Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Online International Journal Available at http://www.cibtech.org/jls.htm
2013 Vol. 3 (4) October-December, pp.200-209/Parvaneh Rahsseparpoor
Research Article

AN INVESTIGATION ON *DEOXYNIVALENOL* (DON) PRODUCTION PATTERN AND QUANTITIES BY 24 *ASPERGILLUS* SPECIES FROM NORTHERN STATES OF IRAN

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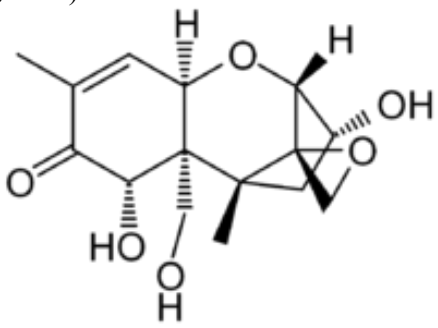
ABSTRACT
Mycotoxins are important chemotoxicant since they are mainly produced in large feed stock substrates where fungus producing these toxins finds optimum conditions for growth and development and they may become the cause of occurrence of some disease in humans and animals. Thus, their presence in foods and animals feed is a subject of global or national importance. Among them, *Aspergillus* has particular importance. In present study we have studied the production of deoxynivalenol toxin in cell extract of *Aspergillus* isolated indigenously in North of Iran. Firstly, sampling, culture and isolation was performed in Gilan and Mazandaran provinces. After recognition of the species, using ELISA and Ridascreen kit®, we quantitatively analysed deoxynivalenol produced by available species. Some of species have toxin production but amount of toxin production in a large number of isolates was small. The greatest number of isolated had produced toxin in 0-10 ppb range and given the fact that in this same range, the greatest amount of toxin production has taken placed in 0-3 ppb range. Despite its low concentration, we can take it seriously due to the collective effect of the toxins, as a critical risk factor, is not negligible. Since deoxynivalenol standard rate in foods is 3-5 ppb, therefore we can't overlook more than 6 % of isolates producing the toxin in 10-20 ppb and 20-30 ppb range or more than 3 % of isolates producing the toxin in 30-40 ppb and 80-90 ppb range. This can cause a serious biologic hazard by effecting public health.

Key Words: *DON, Aspergillus, Species, IRAN*

INTRODUCTION
Vomitoxin (IUPAC name: (3α,7α)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one), also known as deoxynivalenol, is a type B trichothecene, an epoxy-sesquiterpenoid (Gautam and Dill-Macky, 2011), occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale(Gautam and Dill-Macky, 2011).
Trichothecene mycotoxins are a group of structurally similar fungal metabolites that are capable of producing a wide range of toxic effects. Although deoxynivalenol is one of the least acutely toxic trichothecenes, it should be treated as an important food safety issue because it is a very common contaminant of grain (Rotter *et al.,* 1996).



Deoxynivalenol (Vomitoxin)

Vomitoxin (also known as DON, or refusal factor) gets its name from the resulting vomiting and feed refusal. It is produced by *Fusarium* species (*F. graminearum*) and is commonly found on above mentioned grains (Gautam and Dill-Macky, 2011).

Deoxynivalenol is a protein synthesis inhibitor, affecting the digestive tract and immune system. Cattle (may be the other Ruminants) are quite tolerant apparently due to rumen microbial activity of which have consumed up to 10 ppm (parts per million) of toxin with no adverse effects where as dietary dry matter concentrations of up to 21 ppm in growing diets were demonstrated to have no adverse effects on health or production performance. In cows 6.4 ppm deoxynivalenol fed for 70 days, showed no toxin residue founded in the milk (Jane, 2008)

FDA (U.S. Food and Drug Administration) advisory levels regarding feeds indicate that, in ruminating cattle deoxynivalenol contaminated grain and by-products should not exceed 50% with maximum deoxynivalenol levels of 10 ppm and 5 ppm in finished feed (Jane, 2008).

FDA's(U.S. Food and Drug Administration) Advisory Levels for Deoxynivalenol (vomitoxin) For grain and grain byproducts destined for commodities containing this level of vomitoxin not exceed 20percent of the ration 5 p.p.m, 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 percent of the ration for these species.) 10 p.p.m, For grain and grain byproducts destined for all other animal species. (FDA recommends that commodities containing this level of vomitoxin not exceed 40 percent of the ration.) 5 p.p.m.

EU DON Regulatory Levels Based on Tolerable Daily Intake X Uncertainty

Product	**Greatestleve (µg/kg)**
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

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 (Rotter *et al.,* 1996).

Deoxynivalenol is known to alter brain neurochemicals. The serotoninergic system appears to play a role in mediation of the feeding behavior and emetic response (Rotter *et al.*, 1996).

The main overt effect at low dietary concentrations appears to be a reduction in food consumption (anorexia), while higher doses induce vomiting (emesis) (Rotter *et al.*, 1996).

At low dosages of deoxynivalenol, hematological, clinical, and immunological changes are also transitory and decrease as compensatory/adaptation mechanisms are established, in part because of differences in deoxynivalenol metabolisms, with males being more sensitive than females. The capacity of deoxynivalenol to alter normal immune function knows a days has been of particular interests. There is extensive evidence that deoxynivalenol can be immuno modulating agents, immuno suppressive or immunostimulatory, depending upon the dose and duration of exposure (Rotter *et al.*, 1996).

While immunosuppression can be explained by the inhibition of translation, immunostimulation can be related to interference with normal regulatory mechanisms. In vivo, deoxynivalenol suppresses normal immune response to pathogens and simultaneously induces autoimmune-like effects which are similar to human IgA nephropathy (Rotter *et al.,* 1996).

Other effects include super induction 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). Further toxicology studies and an assessment of the potential of deoxynivalenol to be an etiologic agent in human disease are warranted (Rotter *et al.,* 1996).

MATERIALS AND METHODS

Sampling, Culture and Isolation

From the first 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 six 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 100ppm Chloramphenicol and 50ppm tetracycline, "a sample group" plates were withdrawn after 30, 60, 90 minutes and 15, 30, 60 minutes. All plates were incubated aerobicaly in 25±2 $^{\circ C}$ (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, 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$^{\circ C}$ 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, 2002b; Kozakiewicz, 1989; Samson *et al.*, 2001).

Morphological Studies

For morphological studies and macro and microscopic photobiometery the front and back of one week or two weeks aged colonies (two to four 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 in to 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 seven days of float or sink in the tubes of fluid and small Germ tube were purified by centrifuging at around 3000 rpm to 15 minutes and cellular biomasses were harvested. Masses washed for three 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 hours 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 minutes) with 5 ml sampling buffer using a tube mixer and glass globes (pearl) and each time 25 minutes grinding was performed. Mouldy mixture to each tube filtered samples and one ml of cold acetone added and of around 3,000 Rpm centrifuged (15 minutes) remaining a larger separation deposited (Moallaei *et al.*, 2006; Shadzi *et al.*, 1993). Supernatant samples treated by 1 to 5 ratio with cold acetone and then meintand in a cold 20 ° C for one to three days and finaly were centrifuged at around 20 000 RPM to 20 minutes 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 was 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 AND DISCUSSION

Results

Of totally 107 *Aspergillus* isolates, in the study of obtained, the maximum frequent was belonged to subgenus *Circumdati* with 66 isolates (%61.7) and the minimum frequent of subgenus *Fumigati* with 5 isolates (%4.7) (Table 1- Figure 1).

Table 2 and Figure 2 showing statistically frequency of identified species. According to table the greatest frequency species was *A. flavus* in contrast *A. af flavus* and *A. sp*V which were the lowest frequency species.

Aspergillus Isolates frequencies in conducted geographical areas showed that the most habitat was the East of Gilan with 68 samples (63/6%) and the lowest frequent was the West of Mazandaran with 9 samples (%8.4)(Table 3- Figure 3).

Amongst *Aspergillus* isolates 56 samples were from plants and 51 samples were obtained from farms (Table 4- Figure4).

In our study Biomass basic DON concentrations in the intervals 0-100 ppb showed that the highest frequent belongs to range of 0-10 ppb with 89 samples and 60-70 ppb with frequency of the only 1 sample was the lowest. Of range of 40-50, 50-60 , 70-80 and 90-100 ppb have been zero in frequency and no samples were founded (Table 5- Figure5). .

In the 89 biomass of *Aspergillus* isolates, study on basic DON concentration in the intervals 0-10 ppb showed that %81.3 of the toxin quantity were in the range of 0-3 ppb and range of 5-10 ppb was lower than the others with a zero frequency(Table 6- Figure6).

Table 1: Frequency of *Aspergillus* isolates at the subgenus

	Count of isolates	Percent	Cumulative Percent
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 2: Statistically frequency of identified species on samples

Species	Frequency	Percent	Cumulative Percent
A. af flavus	1	.9	.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	.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: Distribution of *Aspergillus* isolates in geographic areas

	Count of isolates	Percent	Cumulative Percent
East of Guilan	68	63.6	63.6
West of Guilan	30	28.0	91.6
West of Mazandaran	9	8.4	100.0
Total	107	100.0	

Table 4: Distribution of *Aspergillus* isolates in separate sampling locations

	Count of isolates	Percent	Cumulative Percent
Farm	51	47.7	47.7
Plant	56	52.3	100.0
Total	107	100.0	

Table 5: Distribution of DON concentrations in the intervals 0-100 ppb in fungal biomass

		Count of isolates	Percent	Cumulative Percent
Biomass/ELISA-DON	0_10	89	83.2	83.2
	10_20	6	5.6	88.8
	20_30	6	5.6	94.4
	30_40	2	1.9	96.3
	40_50	0	0	96.3
	50_60	0	0	96.3
	60_70	1	0.9	97.2
	70_80	0	0	97.2
	80_90	3	2.8	100.0
	90_100	0	0	100.0
	Total	107	100.0	

Table 6: Distribution of DON concentrations in the intervals 0-10 ppb of biomass

		Count of isolates	Percent
Biomass/ ELISA-DON	0_3	87	81.3
	3_5	2	1.9
	5_10	0	0
	Total	89	

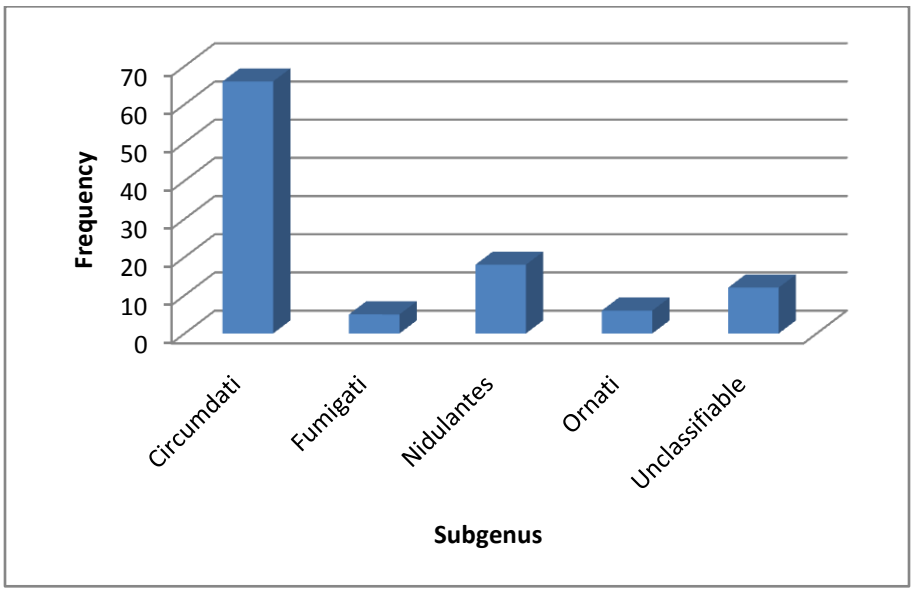


Figure 1: Frequency of *Aspergillus* isolates at the subgenus states

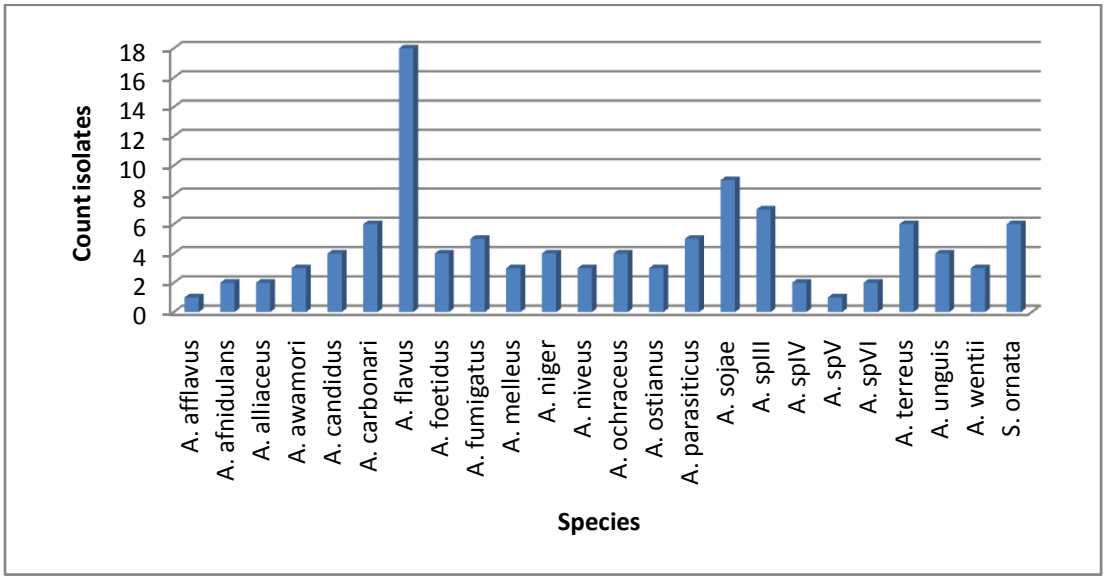


Figure 2: Statistically frequency of identified species on samples

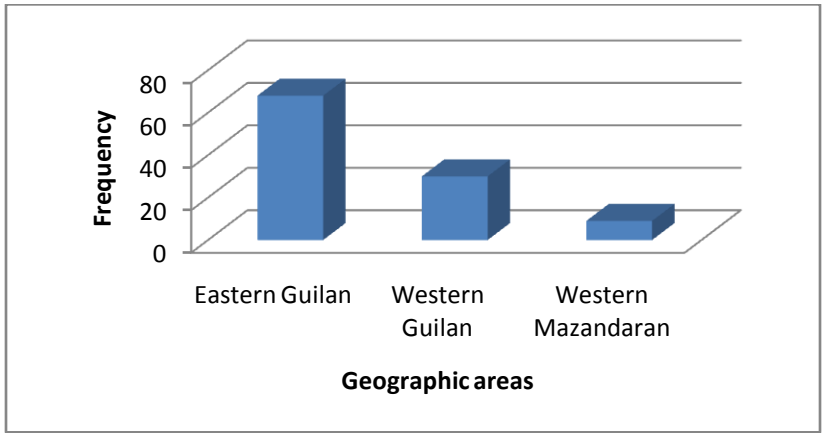


Figure 3: Distribution of *Aspergillus* isolates in geographic areas

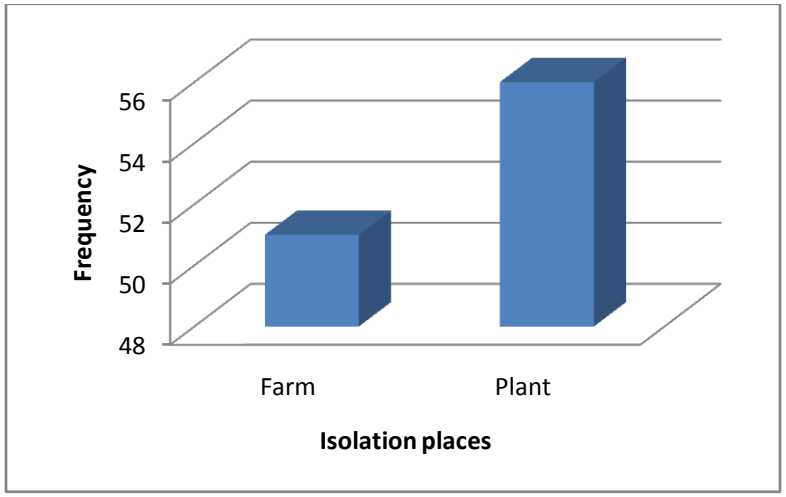


Figure 4: Distribution of *Aspergillus* isolates in separate locations

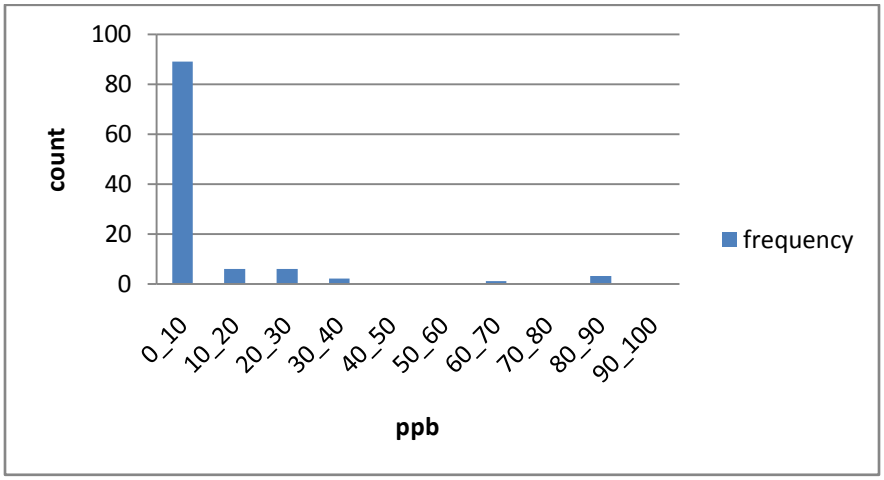


Figure 5: Distribution of DON concentrations in the intervals 0-100 ppb in biomass extracts

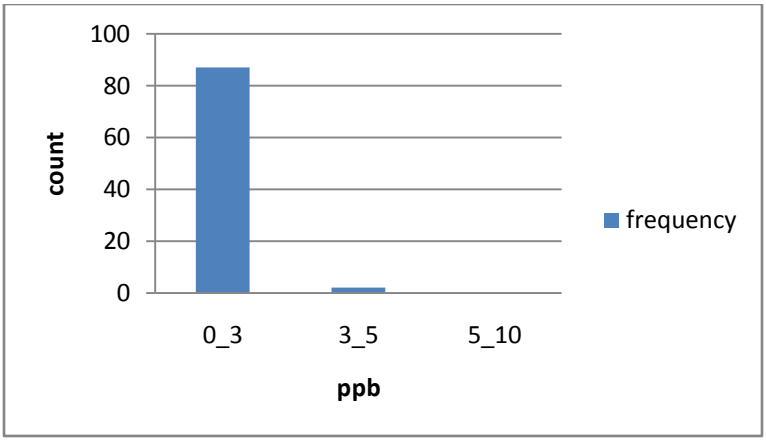


Figure 6: Distribution of DON toxin concentrations in the intervals 0-10 ppb of biomass

Discussion

Fortunately, among all isolates generating the toxin in 0-10 ppb range , more than 85% of them produced their toxin in 0-3 ppb range and lower than 2.5 in 3-5 ppb range. No one had measurable toxin genesis in the range of 5-10 ppb (table 6 and figure 6).

According to the fact that the greatest number of isolates produced the toxin in 0-10 ppb and according to the fact that in this range, the greatest amount of toxic genesis was in the range of 0-3 ppb, despite the low amount of produced toxin, it is possible to take as a serious danger by taking its cumulative effect in to account. Since DON standard amount in the food stocks is 3-5 ppb but more than 6% of isolated producing the toxin in the range of 10-20 and 20-30 or more than 3 % of isolates producing the toxin in the range of 30-40 and 80-90 ppb must not be overlooked(table 5, 6 and figure 5, 6).

Conclusion

Amounts of DON concentration obtained of *Aspregillus* species in our 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 of Al-Hazmi's (2010) finding working on wheat samples from Jeddah, Saudi Arabia ,could be accepted that without the presence of *Fusarium* species by which DON and its related compounds might to be produce, have more popularity, even *Aspergillus* species isolates have seriously

considered of view to produce DON and same of compounds, So imagine us the fact that proves at the beginning of *Aspergillus* species the ability to produce DON and also determines the amount of toxin produced by them in their biomasses and toxin Leakage size or the in growth medium(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.,* (2004-2005), 76.7% of samples in were a range of 54.4-518.4ng/ g) while and the amount of toxin measured in samples of wheat in Jeddah, Saudi, were in a range of 15 to 800 µg/kg 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 our 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). So our advantages about DON producing *Aspergilli* could be beleaved simply.
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 *et al.,* (2003), Queensland and northern New South Wales) and after the days then carefully we can review or compare our research data with the data obtained in their researches (Akinsanmi *et al.,* 2003).
According to genes the promote or regulate toxin production in *Aspergillus* and *Fusarium* species, special those that are pathogenic effects on plants, and their known decisive role in toxin production could be suggested that Same genes and regulatory process similarly to what exists in *Fusarium* species and provides the possibility production of DON and family molecules in *Aspergillus* considered, might prove by which DON or their similarities and differences, until be To exploit the inhibition of toxin production in food products (McDonald *et al.,* 2005).
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 Ibáñez-Vea *et al.,* (2007), (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 basically prove our beleaving ideas about *Aspergilli* potent DON production activities(Ibáñez-Vea *et al.,* 2011).
Thus it could believe that species of the *Aspergillus* spp have to be more considered as well as the most well known potent DON producers the generates such as *Fusarium* spp, which can be related to some of gene mutation and gene diversity in the *A. spergillus* spp and needing to genomicorbiochemical techniques.

ACKNOWLEDGMENT
With thanks a lot of Division of Research's Lahijan Islamic Azad University in Iran, that was supported my study.

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