Occurrence of *Aspergillus* spp. and aflatoxin B1 in stored barley grains marketed in west of Iran

Khosrow Chehri[1] and Hassan Younesi[2]

[1]*Department of Biology, Faculty of Sciences, Razi University, Kermanshah, Iran*
[2]*Agricultural and Natural Resources Research Center, Kermanshah, Iran*
[*]*Corresponding author's email: khchehri@gmail.com*

Abstract

Barley is one of the most important cereals consumed in the world, which is well-known to be invaded by *Aspergillus* spp. under storage conditions and contaminated with mycotoxins. Aflatoxin (AFL) contamination of cereal grains especially barley is one of the serious economic and food security issues. This study aimed to assess the mycological and AFL contamination of barley collected from market in Kermanshah province, in west of Iran. Forty samples were collected at informal markets in ten cities and analysed for aflatoxin B_1 using enzyme-linked immune sorbent assay (ELIZA) method. Sixty *Aspergillus* isolates were collected and four species were identified. All these strains belonged to *A. niger* (50%), *A. flavus* (30%), *A. japonicus* (15%), and *A. sclerotiorum* (5%). Further confirmation of all the *A. flavus* isolates was carried out on molecular basis through species specific primers of AFLA-F/AFLA-R. Natural occurrence of AFB_1 was detected in 18 samples with a range between 0.056–10.564 μg kg^{-1}. The highest AFB_1 level was found in samples from Sarpol-e-Zahab (10.564 μg kg^{-1}). This is the first report of the natural occurrence of AFB1 from stored barley samples collected from west of Iran.

Keywords: *Aspergillus,* Aflatoxin B_1, Barley, Iran, Species specific primers.

Introduction

Barley (*Hordeum vulgare* L.) is strategic agricultural crops worldwide, serving as food and feed for man and livestock. Different storage fungi especially species of *Aspergillus* and *Penicillium* contaminate grains of cereals (Shafique *et al.,* 2005, 2007), pulses (Javaid and Khan, 2019), and trees (Javaid *et al.,* 2010) during storage. Like any other cereal grains, barley is also contaminated with toxigenic fungi especially *Aspergillus* spp. (Klich, 2007; Ibrahim *et al.,* 2023). Kermanshah is one of the largest production areas of planting barley in Iran and annual average of over 174000 ha of barley was harvested in this province (Anonympus, 2020). In cereals, infection with *Aspergillus* spp. reduces the quality of grains (FAO, 2004; Lasram *et al.,* 2016).

Aflatoxins constitute a class of highly carcinogenic secondary metabolites which are produced by various strains of *A. parasiticus* and *A. flavus* (Lasram *et al.,* 2016; Ráduly *et al.,* 2020). Aflatoxins based on their chemical structure are classified into two broad groups: aflatoxins B_1 (AFB_1) and B_2 (AFB_2) (Rasch *et al.,* 2010). AFB_1 was evaluated as the first class of human and animal carcinogen and has been found in most staple feed and foods, *e.g.* cereal grains and nuts (Raduly *et al.,* 2020).

Many studies have shown that Aflatoxin B_1 (AFB_1) is repeatedly present in different ceops especially barley seed (Ráduly *et al.,* 2020). Hence different researches reported the eventuality of toxigenic fungal contamination during storage of cereals grains, However, until now, not much study has been done in the field of identifying Aspergillus species associated with barley in Kermanshah province in Iran. Therefore, our purpose was to isolate and molecular identification of *Aspergillus* spp. and detection of AFB_1 in stored barley grains collected from different areas in Kermanshah province.

Materials and Methods

Isolation and identification of *Aspergillus* spp.

During 2014-2015 growing seasons, barley grains samples were obtained from 40 differnt markets and in west of Iran. Isolation of *Aspergillus* spp. were done from the barley grains by the method of Chehri *et al*. (2015). The barley seeds were plated on half-strength potato dextrose agar (PDA) amended with rose bengal (50 ppm) and incubated at 25 °C for one week. Then *Aspergillus* species were identified on single-spore basis according to Klich (2002). Morphological identification of *Aspergillus* spp. were done by the method of Chehri *et al.* (2015).

Extraction of DNA and molecular identification

The isolation of DNA from *Aspergillus* strains were performed according to Chehri *et al.* (2014). Extraction of DNA was carried out using the DNeasy® Plant Mini Kit (Qiagen). The identification of all *A. flavus* isolates was confirmed with species-specific PCR assay (Hue *et al.,* 2013).

Enzyme-linked immune sorbent assay (ELISA)

analysis

Content of AFB_1 in the 40 subsamples was analyzed by Quantitative Aflatoxin Test Kit (EuroProxima, Netherland) as described by Chehri *et al*. (2015). All subsamples of the whole grain were ground and 3 g of barley powder extracted with 9 mL methanol:water (80:20 v/v) and blended for 10 min. The mixture was filtered through filter paper (Whatman No. 1, England). The AFB_1 standard solution was placed in microtiter plate. Fifty µL of filtrate samples and 150 µL of dilution buffer were mixed together and 50 µL of the mixture placed into the each mixing well of the microtiter plate (H_1 to G_{11}). Then Microtitre plates were coated by adding 25 µL of antibody solution and 25 µL of conjugate (aflatoxin-HRP) to each well and incubated for 1 hr in the dark at 37 °C in a moist chamber. The plates were emptied, washed thrice with rinsing buffer and then 100 µL of substrate was added to each well and incubated for 30 min and finally 100 µL of stop solution added to each well and mixed prior to absorbance reading at 450 nm for a few seconds.

Results and Discussion

A total of sixty *Aspergillus* isolates were obtained from 40 barley grain samples from ten districts (Table 1) in Kermanshah province, in west of Iran during 2014-2015 growing seasons. Based on morphological characteristics the isolates were identified as *Aspergillus flavus*, *A. japonicus*, *A. niger*, and *A. sclerotiorum* (Table 1). *Aspergillus niger* (50%), *A. flavus* (30%), *A. japonicus* (15%) and *A. sclerotiorum* (5%) were the most prevalent as shown in Table 1. Morphological characteristics of all *Aspergillus* spp. cultured in MEA are summarized in the Table 2.

Molecular identification demonstrated that amplicon size of *A. flavus* isolates was about 413 bp (Fig. 1). Expect from the positive control isolate, no other *Aspergillus* spp. provided the target band.

Out of 40 analysed samples, 18 were positive for AFB_1 with a range between 0.056–10.564 µg kg^{-1} (Table 1). The highest AFB1 levels were found in Sarpol-e-Zahab (10.564 µg kg^{-1}) samples followed by samples from Bisotun ranging from 0.304–8.446 µg kg^{-1} (Table 1). All samples (100%) of Ravansar were contaminated with AFB_1 ranging from 0.460–7.224 µg kg^{-1} (Table 1).

The primary aim of our research was to isolate and identify *Aspergillus* species in stored barley grains in west of Iran. Four *Aspergillus* species were isolated and identified on their morphological characteristics. *Aspergillus niger* and *A. flavous* had the highest frequency of isolation in all investigated samples. *A. japonicus* and *A. sclerotiorum* were the least frequent isolates. *A. flavus* is the most prevalent aflatoxin, with increasing importance in feed poisoning (Goldblatt, 2012; Gholami-Shabhani *et al*., 2017).

In this research both morphological and molecular were applied for identification of all *Aspergillus* spp. (Fig. 1). *A. flavous* specific-species primers specifically identified only isolates of the *A. flavous* species, and no PCR products were generated for other target species. This finding shows the ability of using the primers designed by Hu *et al.* (2013) in the specific detection of the *A. flavous* species among other investigated species. Morphological identification is time-consuming and does not have enough accuracy and requires high skill.

According to the result of this study, using ELISA, 18 of the 40 analyzed samples (45%) were positive for AFB_1 (0.056–10.564 µg kg^{-1}) and *A. flavus* was one of the most prevalent with a frequency of 30%. It can be concluded that there is relationship between the produced AFB_1 and a frequency of *A. flavus*. Predominance of *A. flavus* in stored products has been determined in the most cereals-producing areas of the world by ELISA (Adanyi *et al.* 2006; Raduly *et al.* 2010). The occurrence of aflatoxins in cereals especially barley grains is one of the great concerns worldwide, because barley grains are chiefly used for animal feeds. Thus, the maximum permissible level of AFB_1 should be established in all the countries where barley is one of the major staple feeds (Raduly *et al.*, 2020).

The occurrence of AFB1 in the grains of various stored grains, including corn, rice and wild grasses, has been reported from different regions of Iran (Ghiasian *et al.*, 2011; Karami-Osboo *et al.*, 2012; Chehri *et al.*, 2015). However, limited information is available on the contamination of barley seeds with aflatoxin. Yazdan Panah *et al.* (2001) did not find aflatoxins and ochratoxins in barley samples collected from the northern regions of the country. Therefore, to the best of our knowledge, this is the first report of the natural occurrence of AFB1 from stored barley samples collected from west of Iran. Aflatoxin B1 is the most carcinogenic and best-studied aflatoxin (Raduly *et al.*, 2020). However, it is demonstrated that in some cases, the PCR detection of *A. flavus* is no guarantee of aflatoxin production (Levin, 2012), but in our study in all samples that identified as *A. flavus* based on PCR detection, AFB_1 was detected. This results show that the frequency of toxigenic strains in *A. flavous* isolates is high as reported by different researcher in the world (Lasram *et al*., 2016).

Conclusion

This study exposed high occurrence of AFB_1 contaminants in the barley grains in west of Iran and hopefully useful in feasible management strategies for reduction of mycotoxin contamination by several means including biocontrol agents.

Acknowledgements

Authors acknowledge the Department of Biology, Faculty of Science, Razi University, Kermanshah, Republic Islamic of Iran and Agricultural and Natural Resources Research Center, Kermanshah, Iran for providing necessary facilities to carry out this research.

Author's contributions

KC designed this experiment. HY carried out this experiment, analyzed data and drafted the manuscript. Both the authors revised the article.

Conflict of interests

The authors declare that there is no conflict of interest.

Table 1: Frequency of *Aspergillus* spp. and AFB_1 detected in barley grains from western Iran.

Sampling site	Total no. of samples	Number of samples infected with *A. flavus*	AFB_1 levels in positive Samples (µg kg^{-1})	*Aspergillus* spp.
Sarpol-e Zahab	4	2	0.056–10.564	*A. niger* (4), *A. flavus* (2), *A. japonicas* (2), *A. sclerotiorum* (1)
Gilan-e Gharb	4	2	0.690–7.246	*A. niger* (3), *A. flavus* (2), *A. japonicas* (1)
Kamyaran	4	1	7.448	*A. niger* (4), *A. flavus* (1), *A. japonicas* (1)
Kermanshah	4	2	0.408–6.454	*A. niger* (3), *A. flavus* (2), *A. japonicas* (2)
Kangavar	4	1	6.625	*A. niger* (1), *A. flavus* (1)
Qasr Shirn	4	2	0.110–0.546	*A. niger* (3), *A. flavus* (2)
Ravansar	4	4	0.460–7.224	*A. niger* (4), *A. flavus* (4), *A. japonicas* (1), *A. sclerotiorum* (1)
Bisotun	4	2	0.304–8.446	*A. niger* (3), *A. flavus* (2), *A. japonicas* (1), *A. sclerotiorum* (1)
Sahneh	4	1	4.826	*A. niger* (2), *A. flavus* (1), *A. japonicas* (1)
Sonqor	4	1	0.332	*A. niger* (3), *A. flavus* (1)

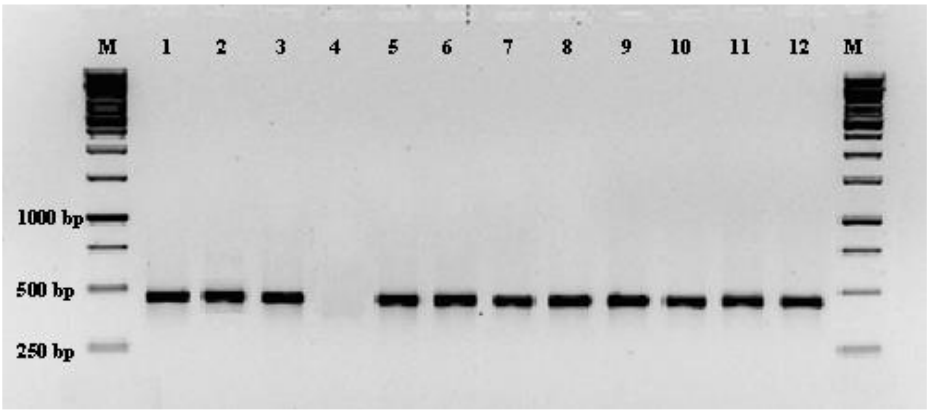

Fig. 1: PCR products obtained with specific primer pair AFLA-F/AFLA-R (band, 413 bp) from 11 isolates of *A. flavus*. **M:** GeneRuler 1 kb DNA Ladder. **1** =Asperflav101barley, **2** = Asperflav102barley, **3** = Asperflav103barley, **4** = *A. fumigates*, **5** = Asperflav111barley, **6** = Asperflav112barley, **7** = Asperflav113barley, **8** = Asperflav121barley, **9** = Asperflav122barley, **10** = Asperflav123barley, **11** = Asperflav124barley, **12** = Asperflav125barley.

Table 2: Morphological characters of *Aspergillus* spp. cultured on malt extract agar.

Characters	*A. flavus*	*A. niger*	*A. japonicus*	*A. sclerotiorum*
Conidiophore length (µm)	430–520	1020–1170	1220–1400	430-520
Conidiophore breadth (µm)	10.2–11.00	11.8–12.8	14.2–15.0	6.8–7.2
Conidiophore color	Uncolored to pale brown	Hyaline to slightly brown	Hyaline to slightly brown	Uncolored to pale brown
Surface texture	Finely rough	Smooth	Smooth	Rough
Conidiosphore size (µm)	2.5–3.6	3.1–4.2	2.1–4.7	2.4–2.7
Conidiospore shape	Globose to ellipsoidal	Globose	Globose, irregularly ellipsoidal	Spherical
Surface texture	smooth to finely roughened	Very rough to finely rough	Rough, sometimes echinulate	Finely rough
Vesicle diameter (µm)	22.0–44.0	34–40	42.7–50.0	21.2–35.0
Vesicle shape	Spherical to elongate	Globose to subglobose	Globose to elongate	Spherical to pyriform
Phialides	Uniseriate	Biseriate	Uniseriate	Biseriate
Colony diameter (mm)	70	82	80	58
Colony color	Deep green	Dark black	Dark brown	Pale yellow to light brown
Conidia color	Deep green	Black	Dark brown	Light yellow
Colony texture	Floccose especially centrally	Slightly floccose	Floccose, light concentric rings formed	Granular, light concentric rings formed
Mycelium	White to parrot green	White, inconspicuous	White	White
Exudates	Present, uncolored	Occasionally present, uncolored	Occasionally present, uncolored	Absent
Reverse	Yellow to pale brown, wrinkled mycelial growth	Pale brown, wrinkled mycelial growth	Pale brown, wrinkled mycelial growth	Light yellow shades to dark brown shades, wrinkled mycelial growth
Sclerotia	Present	Absent	Present	Present
Sclerotia shape	Oval	-	Oval	Oval
Sclerotia color	Dark brown to black shiny	-	Light brown	White to buff

References

Adanyi N, Levkovets IA, Rodriguez-Gil S, Ronald A, Varadi M, Szendr I, 2007. Development of immunosensor based on OWLS technique for determining aflatoxin B1 and ochratoxin A. *Biosens. Bioelectron.*, **22**: 797-802.

Anonympus, 2020. Agricultural statistics of the crop year 2018-2019. Information and Communication Technology Center. Ministry of Agriculture-Jahad. Iran. pp. 97.

Chehri K, Ghasempour HR, Karimi N, 2014. Molecular phylogenetic and pathogenetic characterization of *Fusarium solani* species complex (FSSC), the cause of dry rot on potato in Iran. *Microb. Pathogen*., **65**: 14-19.

Chehri K, Azami E, Mosaber A, 2015. *Aspergillus* and aflatoxin B1 contamination of stored corn grains in Western Iran. *Glob. Vet.*, **14**: 39-42.

Food and Agriculture Organization of the United Nations (FAO), 2004. Food Quality and Standards Service (ESNS), 81th worldwide regulations for mycotoxins in food and feed in 2003. Rome, Italy.

Hue NT, Nhien NH, Thong PM, Thanh CN, An PV, 2013. Detection of toxic *Aspergillus* species in food by a multiplex PCR. In: *Proceedings of 4th International Conference on Biomedical Engineering in Vietnam*. pp. 184-189.

Ibrahim SRM, Abdallah HM, Mohamed GA,

Deshmukh SK, 2023. Exploring Potential of *Aspergillus sclerotiorum*: secondary metabolites and biotechnological relevance. *Mycol. Prog.,* **21**: 8.

Javaid A, Shafique G, Bashir U, 2010. Mycoflora associated with stored seeds of different varieties of shisham (*Dalbergia sissoo* Roxb.). *Pak. J. Phytopathol*., **22**: 9-12.

Javaid A, Khan IH, 2019. Mycoflora associated with seeds of different varieties of chickpea and its effect on germination and radicle growth. *Int. J. Biol. Biotechnol.*, **16**: 175-179.

Karami-Osboo RM, Mirabolfathy R, Kamran M, Shetab-Boushehri A, Sarkari S, 2012. Aflatoxin B1 in maize harvested over 3 years in Iran. *Food Control*, **23**: 271-274.

Klich MA, 2002. Identification of common *Aspergillus* species. CBS, Utrecht, The Netherland. pp. 116.

Klich MA, 2007. *Aspergillus flavus*: the major producer of aflatoxin. *Mol. Plant Pathol*., **8**: 713-722.

Lasram S, Hamdi Z, Chenenaoui S, Mliki A, Ghorbel A, 2016. Comparative study of toxigenic potential of *Aspergillus flavus* and *Aspergillus niger* isolated from Barley as affected by temperature, water activity and carbon source. *J. Stored Prod. Res*., **69**: 58-64.

Levin ER, 2012. PCR detection of aflatoxin production fungi and its limitations. *Int. J. Food Microbiol*. **156**: 1-6.

Raduly Z., Szabó Lf., Madar A., Pocsi I., Csernoch L. 2020. Toxicological and medical aspects of *Aspergillus*-derived mycotoxins entering the feed and food chain. *Front*. *Microbiol*., **10**: 1-23.

Rasch C, Böttcher M, Kumke M, 2010. Determination of aflatoxin B_1 in alcoholic beverages: comparison of one- and two photon- induced fluorescence. *Anal. Bioanal. Chem*., **397**: 87-92.

Shafique S, Shafique S, Javaid A, 2005. Fungitoxicity of aqueous extracts of allelopathic plants on seed-borne mycoflora of maize. *Mycopath*, **3**: 23-26.

Shafique S, Javaid A, Bajwa R, Shafique S, 2007. Effect of aqueous leaf extracts of allelopathic trees on germination and seed-borne mycoflora of wheat. *Pak. J. Bot.,* **39**: 2619-2624.