Egypt. J. Bot., **Vol. 56**, No. **2** pp. 451- 469 (2016)

Ecophysiological Conditions Affecting Growth and Ochratoxin A Production by *Aspergillus terreus* HA2 and *Aspergillus fumigatus* HA1 Isolated from Egyptian Rice Grains

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OCHRATOXIN A (OTA) is one of the most significant mycotoxins of worldwide concern for human health. Various fungi which are ubiquitous contaminants of Egyptian cereals, pre- and post- harvest can produce OTA under ecophysiological conditions. Sixty two fungal isolates belonging to four genera have been isolated from Egyptian cereals. Nine isolates have the ability to produce OTA namely; *A. ochraceus*, *A. terreus*, *A. fumigatus*, *A. alliaceus*, *A. carbonarius*, *A. niger*, *A. sclerotioniger*, *A. versicolor* and *P. verrucosum*. The highest OTA producer strains which isolated from Egyptian rice grains are, *A. ochraceus*, *A. terreus* and *A. fumigatus*. *A. terreus* and *A. fumigatus* were selected for this study. Both strains show optimum growth rate using Yeast Extract Sucrose (YES) culture medium, incubation temperature at 30^{O}C with 0.98 water activity (a_W) after 7 days. Maximum OTA production has been obtained for both strains at 0.98 a_W at 25-30 ^{O}C after 14 and 21 days in the culture medium and rice grains respectively. The two strains failed to grow and to produce OTA at 8^{O}C on YES medium and rice grains at all tested conditions. No OTA was detected at 0.85a_W in culture medium, while the two fungal strains couldn't grow on rice at the same a_W and failed to produce OTA at 0.90 a_W at all tested temperature during the 21 days incubation period. Conclusively our results show that the use of different storage practices of water activity (a_W) and temperature levels below 0.90 and 20°C, respectively, enhance controlling fungal contamination and minimizing the OTA production in rice grains.

Keywords: Ochratoxin A, *A. fumigatus*, *A. terreus*, Ecophysiological Conditions, Grains, Water activity, Temperature, Incubation Time.

Ochratoxins, considered the most critical mycotoxins of concern for human health, come after the famous aflatoxins. They include at least nine metabolites that are similar in structural terms, of which Ochratoxin A (OTA) is the most studied metabolite due to its high occurrence in food, feed and toxicological significance in human and animal diets. This toxin exerts immunotoxic, neurotoxic and teratogenic effects at higher dose levels. The genotoxicity of OTA remains

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controversial (EFSA, 2006), but evidence on the structures of DNA adducts has been recently reported (Mantle *et al*., 2010). The contamination of foods with Ochratoxins is a significant problem for the adverse effects on humans, animals and crops that result in illnesses and economic losses. It has been extensively found in food items like grains, bread, nuts, spices, coffee, beer wine, grapes, and with high levels in animal feedstuff (Cela *et al*., 2014). The most suspected foods susceptible to be contaminated by OTA in Egypt are essentially domestic and imported cereals.

Human exposed to OTA directly by the consumption of contaminated cereal food products and their derivatives (plant sources) as well as derived from meat (animal sources) consume the OTA contaminated feed (Dehelean, 2011). Consumption of these contaminated foods could cause adverse effects on human health. It is implicated in a diverse range of pathological effects in poultry (Hassan *et al*., 2012). Ochratoxins are produced mainly by several species of fungi from *Aspergillus* and *Penicillium* genera (Ali *et al*., 2013 and Cela *et al*. 2014). OTA was isolated, for the first time, from the *Aspergillus ochraceus* as a secondary metabolite (Van der Merwe *et al*., 1965). In spite of *Aspergillus ochraceus* is known to be the best for OTA production, *A. terreus, A. fumigatus, A. alliaceus, A. versicolor, A. carbonarius, A. niger, A. melleus,* and *A. sulphureus* are also considered as sources of OTA production in cereals (Astoreca *et al*, 2009 and Ali *et al*., 2013). In a recent study conducted by Alborch *et al*. (2011), two new OTA producing species; *A. sclerotioniger* and *A. lacticoffeatus* were investigated for the OTA fabrication.

The knowledge of the ecophysiological of OTA producing fungi and ecological factors that influence OTA production is essential to optimize the implementation of preventive strategies aimed at controlling the sanitary quality of raw materials and/or products susceptible to fungal colonization. There are multiple factors significantly involved in the development of *Aspergillus* species and secondary metabolites biosynthesis. These factors include humidity, temperature, presence of oxygen and carbon dioxide, incubation time, substrate composition, loss of grain integrity caused by insects or mechanical thermal damage, fungal inoculum, and the competition between other contaminated fungal species. Water activity (a_W), incubation time and temperature have shown the greatest effects on growth and OTA production.

In general, the toxigenic species are not aggressive pathogens, but are often well adapted to substrates with low humidity. At the same time, they can easily colonize cereal grains and oilseeds that are stored under inappropriate environmental conditions. Several authors have studied the effect of water activity and temperature on fungal growth and OTA production by the most OTA- producing species as *A. carbonarius, A. ochraceus, A. niger aggregate, A. sclerotioniger, A. lacticoffeatus, A. tubingensis* (Alborch *et al*., 2011 and Cela *et al*., 2014) and *P. verrucosum* (Czaban *et al*., 2006). As there is no knowledge of the influence of ecophysiological factors on the two OTA producing species

A. terreus and *A. fumigatus*, the aim of this study has been to evaluate the effect of environmental factors and the interactions of these parameters on growth and OTA production in culture medium and rice grains.

Materials and Methods

Isolation of Grain Colonizing Fungi

Sixty two strains belonging to 4 genera and 19 species were isolated from different Egyptian cereals including, legume, rice and wheat. Dilution plating assay was used as enumeration technique according to Pitt and Hocking (1997). The isolated fungi were serially purified for complete identification and further investigations.

Morphological and Molecular Identification of Isolated Fungi

The purified isolates were sub-cultured on Czapek Yeast extract Agar and allowed to grow at 30 ^{O}C for 7 days. The morphological identification was made according to Raper and Fennell (1965) and Pitt and Hocking (1997). Molecular analyses of the two selected OTA producer strains were also conducted. The genomic DNA was extracted from 3-day- old cultures by freeze fracturing in liquid nitrogen (Sharma *et al*., 2007). Briefly, 0.2 g of the mycelia was placed in liquid nitrogen for 10 min and vigorously homogenized using a mechanical pestle. Next, 500 µl of a DNA extraction buffer (200 mM Tris-HCl, pH 8.0, 240 mM NaCl, 25 mM EDTA, and 1% SDS) has been pipetted into the tube and the mixture then vortexed for 5 min and centrifuged for 5 min at 10,000 rpm. Thereafter, the supernatant has been gently mixed with an equal volume of 1:1 (v/v) phenol:chloroform for 30 min and then centrifuged at 12,000 rpm. The upper phase has been gently withdrawn and mixed with a 0.1 volume of 3 M Na-acetate (pH 5.2) and 2 volume of 96% ethanol for 60 min at -20^{o}C. After centrifugation, the collected DNA pellets have been washed with 70% ethanol, dried, and resuspended in 100 µl of distilled water.

PCR Amplification

The fungal isolates have been identified based on ITS rDNA sequence (18S-28S rRNA, flanking ITS 1, 5.8S rRNA, and ITS 2 according to White *et al*., (1990). The sequences of the ITS1 and ITS4 primers were; 5‘-TCCGTAGGTGAACCTGCGG-3‘and 5′- TCCTCCGCTTATTGATATGC-3′, respectively. The PCR analysis has been don according to Sambrook *et al*. (2001).

Molecular Evolutionary Genetic Analysis (MEGA version 6) software was used for phylogenetic analyses (Tamura *et al*., 2013). The closet homologous to the sequences were selected and multiple sequence alignments were carried out using the Clustal W program in the MEGA6 software. Phylogenetic tree was constructed, using neighbor joining method with 1,000 bootstrap replicates based on ITS gene sequences, to show the phylogenetic relationships between fungal the two fungal isolates used for OTA production and the closely related strains retrieved from NCBI Gen Bank.

Screening Study

For OTA analyses *in vitro*, the isolated fungi were grown in 50 ml of liquid medium in 250 ml Erlenmeyer flasks. The screening study was carried out in yeast extract sucrose agar (YES) according to Samson *et al*. (2000). Cultures were incubated at 30 ^{O}C for seven days in the dark. The culture filtrate was obtained by filtration through filter paper, and extracted with 100 ml of chloroform. Organic phase was collected, evaporated, and resuspended in 1 ml of methanol., 10 µl portions were spotted on a TLC plate with a micro syringe according to Yamazaki *et al*. (1970). 20 µl was analyzed by high-performance liquid chromatography apparatus (HPLC). The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Watres 2475 Multi- Wavelength Fluorescence Detector, and a data workstation with software Breeze, a phenomenex C18 (250 x 4.6 mm i.d.), 5 µm from Waters corporation (USA). This methodology was described by Bragulat *et al*. (2001). The most potent OTA producer's strains were selected for further ecophysiological analysis in culture medium and rice grains.

Ecophysiological studies and OTA production

In Culture Medium

The study of the ecophysiological and OTA production was carried out in YES culture medium. Water activity (a_w) of the medium was modified to aw 0.85. 0.90, 0.95 and 0.98 using glycerol, as reported by Pardo *et al*. (2005). The inoculated flasks were incubated at five different temperatures; 8, 20, 25, 30 and 40^{o}C. Growth assessment and OTA production were determined after 7, 14 and 21 days of incubation at each temperature and water activity. The study was carried out with two replicates per treatment. Ochratoxin A was extracted as previously described and analyzed using HPLC screening method consistent with Bragulat et al. (2001). 20 µl was injected into a HPLC system. OTA detection and quantization were made by the Waters Alliance 2475 (excitation wavelength: 330 nm / emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 × 4.6 mm (Waters Cromatografia). The mobile phase, with a flow rate of 1 ml/ min, consisted of a following linear gradient: acetonitrile, 57%; water, 41% and acetic acid, 2% as stated by Bauer and Gareis (1987). The extracts with the same retention time as OTA (around 6.8 min) were considered positive.

In Rice Grains

Fifty grams of dried white rice grains were weighted into sterile flasks and rehydrated to the required water activity (a_w) levels of (0.98, 0.95, 0.90, 0.85) by adding sterile distilled water using a moisture absorption curve according to Trenk *et al*. (1971). 1ml of each fungal spore suspension was added to each flask containing substrates under sterile conditions. All flasks were analyzed for OTA production each 7 days of incubation using HPLC system. Also, mycelial growth was observed and determined during the experiment.

OTA Analysis from Rice

All of the rice substrate samples were analyzed for OTA production according to the method of Ali *et al*. (2013). Five working solutions were prepared from the standard solution (10.07µg/ml) with the concentrations of 0.015, 0.025, 0.050, 0.10 and 0.20 µg/ml. Then the limit of detection (LOD) was observed that was 0.025µg/ml after the optimization (Bayman *et al*., 2002).

Statistical analysis

All statistical calculations were done using computer programs Microsoft excel version 10 and spss (statistica package for the social science version 20.00) statistical program. At 0.05, 0.01 and 0.001 level of probability (Snedecor and Cochran, 1982) the One-way ANOVA and Post hoc-LSD tests (the least significant difference) was presented using percentage, mean ± standard error. The discrement , pearson corrlation and automatic linear models analysis were estimated to show the relationship of the physiological parameter to each other.(Härdle and Simar, 2007).

Results and Discussion

Mycological Survey and OTA Production

In this study, all isolated mold genera were found to have the ability for Ochratoxin A production (Table 1). The sixty two isolates were obtained in pure culture and identified morphologically as well as on molecular basis to the species level. *Aspergillus* and *Penicillium* were the most distributed genera isolated with high frequency from all of the samples. 57 isolates of *Aspergillus* were isolated comprising 91% of the total isolates including 12 species namely; *A. fumigatus, A. terreus, A. ochraceus, A. alliaceus, A. flavus, A. sclerotioniger, A. candidus, A. carbonarius, A. versicolor, A. clavatus, A. niger, A. glaucus*. While, 15 *Penicillium* isolates comprising 9% of the total isolates including 6 species are determined. Only 8 species of *Aspergillus* and 1 species of *Penicillium* isolates were discovered to be able to produce OTA. The distribution of *Aspergillus* and *Penicillium* genera in grains were previously reported by Riba *et al*. (2008). The potentiality of *Aspergillus* and *Penicillium* genera to produce OTA was previously recorded by several authors (Fernández-Baldo *et al*., 2011 and Nuhu, 2015). Concerning the current outcomes of fungal screening for the OTA production, it was found that all isolates of both *A. ochraceus* and *A. alliaceus*, as well as 60% of *A. niger*, 40% of *A. versicolor* 50% of *A. carbonarius*, 33% of *A. sclerotioniger*, 33% of *A. terreus*, 25% of *A. fumigatus* and 66% of *P. verrucosum* isolates were able to produce OTA. The consistent ability of these species to produce OTA has been reported by other authors such Riba *et al*. (2008). The OTA production by *A. terreus* was mentioned by Ueno *et al*. (1991). *A. fumigatus* was cited by Atalla and El-Din (1993) as OTA producer. Riba *et al*. (2008) indicated that *A. terreus* and *A. fumigatus* isolated from cereal products were capable to produce OTA.

TABLE 1. Ochratoxin A production by fungal strains isolated from different Egyptian seeds in laboratory culture (isolates were grown for 7 days at 30 ºC, on YES medium, tested by HPLC).

Fungal Species	Screened Strains	Number of OTA Producing Isolates	Isolation Source	OTA Production (µg/ml YES)	Ochratoxigenic Strain %
A. fumigatus	4	1	Rice	0.35	25
A. terreus	6	2	Rice	0.29-0.42	33
A. ochraceus	5	2 1 2	Rice Legume wheat	0.9-4.20 2.1 0.71-3.5	100
A. alliaceus	2	2	Rice	0.15-0.09	100
A. carbonarius	4	2	legume	0.11-0.15	50
A. versicolor	5	2	wheat	0.08-0.12	40
A. niger	5	3	legume	0.01-0.03	60
A. sclerotioniger	3	1	legume	0.17	33
A. flavus	4	-	legume	ND	0.0
A. candidus	4	-	Wheat	ND	0.0
A. clavatus	2	-	wheat	ND	0.0
A. glaucus	3	-	corn	ND	0.0
P. verrucosum	3	2	Rice	0.05-.09	66
P. chrysogenum	2	-	Legume	ND	0.0
P. corylophilum	1	-	Corn	ND	0.0
P. notatum	1	-	Legume	ND	0.0
P. citrinum	4	-	Rice	ND	0.0
P. expansum	4	-	legume	ND	0.0
Total Strains Screened	62	20	-	-	32

The most potent OTA producer strains throughout this study were *A. ochraceus* (0.9-4.20 µg ml^{-1}), followed by *A. terreus* (0.29-0.42 µg ml^{-1}) and *A. fumigatus* (0.35 µg ml^{-1}). Similarly, Riba *et al*. (2008) reported that *A. ochraceus*

produced the highest OTA amounts (0.23-11.5 µg g^{-1}) of culture medium, while the level of OTA production by *A. terreus* and *A. fumigatus* ranging from 0.01 to 0.07 µg g^{-1}. The levels of OTA of other Aspergillus isolates in this work were ranged from 0.09-0.18 µg ml^{-1}.; only 2 *P. verrucosum* strains of amongst the15 *Penicillium* isolates have the ability to produce OTA ranging from 0.05-.09 µg/ml. Several authors recorded the levels of OTA produced in culture media ranging from 0.16-0.254 µg /ml (Hajjaji *et al*. 2006). Similarly, Bragulat *et al*. (2001) and Romero *et al*. (2005) recorded that the levels of OTA produced by different *Aspergillus* species in culture media ranging from 0.5 to 234 µg g^{-1}. All previous studies were concentrated on ecophysiological conditions of OTA production by *A. ochraceus*, while there is no any knowledge about the effect of these conditions on OTA production by *A. terreus* and *A. fumigatus*. So these two strains were selected for further studies throughout the current work.

Molecular identification of A. fumigatus HA1 and A. terreus HA2

The morphologically identified *A. fumigatus* and *A. terreus*, based on the 18S-28S rRNA sequences, were further studied (Henry, 2000). The genomic DNA of both species was extracted and its purity checked on a 0.8% Agarose gel electrophoresis. The PCR amplicon was 775 bps for the 18S-28S rRNA for *A. fumigatus* and 570 bps for *A. terreus* flanking the internal transcribed spacer ITS1, ITS2, and ITS5.8S rRNA. After purifying the PCR products, the amplicon was sequenced and the retrieved sequence deposited in the Gene Bank under Accession Number of KP081777 and KM820839 for *A. fumigatus* HA1 and *A. terreus* HA2, respectively.

A phylogenetic tree was constructed using the neighbout joining algorithm with 1000 bootstrap replications for the identification of the mycotoxegenic fungal isolates using the sequence obtained from the molecular marker used. The 18S rRNA-28S rRNA sequence of the two isolates was BLAST-searched on a database using the multiple sequence alignment in MEGA 6 software. From the alignment profile results, it has been clear that the *A. fumigatus* HA1 (KP081777) and *A. terreus* HA2 (KM820839) 18S- 28S rRNA amplicon exhibit > 98% identity with other *A. fumigatus* and *A. terreus* isolates respectively. Constructing the phylogenetic relatedness (Fig.1) of the whole sequence of *A. fumigatus* HA1 and *A. terreus* HA2 18S rRNA-28S rRNA with the closely related strains from the database (http://blast.ncbi.nlm.nih.gov/Blast.cg), have revealed the molecular identity of the current isolates in comparison with the other isolates of *A. fumigatus* and *A. terreus*, respectively. Thus, the molecular analyses have confirmed the morphological criteria displayed by the universal keys like Raper and Fennell (1965) displayed a strong similarity with other *A. fumigatus* and *A. terreus*.

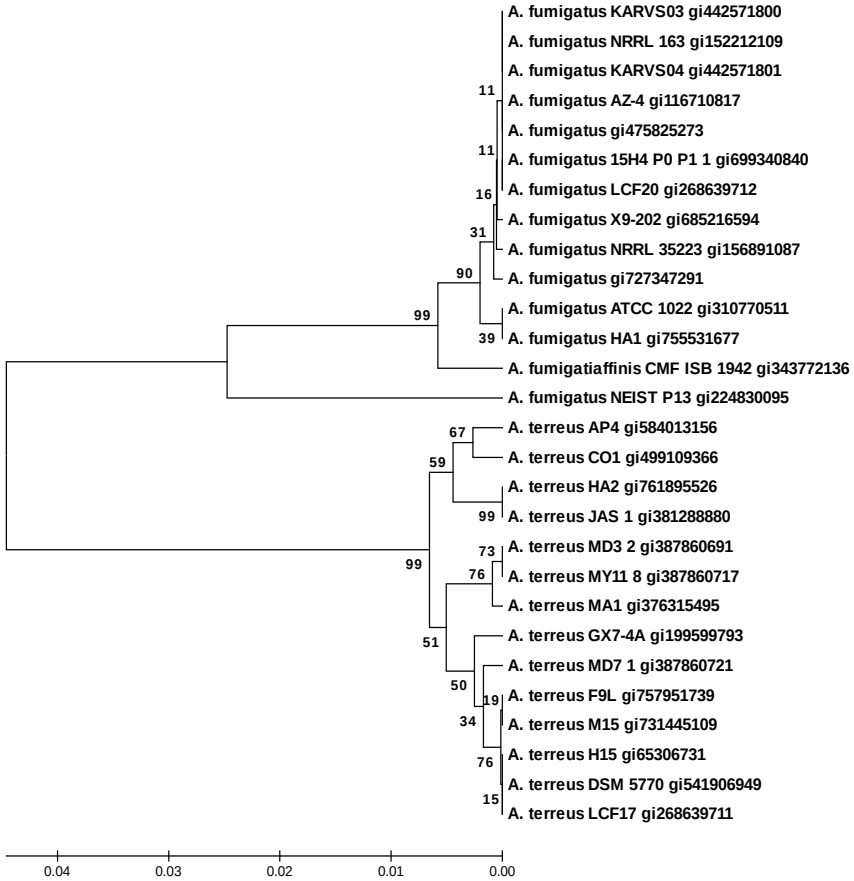

Fig 1. Phylogenetic relationships between the two ochratoxigenic fungi (*A. fumigatu* HA1 and *A. terreus* HA2) and the ITS sequences of closely related fungal strains retrieved from NCBI GenBank.

Ecophysiological Analysis of OTA Producing Strains

Effect of ecophysiological factors on fungal growth and OTA production on YES medium

Fungal growth and mycotoxin production are affected by numerous abiotic and biotic parameters and their complex interactions. Fungal growth and OTA production of *A. terreus* HA2 (KM820839.1) and *A. fumigatus* HA1 (KP081777.1) were determined on a weekly basis for total period 21 day in the culture media (Fig. 2, 3). The two strains were inoculated in YES medium adjusted at different a_W (0.85, 0.90, 0.95 and 0.98) and incubated at five different temperature ranging from 8 to 40 ^{o}C. It was observed that the growth and OTA level of both strains increased significantly with increasing the temperature and water activity which reached till reach its maximum amounts at 0.98 a_W and 25-30^{o}C. Similar results

were reported in YES culture medium by Pardo *et al*. (2006). Esteban *et al*. (2006) showed that the maximum OTA production by different isolates of *A. ochraceus* was detected at 15-30^{O}C at a_W 0.98. In spite of OTA being a stable metabolite, it has been observed that its level decreased significantly ($p < 0.001$) after 14 days of incubation in the culture medium. This decrease in OTA production may be due to breakdown of the OTA to Ochratoxin α, as it has been observed for some other toxigenic species (Varga *et al*., 2002). Some authors have suggested that this decrease may be due to the fact that the strains remove and assimilate the phenylalanine moiety from the OTA molecule as other nitrogen sources in the culture medium become exhausted (Varga *et al*., 2002).

Our results reported that the maximum levels of OTA was 0.45 (±0.014) at 25^{O}C and 0.34 µg ml^{-1} (±0.02) at 30^{O}C after 14 days of incubation at a_W 0.98 for *A. terreus* HA2 and *A. fumigatus* HA1, respectively. According to Bayman and Baker (2006), there is a large intraspecific variation in OTA production, which is a combination of phenotypic plasticity and genetic variation (Riba *et al*., 2008). There is no observed growth was detected at 8^{O}C for our species, whereas it showed weakly growth and failed to produce OTA at a_W 0.85 after all incubation time at all temperature. Statistical analysis of the data in Fig. 5, showed that the OTA production not accompanied with mycelial growth. The minimal a_W needed for fungal growth is 0.85 where the minimal a_W needed for OTA production is 0.90 for both species. These results agree with Esteban *et al*. (2006). They investigated the effect of different water activity values on OTA production using twelve *A. niger* isolates, cultured on Czapek Yeast Agar (CYA) and on Yeast Extract Sucrose agar (YES) where a_W ranged from 0.82 to 0.99. They found that the minimal a_W for fungal growth of *A. niger*, is 0.77, but regarding OTA production, it was found that the optimal a_w is 0.90- 0.99, depending on fungal species and culture medium. To understand the potentiality of fungal isolates to produce OTA in food stuff, it is necessary to understand the physiology and ecology of these species and the difference between themso, this work extend to study the ecophysiological analysis in rice grains.

Effect of ecophysiological factors on fungal growth and OTA production on rice grains

Figure 4 gives a diagrammatic representation of the interaction of a_W, temperature and incubation time on OTA production (µg g^{-1}) of A. *terrreus* HA2 and *A. fumigatus* HA1 in rice grains. Production of OTA has occurred over a range of temperatures; 20-40^{O}C. The optimum temperatures for OTA production has been 25 ^{o}C and 30 ^{o}C, while the minimal temperatures has been 20^{o}C and 40^{o}C for *A. tereus* HA2 and *A. fumigatus* HA1, respectively. It has been observed that OTA production increased significantly as incubation time and a_W increased. At 0.85 and 0.90 a_W both strains failed to produce OTA in rice at all temperature during the 21 days.

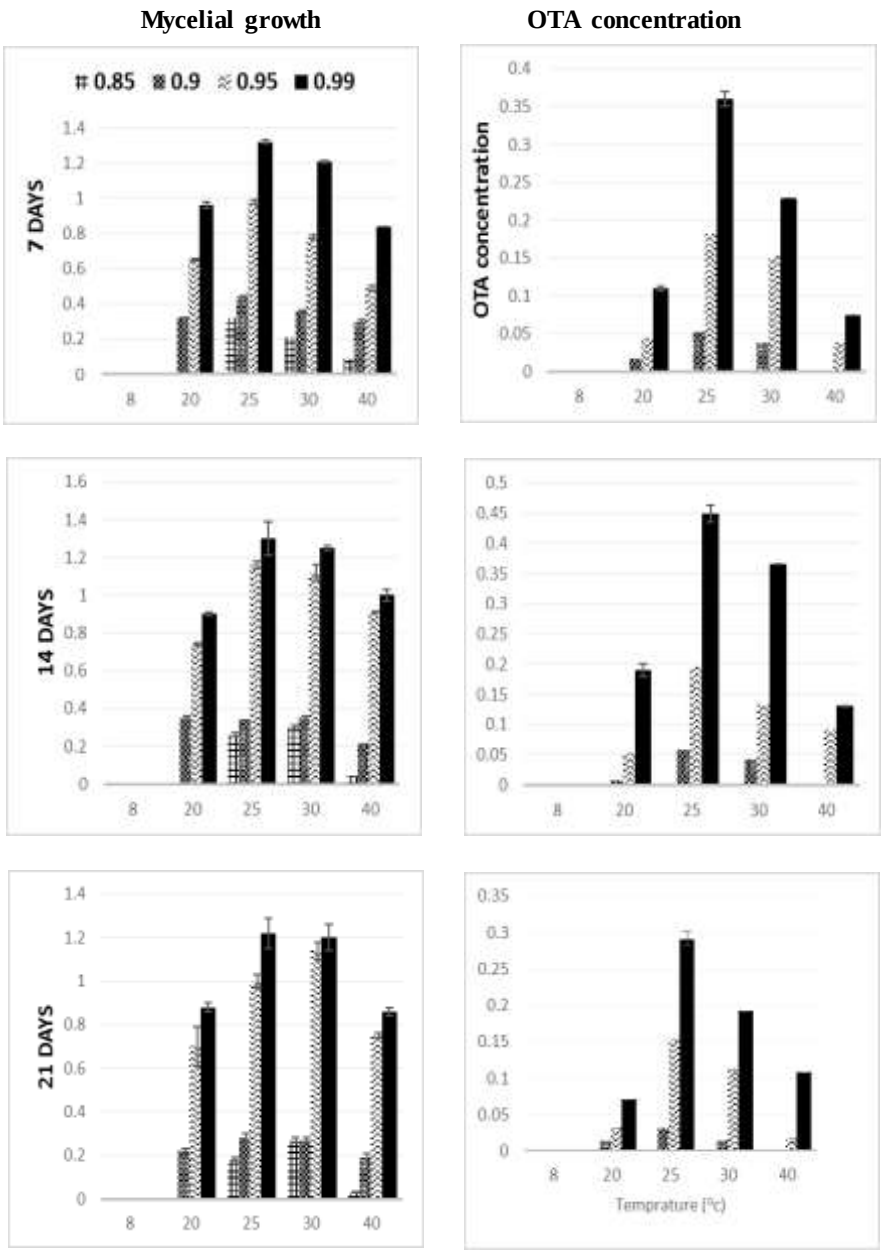

Fig. 2. Mycelial dry weight (g/100 ml) and OTA production (µg/ml) of *A. terreus* HA2 (KM820839) assayed in YES medium with different w_a incubated at different temperatures (^{0}C) for different incubation time (days).

***A. fumigatus* HA1 (KP081777)**

Mycelial growth **OTA concentration**

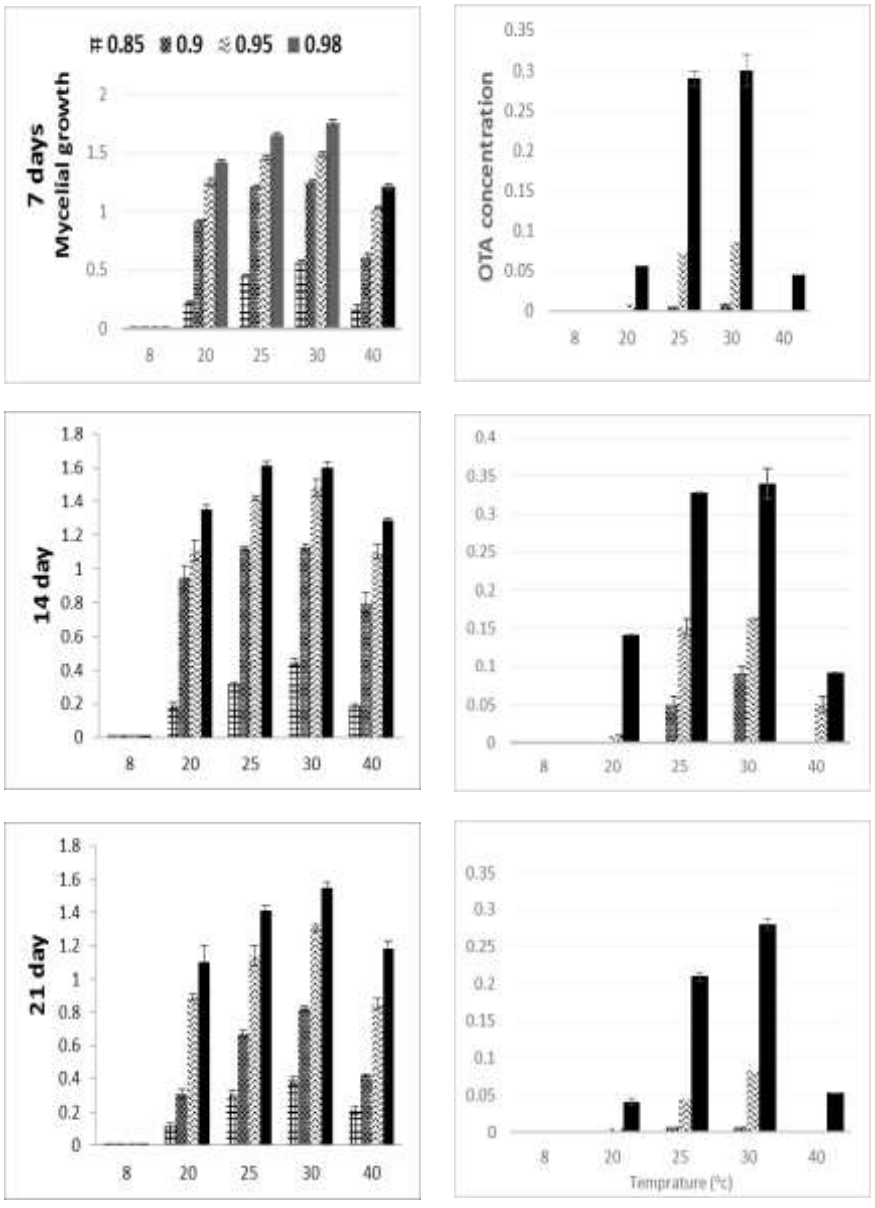

Fig. 3. Mycelial dry weight (g/100 ml) and OTA production (µg/ml) of *A. fumigatus* HA1 *(*KP081777*)* assayed in YES medium with different wa incubated at different temperatures (^{0}C) for different incubation times (days).

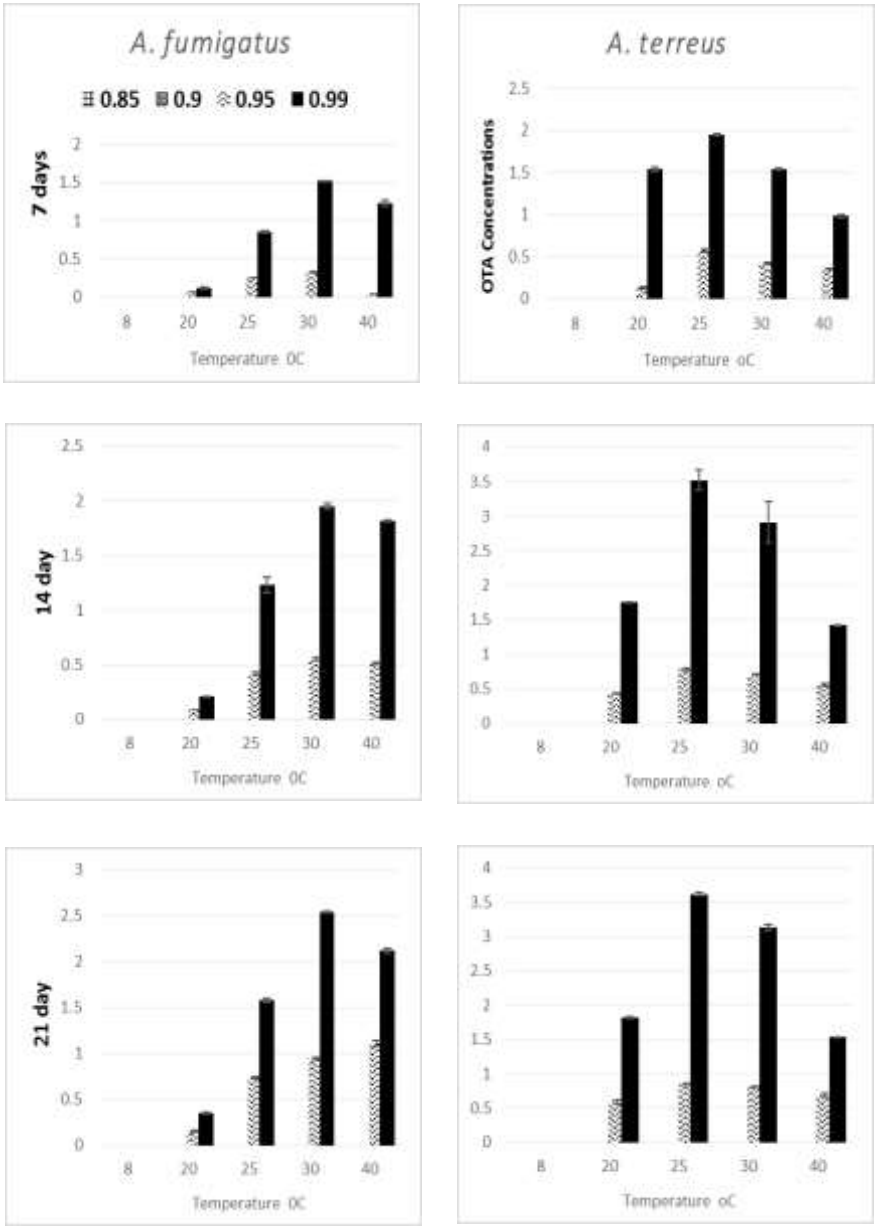

Fig. 4. OTA concentration (µg/g) produced by *A. fumigatus* HA1 (KP081777) and *A. terreus* HA2 (KM820839) on rice grains after different incubation time (days) at different temperature (^{0}C) and different water activity (wa),.0.85,0.90,95 and0.98.

The maximum OTA levels of *A. terreus* HA2 and *A. fumigatus* HA1 were 3.62 (±0.02) and 2.54 (±0.01), respectively, at 25-30 C and 0.89 a_W after 21 days of incubation. Whereas, the minimal OTA levels were 0.041 µg gm^{-1} (±0.001) at a_W 0.95 and 40 C for *A. fumigatus* HA1 and 0.13 µg gm^{-1} (±0.01), at a_W 0.95 and 20°C for *A. terreus* HA2 after 7 days of incubation for both species. The two strains failed to grow at 8 C at all conditions. The impacts of a_W, temperature, incubation time and their interaction on OTA production by different *Aspergillus* species on peanut seeds and corn grains was also studied by Astoreca *et al.* (2009). They reported that the optimal conditions of OTA production were at 0.973 a_W and 25 C, whereas the minimum production was obtained at 0.951 a_W and 15°C, after ~~at~~ 14 and 21 days of incubation for RCP42 and RCP *Aspergillus* species, respectively.

There is no previous knowledge about ecophysiological conditions of *A. terreus* and *A. fumigatus*. But according to our data, it has been observed that the optimum conditions for OTA production in grains by these two strains have been very similar to various ochratoxigenic Aspergillus species. Joosten *et al.* (2001) studied the OTA production by *A. carbonarius* on coffee cherries and found that the maximum OTA concentration (4.81 µg kg^{-1}) was detected at 0.99 a_W and 25 C after 2 weeks of incubation. Pardo *et al.* (2006) reported that the maximum OTA amounts were detected at 25 C and 0.98 a_W on all substrate tested. In addition, Astoreca *et al.* (2007) studied the effect of different a_W (0.85-0.95), temperature (15, 25 and 30 C), incubation time (7, 14 and 21 days) and their interaction on OTA production in different products such as maize kernels, peanuts, dried grapes and coffee beans. They found that the optimum temperature for OTA production was 25 C or 30 C related to strains assayed. They also reported that OTA concentration increases as a_W increases, regardless of changing the temperature. Astoreca *et al.* (2009) reported that the optimum condition for maximum production of OTA by *A. niger* aggregate on corn grains was obtained at 0.97 a_W and 25-30 C after 7 days of incubation. These differences in the production could be attributing to the species and the substrate analyzed.

Our results revealed that *A. terreus* HA2 has been the highest OTA producer as compared with *A. fumigatus* HA1 at all conditions. This variation in OTA level may be perhaps due to the phenotypic plasticity and genetic variation of the two species. According to Bayman and Baker (2006), there was a large intraspecific variation in OTA production, which is a combination of phenotypic plasticity and genetic variation, amongst the tested species in their study.

According to our study, it was observed that, the substrate plays a significant role in OTA production, since the OTA production using YES medium and rice grains have been quite different even under the same environmental conditions. Although the carbon source of the YES medium (sucrose), comprising 40% of the components, the OTA levels found in this medium were lower than those detected on rice grains. Maximum OTA accumulation was higher on rice grains medium than on YES medium with values higher than 2-3µg g^{-1}. These differences in OTA yield could be due to the nature of the carbon sources. For the first time Medina *et al.* (2004) reported that bee pollen can be regarded as a natural medium for fungal growth superior than many cereals.

The dependence of OTA production on nature of carbon sources have been also studied by Muhlencoert *et al*. (2004). They observed the highest OTA yields with sucrose, considerably less with glucose, and none with fructose or lactose as carbon source. Pardo *et al*. (2006) studied the non-specificity of nutritional substrate for OTA production by different isolates of *A. ochraceus*. They observed that the highest OTA production by *A. ochraceus* isolates were on barley grains (1mg g^{-1}), green coffee beans (2mg g^{-1}) while the amount of OTA in YES medium and grape was 13.9 µg ml^{-1} and 3 ng g^{-1}, respectively. The excess of sugars in YES medium and grape could inhibit the OTA synthesis by catabolite repression. This phenomenon has been reported in studies with fungi, in which the pattern of sugar utilization was modified, affecting the expression of genes involved in other metabolic pathways (Flipphi *et al*., 2003).

The canonical discriminant function of ecophysiological analyses (Fig. 5) has been showed that *A. terreus* was the highest OTA producer strain in rice grain as compared with *A. fumigatus* under all tested conditions. The two mycotoxigenic strains produce higher significant amount of OTA in rice grain than in YES culture medium. There is no relation between the OTA production and mycelial growth of both strains.

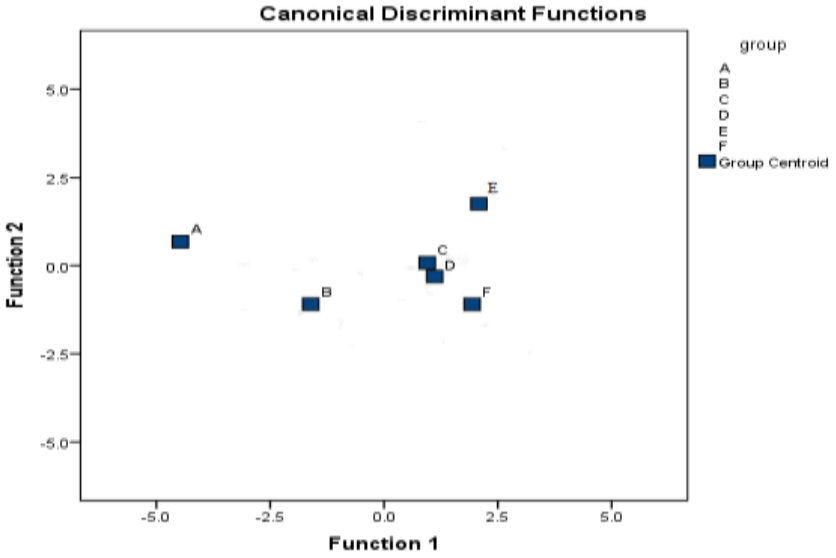

Fig. 5. Canonical Discriminant Function of ecophysiological analyses: mycelial dry weight of *A. fumigatus* in YES medium (A) Mycelial dry weight of *A. terreus* in YES medium (B) OTA production by *A. fumigatus* in YES medium (C) OTA production by *A. terreus* in YES medium (D) OTA production by *A. terreus* in Rice grains (E) OTA production by *A. fumigatus* in Rice grains (F).

The results of this investigation have reported that significant amounts of OTA can be produced in only seven days of bad storage conditions. Therefore, it

is recommended that the use of certain ecophysiological storage practices, such as water activity (a_w) and temperature levels below 0.90 and 20°C, respectively, allow controlling fungal contamination and minimizing the OTA production in culture medium as well as rice grains. We can also conclude that, the OTA production depend on the fungal strains and the growth medium.

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(*Received* 3/11/2015;
accepted 7/12/2015)

دراسة العوامل البيئية و الفسيولوجية المؤثرة على نمو و انتاج الاوكراتوكسين بواسطة الاسبيرجيللس فيوميجاتس HA1 والاسبيرجيللس تيرس HA2 المعزولتين من حبوب الأرز المصرى

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الأوكراتوكسين (OTA) هي واحدة من أهم السموم الفطرية لصحة الإنسان في جميع أنحاء العالم. عديد من الفطريات التي تصيب الحبوب المصرية، قبل وبعد الحصاد لها القدرة على انتاج الاوكراتوكسين فى ظل الظروف البيئية و الفسيولوجية المختلفة . اثنان وستون عزلة تنتمي إلى أربعة أجناس قطرية تم عزلها من الحبوب المصرية. من بين هذه العزلات، تسع عزلات لديها القدرة على انتاج الاوكراتوكسين وهي: الأسبيرجيللس اوكراشيس، الأسبيرجيللس تيرس، الأسبيرجيللس فوميجاتوس، الأسبيرجيللسا الياشيس، الأسبيرجيللس كربوناريس، الأسبيرجيللس نيجر، الأسبيرجيللس اسكلريشيونيجر، الأسبيرجيللس فيرسكلر و البنسيليوم فيركوسم. سلالات الأسبيرجيللس اوكراشيس، الاسبيرجيللس تيريس و الاسبيرجيللس فيوميجاتس المعزولة من حبوب الارزهم أعلى السلالات انتاجا للاوكراتوكسيين. لعدم تواجد اى دراسات سابقة على تأثير العوامل البيئية والفسيولوجية على انتاج الوكراتوكسين بواسطة الأسبيرجيللس ترس و الأسبيرجيللس فيوميجاتوس، فان الهدف من هذه الدراسة هو تقييم تأثيرهذه العوامل المؤثرة و تداخلها معا على النمو و انتاج الاوكراتوكسين في الوسط الغذائى و فى حبوب الأرز لتلك السلالتين.النتائج اظهرت ان أحسن معدل نمو للسلالتين لوحظ عند درجة حرارة ٣٠ درجة مئوية مع نسبة النشاط المائى بعد ٧ أيام من التحضين٠.٩٨ لكلتا السلالتين بينما اعلى انتاج للاوكراتوكسين لوحظ عند درجة حرارة تتراوح بين ٢٥-٣٠ عند درجة النشاط المائى ٠.٩٨ بعد ١٤ يوم من التحضين فى مستنبت مستخلص الخميرة-سكروز، بينما أعلى انتاج للاوكراتوكسين لوحظ بعد ٢١ يوم من التحضين فى الارز. لقد فشل كلتا السلالتين فى قدرتها على النمو و انتاج الاوكراتوكسن عند تحضينهما فى درجة ٨ درجة مئوية فى الوسط الغذائى و الارز تحت كل اظروف البيئية المختبرة. فى درجة الحيوية المائية ٠.٨٥ فقدة كلتا العزلتين قدرتهما على انتاج الاوكراتوكسين فى الوسط الغذائى بينما فشلت فى النمو فى الارز وفقدت قدرتها على انتاج الاوكراتوكسين عند درجة ٠.٩ من النشاط المائى. من هذه النتائج يمكننا استنتاج ان تخزين الارز فى درجات حرارة اقل من ٢٠ درجة مئوية عند درجة نشاط مائى اقل من ٠.٩٠ تقلل من التلوث الفطرى و تساعد على تثبيط انتاج الاوكراتوكسينن فى حبوب الارز.