ردیابی کموتیپ فومونیزین تولید شده توسط قارچ *فوزاریوم پرولیفراتوم* جدا شده از آجیل در عراق با استفاده از روش *پی سی آر* اختصاصی

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چکیده

مقدمه: پژوهش حاضر به منظور ارزیابی وقوع جدایه‌های قارچ *فوزاریوم پرولیفراتوم* تولیدکننده مایکوتوکسین در آجیل در عراق انجام شد.

مواد و روش‌ها: در این پژوهش ۱۰۸ نمونه آجیل از فروشگاه‌های مختلف در عراق جمع آوری شد. ویژگی‌های ریخت شناسی گونه‌های *فوزاریوم* با استفاده از محیط کشت‌های برگ میخک-آگار و عصاره سیب زمینی-دکستروز-آگار بررسی شد. جدایه‌های *فوزاریوم پرولیفراتوم* با استفاده از آغازگرهای اختصاصی PRO1/PRO2 تایید شد. در این پژوهش همچنین قابلیت تولید زهرابه فومونیزین توسط جدایه‌های *فوزاریوم پرولیفراتوم* با استفاده از آغازگرهای طراحی شده برای ژن FUM1 (FUM1 F/FUM1 R) بررسی شد.

نتایج: در این تحقیق ۲۸ جدایه قارچ جداسازی شد که بر اساس بررسی‌های ریخت‌شناسی ۳ گونه قارچی شناسایی شد که شامل گونه‌های *فوزاریوم پرولیفراتوم* (۱۲)، *آسپرژیلوس نایجر* (۸)، *آسپرژیلوس فلاووس* (۵) و گونه نامشخصی از *پنیسلیوم* (۳) بودند. آغازگرهای اختصاصی PRO1/PRO2، قطعه ۵۸۵ جفت بازی را در همه جدایه‌های *فوزاریوم پرولیفراتوم* را تولید کردند. تکثیر قطعه دی‌ان‌ای (۱۸۳ جفت بازی) ژن FUM1 با استفاده از آغازگرهای FUM1 F/FUM1 R تقریبا در ۴۲ درصد از جدایه های *فوزاریوم پرولیفراتوم* بدست آمد.

بحث و نتیجه‌گیری: از بین ۱۲ جدایه *فوزاریوم پرولیفراتوم*، ۵ جدایه (۴۲~) قابلیت تولید فومونیزین داشتند. این پژوهش برای نخستین بار در عراق انجام شد و بر اساس مطالعات مولکولی قابلیت جدایه‌های گونه مرکب *فوزاریوم فوجیکوروی* (*FFSC*) ارزیابی شد.

واژه‌های کلیدی: *FFSC*، آغازگرهای اختصاصی، آجیل، عراق

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تاریخ دریافت: ۱۳۹۶/۰۹/۰۲- تاریخ پذیرش: ۱۳۹۶/۰۴/۳۱

Biological Journal of Microorganism
6th Year, Vol. 6, No. 24, Winter 2018
Received: April 29, 2017/ **Accepted:** July 22, 2017. **Page:** 21- 27

Detection of fumonisin chemotype produced by *Fusarium proliferatum* isolated from nuts in Iraq using specific PCR assays

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Abstract

Introduction: The present study was carried out to evaluate the occurrence of toxicogenic *Fusarium proliferatum* strains isolated from nuts in Iraq.

Materials and methods: A total of 108 nut samples collected from different markets in Iraq. Strains of *Fusarium* spp. isolated from nuts seeds and their morphological characterization of the strains were examined based on their growth on carnation leaf agar (CLA) and potato dextrose agar (PDA). The identification of *F. proliferatum* isolates were confirmed molecularly using species specific primers of PRO1/PRO2 primers. PCR-based detection of fumonisin-synthesis-pathway gene was also used to determine the potential of *F. proliferatum* isolates to produce fumonisin using FUM1 gene-based (FUM1 F/FUM1 R) primers.

Results: Based on morphological features 28 fungal isolates were obtained from nuts and identified into four species *F. proliferatum* (12), *Aspergillus niger* (8), *Aspergillus flavus* (5), and *Penicillium* sp. (3). The primers PRO1/PRO2 produced DNA fragments 585 bp in all *F. proliferatum* strains. PCR assays also showed DNA fragments (183 bp) were amplified in nearly 42% of *F. proliferatum* strains.

Discussion and conclusion: Of 12 tested isolates, 5 isolates (~42%) being fumonisin chemotype. To our knowledge, this is the first report on molecular identification and mycotoxigenic capacity of *Fusarium fujikuroi* species complex (FFSC) isolated from nuts in Iraq.

Key words: FFSC, species specific primers, nuts, Iraq

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Introduction

Agricultural products and foods are proper media for growing fungi. There are many studies that show fungi belong to various genera are present in different stages of agricultural products such as seeds, whole plants, yields, and foods (1-4). Nuts are classified as highly important agricultural products that in addition to direct consumption are also economically important due to their widespread usage in food industry. Nuts contain different nutrients and high level of condensed energy (5). High protein and fat contents as well as low amount of water turned nuts into products with extreme potency to be invaded by fungi. Several species of fungi have been isolated from nuts such as pistachio, peanut, hazelnut, almond, pecan, pine nut, walnut (6-9). Most of toxic species belong to *Aspergillus, Penicillium* and *Fusarium* species (9). Fungal contamination of nuts makes economically massive losses. As a matter of fact, growth of fungi on nuts leads to mycotoxin contamination which results in severs health problems in consumers (10).

Fusarium fujikuroi species complex (FFSC) encompasses important pathogenic fungi which their anamorphs are found within *Fusarium*. At least 36 described species are classified in FFSC such as *F. circinatum*, *F. fujikuroi*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* (11). Members of FFSC are responsible for some diseases in plants such as Bakana on rice seedlings, root rot of Soybean, and ear and stalk rot of maize (12-14).

Members of FFSC are potential producers of mycotoxins such as fumonisins (15). Although, in most of studies *Aspergillus* members are reported as major contaminants of nuts, *Fusarim* spp. have been isolated from nuts, as well (6, 8, 16). Toxins produced by these fungi are a major concern in the production and storage of nuts (8, 17).

Mycotoxins as secondary metabolites are produced by some species of fungi and make harmful effects on consumers either human or other organisms. *Fusarium* and other genera such as *Aspergillus*, *Penicillium*, *Alternaria* and *Claviceps* encompass species that produce important mycotoxins such as trichothecenes, fumonisins, moniliformin, aflatoxins, altenuene, and ergot alkaloids (18). Depends on the type, mycotoxins have different effects on consumer health, therefore using ways to detect them through a rapid procedure is inevitable (19).

Fumonisins are from highly important mycotoxins that influence agriculture and the food industries. Accumulated fumonisins in plants such as maize and consequently consuming with human and animals result in hazardous affects such as cancer and neural tube defects (20, 21). At least 16 defined substances have been detected that are classified as fumonisins (10). A rapid procedure based PCR of specific genes encoding for proteins involving in mycotoxins biosynthesis is developed which help to identify the chemotypes of fungal species through a rapid way. Molecular studies of *F. verticillioides* revealed that a gene cluster (*FUM*) consist of 15 genes is responsible for fumonisins production (22). Their presence in a species can be investigated using PCR to identify ability of a particular maycotoxin biosynthesis and also chemotypes of the fungi (23).

As nuts are valuable and widely used agricultural products and are also absolutely susceptible to toxic fungi, it is necessary to be studied for fungal flora and potential toxins producers in them. Therefore, aims of this investigation are firstly, identification of fungal species on nuts samples collected from markets in Iraq and secondly, determination of chemotypes of the *Fusarium* isolates using the PCR-based molecular analyses.

Materials and methods

Isolation and Identification of *Fusarium* spp.: Nuts seeds were collected from different markets of Baghdad city and transferred to the lab in plastic bags. Strains of *Fusarium* spp. isolated from nuts seeds and their morphological characterization of the strains was examined based on their growth on carnation leaf agar (CLA) and potato dextrose agar (PDA) (24).

DNA extraction: Briefly, following culturing *Fusarium* isolates in Potato Dextrose Broth (PDB, Sigma) shaking at 150 rpm at 25±2^{o}C for 5 days, mycelia were harvested by filtration through Whatman paper 1 and freeze-dried for 20 h. DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's protocol to extract DNA.

Molecular identification using species-specific PCR: To confirm identification of *F. proliferatum* isolates were considered molecularly using species-specific primers of PRO1 (CTTTCCGCCAAGTTTCTTC) and PRO2 (TGTCAGTAACTCGACGTTGTTG) (25). Amplification reactions were done in a total volume of 25 µl, by mixing 1 µl of template DNA with 17.8 µl ddH_2O, 1 µl of deoxynucleotide triphosphate (dNTP) (Promega); 0.5 µl of $MgCl_2$ (Promega); 1 µl of each primer; 0.2 µl of *Taq* DNA polymerase (Promega) and 2.5 µl of PCR 5X reaction buffer (Promega, Madison, Wl, USA). PCR amplification was performed in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) with the following programs: an initial denaturation step at 94^{o}C for 5 min, 35 cycles of 94°C (1 min), 56°C (1 min), 72°C (3 min), and a final extension step at 72°C for 10 min. To visualize the PCR products 1×TBE electrophoresis in ethidium-bromide-stained and 1% agarose gel were used.

Molecular analyses for fumonisin-producing *Fusarium* strains: To investigate potential ability of fumonisin production in the *Fusarium* strains, FUM1 F (CCATCACAGTGGGACACAGT) and FUM1 R (CGTATCGTCAGCATGATGTAGC) primers were applied. PCR amplification was carried out in the Peltier Thermal Cycler, PTC-100® (MJ Research, Inc. USA) according to temperature profiles described by bluhm and colleagues (26). To visualize PCR products 1×TBE electrophoresis in ethidium-bromide-stained and 1.8% agarose gel were used.

Results

One hundred and eight nut samples were collected from different markets in Baghdad city. Based on morphological features, 28 fungal isolates were recovered from infected nut seeds. Macroscopic and microscopic characteristics showed that all isolates belonged to *F. proliferatum* (12) as the known FFSC members, *Aspergillus niger* (8), *Aspergillus flavus* (5), and *Penicillium* sp. (3). Twelve strains of *F. proliferatum* were characterized by the production of abundant aerial mycelium. Also they produced slightly straight macroconidia with 3-5-septate and club shaped microconidia. The conidiogenous cells producer false head and chain microconidia were monophialides and polyphialides (Fig. 1). The identification of *F. proliferatum* isolates were confirmed molecularly using species specific primers of PRO1/PRO2 primers, which selectively amplified the partial calmodulin gene of rDNA. The primers PRO1/2 produced DNA fragments 585 bp in all *F. proliferatum* strains (Fig. 2). PCR-based detection of fumonisin-synthesis-pathway gene was also used to determine the potential of *F. proliferatum* isolates to produce fumonisin using FUM1 gene-based primers. PCR assays showed DNA fragments (183 bp) were amplified in nearly 42% of *F. proliferatum* strains (Fig. 3).

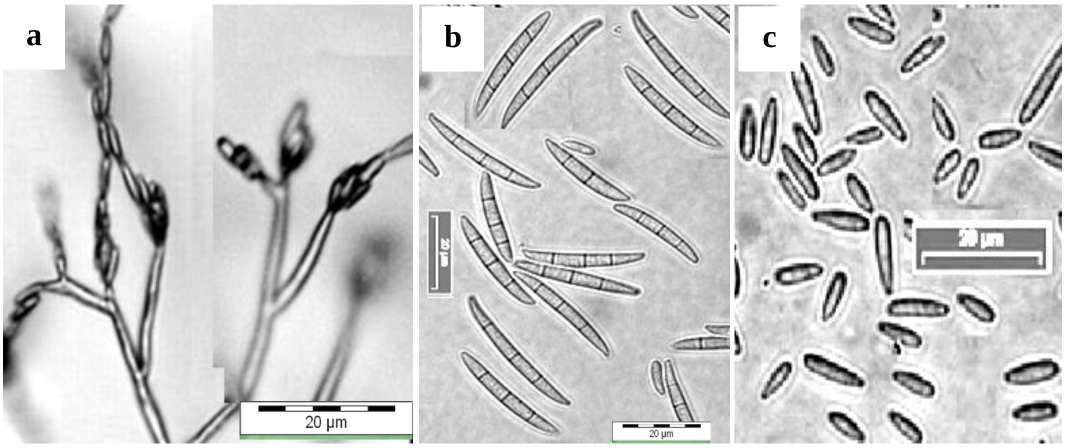

Fig. 1. Monophialidic and polyphialidic conidiogenous cells (a), macroconidia (b), and microconidia (c) shapes of *Fusarium proliferatum* isolated from nuts in Iraq. Bar= 20 µm for all figures.

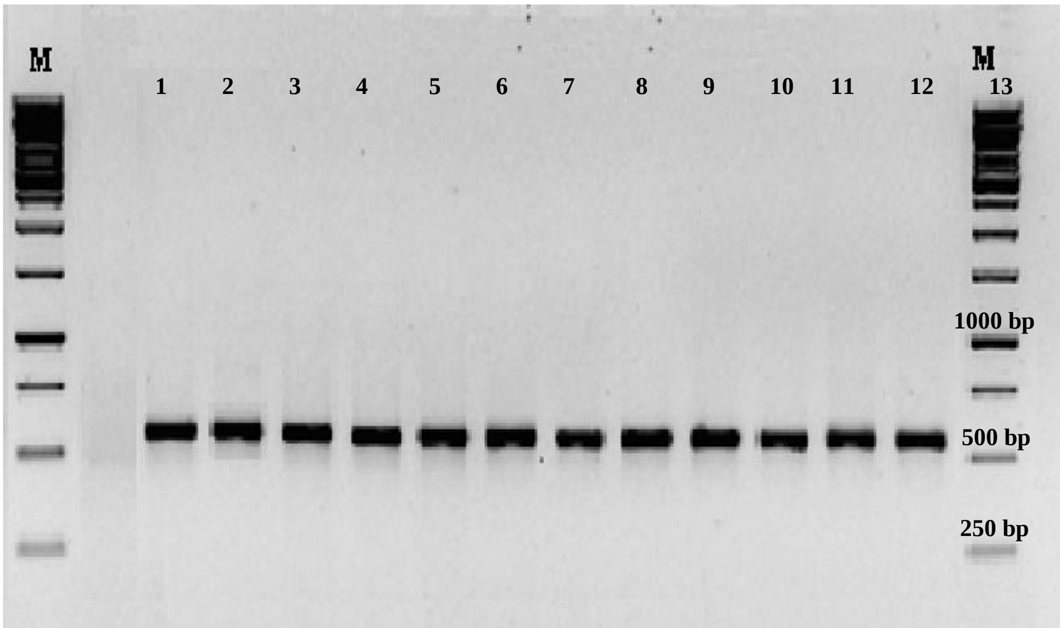

Fig. 2. PCR products obtained with specific primer pairs PRO1/2 (band, 585 bp) from 12 isolates of *F. proliferatum*. Lane M: GeneRuler 1 kb DNA Ladder. Lane 1: *F. subgultinans* (negative control).

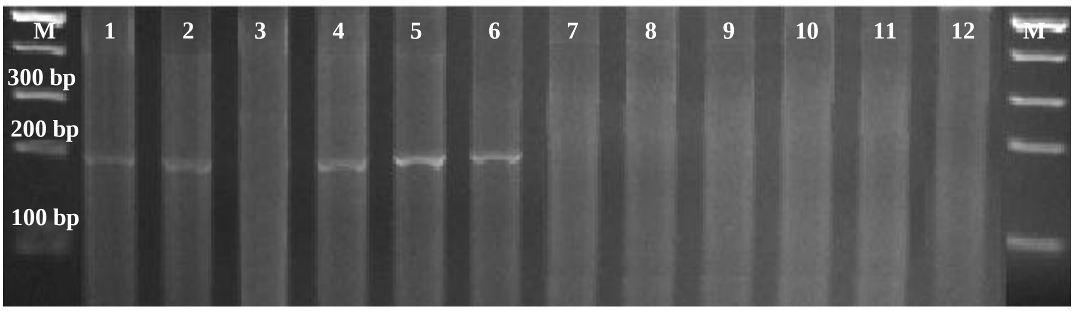

Fig. 3. PCR products obtained with specific primer pairs FUM1 F/FUM1 R (band, 183 bp) from 5 isolates of *F. proliferatum*. Lane M: GeneRuler DNA Ladder Mix, 100–10,000 bp Ladder.

DISCUSSION

Nuts are classified as highly important agricultural products, which are commonly invaded by fungi. In the present study, we considered the nuts collected from markets in Baghdad, Iraq, to identify fungal species in them and more deeply to study fuasaria isolates using molecular techniques to detect their species and chemotypes.

From 108 samples of nuts, 28 isolates were recovered that encompass 12 isolates of *F. proliferatum*, 8 of *Aspergillus niger*, 5 of *Aspergillus flavus*, and 3 of *Penicillium* sp.. Identification of these fungi is accorded to previous researches on nuts. Abdulla (8) considered the fungal flora and mycotoxins in the nuts (almond, cashew nut, hazelnut, Peanut and pistachio) collected from local stores of Erbil, Iraq and species from *Aspergillus* and *Penicillium* were identified in the nuts. Our results confirmed his studies (8). To our knowledge, this is the first report on molecular identification *F. proliferatum* isolated from nuts in Iraq.

A study evaluated the mycobiota and mycotoxins in almond samples collected from different region in Brazil. They detected that the most frequent species belonged to *Phialemonium* spp. (54%), *Penicillium* spp. (16%), and *Fusarium* spp. (13%) (27). Khosravi and colleagues (6) investigated the fungal flora in nuts including pistachio, peanut, hazelnut and almond collected from different regions of Tehran, Iran and reported *Aspergillus* spp. (32.2%), *Penecillium* spp. (30.3%) *Mucor* spp. (17.1%), and *Fusarium* spp. (18.2%) as the most frequent fungi (6). Tournas et al. (9) revealed that *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* species contaminated the nut samples purchased from local markets in Washington, D.C., the United States of America. Our results were in agreement with the previous studies, but in our studies, the frequency of *Fusarium* species were higher compared to *Aspergillus* spp. and *Penicilllium* spp..

Through other part of this study, we detected chemotype of fumonisins produced by *Fusarium* isolates based on PCR analysis. Considering genes related to biosynthesis of the fumonisins are performed in previous studies of fusaria (25, 28). Mateo and Jiménez (29) considered production of fumonisins by strains of FFSC in autoclaved tiger nuts. They revealed that amount of produced fumonisin B1 was similar to level of that on rice and wheat (28). Another study was done to consider production of fumonisin B1 in pine nuts which showed some isolates of *F. proliferatum* can biosynthesis fumonisin B1 in the husk of pine nuts (7). In our study, we considered potency of *F. proliferatum* as the known members of FFSC to produce fumonisin that according to our results about 42% (5 of 12) of them were fumonisin chemotypes. Fumonisins are classified as very important mycotoxins with severe impact on consumers' health, therefore, detecting chemotypes of fungi can be a very helpful tool to manage their contaminations (21). To the best of our knowledge, this is the first report of fumonisin chemotypes of *Fusarium* spp. in the nuts collected from Iraq.

In conclusion, through this study, the mycoflora of the nuts collected from Baqdad, Iraq was identified morphologically and *F. proliferatum* were confirmed using molecular analysis. We revealed that *F. proliferatum* are forming a major part of contaminants in the samples. Moreover, we detected fumonisins chemotypes molecularly which to the best of our knowledge is reported for the first time in Iraq.

References

(1) Priyanka SR., Venkataramana M., Kumar GP., Rao VK., Murali HCS., Batra HV. Occurrence and molecular detection of toxigenic *Aspergillus* species in food grain samples from India. *Journal of the Science of Food and Agriculture* 2014; 94(3):537-543.

(2) Wisniewska H., Stepien L., Waskiewicz A., Beszterda M., Goral T., Belter J. Toxigenic Fusarium species infecting wheat heads in Poland. *Central European Journal of Biology* 2014; 9(2):163-172.

(3) Del Palacio A., Bettucci L., Pan D. *Fusarium* and *Aspergillus* mycotoxins contaminating wheat silage for dairy cattle feeding in Uruguay. *Brazilian Journal of Microbiology* 2016; 47(4):1000-1005.

(4) Han X., Zhao X., Zhang J., Zhang H., Yu D., Jiang T., Li F., Han C. Survey on fungi contamination of feed ingredients in China. *Journal of hygiene research* 2014; 43(3):430-434.

(5) Tan SY., Dhillon J., Mattes RD. A review of the effects of nuts on appetite, food intake, metabolism, and body weight. *The American journal of clinical nutrition* 2014; 100(Supplement 1):412-422. doi: 10.3945/ajcn.113.071456.

(6) Khosravi AR., Shokri H., Ziglari T. Evaluation of fungal flora in some important nut products (Pistachio, Peanut, Hazelnut and Almond) in Tehran, Iran. Pakistan Journal of Nutrition 2007; 6(5):460-462.

(7) Marin S., Ramos AJ., Vazquez C., Sanchis V. Contamination of pine nuts by fumonisin produced by strains of *Fusarium proliferatum* isolated from *Pinus pinea*. *Letters in applied microbiology* 2007; 44(1): 68-72. doi:10.1111/j.1472-765X.2006.02028.x.

(8) Abdulla NQF. Evaluation of fungal flora and mycotoxin in some important nut products in Erbil local markets. *Research Journal of Environmental and Earth Sciences* 2013; 5(6):330-336.

(9) Tournas VH., Niazi NS., Kohn JS. Fungal presence in selected tree nuts and dried fruits. *Microbiology insights* 2015; 8:1-6. doi: 10.4137/MBI.S24308.

(10) da Rocha MEB., Freire FDCO., Maia FEF., Guedes MIF., Rondina D. Mycotoxins and their effects on human and animal health. *Food Control* 2014; 36(1):159-165.

(11) O'Donnell K., Cigelnik E., Nirenberg H. Molecular systematics and phylogeography of the *Fusarium fujikuroi* species complex. *Mycologia* 1998; 90(3):465-493.

(12) Wulff EG., Sorensen JL., Lubeck M., Nielsen KF., Thrane U., Torp J. *Fusarium* spp. associated with rice Bakanae: ecology, genetic diversity, pathogenicity and toxigenicity. *Environmental Microbiology* 2009; 12(3):649-657. doi: 10.1111/j.1462-2920.2009.02105.x.

(13) Arias MMD., Munkvold GP., Leandro LF. First report of *Fusarium proliferatum* causing root rot on soybean (*Glycine max*) in the United States. *Plant Disease* 2011; 95(10):1316-1316.

(14) Scauflaire J., Gourgue M., Callebaut A., Munaut F. *Fusarium temperatum*, a mycotoxin-producing pathogen of maize. *European Journal of Plant Pathology* 2012; 133(4):911-922. doi: 10.1007/s10658-012-9958-8.

(15) Hyung LS., Hye KJ., Wan SS., Theresa L., Hwan YS. Fumonisin production by field isolates of the *Fusarium fujikuroi* species complex and *Fusarium commune* obtained from rice and corn in Korea. *Research in Plant Disease* 2012; 18(4):310-316.

(16) Baquiao AC., Zorzete P., Reis TA., Assuncao E., Vergueiro S., Correa B. Mycoflora and mycotoxins in field samples of Brazil nuts. *Food Control* 2012; 28(2):224-229.

(17) Molyneux RJ., Mahoney N., Kim JH., Campbell BC. Mycotoxins in edible tree nuts. *International journal of food microbiology* 2007; 119(1-2):72-78.

(18) Marin S., Ramos AG., Cano-Sancho G., Sanchis V. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology* 2013; 60(October):218-237. doi: 10.1016/j.fct.2013.07.047.

(19) Eskola M., Parikka P., Rizzo A. Trichothecenes, ochratoxin A and zearalenone contamination and *Fusarium* infection in Finnish cereal samples in 1998. *Food Additives and Contaminants* 2001; 18(8):707-718.

(20) Voss KA., Riley RT., Waes JGV. Fumonisin B1 induced neural tube defects were not increased in LM/Bc mice fed folate-deficient diet. *Molecular Nutrient Food Research* 2014; 58(6):1190-1198.

(21) Ruyck KD., Boevre MD., Huybrechts I., Saeger SD. Dietary mycotoxins, co-exposure, and carcinogenesis in humans: Short review. *Mutation Research* 2015; 766(October–December):32-41. doi: 10.1016/j.mrrev.2015.07.003.

(22) Proctor RH., Brown DW., Plattner RD.,

Desjardins AE. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Fusarium moniliformis*. *Fungal Genetics and Biology* 2003; 38(2):237-249. doi: 10.1016/S1087-1845(02)00525-X.

(23) Yoruk E., Tunali B., Kansu B., Olmez F., Uz G., Zumrut IM., Sarıkaya A., Meyva G. Characterization of high-level deoxynivalenol producer *Fusarium graminearum* and *F. culmorum* isolates caused head blight and crown rot diseases in Turkey. *Journal of Plant Diseases and Protection* 2016; 123(4):177-186.

(24) Leslie JF., Summerell BA. The Fusarium Laboratory Manual. UK: Blackwell Publish Ltd (2006).

(25) Mulé G., Susca A., Stea G., Moretti A. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides, F. proliferatum* and *F. subglutinans*. European Journal of Plant Pathology 2004; 110(5-6): 495-502.

(26) Bluhm BM., Cousin MA., Woloshuk CP. Multiplex real-time PCR detection of fumonisins producing and trichothecene producing groups of *Fusarium* species. *Journal of Food Protection* 2004; 67(3):536-543.

(27) Reis TA., Oliveira TD., Baquiao AC., Goncalves SS., Zorzete P., Correa B. Mycobiota and mycotoxins in Brazil nut samples from different states of the Brazilian Amazon region. *International journal of food microbiology* 2012; 159(2):61-68.

(28) Ivic D., Kovacevik B., Vasilj V., Idzakovic N. Occurrence of potentially toxigenic *Fusarium verticillioides* and low fumonisin B1 content on barley grain in Bosnia and Herzegovina. *Journal of Applied Botany and Food Quality* 2001; 84(2):121-124.

(29) Mateo JJ., Jiménez M. Trichothecenes and fumonisins produced in autoclaved tiger nuts by strains of *Fusarium sporotrichioides* and *Fusarium moniliforme*. *Food microbiology* 2000; 17(2): 167-176. doi: 10.1006/fmic.1999.0301.