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Comparison of the aflatoxin B1 production pattern in fungal biomass and growth medium in toxigenic *Aspergillus* species of northern isolates of Iran

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Abstract

Aflatoxin is the most economically important mycotoxin in the world. The most toxic form of aflatoxin is aflatoxin B_1. It is one of the few fungal toxins that is regulated by the US food and drug administration; moreover, compared with other mycotoxins, more is known about its biology and biosynthesis. In the present study, our aim was to detect the ability of aflatoxin B_1 to be produced in different *Aspergillus* species that have been isolated from agro-ecological zones and processing plants in the North of Iran. The concentration of AFB_1 in the samples was investigated by competitive enzyme-linked immunosorbent assay (ELISA). Also, through using the ELISA method, the aflatoxin B1 production pattern was compared between fungal biomass and growth medium in toxicogenic isolates. The results showed that all of the studied *Aspergillus* species isolates tended to produce aflatoxin B_1. Although there was a statistically significant difference in the mean of aflatoxin B1 in growth medium and cell extract in several species, in *A. flavus, A. parasiticus, A. sojae, A. terreus, A. ostianus, A. carbonarius, A. wentii,* storing the toxin in fungal cell was more than that of releasing toxin in matrix culture medium. In the other isolates including *A. niger, A. foetidus* and *A. ocraceus*, export of toxin was more than that of cell storage. We concluded that toxicogenic isolates of *Aspergillus* spp can be found in all the studied geographic areas of Northern Iran. Although the behavior of species in releasing of aflatoxin into the growth environment was different, we hypothesized that it depends on different mechanisms that effect export toxin into the external environment.

Key words: Aflatoxin, *Aspergillus*, Fungal biomass, Growth medium, Toxigenic

Introduction

Several fungal species, especially the genus *Aspergillus*, are ubiquitous in the environment. Toxigenic Aspergillus strains are indeed opportunistic pathogens of plants, animals, and humans that are found in the soil and many agricultural commodities (Klich 2002, Klich 2007, Adhikari et al. 2016).

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Aflatoxins, as the secondary metabolites of mycotoxin-producer fungi in contaminated foods and feeds storage, are important problems for the scientists to consider, because they lead to undesirable effects to the public health. Annually, a considerable amount of crops those are susceptible to being infected by fungus and subsequently Aflatoxins, cause huge economic losses worldwide (Cotty 1994, Cotty and Jaime-Garcia 2007, Afsah-Hejri et al. 2013).

The most common aflatoxins (i.e., B1, G1, B2 and G2) are toxic carcinogens and concomitantly Aflatoxicosis causative agents. The severity of toxicity can be reduced in such aflatoxins, although (Yin et al. 2008, Alshannaq and Yu2017). Among these, aflatoxin B_1 is one of the most important and well-known mutagenic, teratogenic, and hepatocarcinogenic factors with its natural origin (McKean et al. 2006, Fox and Howlett 2008, Lai et al. 2015).

Numerous factors influence the levels of aflatoxin production by *Aspergillus* spp. Among these soil types, hosts, environmental nutrients, and climate conditions are some of the major agents. In this respect, the humid areas of northern Iran are suitable fungal habitats; therefore, identification of predominant fungal genera and probability of toxin production is considered as vital at these zones.

Looking into the literature reveals that aflatoxigenic species of *Aspergillus* are *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillu snomius*, while other species have been referred to as non-aflatoxigenic by scholars (Varga et al. 2011, Levin 2012). Hence, due to the probable presence of other species and the common, consequential risk of toxic aflatoxin generation in foods, in this research, isolation and detection of the aflatoxin-producing potential of native (indigenous) *Aspergillus* populations among different fields and geographical regions in the Northern provinces of Iran has been investigated.

Previous studies showed that more than 90% of the aflatoxin that is produced by toxigenic *Aspergillus* species is exported to the cell exterior while some studies on the regulation of aflatoxin export mechanism also suggest that modifying the growth medium composition can increase or decrease the amount of aflatoxin exocytose (Tanaka et al. 2008, Chanda et al. 2010).

In the current study, through using the ELISA method, in order to investigate the "aflatoxin export patterns" in isolated species, the amount of aflatoxin B_1 has been measured in fungal growth medium and cell extract. Considering the differences between the average of toxin in growth environment and fungal biomass, the tendency of the fungal cell to release aflatoxin into the cell exterior has been investigated.

Materials and Methods

Through using settle plates based on CBS rules and according to "CBS" instruction from the indoor and outdoor stations, 61 presumptive *Aspergillus* species have been isolated from four zones in three different geographic areas (East of Guilan, west of Guilan, and west of Mazandaran).

Isolates

The strains used in this study are listed in Table 1.

Table 1: *Aspergillus* **isolates examined**

Name	strain no	source
A. terreus	40,64,66 33,7 24,27	Plain areas, West of Guilan Plain areas, East of Guilan Processing plants, East of Guilan
A. wentii	100,101 102	Forest areas, East of Guilan Processing plants, East of Gilan
A. flavus	15,16,36,55 68 34 20,22 9 60,61 4,17,80 62,63 11	Plain areas, East of Guilan Plain areas, West of Guilan Forest areas, West of Guilan Plain areas, West of Mazandaran Forest areas, West of Mazandaran Forest areas, West of Guilan Processing plant, East of Guilan Processing plant, West of Guilan Processing plant, West of Mazandaran
A.sojae	5 41,18 19 1,29,30 69	Forest areas, West of Guilan Plain areas, East of Guilan Plain areas, West of Guilan Processing plant, East of Guilan Processing plant, West of Guilan
A. parasiticus	95 94,96 43,45	Forest areas, West of Guilan Processing plant, West of Guilan Processing plant, East of Guilan
A.niger	84 47 78,79	Plain areas, East of Guilan Processing plant, East of Guilan Processing plant, West of Guilan
A.carbonarius	73,75 71,72,74 3	Plain areas, East of Guilan Processing plant, East of Guilan Processing plant, West of Guilan
A.foetidus	83 39,46 77	Plain areas, West of Guilan Processing plant, East of Guilan Processing plant, West of Guilan
A.ocraceus	107 32 70 106	Plain areas, East of Guilan Plain areas, West of Guilan Processing plant, West of Guilan Processing plant, East of Guilan
A.ostianus	35,37 26	Plain areas, West of Guilan Plain areas, East of Guilan

Morphological analysis

The preliminary study has been carried out using six plates including malt extract agar (MEA), yeast extract agar (YEA), czapek's agar (CZA), czapek yeast extract agar (CZYA), sabouraud dextrose agar (SDA), and potato dextrose agar (PDA), all containing 100 ppm chloramphenicol and 50 ppm tetracycline. After this, the plates have been removed after 30, 60, and 90 minutes for outdoor and 15, 30, and 60 minutes for indoor sites, respectively.

After aerobic incubation at 25°C, all plates have been observed in the periods of 3, 7, 10, and 14 days to recognize growth in them, and then they have been harvested, subcultured, marked, and cultivated in the prepared plates. The 61 distinct *Aspergillus* colonies have been cultivated and grown at 25±2°C for macroscopic and microscopic morphotaxonomy examinations. For detection of *Aspergillus* species, various methods based on ICPA (IUMS

International Commission on *Penicillium* and *Aspergillus*), ICTF (International commission on the taxonomy of fungi), NCF (Nomenclature Committee for Fungi) rules was used (Klich 2002, Pitt and Samson 2007).

Fungal biomasses have been obtained from all isolates grown in czapek's broth shaking medium, then dried out in a desiccator, then converted to powder by pearl/vortex and finally packaged in plastic pocket to preserve from any moisture that might cause fungal growth and increasing aflatoxin formation (Lin et al. 1980, Shuaib et al. 2010, Ma et al. 2015).

ELISA Determination

To detect aflatoxin B_1 levels in the fungal biomass and the growth medium samples, competitive enzyme immunoassay using RIDASCREEN Aflatoxin B_1 30/15 (ART.NO: R1211, R-Biopharm, Darmstadt, Germany) test kit, has been utilized (Aycicek et al. 2005, Ardic et al. 2008). Each kit contains sufficient materials for 96 measurements including standard analyses, wash buffer salt tween, conjugate, antibody, substrate/chromogen and stop solution.

As the basis of the test was the antigen-antibody reaction, microtiter wells have been coated with capture antibodies, directed against anti–aflatoxin antibodies and used for Aflatoxin standards and sample solutions. Then aflatoxin enzyme conjugate and anti–aflatoxin antibodies have been added; thus, free aflatoxin and aflatoxin enzyme conjugate competed for the aflatoxin antibody binding sites (competitive enzyme immunoassay). While it has not been possible to conduct simultaneously, the aflatoxin antibodies have been also bound by the immobilized capture antibodies. Next, any unbound enzyme conjugate has been removed in a washing step, and then substrate/chromogen have been added to the wells, so the bounded enzyme conjugate has converted the chromogen into a blue product. Adding the stop solution leaded to a color change from blue to yellow then. The measurement has been made photometrically at k=450 nm by ELISA plate reader (ELx800, Bio-Tek instruments, USA) within 10 minutes. The absorbance was inversely proportional to the aflatoxin concentration in the samples (Rosi et al. 2007, Atasever et al. 2010).

evaluation of samples was performed according to the RIDASOFT Win computer program prepared by R-Biopharm (Atasever et al. 2010).

Statistical analyses

In order to analyze the data through using the SPSS software version 21, Pearson and Kolmogorov–Smirnov tests have been used. Statically significant differences have been noted through using Kruskal–Wallis and Wilcoxon tests at $P<0.05$.

Results

Results obtained from Pearson analysis showed that there was not any statistically significant correlation between aflatoxin B_1 productions of different species ($p\geq0.05$). Therefore, it seems that the studied isolates, belonging to the different *Aspergillus* subgenus and sections, are independent of each other, even though these results are confirmed in non-parametric statistical analyses such as the Kolmogorov–Smirnov test.

The numerical difference between the measured values of toxin in fungal growth medium and cell extract has been measured through using the nonparametric Wilcoxon test. As a result, numerical values of all variables that have been measured by indirect competitive ELISA method have been significantly varied ($Z = 3/29$, sig=0/00).

Table 2 shows the average aflatoxin B_1 production (µg kg-1), compared between growth medium and fungal biomass.

Table 2. Average aflatoxin B1 production (µg kg-1) by *Aspergillus* isolates

Name	Growth medium	Fungal biomass
A.flavus	6.846	20.659
A.parasiticus	6.465	30.837
A.sojae	7.748	21.609
A.terreus	4.86	18.932
A.ostianus	7.231	32.787
A.carbonarius	6.151	6.352
A.wentii	12.499	23.996
A.niger	9.324	0
A.foetidus	16.658	11.84
A.ocraceus	19.362	17.948

For *Aspergillus flavus* isolates, the average of aflatoxin B_1 estimated by competitive ELISA method in fungal biomass was 20.659 µg kg-1, in contrast with the mean of aflatoxin B1 in growth medium determined by ELISA as 6.846 µg kg-1. For *Aspergillus parasitticus* isolates, the mean aflatoxin B1 in growth medium and cell extracts were 6.465 µg kg-1 and 30.837 µg kg-1, respectively.

Similar results have been obtained for other isolates including *A.sojae, A.terreus, A.ostianus, A.carbonarius*, and *A.wentii* with the mean amount of aflatoxin B_1 7.748, 4.86, 7.231, 6.151, 12.499 µg kg-1 in growth medium and 21.609, 18.932, 32.787, 6.352, 23.996 µg kg-1 in fungal biomass, respectively. Moreover, the average of toxin in other isolates including *A.niger, A.foetidus and A.ochraceus* in growth medium were 9.324, 16.658, 19.362 µg kg-1, and the amount of toxin in cell extracts in these isolates were 0, 11.84, and 17.948 µg kg-1, respectively.

Discussion

Aflatoxins, as secondary metabolites, are low-molecular weight natural products that are generated by filamentous fungi such as *Aspergilluses* (Frisvad et al. 2007, Frisvad et al. 2008, Roze et al. 2010).

In the current study, 24.9% of aflatoxin produced by *Aspergillus flavus* was secreted out of the cell, while 75.1% of toxin was remained in the fungal cell. According to obtained data, it can be concluded that storing the toxin in this fungal cell has exceeded its permeability to the outside of the cell.

In the case of *Aspergillus parasitticus,* 82.7% of the produced toxin has been stored in the cell, but 17.4% has been exported to the cell exterior. Also, in the case of *A. sojae, A.terreus, A.ostianus, A.carbonarius and A.wentii,* the amount of the released aflatoxin were 26.4%, 20.43%, 18.07%, 49.2% and 34.25%, while 73.60%, 79.57%, 81.93%, 50.80% and 65.75% of the produced aflatoxin have remained in the fungal biomass.

All of the data obtained from measuring the amount of toxin in the ELISA method as mentioned above indicated that, generally, the average of poison released from the fungal cell is lower compared with the storage of toxin within the cell. On the other hand, with regard to other studied isolates including *A.niger, A.foetidus and A.ochraceus*, 100%, 58.45% and 51.9% of the produced toxin were exported to the exterior of cell, while 0%, 41.55% and 48.1% were stored. Therefore, in contrast to other species in this study, results showed that in the case of *A. niger, A.foetidus and A.ochraceus* species, export of toxin is more than of storage within the cell.

Regulation of fungal secondary metabolism is a very diverse and complex process. Aflatoxin export processing is mediated by subcellular organelles, called aflatoxisomes, which is a specialized vesicle that the middle and late steps in aflatoxin biosynthesis take place in it. A large number of studies have been conducted on the regulation of the fungal secondary metabolism at molecular levels. However, little is known about the mechanisms that mediate aflatoxin export or why it is happening (Chanda et al. 2010).

Theoretically, vesicle –mediated export could occur in at least three types of mechanisms namely (a) Shuttle, in which aflatoxisomes pass across the cytoplasmic

membrane and shuttle their contents into the cell exterior, (b) Pump, in which transmembrane transporter proteins play an important role in the release of toxin to out of fungal cell, and (c) Burst and blast, in which vesicles fuse with the cytoplasmic membrane and similar to exocytosis, secret the toxin into growth environment (Chang et al. 2004, Shoji et al. 2008, Sirikantaramas et al. 2008, Chanda et al. 2010). Among these three theories, the probability of occurrence of the exocytosis (blast) mechanism is more than other mechanisms; however, the possibility that transporter protein system might play an important role in aflatoxin secretion must not be neglected. Since removal of transmembrane transporter proteins by gene disruption cannot block export toxin completely, the exocytosis (blast) mechanism can be an alternative to the impaired pump system (Chang et al. 2004, Chanda et al. 2010).

Unlike most previous studies that was reported notable aflatoxigenic species of *Aspergillus* are *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius,* observations of this study showed that other species of indigenous *Aspergillus* could be referred as aflatoxigenic. The ability of producing aflatoxin in native *Aspergillus* species can be attributed to the recombination both sexually and asexually (Adhikari et al. 2016, Islam et al. 2018).

With The presence of mating type genes in *Aspergillus* spp, recombination has been occurred among fungi. Sexual recombination between toxigenic and non-aflatoxigenic *Aspergillus* Strains probably alter composition of toxigenic – fungal population and lead to the diversity of distribution of indigenous species of aflatoxigenic *Aspergillus* (Islam et al. 2018).

These results suggest a potential health risk of aflatoxin hazards under favorable conditions within these areas, thus calling for more investigations.

In accordance to our observations, the aflatoxin B_1 production pattern in fungal biomass and growth medium in toxigenic *Aspergillus* species is different. Geographical distribution and different nutrient compositions are potential epigenetic factors that might play key roles in aflatoxin B1 accumulation and toxin export (Sirikantaramas et al. 2008, Chanda et al. 2010).

Indeed, aflatoxin biosynthesis can be modulated by different genes and that are themselves induced or repressed by environmental variables. One of the most important factors that have a significant influence on the biosynthesis of fungal secondary metabolites is fungal growing conditions. Water activities and temperature play key roles for fungal development and toxin production. Changes in temperature and water activity as external factors can have effected on some genes located in aflatoxin cluster such aflR that is required for aflatoxin biosynthesis under most growth conditions. Also type of nutrient sources and bioavailability can regulate the number of secondary metabolites. Another extracellular condition that strongly modulates the production of secondary metabolites is pH. The effect of pH has been related to fungal colony growth and aflatoxin production in *Aspergillus* species (Caceres et al.2020).

On the other hand, variety of mutation shape gene and genomes may be effect on aflatoxin production pattern in *Aspergillus* species (Bandyopadhyay et al. 2016).

Therefore, according to the mentioned assumptions in the current study, it can be concluded that the numerical differences in average of aflatoxin B_1 measured in fungal biomass and growth medium in different studied *Aspergillus* isolates depends on various probable mechanisms and factors that affect exertion or retention of toxin in fungal cell. And last but not least, to confirm these findings, more sensitive methods such as HPLC might be a useful diagnostic tool to monitor the level of aflatoxin export in Growth medium and fungal biomass.

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Conflict of interest

The authors have declared that no competing interests exist.

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