positive samples in Table (2).

Data of the average of fumonisin B1 and zeralenone concentration in maize samples from different governorates during 2011, 2012 and 2014 are showed in Fig. 5 (A and B). In 2011, the highest average of fumonisin B1 was recorded in 2012 in El-Beheira (2.6mg kg^{-1}) and in El-Dakahlya (2.2 mg kg^{-1}) (Fig 5a). While, the highest average of zeralenone was recorded in El-Qalyubia (2.5 mg kg^{-1}) in 2014 (Fig. 5 b). On other hand, Deoxynivalenol were not presented in all samples from the three Governorates during three years. It is work noting that 29% of the samples were free from *Fusarium* toxins (Fig. 6) in spite of that it prove that it has *Fusarium* spores in the kernels.

Table (2) Occurrence of Fusarium toxins (mg/kg) in three years (% of contaminated samples, ranges and mean)

Fumonisin B1 (mg/kg)				
Years	**No. of sample**	**% of contamination sample**	**Range (mg/kg)**	**Mean (mg/kg)**
2011	31	32.3	0.01- 1.4	0.3
2012	19	36.8	0.01- 9.8	2.2
2014	15	93.3	0.02- 1.6	0.2
Total	65	47.7	0.01- 9.8	0.32
Zeralenone (mg/kg)				
Years	**No. of sample**	**% of contamination sample**	**Range (mg/kg)**	**Mean (mg/kg)**
2011	31	38.7	0.04-3	0.8
2012	19	36.8	0.03-0.2	0.1
2014	15	33.3	0.02- 7.2	0.5
Total	65	37.0	0.02- 7.2	0.3

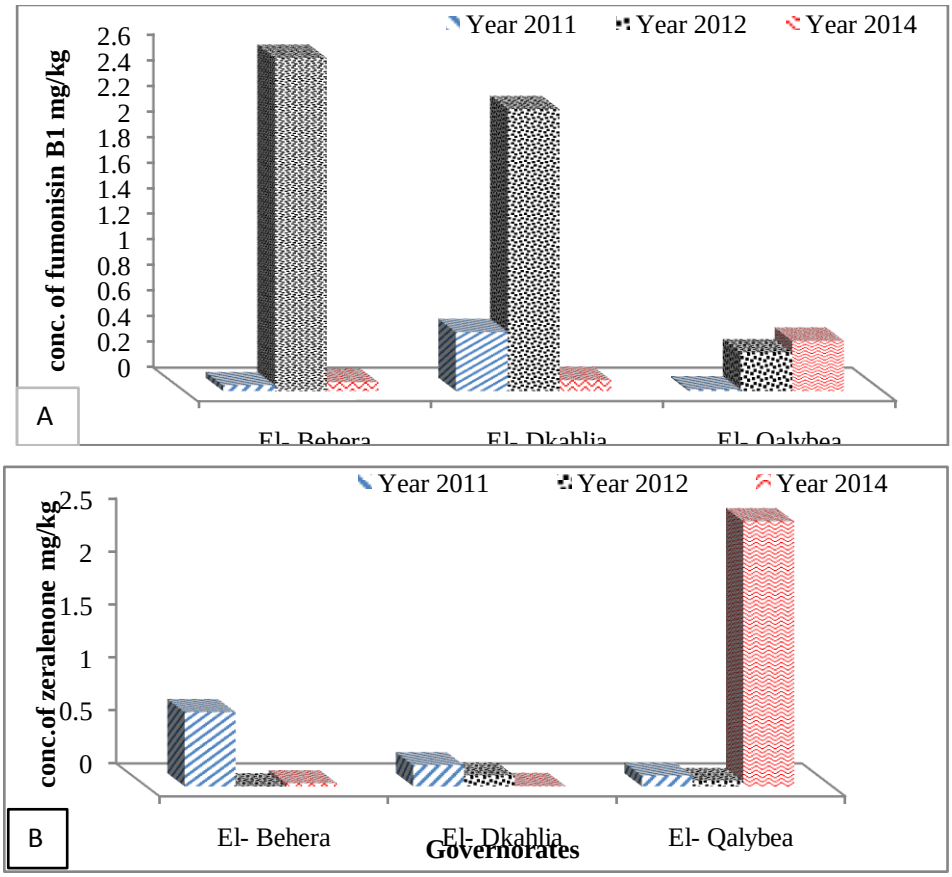

Fig. (5) The average of fumonisin B1 (A) and zeralenone (B) concentration in three governorates during three seasons

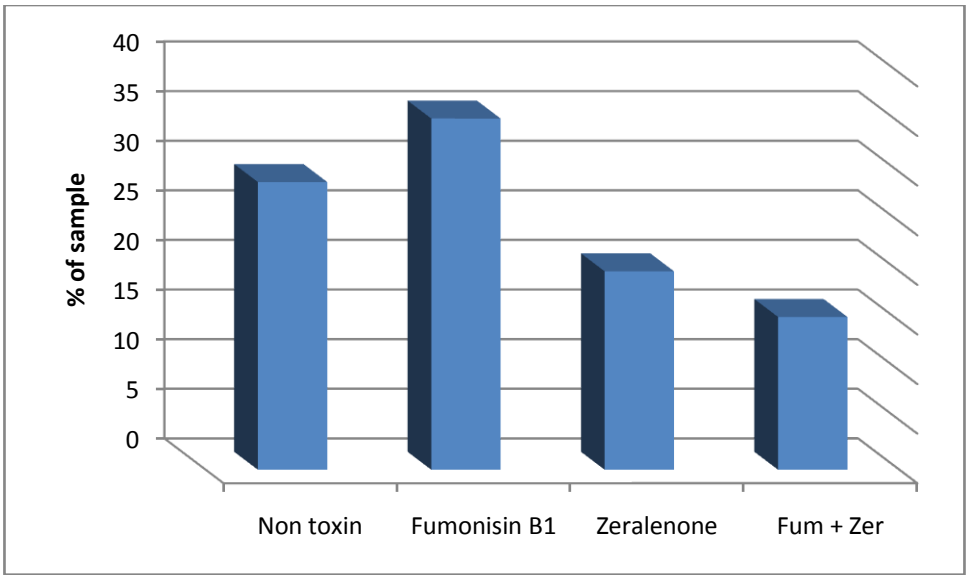

Fig (6) Occurrence percentage of *Fusarium* toxins in samples

Discussion:

The present study focused on the risks of *Fusarium* contamination and the potential accumulation of its mycotoxin in maize. The major three species of *Fusarium* are *F. verticilloids*, *F. proliferatum* and *F. subglutinus*. The main *Fusarium* toxins are fumonisinB1, zeralenone and deoxynivalenol. The study was carried out in three main Governorates in Delta region. Isolation had done using two different culture media composition are essential to reflect the fungal profile. Accordingly, Potato Dextrose Agar medium for the isolation of Fusarium species versus Deep Freezing Blotter methods were used. The obtained results indicated that *Aspergilli* species infection was most dominant fungi in maize kernels seeded on the PDA, followed by *Penicillium* species and *Eurotium. Fusarium* species was the least detected fungi. There was no difference between the Governorates and respectively as well as between different species. To the contrary, using Deep Freezing Blotter as a specific method, *Fusarium* species were the predominant fungus isolated from maize kernels. The present work was in agreement with **Elshabrawi (2001 and 2007) and El-sheikh *et al*. (2009)** who found that *Fusarium spp* were the major residents that isolated from the maize ears from different localities of El-Beheira governorate. In other Egyptian areas were the predominant *Fusarium* species (**Fadl Allah 1998, El-Shabrawy 2001 and Aboul-Nasr and Obied-Allah, 2013**). Globally, Fusarium infection maize revealed that *F. verticilloides* was the most predominant pathogen of maize in the examined regions followed by *F. proliferatum* and *F. subglutinans* (**Samson 1991; Ominski *et al*., 1994; Munkvold and Desjardins 1997; Orsi *et al*., 2000**; **Logrieco *et al*., 2002; Seyed *et al*., 2005; Rahjoo *et al*., 2008; Edson *et al*., 2011 and Lorenzo *et al*., 2011).** Regarding to *Fusarium* toxins which produced by several *Fusarium* species as *F. verticillioides* and *F. proliferatum* **(Marasas 2001)**. Fumonisins can contaminate maize foods and feeds as a result of the *Fusarium* invasion before and after harvest **(Doko *et al*., 1995).** Fumonisin B1 was the predominant *Fusarium* toxins. All samples were in the permissible limit except one maize sample contained high amount of FB1 (9.8 mg kg^{-1}) which exceeded the permissible limit **(FDA 2001)**. These results were agreement with **Lorenzo *et al*., (2011)** in Italy, reported that Fumonisins were the predominant *fusarium* toxins with values, an average, of 4.3 and 5.7 mg kg^{-1}, in 2006 and 2007, respectively, with a maximum of 76.3 mg kg^{-1} in the second year which is in agreement with our results. In south Africa **Edson *et al*.**, **(2011)** reported that the levels of contamination maize samples in South Africa with fumonisins ranged from 0 µg g^{-1} to 21.8 µg g^{-1}, depending on the region where samples were collected. Levels of fumonisins were highest in northern KwaZulu-Natal where 52% and 17% of maize samples collected in 2006 and 2007, respectively, exceeded 2 µg g^{-1}. zeralenone was detected in 37% of maize samples with different concentration but 13 of positive samples were exceeded the permissible limit **(EC 2007).** That was agreement with, **Beatriz *et al*., (2005)** detected one (0.8%) of 121maize samples was contaminated by ZEA (448 µg kg^{-1}). **Sabino *et al*. (1989)** found different levels of ZEA, from not detected to 9.83 mg kg^{-1} in 358 maize samples. In contrary, **(Lorenzo *et al*., 2011)** found that zearalenone content was always very low. Deoxynivalenol (DON) was ranged from 0.2 to 3.98 mg kg^{-1} in 2006 (average 1.04 mg kg^{-1}) and from undetectable levels to 14 mg kg^{-1} in 2007 (average 0.86 mg kg^{-1}) that was disagreement with our results that referred to the absence of DON in all samples **(Lorenzo *et al*., 2011)**.

It is to be concluded that the climate condition prefer in Egypt did not allow *Fusarium* species to grow and accumulate in toxins in the kernels. Some samples contain the spores when analysed by deep freezing blotter method and in the meantime it was free from *Fusarium* toxins; even the samples which showed fumonisin B1 accumulation have concentration less than the permissible limits. To the contrary, the conditions were more favour to the accumulation of the zeralenone, as 20% of samples, exceeded the permissible limit. In the meantime non analysed samples contain deoxynivalenol. It is suggested that the factories producing feed give the almost concern for the analysis of *Fusarium* toxins and blend the kernels lots to maintain the animal feed with *Fusarium* toxins less than the permissible limits.

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