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Determination of Deoxynivalenol (DON) Producing *Aspergillus* Subgenus Related Species in the Iran's Northern Agriculture Shores

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Abstract: Mycotoxins are harmful substances produced by fungi in various foods and are estimated to affect, as much as 25% of the world's crop each year. Most of these mytocoxins belong to the 3 genera of fungi: *Aspergillus*, *Penicillium* and *Fusarium*. Gilan and Mazandaran provinces are located in the North of Iran with favorite conditions for Aspergillus growth. In present study, researchers will study the production of Deoxynivalenol toxin in cell extract of Aspergillus species isolated indigenous in North of Iran. After recognition of the species using ELISA, researchers quantitatively analysis Deoxynivalenol produced by available species. Finally, the greatest DON mean concentration in the biomass based on the farms isolates was related to mountainous region with maximum amount and followed by related to the forestrial and interstitial regions, respectively.

Key words: Aspergillus, species, fungi, DON, Iran

INTRODUCTION

Deoxynivalenol is one of trichothecenes known to be produced mainly by *Fusarium* species. *Fusarium graminearum* (Teleomorph: *Gibberella zeae*) isolates are considered, as major producers of Deoxynivalenol (DON), Nivalenol (NIV) and their derivatives including 3-Acetyldeoxynivalenol (3-ADON), 15-Acetyldeoxynivalenol (15-ADON), 4-Acetylnivalenol (4-ANIV) in wheat, barley and corn grains (Kimura *et al*., 2003).

Vomitoxin (IUPAC name: (3, 7α)-3, 7, 15-trihydroxy-12 and 13-epoxytrichothec-9-en-8-one)), also known as deoxynivalenol is a type B trichothecene, an epoxy-sesquiterpenoid, occurs predominantly in grains such as wheat, barley, oats, rye and maize and less often in rice, sorghum and triticale (Gautamand and Dill-Macky, 2011). Although, DON is among the least toxic trichothecenes, it is the most frequently detected throughout the world and its occurrence is considered to be an indicator of the possible presence of other more toxic trichothecenes (Krska *et al*., 2007).

At the cellular level, the main toxic effect is inhibition of protein synthesis via binding to the ribosome. In animals, moderate to low ingestion of toxin can cause a number of as yet poorly defined effects associated with reduced performance and immune function. The main overt effect at low dietary concentrations appears to be a reduction in food consumption (anorexia) while higher doses induce vomiting (emesis). DON is known to alter brain neurochemicals, such as serotoninergic system appeared to play a role in mediation of the feeding behavior and emetic response. Animals fed low to moderate doses are able to recover from initial weight losses while higher doses induce more long-term changes in feeding behavior. At low dosages of DON, hematological, clinical and immunological changes are also transitory and decrease as compensatory/adaptation mechanisms are established. The capacity of DON to alter normal immune function has been of particular interest. There is extensive evidence that DON can be immunosuppressive or immunostimulatory, depending upon the dose and duration of exposure. While immunosuppression can be explained by the inhibition of translation, immunostimulation can be related to interference with normal regulatory mechanisms. Other effects include superinduction of cytokine production by T helper cells (*in vitro*) and activation of macrophages and T cells to produce a proinflammatory cytokine wave that is analogous to that found in lipopolysaccharide-induced shock (*in vivo*). To what extent the elevation of cytokines contributes to metabolic effects, such as decreased feed intake remains to be established. Further toxicology, studies and an assessment of the potential of DON to be an etiologic agent in human disease are warranted (Rotter *et al*., 1996).

In cows fed 6.4 ppm vomitoxin (diet dry matter) for 70 days, no vomitoxin residue was found in their milk.

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Table 1: EU DON regulatory levels based on tolerable daily intake X uncertainty

Products	Greatestleve ($\mu g\ kg^{-1}$)
Unprocessed cereals other than durum wheat, oats and maize	1250
Unprocessed durum wheat and oats	1750
Cereal flour including maize flour, maize grits and maize meal	750
Breads, pastries, biscuits, cereal snacks and breakfast cereals	500
Pasta (dry)	750
Processed cereal-based food for infants and young children and baby food	200

Advisory levels from FDA regarding vomitoxin in livestock feeds indicate that in ruminating beef and feedlot cattle older than 4 months, vomitoxin contaminated grain and grain by-products should not exceed 50% of the diet with maximum vomitoxin levels of 10 ppm in grains and grain by-products and 5 ppm in finished fee (Parish, 2008).

FDA's (US food and drug administration) advisory levels for deoxynivalenol (vomitoxin) for grain and grain by products destined for commodities containing this level of vomitoxin not exceed 20% of the ration 5 ppm, for grain and grain byproducts destined for beef cattle and feedlot cattle older than four months, as well as for chickens (FDA recommends that commodities containing this level of vomitoxin not exceed 50% of the ration for these species) 10 ppm, for grain and grain byproducts destined for all other animal species (FDA recommends that commodities containing this level of vomitoxin not exceed 40% of the ration) 5 ppm (Table 1).

MATERIALS AND METHODS

Sampling, culture and isolation: From the 1st May to late October (2011) in the provinces of Gilan and Mazandaran (Northern states of Iran), following the agenda, the sampling process on indoor and outdoor sites by (CBS firms) was performed (Klich 2002a; Kozakiewicz, 1989; Samson *et al*., 2001).

A group of sample was applied using settle plates technique by 6 plates with malt extract agar, yest extract agar, czapek-yest extract agar, czapek-agar, sabouraud dextrose agar and potato dextrose agar while all impregnated with 100 ppm chloramphenicol and 50 ppm tetracycline, a sample group plates were withdrawn after 30, 60 and 90 min and 15, 30 and 60 min. All plates were incubated aerobicaly in 25±2°C (Klich, 2002a; Kozakiewicz, 1989; Odds *et al*., 1983; Samson *et al*., 2001).

Till 15 days all plates were investigated for all the young colony to be identified, marked, newly growth colonies are harvested and planted in prepared malt extract agar, yeast extract agar, potato dextrose agar, corn male agar, saboraud's dextrose agar, Czapek-Yeast agar and Czapek-Dox agar plates all the new found mould samples were restored and were followed by prestove program like macro and microscopic properties in the 5, 10 and 15 days span and then were recorded (Klich, 2002a; Kozakiewicz, 1989; Pittet, 1998; Rodger, 2001).

At the end, of 300 Aspergillus colonies the 150 ones randomly selected colonies transfer to in plates with malt extract agar, Czapek-Doux agar, Czapek-Yest extract (with and without sucrose 20%), Czapek-Dox agar (with and without sucrose 20%) which has been examined for morphological macro and microscopic incubation at 37°C and after 3, 7, 14 and sometimes 25 or 30 days examination and simultaneous slide culture from each sample on the Czapek-Dox agar, Czapek-Yest extract 20% sucrose for growth normaly by perverse model was provided (Klich, 2002a, b; Kozakiewicz, 1989; Samson *et al*., 2001).

Morphological studies: For morphological studies and macro and microscopic photobiometery the front and back of 1 or 2 weeks aged colonies (2-4 weeks for black Aspergillusy colonies) were selected. Measureing the width, check out the colors, pigments and extrolits, taking photographes, cells and umbrellas, hyphae, stypes, the conidies crown and micrometerics on conidiophores, vesicles and conidies and also the emergence and micrometery of sclertia or Ascs were done (Klich, 2002a; Kozakiewicz, 1989; Samson *et al*., 2001).

Providing cellular extracts: A loop full of the mixture of PBS and each isolate in each agar plate been harvested and transferred into 50 mL falcon tube with a fluid bed czapek-dox broth containing one per cent malt extract agar and then subcultured. With 200 rpm, 25±2°C in and photo periodic conditions incubated and inspected daily (Green *et al*., 2003; Oda *et al*., 2006; Odds *et al*., 1983). After 7 days of float or sink in the tubes of fluid and small germ tube were purified by centrifuging at around 3000 rpm to 15 min and cellular biomasses were harvested. Masses washed for 3 consecutive times with 25 mL of PBS with centrifugation (3000 rpm for 15 min) and stoked in a -20°C were stored (Ausubel *et al*., 2002; Shadzi *et al*., 1993).

Defrosting the samples soaked in ice fields, 48 h each in a desiccator and then 2 g of it was harvested. Mass of every dry mould filament was mixed 3 times in a 15 mL Falcan tube, each time with 3 subsequent replication (each 7 min) with 5 mL sampling buffer using a tube mixer and glass globes (pearl) and each time 25 min grinding was performed. Mouldy mixture to each tube filtered samples and 1 mL of cold acetone added and of around 3.000 rpm centrifuged (15 min) remaining a larger separation deposited (Moallaei *et al*., 2006; Shadzi *et al*., 1993). Supernatant samples treated by 1-5 ratio with cold

acetone and then meintand in a cold 20°C for 1-3 days and finaly were centrifuged at around 20000 rpm to 20 min in the cold -20°C fridged centrifuged. Deposits and with drawals made from the concentrated samples were diluted in dilution of the concentrated extracts of the same method was applied to all samples (Ausubel *et al*., 2002; Medina *et al*., 2005; Puente *et al*., 1991). Then, detection of DON were done by direct competitive ELISA in *Aspergillus* species using RIDASCREEN® DON (Art. No.: R5906) which is a competitive enzyme immunoassay for the quantitative analysis of DON in feed and foods.

ELISA assay: As the basis of the test was the antigen-antibody reaction, microtiter wells were coated with capture antibodies directed against anti deoxynivalenol antibodies used for deoxynivalenol standards and sample solutions then deoxynivalenol enzyme conjugate and anti-deoxynivalenol antibodies were added, thus free deoxynivalenol and deoxynivalenol enzyme conjugate to be competed for the deoxynivalenol antibody binding sites (competitive enzyme immunoassay). Anable the same time, the deoxynivalenol antibodies to be also bound by the immobilized capture antibodies. Any unbound enzyme conjugate were then removed in a washing step. Then substrate/chromogen were added to the wells, bounded enzyme conjugate converted the chromogen into a blue product. Addition the stop solution leaded to a color change from blue to yellow. The measurement was made photometrically at 450 nm. The absorbance was inversely proportional to the deoxynivalenol concentration in the samples.

RESULTS

Table 2 and Fig. 1 showing statistically frequency of identified species. According to Table 2, the greatest frequency species was *A. flavus* in contrast *A. afflavus* and *A. sp* V which were the lowest frequency species.

Table 3 and Fig. 2 showing of totaly 107 *Aspergillus* isolates in the study of obtained, the greatest frequency was belonged to subgenus *Circumdati* with 66 isolates (61.7%) and the least frequency of subgenus *Fumigati* with 5 isolates (4.7%).

Table 4 and Fig. 3 showing frequency ratio of the farms. The greatest frequency was belonged to plate (19/6%) and the least frequency was mountainous (2/8%).

Table 5 showing frequency ratio of subgenus in the farms. In forestrial, the greatest frequency was belonged to *Circumdati* (8/4%) and the lowest frequency was *Ornati* and unclassifiable. In interstitial, the greatest frequency was belonged to *Circumdati* (9/3%) and the remaining subgenus had the equal frequencies. In mountainous, there were only 2 subgenus, *Fumigati* (1/9%) and *Nidulantes* (0/9%) also in plate the greatest frequency was belonged to *Circumdati* with (10/3%) and the lowest frequency were *Fumigati* and unclassifiable (1/9%).

Table 6 showing DON concentration frequency in the biomass based on specific farms isolates in the range of 0-100 ppb. According to Table 6 in forestrial the greatest frequency (8/4%) was in 0-10 ppb range and least frequency (0/9%) was 20-30 and 60-70 ppb range. In interstitial the greatest frequency (12/1%) was in 0-10 ppb range and least frequency (0/9%) was in 80-90 ppb. In Mountainous, the greatest frequency (1/9%) was 0-10 ppb and least frequency (0/9%) was in 10-20 ppb in plate the greatest frequency (15/0%) was 0-10 ppb

Table 2: Statistically frequency of identified species on samples

Species	Count isolates	Percentage	Cumulative (%)
A. af flavus	1	0.9	0.9
A. af nidulans	2	1.9	2.8
A. alliaceus	2	1.9	4.7
A. awamori	3	2.8	7.5
A. candidus	4	3.7	11.2
A. carbonari	6	5.6	16.8
A. flavus	18	16.8	33.6
A. foetidus	4	3.7	37.4
A. fumigatus	5	4.7	42.1
A. melleus	3	2.8	44.9
A. niger	4	3.7	48.6
A. niveus	3	2.8	51.4
A. ochraceus	4	3.7	55.1
A. ostianus	3	2.8	57.9
A. parasiticus	5	4.7	62.6
A. sojae	9	8.4	71.0
A. spIII	7	6.5	77.6
A. spIV	2	1.9	79.4
A. spV	1	0.9	80.4
A. spVI	2	1.9	82.2
A. terreus	6	5.6	87.9
A. unguis	4	3.7	91.6
A. wentii	3	2.8	94.4
S. ornata	6	5.6	100.0
Total	107	100.0	

Table 3: Frequency of Aspergillus isolates at the subgenus states

Subgenus	Count isolates	Percentage	Cumulative (%)
Circumdati	66	61.7	61.7
Fumigati	5	4.7	66.4
Nidulantes	18	16.8	83.2
Ornati	6	5.6	88.8
Unclassifiable	12	11.2	100.0
Total	107	100.0	

Table 4: The frequency of the sample isolation places

Isolation places	Count isolates	Percentage	Cumulative (%)
Factory	56	52.3	52.3
Forestrial	13	12.1	64.5
Interstitial	14	13.1	77.6
Mountainous	3	2.8	80.4
Plate	21	19.6	100.0
Total	107	100.0	

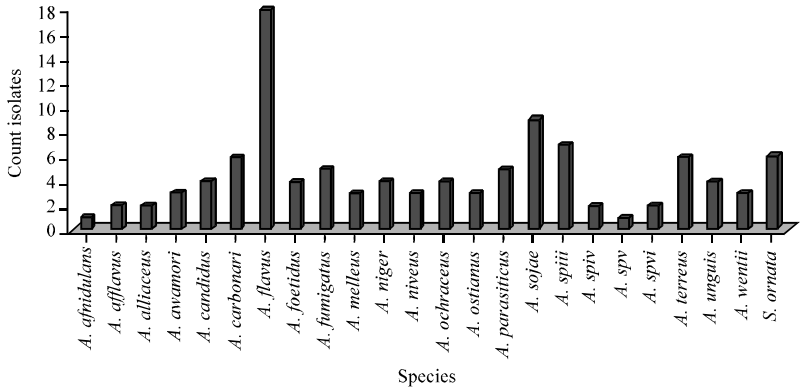

Fig. 1: Statistically frequency of identified species on samples

Table 5: The subgenus frequency in the farms

	Subgenus					
Farms	*Cirumdati*	*Fumigati*	*Nidulantes*	*Ornati*	Unclassifiable	Total
Factory						
Count	36.0	0.0	10.0	3.0		56.0
Within farm (%)	64.3	0.0	179.0	5.4	12.5	100.0
Within subgenus (%)	545.0	0.0	55.6	50.0	5.3	52.3
Total (%)	3.6	0.0	9.3	2.8	6.5	52.3
Forestrial						
Count	9.0	0.0	0.0	2.0	2.0	13.0
Within farm (%)	69.2	0.0	0.0	15.4	15.4	100.0
Within subgenus (%)	13.6	0.0	0.0	33.3	16.7	12.1
Total (%)	8.4	0.0	0.0	1.9	1.9	12.1
Interstitial						
Count	10.0	1.0	1.0	1.0	1.0	14.0
Within farm (%)	71.4	7.1	7.1	7.1	7.1	100.0
Within subgenus (%)	15.2	20.0	5.6	16.7	8.3	13.1
Total (%)	9.3	0.9	0.9	0.9	0.9	13.1
Mountainous						
Count	0.0	2.0	1.0	0.0	0.0	3.0
Within farm (%)	0.0	66.7	33.3	0.0	0.0	100.0
Within subgenus (%)	0.0	40.0	5.6	0.0	0.0	2.8
Total (%)	0.0	1.9	0.9	0.0	0.0	2.8
Plate						
Count	11.0	2.0	6.0	0.0	2.0	21.0
Within farm (%)	52.4	9.5	28.6	0.0	9.5	100.0
Within subgenus (%)	16.7	40.0	33.3	0.0	16.7	19.6
Total (%)	10.3	1.9	5.6	0.0	1.9	19.6
Total						
Count	66.0	5.0	18.0	6.0	12.0	107.0
Within farm	61.7	4.7	16.8	5.6	11.2	100.0
Within subgenus (%)	100.0	100.0	100.0	100.0	100.0	100.0
Total (%)	61.7	4.7	16.8	5.6	11.2	100.0

Table 6: DON concentration frequency in the biomass based on the farms in the range of 0-100 ppb

	Biomass/ELISA-DON									
Farm	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	Total
Factory											
Count	49.0	2.0	2.0	2.0	0.0	0.0	0.0	0.0	1.0	0.0	56.0
Within farm (%)	87.5	3.6	3.6	3.6	0.0	0.0	0.0	0.0	1.8	0.0	100.0
Within biomass/ELISA-DON (%)	55.1	33.3	33.3	100.0	0.0	0.0	0.0	0.0	33.3	0.0	52.3
Total (%)	45.8	1.9	1.9	1.9	0.0	0.0	0.0	0.0	0.9	0.0	52.3
Forestrial											
Count	9.0	2.0	1.0	0.0	0.0	0.0	1.0	0.0	0.0	0.0	13.0
Within farm (%)	69.2	15.4	7.7	0.0	0.0	0.0	7.7	0.0	0.0	0.0	100.0
Within biomass/ELISA-DON (%)	10.1	33.3	16.7	0.0	0.0	0.0	100.0	0.0	0.0	0.0	12.1
Total (%)	8.4	1.9	0.9	0.0	0.0	0.0	0.9	0.0	0.0	0.0	12.1

Table 6: Continue

	Biomass/ELISA-DON										
Farm	0-10	10-20	20-30	30-40	40-50	50-60	60-70	70-80	80-90	90-100	Total
Interstitial											
Count	13.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	14.0
Within farm (%)	92.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.1	0.0	100.0
Within biomass/ELISA-DON (%)	14.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	33.3	0.0	13.1
Total (%)	12.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9	0.0	13.1
Mountainous											
Count	2.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0
Within farm (%)	66.7	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0
Within biomass/ELISA-DON (%)	2.2	16.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8
Total (%)	1.9	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.8
Plate											
Count	16.0	1.0	3.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	21.0
Within farm (%)	76.2	4.8	14.3	0.0	0.0	0.0	0.0	0.0	4.8	0.0	100.0
Within biomass/ELISA-DON (%)	18.0	16.7	50.0	0.0	0.0	0.0	0.0	0.0	33.3	0.0	19.6
Total (%)	15.0	0.9	2.8	0.0	0.0	0.0	0.0	0.0	0.9	0.0	19.6
Total											
Count	89.0	6.0	6.0	2.0	0.0	0.0	1.0	0.0	3.0	0.0	107.0
Within farm (%)	83.2	5.6	5.6	1.9	0.0	0.0	0.9	0.0	2.8	0.0	100.0
Within biomass/ELISA-DON (%)	100.0	100.0	100.0	100.0	0.0	0.0	100.0	0.0	100.0	0.0	100.0
Total (%)	83.2	5.6	5.6	1.9	0.0	0.0	0.9	0.0	2.8	0.0	100.0

Table 7: DON mean concentration based on the isolation places

Isolation places	Count isolates	DON mean concentration (ppb)
Factories	56	4.22164
Forestrial farms	21	7.52752
Interstitial farms	14	5.80750
Mountainous farms	13	9.32415
Plate farms	3	4.14500
Total	107	5.69574

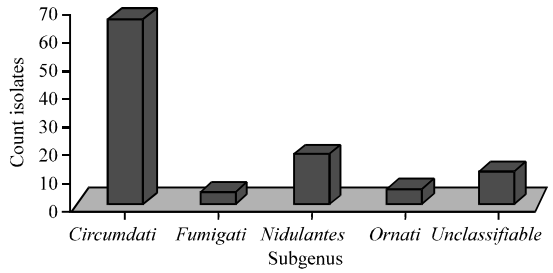

Fig. 2: Frequency of *Aspergillus* isolates at the subgenus states

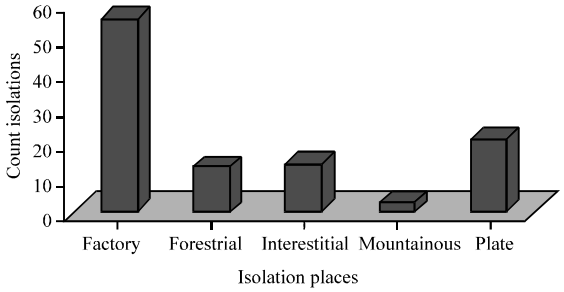

Fig. 3: The frequency of the sample isolation places

and least frequency (0/9%) was in 10-20 and 80-90 ppb. As observed the greatest frequencies exist

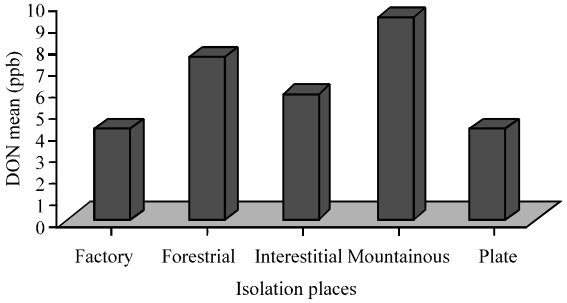

Fig. 4: DON mean concentration based on the isolation places

in the range of 0-10 ppb in plate, interstitial, forestrial and mountainous farms, respectively.

Table 7 and Fig. 4 showing DON mean concentration based on the isolation places. The greatest DON mean concentration in the biomass was in mountainous in 9.324 ppb range and the lowest frequency was in plate in 4.145 ppb.

DISCUSSION

The greatest DON mean concentration in the *Aspergilli* biomasses based on the farms was related to mountainous region with maximum amount and follow was related to the forestrial and interstitial regions, respectively.

Amounts of DON concentration obtained of Aspregillus species in the study was not more than FDA's advisory levels for DON and level the safe limit for baby foods and young children and level of DON in unprocessed wheat according to the European commission.

According to the growing time limits of 14 days has been performed in the lab, the authority of the *Aspergillus* species in compared with same time for *Fusarium* species DON production time that in study Akinsanmi, Queensland and Northern new South wales and after the days then carefully we can review or compare the research data with the data obtained in their researches.

CONCLUSION

The Aspergillus species molds can produce aflatoxin and ochratoxin under stress conditions. Aflatoxins are produced by the Aspergillus species; *A. flavus* and *A. parasiticus*. Ochratoxin is produced by Aspergillus (*A. ochraceus*) and Penicillium species (*P. viridicatum*) (Parish, 2008).

Some studies aimed to explore the fungal flora along with the DON concentration in the collected crop samples from markets to correlate between this flora and the detected DON. Whole collected grain farms or samples were from sampling areas represented imported and locally produced crops. Indicated and showed high incidence of Aspergilli. The High Performance Liquid Chromatography (HPLC) chromatogram of the samples showed high DON resolution. DON was detected in a range of 15-800 μg kg^{-1} in the collected samples although no Fusarium species was detected in these samples. The 200 μg kg^{-1} DON level (the safe limit for baby foods and young children) was exceeded by 50% of some of the imported samples. The presence of some toxigenic fungi in these samples should set the alarm of possible contamination of these samples with other mycotoxins during storage. However, the level of DON in all samples was within the permissible level of DON in unprocessed which is 1750 μg kg^{-1} according to the European commission) (Al-Hazmi, 2011).

According to amount of DON measured in samples of corn in the presence of toxin-producing Fusarium in Golestan and Ardabil (Moqan) provinces, Iran, Karami-Osboo *et al*. (2010), 76.7% of samples were in a range of 54.4-518.4 ng g^{-1}) while and the amount of toxin measured in samples of wheat in Jeddah, Saudi were in a range of 15-800 μg kg^{-1} in the collected samples in the absence of Fusarium specie, shows that when Fusaria are toxin-producing flora toxin then cases of Aspergilluses are toxin-producing amounts are more. So, the guess that some Aspergillus species parallel and play role a simultaneously the same as toxigenic Fusarium isolates produce DON is or like toxicants (Karami-Osboo *et al*., 2010).

According to the possibility of toxin producing Aspergilli isolation their family molecules could be released so toxicology risk assessment and effects on consumers should be considered more and more that of the cumulative effect of these toxins can be much more toxic than pure DON values measured in samples conducted in research or findings Ibanez-Vea *et al*. (2011), Navarra (Spain) by GC-MS prove DON can be index to indicate mycotoxins production proper conditions are to much relied by fungal contamination expectra, specially present in the sample basicly prove the beleaving ideas about Aspergilli potent DON production activities (Ibanez-Vea *et al*., 2011).

Thus, it could believe that species of the *Aspergillus* ssp., have to be more considered, as well as the most well known potent DON producers the generates, such as *Fusarium* ssp., which can be related to some of gene mutation in the *Aspergillus* ssp. and a great need to genomicrobiochemical investigations by related techniques must be regularity conducted till the hypothesis to be confirmed.

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