Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231-6345 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2014 Vol. 4 (3) July-September, pp. 72-76/Moafi and Modiri
Research Article

THE PRODUCTION PATTERN AND THE SIZE OF TOTAL AFLATOXIN IN ASPERGILLUS SPECIES IN NORTHERN IRAN

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ABSTRACT
A lot of efforts, resources and time have been committed to Aflatoxin research work since the early1960s. Sampling was done according to "CBS" instructions from indoor and outdoor stations.in HPLC measuring, we first purified our sample of all Aflatoxins, We used imonoaffinty columns, and finally the device compared average of the results of the samples curve areas with average of the results of standard curve areas and showed the effective material value based on ppb. All samples prepared for an indirect competitive ELIZA assay for quantitation of total Aflatoxin based on manufacturer instructions. Geographical distribution of 107 mentioned samples and The frequency of subgenuses in different regions, The minimum, average and maximum amount of toxins, The average amount of produced toxin in culture medium were determined. In comparison, the average of total aflatoxin with ELIZA method in biomass the highest examined amount is in the range of 20-55 ppb which is produced by fungi such *as A. nidulans, A. fumigates, A. flavus and A. soja*. In biomass, the correlation between total Aflatoxin measured by ELIZA method and the average amount of total B subgroup was reversed relation. In examining the correlation between the average amount of total Aflatoxin measured by ELIZA method and average of Aflatoxin of B1, B2, G1, G2 measured separately by HPLC, there isn't any reverse relation and meaningful numeral differences. In culture medium, checking the average value of total aflatoxin which is measured by ELIZA method with the average of aflatoxin of B1 which is measured by HPLC, we can see a correlation and there is a meaningful numeral difference.

Keywords: *Pattern, Aflatoxin, Aspergillus, Northern Iran*

INTRODUCTION
Aflatoxin derivatives are carcinogenic, mutagenic, teratogenic and immunosuppressive and can be found in grains, nuts, cottonseed and other commodities associated with human food or animal feeds. Aflatoxin are highly toxic and carcinogenic metabolites produced by fungi of *Aspergillus* genus. There are serious problems in many countries of the world, especially developing countries and occur in almost all agricultural products (Shan *et al.,* 2007; Kankar *et al.,* 2005; Bankola, 2004).

A lot of efforts, resources and time have been committed to Aflatoxin research work since the early1960s (Windhom *et al.,* 2002). Aflatoxin often occur in crops in the field prior to harvest. Postharvest contamination can occur if crop drying is delayed or if water is allowed to exceed critical values during storage leading to mold growth (Craufurd *et al.,* 2006). Aflatoxin are produced member of *aspergillus* are common and widespread in nature (Gupta *et al.*, 2002).

They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high humidity environment or damage from stressful conditions such as drought, a condition which brewers the Barrier to entry (Kankar *et al.,* 2005; Ayejuyoo *et al.,* 2008).

In recent years, enzyme linked Immuno sorbent assays have been described, adapted and fully calibrated for aflatoxin determination assays or quantitation.

ELIZA methods potentially have many valuable advantages over the other procedures because of their simplicity, sensitivity, low cost and the use of safe techniques and regents. Extensive studies of aflatoxinin food have validated ELIZA, compared to very accurate, but expensive, low throughput research-oriented techniques, such as HPLC and other primitive chromatography methods (Chiou 2002; Gunterus, 2002).

MATERIALS AND METHODS

From the first days of May to the last days of October 2011, sampling was done according to "CBS" instructions from indoor and outdoor stations. One sample group was taken from among 50000 meter square area fields and also per processing plant using settle plates based on CBS rules too. Six plates including Malt extract agar (MEA), Yeast extract agar(YEA), Czapeks agar (CZA), Czapeks Yeast extract agar (CZYA), Saboruds dextrouse agar (SDA) and Potato dexterous agar (PDA), all with 100 ppm chloramphenicol and 50 ppm tetracycline were applied for one sample group. Plates removed after 30, 60 and 90 minutes for outdoor and 15, 30 and 60 minutes for indoor sites, respectively. All the plates were incubated at 25±2°C aerobically, then examined in the periods of 3, 7 and 15 days. We could identify any growing so that they were marked and harvested then cultivated in the prepared plates. Finally, for macroscopic and microscopic morphology examinations, 107 colonies were cultivated and grown at 25±2°C. In order to identify and rank the colonies, various methods were used. Front and back of one-two week colonies were selected for morphologic examinations and microscopic and macroscopic photography. Measurement of the colonies width, examination of front and back color of pigments, photography of mushroom umbrella, cells and grown masses, and also examination of creation and micrometry of asks were done by stereoscope. In all of the samples, micrometry and photography of conidiophores, wisicole and asks were done by micro analysis microscope.

HPLC Measuring

In order to do HPLC process, we first purified our sample of all *Aflatoxins*. We used imonoaffinty columns. According to the test instructions, extraction of toxin was done by using methanol solution and water. Initially, we injected extracts to the columns. The column includes gels containing antibodies specific for Aflatoxin B and G variants. At the next step, the column was washed by PBS solution. This leads to remaining B and G Aflotoxin which attached to gel and removal of other materials of cell extract. Then, the column was washed by special ethanol solution.

The washing method was a two-phase process; at first phase, 1250 ml and then 500 ml of the solution was added to the column in such a way that whole of it was washed carefully. Finally, the attached toxin, was separated from the column using methanol and collected into particular vial.

Preparation and Work with Device

The mobile phase including one of water, methanol, acetonitrile, phosphate buffers or a mixture of them, was entered in the column and poured into the device bottle. Then the detector was turned on and set to 360 nm wave length and the electronic recorder switched on and speed of mobile phase current justified based on RP. Finally, the device was placed in the current, for 30 to 45 minutes without injection. After 45 minutes, absorption of the mobile phase was stopped, system suitability test was added and the results recorded. As a result, resolution factor, middle peaks, tailing factor, number of sub-pages of the column for measuring test material (theoretical plates), and capacity factor were calculated.

After this step, the standard solution was injected 5 times to the device and its scale value was obtained. This value was bigger than %98. Then each test standard was injected 3 times and reference standard solution injected, afterwards. Finally the device compared average of the results of the samples curve areas with average of the results of standard curve areas and showed the effective material value based on ppb.

ELIZA

All samples prepared as mentioned above for an indirect competitive ELIZA assay for quantitation of total Aflatoxin based on manufacturer instructions. For all samples and standards, we recorded the estimated situation and then corrected the data based on standard curve obtained by an ELIZA reader calibrated by 450 nm UV light for comparing the density of samples and standard OP and preparing final results (Prentice, 1999).

Statistical Analysis

In this trial, we utilized k2 independence test and Anova table, which are used to show correlation or irrelation of two classified variables (Bhat, 2003).

RESULTS AND DISCUSSION

Geographical distribution of 107 mentioned samples is as follows: 68 samples (%63.3) are from Eastern Gilan, 30 ones (%23) from Western Gilan and 9 ones (%8.4) from Western Mazandaran, with the least share of Genus Aspergillus samples.

The frequency of subgenuses in different regions is as follows: *Circumedati* determined in 56 samples (%61.68) - the highest frequency, *Nidulantes* in 14 samples (%13.1), *Ornati* in 6 samples (%5.6) and 16 samples (%15) were grouped as unknown subgenus members as A. sp I-VI. Subgenus *Circumdati* including: *A. alliaceus* (n=2), *A. awamori* (n=3), *A. candidus* (n=4) *A. carbonarius* (n=6), *A. flavus* (n=17), *A. foetidus* (n=4), *A. melleas* (n=3), *A. niger* (n=4), *A. ochraceus* (n=4), *A. ostianus* (n=3), *A. parasiticus* (n=5), *A. soja* (n=8), *A. wentii* (n=3). Subgenus species of *fumigati* include *A. fumigatus* (n=5), subgenus species of *Nidalantes* include *A. niveus* (n=3), A. terreus (n=7), A. unguis (n=4) and subgenus species of ornate include S.ornata (n=6) and unknown subgenus types including *A. Af flavus* (n=1), *A. af nidulans* (n=2), A. sp III (n=7), A. sp IV(n=2), A. sp v(n=2), A. sp VI (n=2).

The minimum, average and maximum amount of toxins in *cirumdati* species in biomass extracts are as follows: for *A. ochraceus* 0, 17.94 and 40.75 ppb; for *A. ostianus* 39.20, 41.53 and 48.42 ppb; for *A. melleus* 33.22, 39.12 and 44.48 ppb; for *A. candidus* 30.92, 36.12 and 42.52 ppb; for *A. flaves* 0, 22.46 and 49.506 ppb; for A. *sojea* 0, 21.61 and 55.06 ppb; for *A. parasticus* 26.79, 30.83 and 35.06 ppb; for *A. allaceus* 0, 14.66 and 29.32 ppb; for *A. awamori* 0, 9.11 and 27.35 ppb; for *A. carbonarius* 0, 6.35 and 38.11 ppb, respectively. The minimum, average and maximum amount of toxins in subgenus *Nidulnates* is as follows: 0, 23.75 and 47.50 ppb, respectively. In *Ornata* subgenus *ornati* minimum, average and maximum were 0, 28.17, 45.62 ppb, respectively. Also for *Fumigati, A. fumigatus*, minimum, average and maximum of toxin was 0, 22.15 and 41.69 ppb. Finally in unknown sample (sp III) the minimum of produced toxin was 38.16 ppb, its average amount was 39.55 ppb and its maximum amount was 39.95 ppb.

The average amount of produced toxin in culture medium was as follows: in subgenus *Circumdati, A. foetidus* 2.58 ppb; in *A. mellus* and *A. ochraceus*, the 0; in *O.ostianus*, 2.74 ppb; in *A. cantidus* 0.3 ppb and in *A. flavus* 0.45 ppb. About subgenus *A. wentii*, the average of toxin was 0; in *A. sojen* 0.39 ppb; in *A. parasiticus* 0.29 ppb; in *A. alliaceus* 0.49 ppb; in *A. niger* and 0.22 ppb; in *A. awamori* 0.25 ppb and in *A. carbonarius* 90.28. The average amount of produced toxin in *Oranati*, subgenus *S.ornati* was 0; in *fomigati* 0.6 ppb; in nidulantes was 1.31 ppb and in unknown genus from 0.40 ppb to 0.92 ppb.

The maximum amount of toxin produced in different species was as follows:

1: *A. carbonarius* from *circumdati* genus with 6 isolates including 18.55 ppb of produced toxin
2:*A. foetidus* from unknown genus whit 2 isolates including 1.91 ppb of produced toxin
3*: A. foetidus* from *circumdati* genus whit 4 isolates including 1.41 ppb of produced toxin
4: *A. sojea* from *circumdati* genus whit 8 isolates including 0.77 ppb of produced toxin
5: *A. ostianus* from *circumdati* genus whit 3 isolates including 0.60 ppb of produced toxin
6*: A. parasiticus* from *circumdati* genus whit 5 isolates including 0.57 ppb of produed toxin
7: *A. niger* from *circumdati* genus whit 4 isolates including 0.43 ppb of produced toxin
8: *A.* af *froavus* from unknown genus whit 1 isolates including 0.35 ppb of produced toxin
9: *A. alliaceus* from *circumdaty* genus whit 2 isolates including 0.34 ppb of produced toxin
10: *A. fumigatus* from *fumigati* genus whit 5 isolates including 0.12 ppb of produced toxin

In comparison, the average of total aflatoxin with ELIZA method in biomass the highest examined amount is in the range of 20-55 ppb which is produced by fungi such *as A. nidulans, A. fumigates, A. flavus* and *A. soja*. The maximum amount in the production of toxin is in the range of 40-45 ppb which is produced by *A. ostianus*, the minimum amount of whole aflatoxin is more than 5 ppb and its average is less than 40 ppb. Also average value of total aflatoxin calculated by ELIZA method in culture medium, was in in the range of 0-2 ppb that has been seen by fungi such as *A. nidulans, A. flavus, A. fumigates* and *A. niger*. The maximum amount of toxin production was in the range of 8-10 ppb. The minimum value of total Aflatoxin was more than 2 ppb and the average of its maximum is less than 4 ppb.

In biomass, the correlation between total Aflatoxin measured by ELIZA method and the average amount of total B subgroup which is confirmed with the amount of Sig: 0/000 and z: -3/621, is a reverse relation. The numeral difference of the above measurements isn't meaningful. Also measuring the correlation between total Aflatoxin with average amount of aflatoxin of B1 measured by ELIZA method, is a reverse relation (z=-3/621and sig=0/000), as well. Numeral difference of above amounts isn't meaningful, too.

In examining the correlation between the average amount of total Aflatoxin measured by ELIZA method and average of Aflatoxin of B1, B2, G1, G2 measured separately by HPLC, there isn't any reverse relation and meaningful numeral differences.

In culture medium, checking the average value of total aflatoxin which is measured by ELIZA method with the average of aflatoxin of B1 which is measured by HPLC, we can see a correlation and there is a meaningful numeral difference (Sig: 0.000, pc: 0.930).

Measuring total Aflatoxin and Aflatoxin B by ELIZA method shows that greater average of measured toxin, more important role of analogue molecules in increment of quantity of the measured toxin. This can explain the non-alignment behavior in total Aflatoxin and Aflatoxin B quantity during ELIZA measurement. According to the fact that numeral difference resulted from toxin measurement in statistical examinations has been meaningful, so we can find out that except the confirmed correlations and non alignment between them, the numeral difference regarding to error tolerance of commercial kits is reasonable and this led to the meaningful difference.

In examining meaningfulness of the numeral difference and the kind of correlation between average values of whole Aflatoxin and Aflatoxin B and B1, each one separately measured by HPLC, no meaningful numeral difference between the cases was seen and also no significant correlation despite reverse relation, we can point to above cases (Khurgami *et al.,* 2012; Vahidi *et al.,* 2012; Mehdikar *et al.,* 2011).

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