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DETERMINATION OF THE DEOXYNIVALENOL (DON) PRODUCTION PROFILE BY THE *ASPERGILLUS* ISOLATES IN THE NORTH OF IRAN

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ABSTRACT

Mycotoxins are important pathogenic since they are mainly over grown in large feed stock substrates where fungus producing some extrolites. Since among them, *Aspergillus* has particular importance due to some guesses, in present study we performed a series of analytical assays on the production of DON toxin in cell extract of *Aspergillus* spp isolated indigenous in Northern Iran states. Firstly, sampling, culture and isolation was performed in Guilan and Mazandaran provinces. After recognition of the species, using ELISA, we quantitatively analyse DON produced by available cellular extracts. In identified species the maximum produced DON is obtained by species *A.sp* VI (subgenus Unclassifiable; 34.93 ppb) and minimum produced DON observe in the *A.carbonarius* (subgenus circumdati ; 1.03 ppb).

KEY WORDS: *Mycotoxin, Aspergillus, DON (Deoxynivalenol), Species, Iran*

Introduction

Fungus are eukaryotic organisms placed in the Kingdom Mycota. They can also producer metabolic products which initial known as metabolites are products of cellular regular metabolism distributed abundantly in the nature and are found in all the genus even. Secondary metabolites are the cell regular metabolism product will limit abundance and they are not present in all the species of a population, Seemingly don't have any benefit for the fungi cell itself (Alborzi et al., 2006; Gams et al., 1998). Mycotoxins along with other secondary metabolites such as antibiotics and so on are compounds produced at final periods of moulds and are recognized as exponential phase metabolites (Alborzi et al., 2006; Allcroft et al., 1963). Generally, toxigenic strains are found in all the major classified groups of fungi. The most known mycotoxins are metabolic products of the genus: *Aspergillus*, *Fusarium*, *Penicillium*, *Klaviseps*, *Althernaria*, Stocky *butris*, *Fuma* and Diploid (Dragacci et al., 1996). Food and Agriculture organization (FAO) estimated that approximately 25 % of world crop yields have mycotoxins and currently, they are considered as a contaminant in foods and feeds (Iheshiulor et al., 2011). American Food and Drug organization (FAD) consider the recommended level of deoxynivalenol in products produced by wheat which it doesn't exceed 5 ppm in lactating cows and is used in lower than 40 % concentrations. In fact a maximum 2 ppb, concentration is recommended in total diet of lactating cows by this organisation (García., 2008).

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EU DON Regulatory Levels Based on Tolerable Daily Intake X Uncertainty

Product	Maximum level (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
Bread, pastries, biscuits, cereal snacks and Breakfast cereals	500
Pasta (dry)	750
Processed cereal-based food for infants and Young children and baby food	200

According to the tendency to the target texture of mycotoxins, they are designed by various names such as hepatotoxin , neurotoxin, nephrotoxin, cardiotoxin, d ermatotoxin, etx (Bennet et al., 2003). Trichotoxins are most important mycotoxins produced through the movalanat path way. Deoxynivalenol (IUPAC: (3α,7α)-3,7,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) is a trichotoxin with lowest toxicity. Deoxynivalenol is a type B trichothecene, an epoxy-sesquiterpenoid, However Deoxynivalenol is a low toxic trichotoxin the common and frequent trichotoxins are in human food and its presence is an indication of possible outbreak of other more toxic trichotoxins, also occurs predominantly in grains such as wheat, barley, oats, rye, and maize, and less often in rice, sorghum, and triticale (Al-Hazmi,٢٠١١ ; Cirilli et a., 1988). Since *Aspergillus* has particular importance due to some guesses, in present study we performed a series of analytical assays on the production of DON toxin in cell extract of *Aspergillus* spp isolated indigenous in Northern Iran states.

Materials and Methods

Sampling, culture and isolation

From the first days of May to late October (2011 years) in the provinces of Guilan 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; Pittet, 1998;Rodger, 2001;Samson et al.,2000; Samson et al.,2006; Samson et al.,2007). 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 mixed with 100ppm Chloramphenicol and 50ppm tetracycline to withdraw "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; Pittet, 1998; Rodger, 2001; Samson et al., 2000; Samson et al.,2006; Samson et al., 2007) . Till 15 days all plates were investigated to 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, Sabro dextrose agar, Czapek-Yeast agar and Czapek-Dox agar plates, all the new found mould samples were restored and they were followed by prestove

program and macro and microscopic properties in the 5 , 10, 15 days span and then were recorded (Klich., 2002a; Kozakiewicz, 1989; Pittet., 1998; Rodger., 2001; Samson et al., 2000; Samson et al., 2006; Samson et al., 2007). At the end of 300 *Aspergillusy* colony the 150 ones selected colonies in plates with czapek-Doux agar, Czapek-yest extract (with and without sucrose 20%) , malt extract agar, Czapek- Dox Agar (with and without sucrose 20%) which has been cultured for morphological Macro and Microscopic incubation at 37 $^{\circ}$C and after 3, 7, 14, 25 and sometimes 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 and keeping in incubator were not performed (Klich., 2002a; Klich., 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 photograph, cells, and umbrellas, hyphae, stypes, the conidies crown and micrometerics 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 were incubated and were 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. 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 filter sample and one ml of cold acetone added and of around 3,000 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 juice 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 and using RIDASCREEN® DON (Art. No.: R5906) which is a competitive enzyme immunoassay for the quantitative analysis of DON in feed and foods.

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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 solution, then deoxynivalenol enzyme conjugate and anti – deoxynivalenol antibodies are added. 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 sample.

Results and Discussion

Of totaly 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 1 - Frequency of *Aspergillus* isolates at the subgenus

		Frequency	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
	Unclassified	12	11.2	100.0
	Total	107	100.0	

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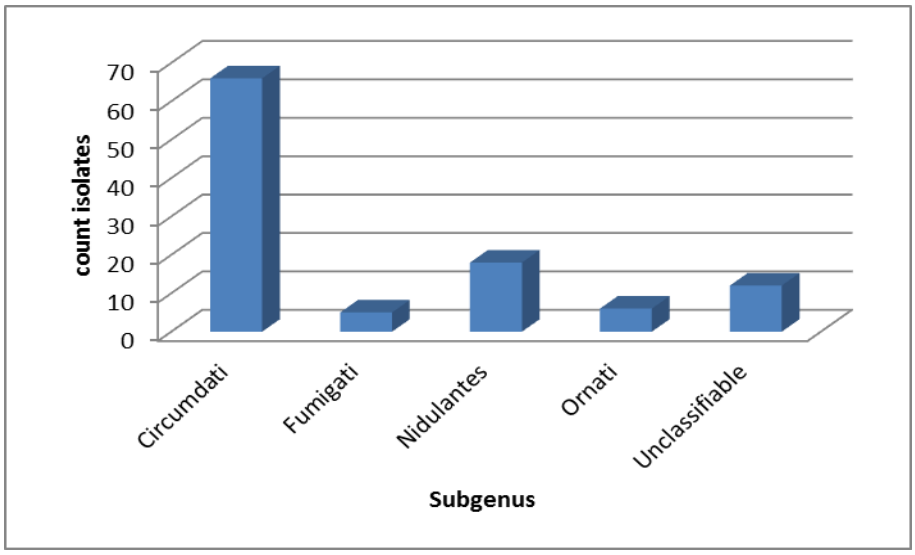

Figure 1 - Frequency of *Aspergillus* isolates at the subgenus states

According to table the most frequent species was *A. flavus* incontrast *A. afflavus* and *A. sp* V which were the least frequent species (Table 2- Figure 2).

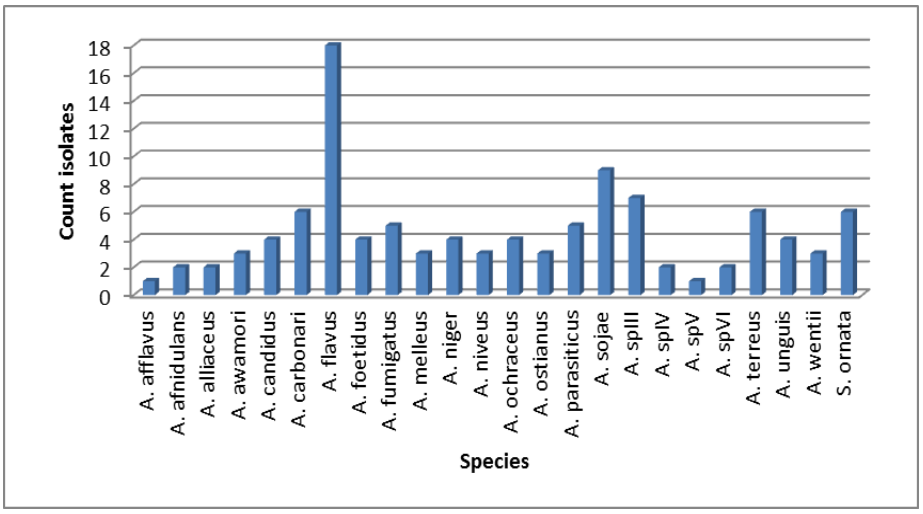

Figure 2- statistically frequency of identified species on samples

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Table 2- statistically frequency of identified species on samples

	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 showing DON concentration frequency based on the subgenus in the range of 0-100 ppb in the biomass. In subgenus *Circumdati* the greatest frequency of DON concentration was in the range of 0-10ppb (54 isolates) and the least DON concentration frequency was in the range of 60-70ppb (1), in subgenus *Funmagati* the greatest frequency was in the range of 0-10ppb (3) and the least frequency was in the range of 10-20ppb (2), in subgenus *Nidulantes* the greatest frequency was in the range of 0-10ppb (16) and the least frequency was in the range of 20-30ppb (2), in subgenus

Ornati the greatest and lowest frequencies, were 0-10ppb (5) and 10-20(1) respectively, in subgenus Unclassifiable the greatest and least frequencies were 0-10ppb (11) and 80-90 ppb (1) respectively. Table 4 the definitive concentration frequency of DON in biomass of the species in the range 0 to 100 ppb indicating that species *A. af flavus* has the only one isolate observed in the range of 0 to 10 ppb, *A. af nidulans* 2 isolates *A. alliaceus* in the range of 0 to 10 ppb and even 60 to 70 ppb has one isolate in each ranged intervales, *A. awamori* showed 3 isolates only at o to 10 ppb the same as *A. candidus* 3 isolates for the range of 0 to 10 ppb and 10 to 20. of *A. carbonarius* 6 isolates observed only in the range of 0 to 10 ppb, *A. flavus* isolates were 16 for 0 to 10 ppb and 2 for 80 to 90 ppb. Isolates of *A. foetidus* were only 4 in the range of 0 to 10 ppb like *A. fumigatus* 3 isolates for the range 0 to 10 ppb and 2 for the range 10 to 20. Reflecting to *A. melleus* 2 isolates were in the range of 0 to 10 ppb and one in the range of 10 to 20 ppb. *A. niger* 4 isolates observed only in the range of 0 to 10 ppb like *A. nievus* one isolate for the 0 to 10 ppb in contrast 2 isolates in the range of 20 to 30 ppb. In the *A. ochraceus* observed cases only 4 in the range 0 to 10 ppb, and *A. ostianus* 3 isolates concomitantly the only one case for *A. parasiticus* in the range of 0 to 10 ppb and 2 isolates for the each range of 20 to 30 ppb and 30 to 40 ppb. About cases *A. sojea* 7 cases in the range of 0 to 10 ppb and 2 isolates in the range of 20 to 30 ppb were observed. In the species of *A. sp* III we observed 6 isolated in the range of 0 to 10 ppb and one in the range of 80 to 90 ppb. *A. terreus* observed isolates were only 4 in the range of 0 to 10 ppb, likely 2 cases of *A. wenti* this range 0 to 10 ppb and one for the range of 10 to 20 ppb. In the species *S. ornata* 5 cases were obtained toxigenic at the range of 0 to 10 ppb and only one isolates in the range of 10 to 20 ppb .

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 3- Figure 3).

Table 3 - 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	

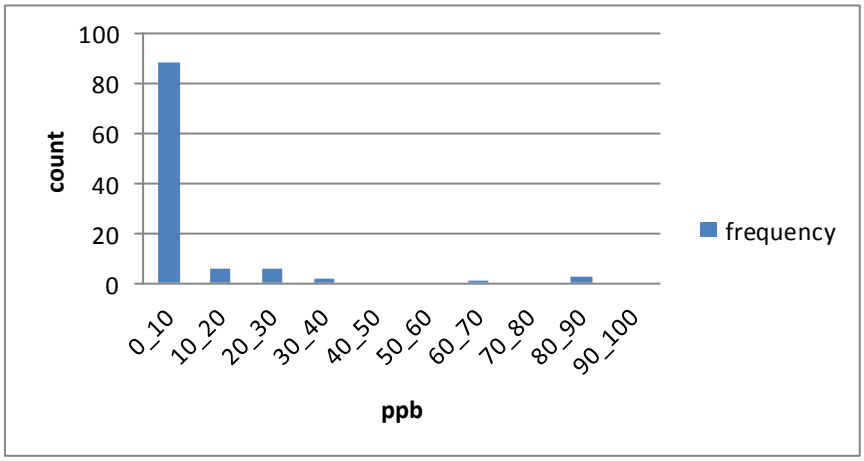

Figure 3 - Distribution of DON concentrations in the intervals 0-100 ppb in biomass extracts.

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 4- Figure 4).

Table 4- 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	

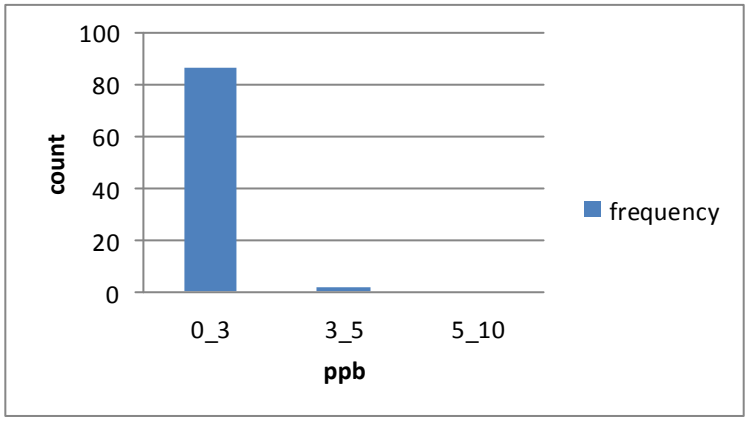

Figure 4- Distribution of DON toxin concentrations in the intervals 0-10 ppb of biomass

Conclusion

It could believed that species of the *Aspergillus* have to considered as a potent DON producers in contrast with the well known Generate such as *Fusarium spp* . Amongst identified species the Maximum produced DON were obtained by species graups; *A. sp* VI with 2 isolates (34.93 ppb), *A. ochraceus* with 5 isolates (21.62 ppb) and *S. ornata* with 3 isolates (15.34 ppb). Minimum DON production observed in the *A. carbonarius* with 6 isolates (1.03 ppb), *A. terreus* with 6 isolates (2.72 ppb), *A. sp* V with 3 isolates (3.99 ppb) concomitantly and DON intermediate producers were only the *A. flavus* with 7 isolates (11.96 ppb), *A. awamori* with 18 isolates (9.03 ppb), and *A. unguis* with 3 isolates (7.72 ppb). In identified species *A. af nidulans* (with 2 isolates), *A. alliaceus* (6 isolates), *A. foetidus* (3 isolates) *A. fumigatus* (4 isolates), *A. melleus* (2 isolates), *A. niger* (4 isolates) *A. niveus* (3 isolates), *A. ostianus* (1 isolates), *A. parasiticus* (4 isolates), *A. sojae* (2 isolates), *A. sp* III *(*1 isolates), *A. sp* IV (4 isolates) never produced DON (0 ppb) or never detected by the used kit (- ppb). Although the amount of toxin produced in a large number of isolates was trace but the cumulative effect of toxin in the body weight should not be overlooked (Table 4).

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 3 and figure 4). 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 3, 4 and figure 3, 4). Amounts of DON concentration obtained of *Aspregillus* species in our study was not more than FDA's Advisory Levels for DON or level the safe limit for baby foods and young children and level of DON in unprocessed wheat according to the European Commission.

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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 O.A.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(O.A. Akinsanmi"et al"2003) . According to the of Al-Hazmi's 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 be produced, have more popularity, even *Aspergillus* species isolates have seriously considered of view to produce DON and same compounds, so based of our findings imagine us the fact that proves the beginning steps of *Aspergillus* species with an 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, R. 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 (R. Karami-Osboo "et al" 2010). So our advantages about DON producing Aspergilli could be beleaved simply. 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 T"et al"2005). Thus it could believe that species of the genus *Aspergillus* have to be more considered as well as the most well known potent DON producers the genera such as *Fusarium* spp, can be related to some of gene mutation and gene diversity in the *Aspergillus* spp needing to genomicorbiochemical investigations by related techniques.

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