ISSN No:-2456-2165

Molecular Characterization of Aflatoxinogenic and Non-Aflatoxinogenic Aspergillus Flavus Group Isolates Collected from Soybean Seeds from El-Beheira Governorate

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Abstract:- A study to distinguish the seed-borne fungi of non-disinfected and disinfected soybean seeds was conducted in Etay El-Baroud Agricultural Research Station, El-Beheira Governorate, Egypt during 2017. A total of seven fungi namely; *Aspergillus flavus, A. parasiticus* (*Aspergillus flavus* group), *A. niger, Rhizopus stolonifer, Pythium* spp., *Fusarium* spp., *Alternaria* spp. and *Penicillium* spp. were secluded which varied in there frequency. Also, qualitative levels of aflatoxin(s) produced by fifty isolates of *A. flavus* group from ten locations at El-Beheira Governorate were described by using fluorescence technique. Blue and green fluorescence surrounding the colonies of A. flavus group was observed in 32 out of the 50 isolates. Three isolates of A. flavus group; 1) highly producer, 2) moderately producer and 3) non producer as examples, were studied genetically using Polymerase Chain Reaction (PCR) based assess with versicolorin A dehydrogenase (ver-1), norsolorinic acid reductase (nor-1) sterigmatocystin O-methyltransferase (omt-A) and aflatoxin R (aflR) primers. Generally, for the tested isolates there is a complete form with four bands obtained in PCR. The occurrence of patterns in the non-aflatoxinogenic isolates shows that PCR is not a satisfactory marker for distinction between aflatoxinogenic and some non-aflatoxinogenic isolates, but PCR exhibited that it is speedy and sensitive technique for revealing fungi.

Keywords:- *Seed Borne Fungi, PCR, Aflatoxins, Aspergillus Flavus Group, Soybean*

I. INTRODUCTION

Diseases caused by seed-borne fungal pathogens are quite difficult to control because of the establishment of fungal hyphae (Butt *et al*., 2011) [1]**.** Several investigators have stated that *Aspergillus flavus, A. parasiticus, A. niger, Macrophomina phaseolina, Cercospora kikuchi Fusarium solani, F. oxysporum , F. moniliforme, Cladosporium* sp., *Phomopsis* sp., *Rhizopus* sp., *Alternaria* sp., *Curvularia* sp., *Colletotrichum dematium* and *Phoma* spp. are the most fungi related with soybean seeds (Venugopal *et al*., 2015 and Aoudou *et al*., 2017) [2, 3].

Aspergillus is an enormous genus consisted of further than 180 recognized species (Pitt *et al*., 2000) [4]. The genus is simply—recognized by its representative conidiophore, but species recognition and variation is very difficult, for it is conventionally based on a range of morphological features which are include conidial and mycelial color, colony diameter, colony reverse color, exudates production, soluble pigments, cleistothecia and sclerotia presence (Klich, 2002) [5]. The toxigenic potential of *Aspergillus* Section *Flavi* has concerned attention of many investigators. Section *Flavi* includes two groups of species, the first (*A. flavus* group), talk about the aflatoxigenic species of *A. flavus* and *A. parasiticus*, which cause severe problems in agricultural commodities in the world, and the second includes the non-aflatoxigenic species (Kumeda and Asao 2001) [6]. The present investigation is focused on the 1st group.

Aflatoxins (B1, B2, G1 and G2) are mycotoxins belong to polyketide-derived furanocoumarins. They are one of the furthermost carcinogenic matters, which cause great polluting of food and agricultural produces. Aflatoxin B1 is human cancer-causing agent (group 1A) as categorized by the International Agency for Research on

Cancer (IARC) (Passone *et al*., 2010) [7]. They have nephrotoxic, carcinogenic, mutagenic and teratogenic effect (Shapira *et al*., 1996) [8]. Two species from genus *Aspergillus*; *A. flavus* and *A. parasiticus* produce AFs (B1, B2) and (B1, B2, G1 and G2), respectively. The B and G terms arise from the blue and green fluorescent colors produced under UV light on culture (Edwards *et al*., 2002) [9].

Great hard works are made to determine the aflatoxigenic fungi in human food and animal feed samples. Polymerase Chain Reaction presents a hopeful mean to recognize and quantify fungi in food and feed industry (Degola *et al*., 2007) [10]. PCR based on enzymatic magnification of an objective segment of DNA distinguished by two oligonucleotides, known as primers, that bind contrasting to complementary DNA strands (Sambrook and Russel 2001) [11].

II. MATERIAL AND METHODS

A. Survey

➢ *Collection of samples*

Randomly, whole of 50 soybean seeds samples were collected from some oil extracting factories, some local feed markets and eight major locations for soybean cultivation at El-Beheira Governorate namely; Damanhour, Kom Hamada, Shubrakhit, Etay El-Baroud, Wadi El-Natroun, Nubaria, Abu El-Matamir and Hosh Issa. Ten samples were collected from factories and markets (5 samples each) and forty samples from the eight regions were collected from farmer's fields at harvest time during 2017 growing season. Samples were carefully investigated according to the procedures published by (ISTA, 1996) [12] to study the associated pathogens specially AFs producing fungi.

➢ *Recognition of fungi related with soybean seeds*

Soybean seeds related fungi were isolated by consuming blotter paper method as recommended by ISTA (1996). Samples were separated for two groups; one was sanitized with NaOCl (1%) for 3 min, while the other was not sanitized (untreated with NaOCl). By autovlaved water, Whole samples were quickly washed one time, after that, seeds were put among two ovened filter papers for drying, then plated on autoclaved Petri dishes containing wet filter papers (blotter test). Five soybean seeds/plate and 3 plates were used as replicates according to the method described by Kumlachew (2014); Mathur and Olga (2003). [13, 14]. Whole plates were incubated at 25±2 ˚C for 7 days. Whole fungal growths were transferred onto PDA medium amended with streptomycin to suppress bacterial growth and purified using hyphal tip technique.

➢ *Identification of Pathogens*

Growing fungi were transferred to PDA slants medium for 7 days, and then identified according to their cultural, morphological and microscopical characters under steriobinoculer microscope. Identification was further confirmed according to Maren and Johan (1988) [15] for *Aspergillus* spp., Booth (1977) [16] for *Fusarium* spp., Pitt and Hocking (1997) [17] for *Penicillium* spp. and Nelson *et al*., (1983) [18] for other fungi. Among the various colonies identified, *A. flavus* and *A. parasiticus (A. flavus* group) were cultured onto the Czapek-Dox Agar media for further studies. Frequency percent of the isolated fungi were calculated according to: the sum of the tested fungal isolates /total sum of all fungal isolates x 100 according to Kumlachew (2014) [13].

B. Screening of Aflatoxins Producing Isolates of A. flavus Group

➢ *Fluorescence Technique*

The ability of AFs producing was tested in 50 isolates of *A. flavus* group by the fluorescence technique mentioned by Hara *et al*., (1974) [19]. Isolates from different locations were streaked on the Czapek-Dox Agar dishes and incubated for 7 days at 25±2 ˚C. Petri dishes with non-fluorescent glass were used. Diffusible areas of AFs were distinguished visually as fluorescent areas under UV (365 nm) light.

➢ *Molecular Technique*

- *Sample Preparation*

Czapek-Dox Broth medium was set and ten conical flasks at size of 250 ml, each contain 100 ml of the mentioned medium were autoclaved at 121 ˚C for 20 min. The flasks had fungal inoculum (one disk 5 mm/flask) of *Aspergillus flavus* group isolates according to their florescence (highly, moderately and non-producer) in sterilized conditions and incubated at 25±2 ˚C for 7 days. The mycelial mats were gathered by centrifugation at 10000 xg for 10 min and stored at 4 ˚C until used (El-Bazza, 1979 and Watanabe *et al*., 2010) [20,21].

- *Extraction and Isolation of DNA*

DNA was taken out and extracted according to the technique mentioned by (Yelton *et al*., 1984) [22] with some modifications. The harvested mycelium of each isolate was transmitted to the mortar, froze in liquid Nitrogen and crushed to become powder. This powder was re-suspended in Lysis buffer (50 mM/L EDTA, 0.2% SDS pH 8.5) and heated at 68 ˚C immediately for 15 min. After sample centrifugation for 20 min at 15000 xg, 10 ml of the molten supernatant was transmitted to a novel centrifuge tube and 2 mM/L of sodium acetate was supplemented. The solution was put on ice for 1 h and centrifuged for 15 min at 15000 xg. After being centrifuged, the supernatant fluid was transmitted to another tube. The solution was taken out by phenol and the sedimentation of the extracted DNA was by adding of 2.5 volumes of ethanol.

- *PCR based Assay*

Polymerase chain reaction was achieved according to Farber *et al*., (1997) [24] with some basic amendments. The extracted DNA was diluted to 2 µg/ml and used as DNA template for AFs biosynthetic specific genes during PCR reaction. A complete of 35 PCR cycles with the following temperature procedure was achieved: 95 ˚C, 1

min; 65 ˚C, 30 s; 72 ˚C, 30 s for the 1st cycle; and 94 ˚C, 30 s; 65 ˚C, 30 s; 72 ˚C, 30 s for the next 34 cycles. Four primers for AFs biosynthetic genes i.e. versicolorin A dehydrogenase (ver-1), norsolorinic acid reductase (nor-1) sterigmatocystin O-methyltransferase (omt-A) and aflatoxin R (aflR) were used. The sequences of the used primers were as created in Table (1).

Primer	Sequence
nor-1	nor1, 5' -ACC-GCTACGCCGGCACTCTCGGCAC-3' nor2, 5' -GTT-GGCCGCCAGCTTCGACACTCCG-3'
ver-1	ver1, 5' -GCCGCAGGCCGCGGAGAAAGTGGT-3' ver2, 5' -GGGGATATACTCCCGCGACACAGCC-3'
omt-A	omt1, 5' -GTGGACGGACCTAGTCCGACATCAC-3' omt2, 5' -GTC-GGCGCCACGCACTGGGTTGGGG-3'
aflR	aflR1, 5' -TATCTCCCCCCGG-GCATCTCCCGG-3' aflR2, 5' -CCGTCAGACAGCCA-CTGGACA-CGG-3'

Table 1:- Sequences of the four primers, nor-1, ver-1, omt-1 and aflR which used in PCR based assess

nor1 primer, including a piece of 400 bp from nucleotide 501 - 900 of the fungal nor-1 gene; ver1, including a piece of 895 bp from nucleotide 623 - 1160 of the fungal ver-1 gene; omt1, including a piece of 1032bp from nucleotide 301 - 1098 of the fungal omt-A gene;

aflR1, including a piece of 1232bp from nucleotide 450 - 1482 of the fungal aflR gene. The primer had A-T and G-C contents further or fewer like in quantities and did not contain palindromic sequences. In total volume of 50 µL reaction mix, amplification was carried out enclosing chemicals in Table (2).

Chemical	Volume (µL)
Primer (2 µM/µL)	8.0
10X Buffer	5.0
2 MmM dNTP Mix	5.0
Taq DNA polymerase (U/µL)	0.5
Template DNA (50 ng)	2.0
Sterile Distilled Water	29.5
Total	50.0

Table 2:- Volume of chemicals used in amplification of PCR based assay.

Two µL of loading dye was supplemented prior to loading of 10 µL per gel well. Electrophoresis was achieved on 2% agarose gel in 1 × TBE as running buffer (45 mM boric acid, 40 mM Tris-Cl, 1 mM EDTA and pH 8.3) at 75 V for 3 h. The agarose gel was marked with (EtBr) and under a UV transilluminator, the improved produces was visualized. Presence and absence of bands produced from use of four primers were scored visually from the resulting photographs. Gel produces were sized to a comparative 1 kb ladder marker (Suanthie, 2009; Lezar and Barros, 2010) [25, 26].

C. Statistical Analysis

The obtained data were analyzed as RCBD. Data were tested using the statistical analysis system (SAS) software (version 9.3) and means were compared using the least significant difference (LSD) at $P < 0.05$ (Steel and Torrie, 1960) [27].

III. RESULTS

A. Survey, Isolation and Recognition of the Related Fungi with Soybean Seeds

Soybean seeds which taken randomly during 2017 season from some oil extracting factories, some local feed markets and eight major regions for soybean cultivation at El-Beheira Governorate were used to survey soybean seed borne fungi. Results obtained in Table (3) show the frequency (%) of seed borne fungi related with non-disinfected and disinfected soybean seeds from ten locations. A total of seven fungi namely; *A. flavus* and *A. parasiticus* (*Aspergillus flavus* group), *A. niger*, *Rhizopus stolonifer*, *Pythium ultimum*, *Fusarium solani*, *Alternaria alternata*. and *Penicillium* spp. were isolated from samples of soybean seeds (Fig. 1). Among the identified fungi, *A. flavus* group was the most common fungal genera recovered from the collected disinfected soybean seeds at the chosen locations followed by *A. niger*, *R. stolonifer*, *Penicillium* spp., *F. solani*, *P. ultimum* and *A. alternate* with general averages of 6.50, 6.30, 6.08, 5.76, 4.48, 2.42 and 2.20 %, respectively. *A. flavus* group had the highest frequency in case of the factory samples followed by *A. niger* and *R. stolonifer* with averages of 12.20, 11.40 and 10.60%, respectively (Fig. 2). The same trend clear with the market samples where *A. flavus* group, *A. niger*, *R. stolonifer* and *Penicillium* spp. had the highest frequency with averages of 10.40, 9.60, 8.80 and 8.80%, respectively. In consideration of the all locations, factories samples had higher frequency of the six fungal genera mentioned before followed by the samples of the markets, Etay El-Baroud, Kom-Hamada, Shubrakhit, Damanhour, Hosh Issa, Abu El-Matamir, Nubaria and Wadi El-Natroun with averages of 8.16, 7.45, 5.10, 4.86, 4.76, 4.75, 3.75, 3.76, 3.11 and 2.52 %, respectively (Fig.3).

In case of disinfected soybean seeds, the factories samples had the highest frequency with *A. flavus* group followed by *A. niger*, *R. stolonifer*, *F. solani* and *Penicillium* spp. with averages of 8.40, 7.90, 7.40, 7.20 and 6.40%, respectively. In this respect, the market samples show higher fungal genera frequency in case of *A. flavus* group than the other fungal genera with an average of 7.60

ISSN No:-2456-2165

followed by *A. niger* and *F. solani*, *R. stolonifer* and *Penicillium* spp. with averages of 6.90, 6.80, 6.20 and 6.20 %, respectively. Generally in concern with locations, it clear that *F. solani* had the highest frequency followed by *A. flavus* group, *A. niger*, *Penicillium* spp., *R. stolonifer*, *A. alternata*. and *P. ultimum* with averages of 4.32, 4.14, 3.86, 3.62, 3.58, 2.18 and 1.66%, respectively. The fungi grand mean for the two treatments (non-disinfected and disinfected seeds) are 5.32, 5.07, 4.83, 4.69, 4.40, 2.19 and 2.04 % for *A. flavus* group, *A. niger*, *R. stolonifer*, *Penicilium* spp., *F. solani*, *A. alternata* and *P. ultimum*, respectively. Also the grand mean of the non-disinfected seeds treatment is 4.81 and 3.33 % in case of disinfected seeds treatment.

Treatment	Location	*Aspergillus flavus* group	*Rhizopus stolonifer*	*Pythium ultimum*	*Fusarium solani*	*Alternaria alternata*	*Penicillium* spp.	*Aspergillus niger*	Mean
Non disinfected surface seeds	Factories	12.20	10.60	3.40	6.60	4.80	8.20	11.4	**8.16**
	Markets	10.40	8.80	3.20	8.00	3.40	8.80	9.60	**7.45**
	Damanhour	7.20	5.40	2.00	4.60	2.20	5.60	6.30	**4.75**
	Kom Hamada	6.40	5.80	2.60	4.80	2.60	5.80	6.10	**4.86**
	Shubrakhit	5.80	6.00	2.40	4.80	2.20	6.20	6.00	**4.76**
	Etay El-Baroud	6.20	6.40	3.20	5.60	1.80	6.20	6.30	**5.10**
	Wadi El-Natrun	3.40	3.20	1.20	2.20	1.00	3.40	3.30	**2.52**
	Nubaria	3.60	4.60	2.80	1.20	1.40	4.00	4.20	**3.11**
	Abu El-Matamir	4.80	5.20	1.60	3.40	1.20	4.60	5.00	**3.67**
	Hosh Issa	5.00	4.80	1.80	3.60	1.40	4.80	4.90	**3.75**
	Mean	**6.50**	**6.08**	**2.42**	**4.48**	**2.20**	**5.76**	**6.30**	**4.81**
Disinfected surface seeds	Factories	8.40	7.40	2.20	7.20	4.60	6.40	7.90	**6.30**
	Markets	7.60	6.20	2.40	6.80	3.80	6.20	6.90	**5.70**
	Damanhour	4.40	3.20	1.60	4.80	2.40	3.20	3.80	**3.34**
	Kom Hamada	4.20	3.40	1.80	4.40	2.40	3.40	3.80	**3.34**
	Shubrakhit	3.60	3.80	1.40	4.60	2.00	3.80	3.70	**3.27**
	Etay El-Baroud	3.20	4.00	2.00	5.40	1.60	4.00	3.60	**3.40**
	Wadi El-Natrun	2.20	1.80	1.20	1.80	1.20	2.20	2.00	**1.77**
	Nubaria	2.00	1.80	1.00	2.00	1.20	1.80	1.90	**1.67**
	Abu El-Matamir	2.80	2.00	1.40	3.20	1.40	2.00	2.40	**2.17**
	Hosh Issa	3.00	2.20	1.60	3.00	1.20	3.20	2.60	**2.4**
	Mean	**4.14**	**3.58**	**1.66**	**4.32**	**2.18**	**3.62**	**3.86**	**3.33**
Fungi grand mean±SE		**5.32[a] ±0.68**	**4.83[a] ±0.59**	**2.04[b] ±0.18**	**4.40[a] ±0.47**	**2.19[b] ±0.28**	**4.69[a] ±0.49**	**5.07[a] ±0.63**	

LSD 5%				
	Treatment (T)	1.82	T × F	1.38
	Location (L)	1.42	L × F	1.57
	Fungi (F)	1.23	T × L × F	2.44
	T × L	1.44		

Table 3:- Survey of different seed-borne fungi related with soybean seeds in ten locations at El-Beheira governorate.

Statistically, the variances between the two treatments are not significant but the variances between the fungi frequency in the tested locations varied statistically in their significance. Also, the differences between the interactions of the tested factors are significant.

ISSN No:-2456-2165

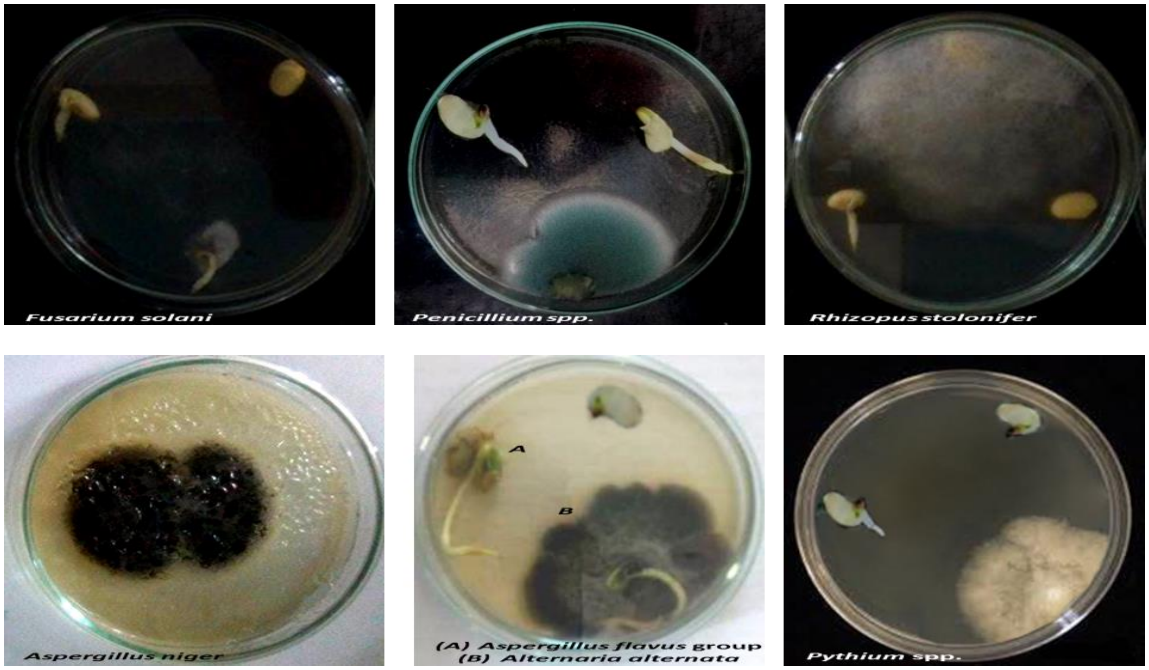

Fig 1:- Example for seed-borne fungi related with soybean seeds.

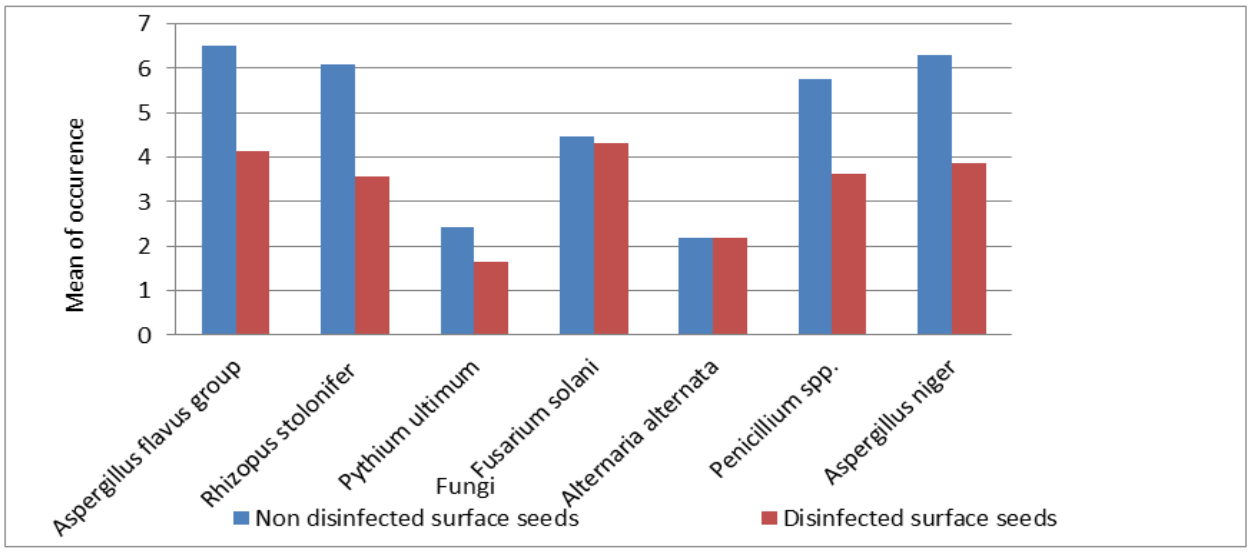

Fig 2:- Frequency of seven associated fungi with soybean seeds at El-Beheira governorate during 2017 season.

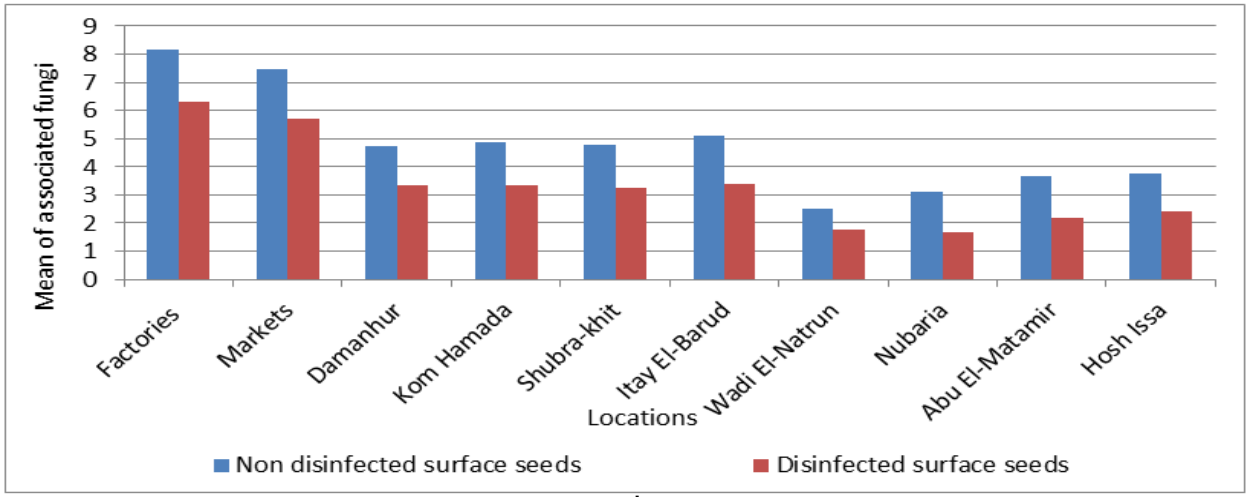

Fig 3:- Frequency of associated fungi with soybean seeds from ten locations at El-Beheira governorate during 2017 season.

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B. Screening of Aflatoxins Producing Isolates of A. flavus Group

➢ *Fluorescence Technique*

Aflatoxins production was observed as shown in some isolates of *A. flavus* group under study as compared with the standard under the UV long and short waves (365 and 254 nm).

Using the UV (365 nm) to do the fluorescence technique described by Hara *et al.*, (1974) [19], blue and green fluorescence surrounding the colonies of *A. flavus* group was observed in 32 out of the 50 isolates as shown in Table (4). This result indicates the probability of the presence of AFs in its culture medium. They differed in their fluorescence intensity where some isolates exhibited high intense fluorescence (++++) and others showed moderate intensity (++). Eighteen isolates were non aflatoxins producers (-) Fig. (4).

Location	Number of isolate				
	1	2	3	4	5
Factories	_	++	++++	++++	++++
Markets	++++	_	++++	++++	++
Damanhur	_	++++	_	++	++++
Kom Hamada	++++	_	++	_	++++
Shubra-khit	_	++	++++	_	++
Etay El-Barud	_	++++	_	++++	++
Wadi El-Natrun	++++	_	++	++++	_
Nubaria	++	_	++++	_	++++
Abu El-Matamir	++++	++	_	_	++++
Hosh Issa	++++	_	++++	++	_

Table 4:- Aflatoxins qualitative levels produced by fifty isolates of *Aspergillus flavus* group from ten locations at El-Beheira governorate during 2017 season.

(++++) = highly producer
(++) = moderate producer
(-) = non aflatoxins producers

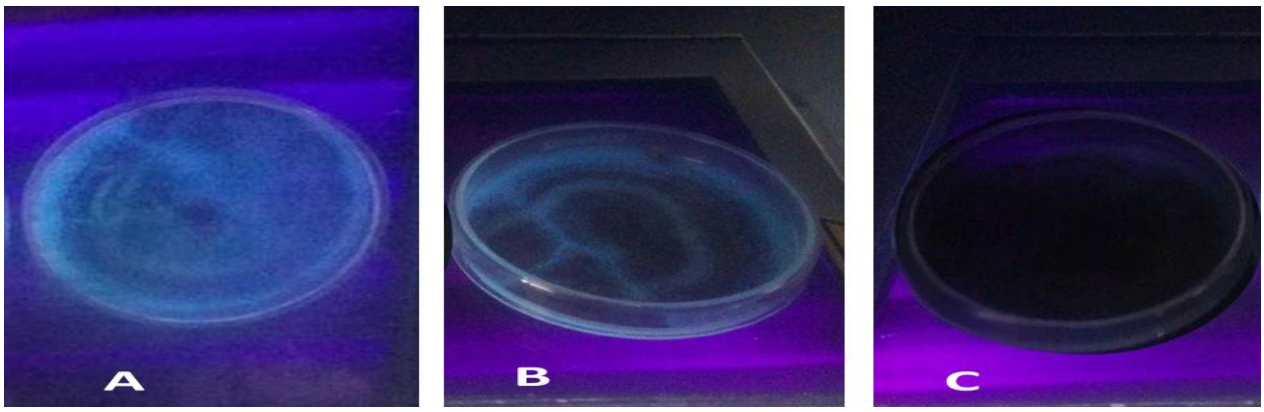

Fig 4:- Aflatoxins fluorescence of three *Aspergillus flavus* group isolates, A) highly producer (++++), B) moderately producer (++) and C) non producer (-) using UV waves at 365 nm.

➢ *Molecular Technique*

The imperative purpose of the present study is to systematize and optimize a PCR technique for identifying the AFs producing fungi, by operative genes in biosynthesis pathway. Ver-1, Nor-1 and Omt-A are three structural genes in biosynthesis AFs pathway that coding for key enzymes in producing of AFs, as well as the functional gene aflR. Thus, they are necessary for AFs producing (Yu *et al*., 2004) [28].

PCR technique was used for investigating the isolated genomic DNA variation between three isolates of *A. flavus* group representing different levels of AFs production, the 1st was highly AFs producer, the 2nd was moderately AFs producer and the 3rd was non-AFs producer according to physical characters. PCR was developed consuming four sets of primers for diverse genes involved in AFs biosynthetic pathway, nor-1, ver-1, omt-A and aflR genes. The results achieved by the molecular methods reported in Fig. (5), show that bands of the segments of aflR, omt-A, ver-1 and nor-1 gene can be visualized at 1232, 1032, 895 and 400 bp, respectively. Lanes 1, 2, 3 is DNA of the highly producer, moderately producer and non-producer isolates, respectively with ver-1 primer at 895 pb. Lanes 4, 5, 6 is DNA of the highly producer, moderately producer and non-producer isolates, respectively with alfR primer at 1235 pb. Lanes 7, 8, 9 is DNA of the highly producer, moderately producer and non-producer isolates, respectively with omt-A primer at 1032 pb. Lanes 10, 11, 12 is DNA of the highly producer, moderately producer and non-producer isolates, respectively with nor-1 primer at 400 pb. Lane M: 500-bp is DNA ladder size marker.

All tested isolates shows parallel pattern demonstrating the presence of the four genes. The results revealed that three primers (ver-1, alfR and nor-1) made shrill and distinctive bands in its specific region, but omt-A did not. PCR using the mentioned four primers has permitted the recognition of aflatoxinogenic isolates of *A. flavus* group by the attendance of a complete pattern with four bands, therefore representing the attendance of all the studied genes which encode for functional produces.

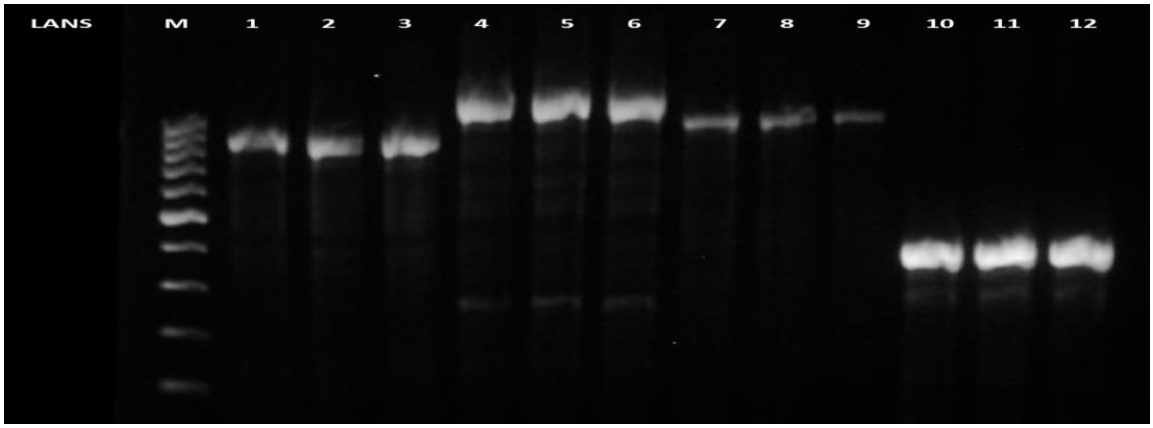

Fig 5:- PCR products analyzed with gel electrophoresis using nor-1, ver-1, omt-A and alfR primers and DNA taken out from 3 strains of *A. flavus* group.

IV. DISCUSSION

Soybean is an important plant which belongs to family Fabaceae, its seeds are a main source of protein, free cholesterol oil (Ash *et al.,* 2006) [29], carbohydrates and minerals (Capeleti *et al.,* 2005) [30]. A whole of seven fungi encompassing six dissimilar genera (*Fusarium, Aspergillus, Alternaria, Rhizopus Penicillium* and *Pythium*) were isolated from soybean seeds in this investigation. These results are in settlement with the findings of Schafer and Kotanen (2004) [31] who exhibited that the essentially saprophytic genera; *Mucor, Rhizopus, Trichoderma, Penicillium, Cladosporium, Chaetomium* and *Aspergillus* moreover the essentially pathogenic genera *Alternaria, Pythium, Fusarium, Phoma* and *Acremonium* are commonly isolated from legume seeds and Abd-Allah (2018) [32] who isolated *A. alternate, A. niger, A. flavus, A. parasiticus, A. terrus, Fusarium* spp., *Penicillium* spp. and *Rhizopus* sp. with frequency of 3.36, 2.56, 5.60, 12.82, 4.16, 2.72, 2.88 and 0.16%, respectively. The most common genus is *Aspergillus*, isolated from soybean seeds in EL-Beheira Governorate. The contamination of legume seeds by fungi is a global problem. Aflatoxins are among the foodborne hazards that are reliant on climatic conditions. Certainly, temperature, relative humidity, insect attack, storage conditions and stress conditions of the plants influence on the ability of fungi to produce aflatoxins (Botast *et al.,* 1981) [33]. Generally, the differences in the incidence of soybean seed borne fungi collected from diverse regions may be due to the variations in moisture content of the seed and storage conditions (Temperature, Relative humidity and Light) implemented by the growers (Rao *et al.,* 2015) [34]. It means that seed borne fungi of seeds diverse from location to location owing to changes in environmental conditions prevalent through seed growth, harvesting and storing (Afzal *et al*., 2010 and Ramesh *et al*., 2013) [35, 36]. In this respect Nasreen (2003) [37] reported that the highest number of fungal species was obtained without surface disinfection of seeds as compared to the seeds treated with Sodium hypochlorite (NaOCl). Most of these fungi have been stated by Ibrahim (2015) and Venugopal *et al.,* (2015) [38, 2].

The recognition of AFs as mediated by fungal colonies fluorescence designated that the AFs were produced by some isolates of *A. flavus* group not the all of isolates, and this has fortified to screen fungi which AFs producing. In this technique, aflatoxinogenic isolates is producing a blue or green fluorescence on the fungal colony under the light of UV wave length, while non-aflatoxinogenic colonies cannot produce any color fluorescence (Davis *et al.,* 1966) [39]. Thirty two out from fifty *A. flavus* isolates described in the present investigation were belonged to AFs producers with (64%).

Molecular characterization of three different *Aspergillus flavus* group isolates using specific primers for estimating the aflatoxinogenic and non-aflatoxinogenic ones. In the present study, four specific primers, ver-1 nor-1, omt-A and aflR genes were used. aflR primer was used because this gene is playing an imperative role in the AFs biosynthetic pathway. It regulates the other structural genes activity (Woloshuk *et al*., 1994; Chang *et al*., 1999) [40, 41]. For all tested isolates there was a complete pattern with four bands achieved in PCR cleared the presence of patterns in the non-aflatoxinogenic isolate. Liu and Chu (1998) [42] found aflR in whole the examined *Aspergillus* section *flavi* non-aflatoxinogenic isolates. Another investigators (Ehrlich *et al*., 2003, Cary *et al.,* 2002) [43, 44] recommended that AFs biosynthesis regulation in *Aspergilli* contains a complex pattern of positive and negative acting factors of transcription regulation, which are influenced by ecological and nutritive conditions. Geisen (1996) [45] advocated that, the reduction of AFs producing may be owed to plain mutations which inactivated the AFs biosynthetic pathway of these isolates leading to the development of non-functional produces. Liu and Chu (1998) [42] proposed that, a variability of different physiological parameters affecting AFs biosynthesis. Sweeney *et al.,* (2000) [46] and Mayer *et al.,* (2003) [47]

recommended that, the induction or reduction of mRNA may license characterization among the two fugal types.

V. CONCLUSION

In the present study, we observed that the PCR method is useful for main isolation screening test due to its speediness and great sensitivity. The presence of patterns in the non-aflatoxinogenic isolates showed that PCR isn't a satisfactory indicator for differentiation among aflatoxinogenic and some non-aflatoxinogenic isolates, but it exposed that it is fast and sensitive technique for recognition fungi.

ACKNOWLEDGEMENT

The investigators express their great thanks to all staff members at (GEBRI), Egypt, for their support and helpful advices.

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