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# Study of the properties of Deoxynivalenol (DON) production in culture medium regarding to *Aspergillus* spp. isolates from processing factories in Northern Iran

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ABSTRACT

According to the increased fungi contaminations and related damages, microbiologist's incentive in considering the fungal contaminations in human habitats had increased. Some of fungi cause disease through production of toxins in animals as well as humans. Since these toxins are not easily distinguishable, then it is crucial to study their characteristics. *Aspergillus* are among the most important toxigenic fungi which are found abundantly in Northern Iran habitats where is one of most important habitats and the main source of many feed and food stuffs in the state. Thus, we have decided to study the Deoxynivalenol (DON) production characteristics in culture medium by collection of *Aspegillus* isolates in Northern Iran. Samples were collected from Northern Alborz and southern Caspian Sea agricultural plants culturing and processing centers. The mould samples were isolated and identified based on CBS environmental sampling rules and ICPA diagnostics standards. They were then cultured to stimulate the toxin production till the targeted toxin to be measured at culturing substrate and fungi biomass. Afterward, they were exposed to extraction and then DON was measured by enzyme linked immunosorbent assay (ELISA). Amongst studied species, *A. melleus* (subgenus Circumdati/82.581 ppb) had the highest DON toxin production followed by *A.* spp. *VI* (subgenus Unclassifiable/50.803 ppb) and *A. parasiticus* (subgenus Circumdati/49.108 ppb). This report gives important information about the presence of DON in foodstuff. DON presence is a threat in Feed-Food processing; storing and packing which may be controllable by regular test and measuring of DON.

## 1. Introduction

Mycotoxins are low molecular weight secondary metabolites produced by certain strains of filamentous fungi such as *Aspergillus*, *Penicillium* and *Fusarium*, which mainly invade crops in the field and may grow on foods during storage under favorable conditions of temperature and humidity (Reddy et al., 2010; Iheshiulor et al., 2011). When ingested, inhaled or absorbed through skin, mycotoxins may reduce appetite, general performances, and cause sickness, immune reduction or death in some cases (Reddy et al., 2010). Mycotoxins have been shown to have

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carcinogenic, immunotoxic, neurotoxic and teratogenic effects on susceptible consumers (Dwivedi et al., 2008). Over 100 different fungal species produce mycotoxins (Trefilov, 2011). Of which the greatest concern include: aflatoxin B1, which is generally produced by *Aspergillus* mould, deoxynivalenol, zearalenone, T-2 Toxin, and fumonisin B1, which are produced by *Fusarium* moulds, ochratoxin A and citrinin produced by *Aspergillus* and *Penicillium* moulds. They are regularly implicated in toxic syndromes in animals and humans (Dwivedi et al., 2008). No region of the world escapes the problem of mycotoxins or mycotoxic syndrome while estimated that there are about 300 harmful mycotoxins (Iheshiulor et al., 2011). In agricultural areas, the contamination of feed stuffs with molds and mycotoxins presents major environmental and health concerns (Lanier et al., 2010). According to Food and Agricultural Organisation (FAO) it is estimated that about 25-35% of the world crops contain mycotoxins. (Iheshiulor et al., 2011). Problems associated with mycotoxin contamination are generally complex. These compounds are highly chemically stable and very difficult to denature. Uncleaned grains are more likely to be contaminated with fungus than cleaned grain. Aflatoxins, ochratoxins, cyclopiazonic acid as well as fusariotoxins such as zearalenone, deoxynivalenol, moniliformin and fumonisins, are common, and all of them can be found in pet foods (Böhm, 2006). Mycotoxins have been detected in various foods commodities from many parts of the world and are presently considered as one of the most contaminants food. Mycotoxins causes mycotoxicoses and their toxicity depends not only on the amounts ingested, exposure time-span, species-breed susceptibility, age, sex, health status, but also other parameters such as density, food-feeding preferences, diseases and environmental conditions like relative temperature or humidity (Iheshiulor et al., 2011). Consumption of food or feed contaminated with mycotoxins may cause various diseases defined as mycotoxicoses are immediate threat to human and hematothermal animals (cows, pigs, dogs, cats and etc.) (Korbas and horoszkiewicz-Janka, 2007). Different types of mycotoxin-producing fungi notably develop in different weather conditions, and therefore types and levels of mycotoxins in cereal grains may vary significantly (Trefilov, 2011). Deoxynivalenol (DON) is one of the least toxic reliable mycotoxins, however, it is the most prevalent trichothecenes in human foods and its presence is an indicator of the possible incidence of other more toxic trichothecenes (Al-Hazmi, 2011). They cause significant economic losses in animals due to reduced productivity, increased disease incidence, chronic damage of vital organs and decreased reproductive performance (Murthy et al., 2009; Iheshiulor et al., 2011). Moreover, the productivity and nutritive value of infected grains and cereals drops after contamination by causative moulds. Animals may have variable susceptibilities to mycotoxins depending on physiological, genetic and environmental factors. Preventing mould growth and subsequent mycotoxin production is essential to the feed manufacturer, livestock producer and for maximum animal performance (Iheshiulor et al., 2011). As these mycotoxins are ubiquitous, the testing of products is required to keep our food and feed safe. For this purpose, sensitive and reliable tests are needed to detect contaminations. One method of detection is an immunoanalytical based test which needs antibodies as reagents (Baumgartner et al., 2010). Level of mycotoxins can be reduced by using various methods, for example: agrotechnical or chemical during vegetation period and physical during grain storage. The chemical methods of reduction of harmful metabolites production is important and consist chemical protection of plants during vegetation period using the most effective plant protection products (Korbas and horoszkiewicz-Janka, 2007). The aim of this study was to investigate the Deoxynivalenol (DON) production characteristics in culture medium by northern Iran's *Aspegillus* isolates collection.

## 2. Materials and Methods

### *2.1. Sampling, Culture and Isolation*

Sampling was performed from indoor and outdoor (based on CBS institute sampling program) from May until mid October in Guilan and Mazandaran (Kozakiewicz, 1989; Samson et al., 2001; Klich, 2002 a; Pitt, 1997; Samson et al., 2001). Sampling was done by a group of six plates containing Malt extract agar (MEA), Yeast extract agar (YEA), Czapeck yeast extract agar (CZYEA),

Czapeck agar (CZA), Saburaud dextrose agar (SDA) and potato dextrose agar (PDA) containing 100 ppm Chloramphenicol and 50 ppm Tetracycline (Pitt and Hocking, 1997; Kozakiewicz, 1989; Samson et al., 2001; Klich, 2002a; Klich, 2002 b). Plates were then harvested with 15-25 $cm^3$ of agar and 10-12 cm diameter after 30, 60 and 90 minutes (451 suitable plates in the farms) and 15, 30 and 60 minutes (441 suitable plates in factories), and then enclosed in mushy polyethylene bags for further analysis in the laboratory (Samson et al., 2001; Klich, 2002a). All plates were incubated aerobically in 25±2°C on the table for natural photoperiodic conversion of light to dark, except one plate from each culture medium in the dark, the other in the light (Odds et al., 1983; Samson et al., 2001; Klich, 2002a). Until 15 days with the intervals of 3, 7 and 15 days, all the plates were examined to identify new grown colonies by a stereomicroscope (Kozakiewicz, 1989; Gams et al., 1998; Samson et al., 2001; Klich, 2002 a). In the plates and tubes with agars from MEA, YEA, PDA, corn mill agar (CMA), SDA, Czapeck yeast agar (CZYA) and Czapeck dox agar (CZDA) medium, all new-found moldy samples subcultured and incubated based on previous program and the macroscopic and microscopic characteristics were followed in the intervals of 5, 10 and 15 days and recorded. Finally, from 300 *Aspergillus* colonies (from more than 600 generative moldy isolates), 150 colonies were selected and cultured in CZDA, CZYEA with and without 20% sucrose, MEA and CZDA with and without 20% sucrose for microscopic and macroscopic studies according to ICPA taxonomic rules.

### *2.2. Morphological Studies*

For morphological studies, microscopic and macroscopic imaging, the surface and back of one - to two weeks mature colonies (in black *Aspergillus* of two to four weeks colonies) were selected. Measuring the colonies diameter, examination of the color of surface and back of colonies, pigments, any extrolits together with aspergils, cells and grown masses, filaments, stipes, corona of conidia and micrometry of conidiophores, vesicles of conidia and examination of the any generations and micrometric imaging of sclerotia or asci was done with stereoscopes linked to Leica® Microanalysis Software Shared Network System (Kozakiewicz, 1989; Samson et al., 2001; Klich, 2002 a). In all samples with slide culture, tease mount and steaky tape of conidiophores (stipe), vesicle, corona of conidia, phialides, metullas, conidia or asci and all accessories, micrometry or imaging were done consequently (Kozakiewicz, 1989; Powell et al., 1994; Gams et al., 1998; Samson et al., 2001; Klich, 2002 a).

### *2.3. Providing media samples/Cellular extracts*

In order to provide prepared isolates extracts, CZ liquid medium + 2% ME was selected. A loop full ($10^5$ phialospores) of PBS-conidies suspension of each grown isolates in Czapeck extract agar (CZEA) plates were picked up and inoculated into one 50 ml falcon tubes containing Czapeck broth (CZB) with 2% malt extract (Shadzi et al., 1993) then incubated in 25±3°C with a natural photoperiodic light-darkness conversion while were examined daily to inhibit any mouldy matt on the liquid even in the third and sixth days, adding liquid medium with 1% MEA until 50 ml liquid medium to be remained in the test tubes (Odds et al., 1983; Green et al., 2003; Oda et al., 2006). After seven days, floating or deposit fungi floating masses that were small and new borne (Germ tubes) mouldy fungal short filaments centrifuged in 3000 rpm for 15 minutes till to be precipitated and harvested (Ausubel et al., 2002). Recognition and estimation of DON were done by direct ELISA method using RIDASCREEN® DON (Art. No.: R5906) which is a competitive enzyme immunoassay for the quantitive analysis of DON in feed and foods. All valuable and trustable data conducted by a variety of statistical softwares using as Excel (version 2007) and SPSS (version15). Chi two tests was used to measure the relation of two classified variants while ANOVA variance analysis was applied for experimental design.

## 3. Results

In the study of culture media in isolates obtained from processing factories, subgenus Ornati (*S. ornata*/mean 48.247 ppb) had the highest DON toxin production followed by subgenus Circumdati

(*A.melleus*, *A. parasiticus*, *A. sojae*/21.988 ppb) and Unclassifiable (*A.* spp. *VI*, *A.* spp. *III*/20. 874 ppb). Subgenus Nidulantes (*A.terreus*, *A. unguis*/3.003 ppb) had the lowest detectable toxin mean (Table-1; Figure-1).

**Table 1.** DON concentration mean in culture medium based on subgenus in processing factories.

| Subgenus/ Factory | Count | DON Mean (ppb) |
|---|---|---|
| Circumdati | 35 | 21.988 |
| Nidulantes | 7 | 3.003 |
| Ornati | 3 | 48.247 |
| Unclassifiable | 11 | 20.874 |

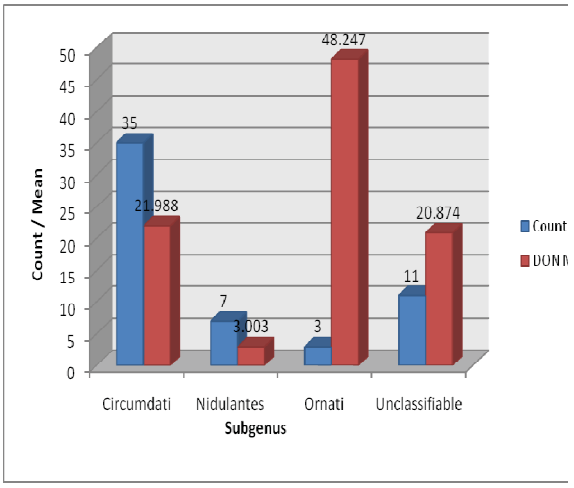


**Figure 1**- DON concentration mean in culture medium based on subgenus in processing factories

*A. melleus* (subgenus Circumdati/82.581 ppb) had the highest DON toxin production followed by *A.* spp. *VI* (subgenus Unclassifiable/50.803 ppb) and *A. parasiticus* (subgenus Circumdati/49.108 ppb). *A. terreus* (subgenus Nidulantes/1.752 ppb) had the lowest detectable toxin and followed by *A. unguis* (subgenus Nidulantes/4.671 ppb) and *A.* af*nidulans* (subgenus Nidulantes/4.807 ppb). *A. carbonarius* (subgenus Circumdati), *A. flavus* (subgenus Circumdati), *A. foetidus* (subgenus Circumdati), *A. niger* (subgenus Circumdati), *A. ochraceus* (subgenus Circumdati) and *A. wentii* (subgenus Circumdati) had distinctly a mean of 0 ppb (Table-2; Figure-2).

**Table 2**- DON concentration in culture medium based on species in processing factories

| Species/ Factory | Count | DON Mean (ppb) |
|---|---|---|
| *A.* af *flavus* | 1 | 19.228 |
| *A.* af *nidulans* | 2 | 4.807 |
| *A. awamori* | 2 | 7.9 |
| *A. candidus* | 4 | 28.701 |
| *A. carbonarius* | 3 | 0 |
| *A. flavus* | 6 | 0 |
| *A. foetidus* | 3 | 0 |
| *A. melleus* | 3 | 82.581 |
| *A. niger* | 3 | 0 |
| *A. ochraceus* | 2 | 0 |
| *A. parasiticus* | 4 | 49.108 |
| *A. sojae* | 4 | 48.696 |
| *A.* spp. *III* | 4 | 21.342 |
| *A.* spp. *IV* | 2 | 6.899 |
| *A.* spp. *VI* | 2 | 50.803 |
| *A. terreus* | 4 | 1.752 |
| *A. unguis* | 3 | 4.671 |
| *A. wentii* | 1 | 0 |
| *S. ornata* | 3 | 48.247 |

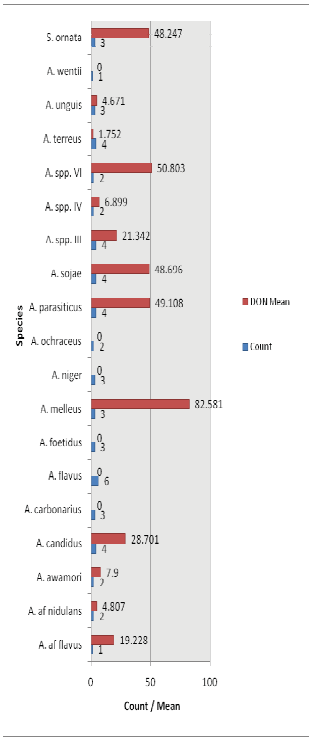


**Figure 2.** DON concentration in culture medium based on species in processing factories

**Table 3.** A brief introductive toxin producer isolated species based on DON acceptable maximum value and Feed and Food standards

| Subgenus | Species | Count of Isolates | DON mean (ppb) | Limits/Std. % | | |
|---|---|---|---|---|---|---|
| | | | | Feed | Food | |
| | | | | | Infant | Adult |
| Circumdati | *A.* af *flavus* | 1 | 19.228 | 0.38 | 1281866.7 | 1.92 |
| | *A. awamori* | 2 | 7.9 | 0.16 | 526666.7 | 0.79 |
| | *A. candidus* | 4 | 28.701 | 0.57 | 1913400 | 2.87 |
| | *A. carbonarius* | 3 | 0 | 0 | 0 | 0 |
| | *A. flavus* | 6 | 0 | 0 | 0 | 0 |
| | *A. foetidus* | 3 | 0 | 0 | 0 | 0 |
| | *A. melleus* | 3 | 82.581 | 1.65 | 5505400 | 8.26 |
| | *A. niger* | 3 | 0 | 0 | 0 | 0 |
| | *A. ochraceus* | 2 | 0 | 0 | 0 | 0 |
| | *A. parasiticus* | 4 | 49.108 | 0.98 | 3273866.7 | 4.91 |
| | *A. sojae* | 4 | 48.696 | 0.97 | 3246400 | 4.87 |
| | *A. wentii* | 1 | 0 | 0 | 0 | 0 |
| Nidulantes | *A.* af *nidulans* | 2 | 4.807 | 0.1 | 320466.7 | 0.48 |
| | *A. terreus* | 4 | 1.752 | 0.04 | 116800 | 0.18 |
| | *A. unguis* | 3 | 4.671 | 0.09 | 311400 | 0.47 |
| Ornati | *S. ornata* | 3 | 48.247 | 0.96 | 3216466.7 | 4.82 |
| Unclassifiable | *A.* spp. *III* | 4 | 21.342 | 0.43 | 1422800 | 2.13 |
| | *A.* spp. *IV* | 2 | 6.899 | 0.14 | 459933.3 | 0.69 |
| | *A.* spp. *VI* | 2 | 50.803 | 1.02 | 3386866.7 | 5.08 |

## 4. Discussion

According to analytic results; 107 *Aspergillus* isolates obtained from processing factories, the subgenus Ornati (*S. ornata*/ mean 48.247 ppb), Circumdati (*A.melleus*, *A. parasiticus*, *A. sojae*/ mean 21.988 ppb) and Unclassifiable is spolates (*A.* spp. *VI*, *A.*spp. *III* mean 20. 874 ppb) had the highest potency for DON production and release mean at all (Figure-1). The *A. melleus* (82.581 ppb), *A.* spp. *VI* (50.803 ppb) and *A. parasiticus* (49.108 ppb) had the highest DON toxin production in culture medium (Figure-2). Among species of subgenus Circumdati, isolates of *A. melleus* (82.581 ppb), *A. parasiticus* (49.108 ppb), *A. sojae* (48.696 ppb), *A. candidus* (28.701 ppb), *A.* af *flavus* (19.228 ppb) and *A. awamori* (7.9 ppb) were shown the decreased level of toxin concomitantly (Table-2). Among species as unclassifiable, isolates *A.* spp. *VI* (50.803 ppb), *A.* spp. *III* (21.342 ppb) and *A.* spp. *IV* (6.899 ppb) were found to be the moderate toxin producers (Table-2). Value of measured toxin by competitive direct ELISA showed that all of the mentioned species had toxin production less than permissive limit by FDA (5 ppm or 5000 ppb) for feed (Table-3) while they had toxin production more than permissive limit by ECS (0.0015 ppb) for infants food (Table-3; Al-Hazmi, 2011) permissive limit by FAO (1 ppm or 1000 ppb) for adults food (Table-3; FAO/WHO, Agenda Item 6, CX/CF 12/6/9).

Mouldy is the result of growth of mixture of species of moulds. DON presence is a danger in Feed-Food processing; storing or packing that is controllable by regular examination and toxin measuring for DON. In terms of processing if DON producer fungi be present, in hot and humid conditions, toxin production without observable mouldy criteria can be a latent danger biorisk. In addition to examination of produced food in processing and packing factories, its better to do regular examination in primal materials by which provide products specially before entrance to processing plants and packing of products.

According to the highest frequency of the DON toxin recognition that obtained from *Fusarium* spp. now we could say this is the first report of DON observation and estimation or measuring in

Aspergilli isolates in the world based on the average levels between the minimum 30 ppb (2.8%) and maximum 80-100 ppb (7.5%) detected toxins which are too much more than the international standards and regulations (Whitlow, PC; Al-Hazmi, 2011; FAO/WHO, Agenda Item 6, CX/CF 12/6/9). It should be noticed that in addition to *Fusarium* ingredients as the second genera of DON production causative agents. Eventually it is noticeable that DON tracer production genes might be introduced by all of fungi isolates in comparison with *Fusarium* while recognized as the specific mentioned toxin faster than other fungi; because there is this possibility that other fungi have potency of producers in which may be transmitted during gene transmission phenomenon. It seems that genomic study is necessary to validate that DON or any other analogue molecules are activated in the conducted toxin producer species or not. Thus, evaluation of these results indicates that in addition to *Fusarium*, genus *Aspergillus* has a potential to produce DON toxin or similar molecules. Validation of this issue needs further studies, in particular, by advanced biochemical or genomic molecular techniques.

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