Assessment of Aflatoxin Gene Cluster and Interaction of Toxigenic and Atoxigenic *Aspergillus Flavus* Strains of Pistachio *in Vitro*

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Abstract

Introduction: Aflatoxin is the most significant mycotoxin hazard to human health, a secondary metabolite characterized by mutagenicity, teratogenicity, and carcinogenicity. It is produced by some species of *Aspergillus* in susceptible agricultural crops under certain environmental conditions. Different strategies, such as physical, cultural, biological, and chemical, have been suggested to manage the contamination of various crops with *Aspergillus* fungus. Using atoxigenic isolates of *Aspergillus flavus* is considered an effective and successful method in reducing aflatoxin contamination.

Materials and Methods: In the present study, 58 atoxigenic isolates of *Aspergillus flavus* obtained from pistachio orchards in different agro-ecological zones of Iran were used. Coconut agar medium (CAM) Petri plates were co-inoculated by an equal number of spores (4×10^3) of 58 atoxigenic and one toxigenic strain. The interactions between toxigenic and atoxigenic strains to produce aflatoxin were evaluated by exposing them to ammonia vapor. Potato dextrose broth (PDB) medium was used to produce mycelium, and the CTAB modified method was used to extract DNA. Species-specific primer was used for molecular identification. Four genes, including *nor*1, *avn*A, *est*A, *afl*R, and C3 region of the aflatoxin gene cluster, were detected in 24 atoxigenic isolates using specific primers and PCR reaction.

Results: Overall, the isolates were divided into six groups based on the color intensity of the back of the colony. Atoxigenic isolates were placed into 12 groups on the basis of the gene deletion pattern.

Conclusion: *In vitro* competition test showed a potential for limiting toxigenic *A. flavus* strains growth and aflatoxin control among atoxigenic strains

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1. Introduction

Different species of *Aspergillus* can grow on various substrates and produce secondary metabolites, including aflatoxins, dangerous to human and animal health. Aflatoxins are a group of important mycotoxins, including aflatoxin B1, B2, G1, and G2, mainly produced by two species of *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus)*. *A. flavus* isolates show large variances in producing aflatoxins and other secondary metabolites [1, 2]. Toxigenic *A. flavus* isolates mainly produce two aflatoxins B1 and B2; however, most *A. parasiticus* isolates are capable of producing all four types of aflatoxins [1]. Several physical, cultural, chemical, and biological methods have been recommended to manage the contamination of crops with *Aspergillus* or Aflatoxin, each of which has its own applicability/efficiency and advantages/disadvantages, depending on the in-country legislation, agricultural commodity, financial and practical capabilities [3]. The efficacy of many different species of microorganisms, such as bacteria, yeasts, actinomycetes, and atoxigenic *Aspergillus* isolates, to mitigate *Aspergillus* and aflatoxin has been studied [4- 6]. Meanwhile, the widespread use of atoxigenic *Aspergillus flavus* isolates on several crops, such as cotton, pistachios, peanuts, almonds, and figs, has been developed as an effective method to reduce toxigenic isolates [7]. The results have shown atoxigenic *A. flavus* isolates to be able to compete with toxigenic isolates successfully in ecological niche occupancy, reducing toxigenic isolates in the soil [1]. The greatest success in the biological control of aflatoxin contamination before and after harvest is obtained by using atoxigenic *A. flavus*. In this strategy, atoxigenic isolates (defined as isolates that do not produce any aflatoxin) of *A. flavus* have been developed as biological control agents directed at competing with and displacing aflatoxin producers [4]. Based on the size of the sclerotium, there are two morphotypes: L-strain referring to a sclerotium diameter greater than 400 micrometers and S-strain referring to a sclerotium diameter less than 400 micrometers. The atoxigenic isolates used in biocontrol are all L-strains. These isolates generally differ in the production of sclerotia, conidia, production of aflatoxins, aflatoxin gene cluster, vegetative compatibility groups, ecological and biological requirements [8]. To obtain atoxigenic *A. flavus* isolates, screening different populations of *Aspergillus* in terms of aflatoxin production, as well as the ability to adapt to the environmental conditions and compete with toxin-producing populations, should be

considered. There are different screening methods, including cultural, analytical, and molecular methods, which can be used depending on the available facilities and conditions [9, 10]. At least 23 enzymatic reactions are involved in the synthesis of aflatoxin; the synthesis occurs through a series of oxidation and reduction reactions. More than 25 genes located in the 75 Kb gene cluster are involved in the biosynthesis pathway. Comparison of gene clusters in toxinogenic and non-toxinogenic isolates has shown that non-toxigenicity may be due to complete deletion of some genes involved in aflatoxin production or single-nucleotide polymorphism and mutations responsible for non-toxinogenicity [11, 12].

There is little information on the aflatoxin gene cluster in atoxigenic isolates for biological control of aflatoxin produced by toxigenic strains in pistachios. Therefore, the present study aims to evaluate the molecular basis of atoxigenicity in atoxigenic isolates and their interaction with those of toxigenic in agar medium. The interaction test result can be used as a primary screening of efficient atoxigenic isolates in biocontrol.

2. Materials and Methods

2.1- *Aspergillus flavus* isolates

58 atoxigenic and (one) toxigenic isolates of *A. flavus* used in the study obtained from pistachio soil and nut of the orchards of Kerman, Yazd, Khorasan Razavi, Isfahan, Qom, Semnan, and Markazi from Technology and Production Management Group of Pistachio Research Center, as well as the Plant Protection Department of Yazd Agricultural and Natural Resources Research and Education Center [12] (Table 1).

Table 1- Characteristics of nontoxigenic *Aspergillus flavus* isolates

No.	ITEM* code	Location	Source	No.	ITEM* code	Location	Source
1	16441	K. Razavi**	Nut	31	16471	K. Razavi	Nut
2	16442	K. Razavi	Nut	32	16472	Semnan	Soil
3	16443	Kerman	Nut	33	16473	Markazi	Nut
4	16444	Kerman	Nut	34	16474	K. Razavi	Nut
5	16445	Kerman	Nut	35	16475	K. Razavi	Nut
6	16446	Kerman	Nut	36	16476	K. Razavi	Nut
7	16447	Kerman	Nut	37	16477	K. Razavi	Soil
8	16448	Yazd	Nut	38	16478	K. Razavi	Nut
9	16449	Yazd	Soil	39	16479	K. Razavi	Nut
10	16450	Yazd	Soil	40	16480	K. Razavi	Nut
11	16451	Kerman	Soil	41	16481	K. Razavi	Nut
12	16452	Kerman	Nut	42	16482	K. Razavi	Nut
13	16453	Kerman	Nut	43	16483	K. Razavi	Nut
14	16454	Kerman	Nut	44	16484	K. Razavi	Nut
15	16455	Esfahan	Nut	45	16485	K. Razavi	Nut
16	16456	Esfahan	Nut	46	16486	K. Razavi	Nut
17	16457	Esfahan	Nut	47	16487	K. Razavi	Nut
18	16458	Esfahan	Soil	48	16488	K. Razavi	Soil
19	16459	Esfahan	Soil	49	16489	K. Razavi	Nut
20	16460	Esfahan	Nut	50	16490	K. Razavi	Nut
21	16461	Qom	Nut	51	16491	K. Razavi	Nut
22	16462	Esfahan	Nut	52	16492	Kerman	Nut
23	16463	Esfahan	Nut	53	16493	Kerman	Soil
24	16464	Esfahan	Nut	54	16494	K. Razavi	Nut
25	16465	Qom	Soil	55	16495	K. Razavi	Nut
26	16466	K. Razavi	Nut	56	16496	K. Razavi	Nut
27	16467	Qom	Nut	57	16497	K. Razavi	Nut
28	16468	Kerman	Soil	58	16498	Kerman	Nut
29	16469	Esfahan	Nut	59	16499***	Kerman	Soil
30	16470	Esfahan	Nut				

*ITEM Microbial Culture Collection of ISPA (Institute of Sciences of Food Production), Bari, Italy
**Khorasan Razavi
***Toxigenic strain with mean aflatoxin production levels of 703.68 and 144.78 ng/Kg for aflatoxin B1 and B2, respectively, in YES medium [12]

2.2- Co-inoculation of isolates on the coconut agar medium (CAM)

200 g of coconut powder was poured into one liter of boiling water. After 5 minutes of stirring, the liquid was filtered through 4 layers of mullein cloth. 20 g of agar powder was added to it and transferred to the autoclave for 20 minutes at 1.5 atmospheric pressure and 120 °C for sterilization. The medium was poured into 9 cm Petri dishes [13]. In all experiments, single spore isolates were used. Fungal isolates were cultured on potato dextrose agar (PDA, per L: 39 g potato dextrose agar, pH 6.5) for 5 days at 30 °C in the dark. Spores were washed with 10 ml of sterile distilled water (containing one gram peptone per liters), counted by a Thoma counting chamber (Hawksley, UK), and adjusted to 4×10^3 spores/ml. Spore suspensions of toxigenic and nontoxigenic isolates were mixed in equal concentration (4×10^3) and volume; then, 20 µl of the mixture was inoculated in the center of 9 cm Petri-plates of CAM. Three replicates were assessed for each atoxigenic isolate. Plates were incubated for 96 hours at 30 °C in the dark.

2.3- Ammonia vapor assay

In this method, four-day-old colonies grown at 30 °C were exposed to ammonia vapor (produced from 200 microliters of liquid sodium ammonium), and the isolates were grouped as pink or red based on the intensity of discoloration behind the colony (14, 15).

2.4- Molecular assays

2.4.1- DNA extraction

Twenty-three isolates were selected based on the interaction test results for molecular assays. Fungal isolates were cultured on potato dextrose broth (PDB) medium; then, it was placed in a shaker at a speed of 150 rpm for 48 hours at 25 °C. The mycelium produced was isolated from the culture medium using a vacuum pump and filter paper (Watman®, No. 4, Sigma-Aldrich, Germany). Mycelium was ground in liquid nitrogen. The modified CTAB (cetyltrimethylammonium bromide) method was used for DNA extraction (16). Purified DNA was stored in the TE (Tris-EDTA) buffer at -20 °C for subsequent experiments.

2.4.2- Molecular identification of isolates

Species-specific primers FLAVIQ1 / FlaQ2 primers (FLAVIQ1: 5 ′ GTCGTCCCCTCTCCGG 3 ′ / FLAQ2: 5 ′ CTGGAAAAAGATTGATTTGCG 3 ′) were used for molecular identification (17). 25 µl PCR reactions, including 18.15 µl of deionized water, 2 µl (5 ng) of pattern genomic DNA, 1 µl (20 µM) of each primer, 2.5 µl 10X PCR buffer, 1 µl MgCl2 (50 mM), 0.2 µl dNTP (100 mM), and 0.15 µL of Taq DNA polymerase (5 U / (Cinna Gen Corp., Iran) in a

thermocycler (Primus, MWG Biotech), according to thermal program, were involved initial denaturalization at 94 °C for 5 min, annealing at 94 °C for 30 sec, hybridization at 55 °C for 30 sec, extension at 72 °C for 1.5 min in 30 cycles, and final extension at 72 °C for 7 min.

Table 2- Primers for aflatoxin genes detection

Genes involved in aflatoxin biosynthesis	Primers	Size of band (bp)
C_3	F: GCGATCTGTAACACTACACA R: GCCATACGATTCCCAAGTCT	622
nor1	F: AGCACGATCAAGAGAGGCTC R: GATCTCAACTCCCCTGGTAG	150
aflR	F: ATGGTCGTCCTTATCGTTCTC R: CCAGACAAAGACGGATCC	608
estA	F: CGATGGGACTGACGGTGATT R: ACCACGCCGCTGACTTTAT	529
avnA	F: GCGATAGAACTGACAAAGGCA R: AATGAGTCTCCAAAGGCGAG	540

After preparing 1% agarose gel, 2 µg/ml ethidium bromide was added to it for DNA staining. To observe the PCR product, electrophoresis with a constant voltage of 85 volts was performed for 1 hour in agarose gel and the TAE (Tris-acetate-EDTA) buffer (hydroxymethyl 242 mg, acetic acid 57 ml, EDTA (Ethylenediaminetetraacetic acid) 100 100 ml, water up to a final volume of 1000 ml) (17). It was photographed under 366 UV (ultraviolet) light using Gel Documentation (UVtec, Cambridge, UK). In order to determine the weight of the fragments, a DNA size indicator called Gene RulerTM DNA ladder mix was used.

2.4.3- Aflatoxin gene detection

*Nor*1 (= *aflF*; encoding a putative aryl alcohol dehydrogenase), *afl*R (= *aflR*; encoding the aflatoxin pathway transcription factor), *est*A (= *aflJ*; encoding an esterase), *avn*A (= *aflG*; encoding a cytochrome P450 monooxygenase), and C3 (part of the 5' flanking region) genes (Table 2) were assayed to confirm presence or absence of deletion in the aflatoxin gene cluster leading to atoxigenicity as described

previously [11]. PCR assays were performed in 25 µL using 2 µL (5-50 ng) genomic DNA [16], 1 µL of each primer (20 µM), 2.5 µL of 10× PCR buffer, 1 µL of $MgCl_2$ (50 mM), 0.2 µL of dNTPs (100 mM), and 0.15 µL of Taq DNA polymerase (5 U/µL) supplied by the manufacturer (Cinna-Gene, Tehran, Iran), with 5 min initial denaturalization at 94 °C followed by denaturalization of 30 cycles of 30 sat at 94 °C, 30 sec annealing at 55 °C, 1.5 min extension at 72 °C, and final 7 min extension at 72 °C. A 5 µl aliquot of each PCR product was visualized under UV light on 1.0% agarose gel (Bio-Rad, Richmond, California, USA) in Tris–borate–EDTA buffer, stained with ethidium bromide (0.5mg/ml) for 10 min.

3. Results

3.1- Competition of atoxigenic isolates with toxigenic strain

Co-inoculation of toxigenic and atoxigenic isolates led to different growth patterns in the CAM medium. Uniform and white-streak green colonies were among the types of fungal growth and sporulation. The white streaks in the green indicate that one of the strains is not sporulating. The growth of one isolate is not simultaneous with the other, thus being delayed due to a factor induced by the other isolate (Fig. 1).

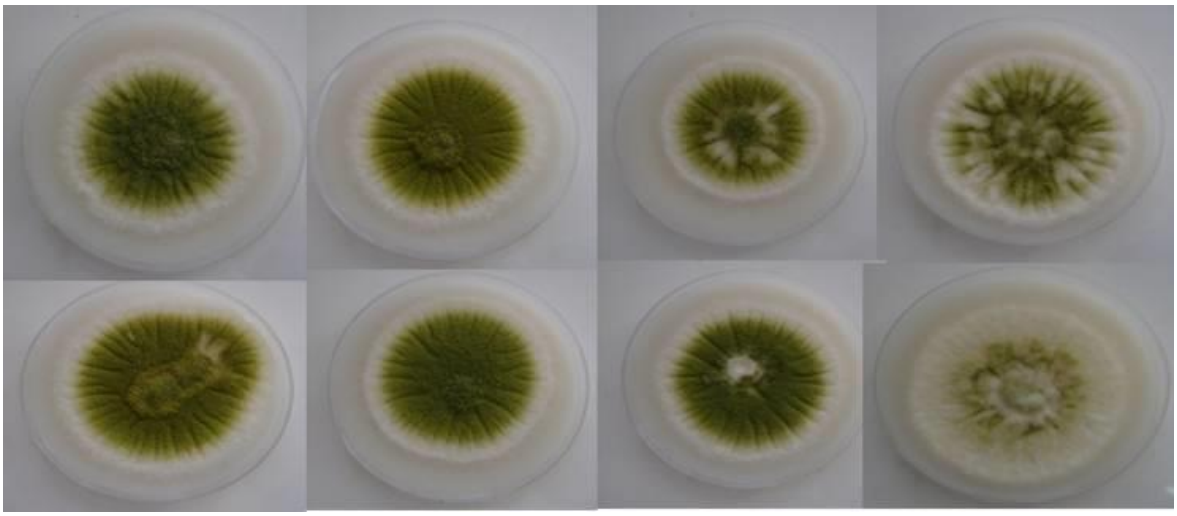

Fig.1- Different types of colony growth in co-inoculation of atoxigenic isolates with toxigenic isolate

3.2- Reactions of cultured colonies to ammonia vapor

The back surface color of the Petri plate with 96-hour fungal culture colonies changed immediately after exposure to ammonia vapor. Atoxigenic isolates were divided into 6 groups: dark red, pale red, pink, pale pink, orange, and without color change (Fig. 2).

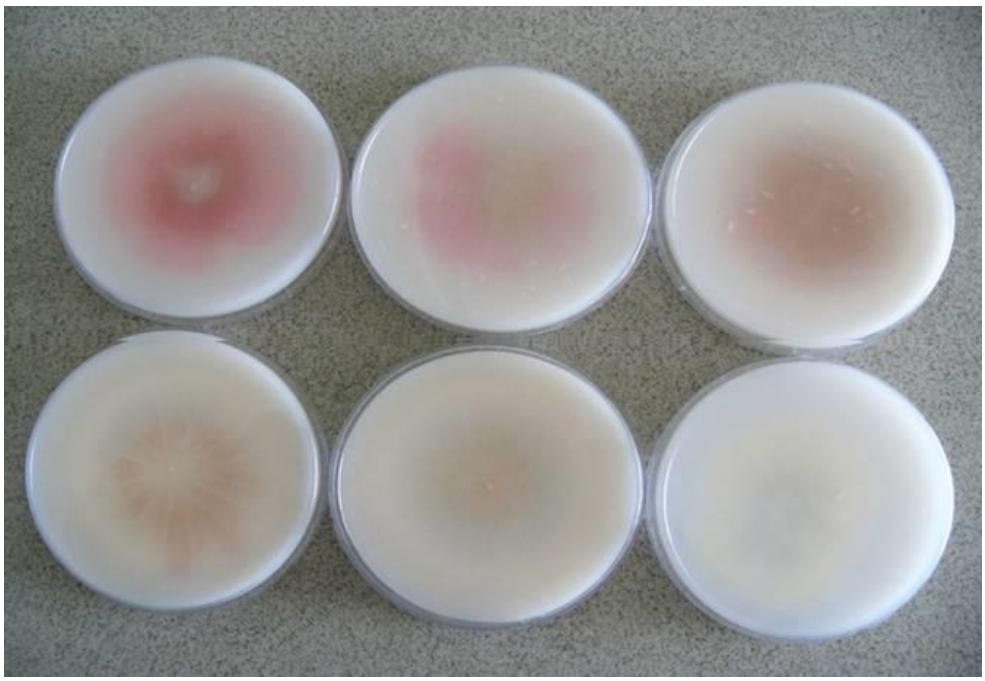

Fig. 2 - Color spectrum of mixed cultures of atoxigenic and toxigenic isolates in coconut agar medium (CAM) after reaction with ammonia vapor (backside)

The results of co-inoculation of atoxigenic and toxigenic isolates showed the color intensity reduction in the atoxigenic isolates after exposure to ammonia vapor. Accordingly, they were divided into 6 groups, as described in Table 4. The color variation ranged from dark red to no color. This suggests interference with the aflatoxin production process by atoxigenic isolates that may be useful in biological control. Comparison of isolates in terms of the color intensity showed 8.33%, 23.33%, 18.33%, 16.66%, 20%, and 13.33% of isolates to change into dark red, pale red, pink, pale pink, orange, and no color, respectively (Table 3).

Table 3- Grouping atoxigenic isolates of *Aspergillus flavus* based on the reaction type with toxigenic isolate (16499) in the presence of ammonia vapor in CAM

Colony discoloration after being exposed to ammonia vapor					
Dark red	**Pale red**	**Pink**	**Pale pink**	**Orange**	**No color**
16497	16467	16454	16495	16459	16471
16470	16477	16492	16463	16460	16446
16473	16472	16444	16457	16443	16495
16450	16490	16465	16475	16468	16445
16462	16483	16476	16484	16452	16497
	16486	16480	16453	16491	16498
	16449	16451	16486	16479	16466
	16455	16442	16447	16493	16464
	16487	16474	16460	16469	
	16478	16458		16481	
	16441	16448		16461	
	16482				
	16456				
	16485				

3.3- Molecular assays

Using species-specific primers and amplification of a 100 bp DNA fragment confirmed the identification of isolates as *A. flavus*. PCR assays using primers specific for 5 genes in the aflatoxin biosynthesis gene cluster were applied to assess genetic deletions explaining atoxigenicity in 23 isolates. The results showed different patterns involved in the deletion of one or more aflatoxin-producing genes to be the reason for the inability to produce aflatoxin. In most atoxigenic isolates, in addition to the difference in aflatoxin gene cluster compared to toxigenic strain isolate, the left side of this cluster also had a negative PCR reaction. For example, in isolates 16482, most of the studied genes and the C3 were deleted; also, in isolate 16478, the C3 of cluster and *avn*A was missed. Based on the results, different isolates were placed in twelve groups, as provided in Table 4.

Table 4- Deletion of genes in the aflatoxin biosynthesis gene cluster (*nor*1, *afl*R, *est*A, *avn*A) or flanking region (C3) among 23 atoxigenic and (one) toxigenic *A. flavus* isolates

No.	ITEM code	C3	*nor*1	*afl*R	*est*A	*avn*A	Deletion pattern
1	16453	o	o	o	o	o	1
2	16479	o	o	o	o	o	1
3	16447	o	o	o	o	o	1
4	16497	o	o	o	o	o	1
5	16498	o	o	o	o	o	1
6	16494	o	o	o	o	o	1
7	16446	o	o	o	o	o	1
8	14469	o	o	o	o	●	2
9	16452	o	o	o	o	●	2
10	16482	o	o	o	o	●	2
11	16493	o	o	o	●	o	3
12	16475	o	o	●	o	o	4
13	16490	o	o	●	o	o	4
14	15459	o	o	●	o	o	4
15	16449	o	o	o	●	●	5
16	16442	o	o	●	o	●	6
17	16481	o	o	●	o	●	6
18	16451	●	o	o	o	●	7
19	16478	●	o	o	o	●	7
20	16456	o	o	●	●	●	8
21	16486	●	●	o	●	o	9
22	16472	o	●	●	●	●	10
23	16491	●	o	●	●	●	11
24	16499*	●	●	●	●	●	12

Absence (empty circle) or presence (filled circle) of specified genetic regions in the aflatoxin biosynthesis gene cluster or flanking region of *Aspergillus flavus* isolates obtained from pistachio nuts and soil in Iran

*Toxigenic strain

4. Discussion

One of the challenges to the safety and health of humans and animals is the presence of aflatoxins in agricultural crops or their products, leading to making strict rules and regulations with different costs to manage contamination at different stages from a field to fork [18, 19]. Analysis of aflatoxin indicates that *Aspergillus* section *Flavi* members contain a combination of highly toxic strains with the ability to produce aflatoxins in large quantities, while others produce moderate aflatoxins in combination with atoxogenic strains [20]. Additionally, reports have suggested high levels of phenotypic diversity in the atoxigenic strains [21]. A study performed on the toxigenicity of *Aspergillus* isolates showed 89.15% of 120 isolates from different pistachio growing areas of *Aspergillus* section *Flavi* to be able to produce one or more types of aflatoxins while 10.83% did not produce any toxins [22]. Different aspects of atoxigenic isolates, including biological and ecological requirements and their evolution compared to all *A. flavus* isolates, have been studied by many researchers [23, 24]. Since the efficiency of biological control in most cases depends on used native isolates of each region, the genetic diversity of atoxigenic *A. flavus* isolates was studied, being useful for field application. However, biocontrol in the field conditions requires studying their competitiveness and vegetative compatibilities to toxigenic isolates. According to the results, the main difference between toxigenic and atoxigenic isolates was observed in the aflatoxin gene cluster, thus requiring further and extensive research. Among 58 atoxigenic isolates, 8 (13.8%) were able to significantly inhibit the toxigenic strain after co-inoculation with toxigenic isolate *in vitro;* 4, 3, and 1 of isolates were from Khorasan Razavi, Kerman, and Isfahan provinces, respectively, all with the source of pistachio nuts. The geographical diversity of the isolates can also be an advantage for field application. The combined use of atoxigenic strains in the aflatoxin biocontrol in maize has been successful in African countries [25]. In 3 efficient isolates, 5 genes in the aflatoxin cluster were deleted. Further research is needed to study the mechanism of atoxigenicity.

The use of atoxigenic *A. flavus* isolates for contamination management in various crops, such as peanuts, maize, pistachios, and cotton [4, 7], has been studied, and now it is being developed and applied extensively. The effectiveness of biological control in most cases depends on used native isolates of each region [1, 7]. In this study, the reaction of a toxigenic *A. flavus* strain isolated from Kerman pistachio orchard soil with 58 atoxigenic *A. flavus* isolates from different

agro-ecological zones is evaluated. These results can be useful in fields. However, the application of atoxigenic strains in field conditions requires evaluating their competitiveness and vegetative compatibility against toxigenic isolates. Although the population of atoxigenic isolates is low under natural conditions, their mass reproduction and release on fields or orchards lead to their predominant population in the environment. As a result, it leads to a significant decline in toxigenic *A. flavus* populations, which, in turn, indirectly affects food security and human health [26].

The main difference between toxigenic and atoxigenic isolates was observed in the aflatoxin gene cluster, which requires further and extensive research. The inability to produce aflatoxin in isolates may be due to the deletion of a gene partially or completely, several genes, or the entire aflatoxin gene cluster [27]. Elimination of genes in atoxigenic isolates is not abnormal and, in most cases, is a specific pattern that appears to have undergone quantitative changes throughout history [11]. Changes in an aflatoxin gene cluster in atoxigenic isolates in other crops, such as cotton, maize, and peanut, have been reported across the world [28, 29]. It should be noted that isolates with different gene deletion patterns have been used for biological control in the field.

5. Conclusion

Aflatoxins are potentially carcinogenic, and their levels in food and feed are closely monitored in most countries. Pistachio contamination with aflatoxin is one of the most important problems in pistachio consumption and export. Biological control of toxinogenic strains using atoxigenic strains is one of the most effective methods known to reduce contamination in different crops globally. Therefore, additional studies on atoxigenic strains to introduce the most effective and safest strains is an essential step in using the aflatoxin biocontrol method practically. Using this method, in addition to significantly reducing aflatoxin in pistachios produced and ensuring the health of domestic consumers, can improve its export to reputable foreign markets.

Conflict of Interest

The authors state that there is no conflict of interest.

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References

1- Amaike S, Keller NP. *Aspergillus flavus*. Annu. Rev. Phytopathol. **2011**; 49:*107-133*.

2- Pourhosseini R, Sedaghati E, Fani SR, Nadi M, Moradi M, Ahmadi Z. Potential of cyclopiazonic acid production in non-aflatoxinogenic strains of *Aspergillus flavus* of iranian pistachio. Pistachio Health J. **2020**; 3 (3): *6-17*.

3- Moradi M, Fani SR. Review of aflatoxin in pistachio and its control strategies. Plant Pathol. **2018**; 7(2): *22-33*.

4- Dorner JW. Biological control of aflatoxin contamination of crops. Toxin Rev. **2004**; 23(2-3): *425-450*.

5- Rouhani M, Moradi M, Hamed-Mosavian MT, Fani SR. Efficacy of saprophytic yeasts on growth of *Aspergillus flavus* and aflatoxin production. Pistachio Health J. **2018**; 1(1): *26-30*.

6- Moradi M, Rohani M, Fani SR, Mosavian MTH, Probst C, Khodaygan P. Biocontrol potential of native yeast strains against *Aspergillus flavus* and aflatoxin production in pistachio. Food Addit. Contam.: Part A. **2020**; 37(11): *1963-73*.

7- Ehrlich KC. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: Advantages and limitations. Front. Microbiol. **2014**; 5(50):*1-9*.

8- Cotty PJ. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathol. **1989**; 79:*808-814*.

9- Abbas HK, Shier WT, Horn BW, Weaver MA. Cultural methods for aflatoxin detection. J. Toxicol. **2004**; 23: *295-319*.

10- Fani SR, Moradi M, Zamanizadeh HR, Mirabolfathy M, Probst C. Distribution of nontoxigenic strains of *Aspergillus flavus* throughout pistachio growing areas in Iran. Appl. Entomol. Phytopathol. **2014**; 81(2): *179-89*.

11- Chang PK, Horn BW, Dorner JW. Sequence breakpoints in the afatoxin biosynthesis gene cluster and flanking regions in nonaflatoxigenic *Aspergillus favus* isolates, Fungal Genet. Biol. **2005**; 42: *914-23*.

12- Fani SR, Moradi M, Probst C, Zamanizadeh HR, Mirabolfathy M, Haidukowski M, Logrieco AF. A critical evaluation of cultural methods for the identification of atoxigenic *Aspergillus flavus* isolates for aflatoxin mitigation in pistachio orchards of Iran. Eur. J. Plant Pathol. **2014**; 140(4): *631-42*.

13- Davis ND, Iyer SK, Diener UL. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. **1987**; 53 (7): *1593-95*.

14- Saito M, Machida SA. Rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus*. Mycoscience. **1999**; 40: *205-208*.

15- Fani SR, Zamanizadeh H, Moradi M. Comparison between cultural methods and TLC for detection of atoxigenic strains of *Aspergillus flavus* isolated from Iranian pistachio. In First International Symposium on Mycotoxins in Nuts and Dried Fruits. **2012**; 963:*33-35*.

16- Moradi M, Oerke EC, Steiner U, Tesefaye D, Schellander K, Dehne HW. Microbiological and SYBR® Green real-time PCR detection of major *Fusarium* head blight

pathogens on wheat ears. Microbiol. **2010**; 79: 646-54.

17- Sardinas N, Vazquez C, Gil-Serna J, González-Jaen MT, Patino B. Specific detection and quantification of *Aspergillus flavus* and *Aspergillus parasiticus* in wheat flour by SYBR® Green quantitative PCR. Int. J. Food Microbiol. **2011**; 145(1), *121-125*.

18- Moradi M, Hokmabadi H. Control of mycotoxin bioactives in nuts: farm to fork. In: Tokusoglu O. ed. Nut and cereal bioactives sources. Chemistry and applications. CRC Press, **2011**;*P. 253-73.*

19- Jafari Nodoushan A, Fani SR, Sajadipour SJ, Fattahi M. HACCP and its effects on aflatoxin contamination of pistachio in Yazd Province. In I International Symposium on Mycotoxins in Nuts and Dried Fruits. **2011**; 963: *213-15*.

20- Moradi M, Fani SR, Masoumi H. Population density of *Aspergillus* species belongs to section *Flavi* and *Nigri* on pistachio nut in Kerman province. J Appl Res. Plant Prot. **2014**; 3 (2): 79- 91.

21- Moradi M, Dargahi R, Sherafati A, Fani SR, Sedaghati E, Mohammadi-Moghaddam M, Mirabolfathy M. Screening of atoxigenic *Aspergillus flavus* isolates in pistachio producing areas of Iran. In I International Symposium on Mycotoxins in Nuts and Dried Fruits. **2011**; 963: *91-3.*

22- Mohammadi-Moghadam M, Rezaee S, Mohammadi AH, Zamanizadeh HR, Moradi M, Fani SR. The potential of aflatoxin production in the *Aspergillus* section *Flavi* isolates of pistachio in Iran. J Nutr Fast Health. **2020**; 8(4): *254-63*.

23- Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. Distribution of *Aspergillus* section *Flavi* in soils of maize fields in three agro-ecological zones of Nigeria. Soil Biol. Biochem. **2009**; 41(1): *37-44*.

24- Donner M, Atehnkeng J, Sikora RA, Bandyopadhyay R, Cotty PJ. Molecular characterization of atoxigenic strains for biological control of aflatoxins in Nigeria. Food Addit. Contam. **2010**; 27(5):*576-90*.

25- Senghor LA, Ortega-Beltran A, Atehnkeng J, Callicott KA, Cotty PJ, Bandyopadhyay R. The atoxigenic biocontrol product Aflasafe SN01 is a valuable tool to mitigate aflatoxin contamination of both maize and groundnut cultivated in Senegal. Plant Dis. **2020**; 104(2): 510-20.

26- Cotty P. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. Phytopathol. **1994**; 84(11):*1270-77*.

27- Ehrich KC, Cotty PJ. An isolate *Aspergillus flavus* used to reduce aflatoxin contamination in cottlnseed has a defective polyketide synthase gene. J Microbial Biotecnol. **2004**; 65(4): *473-478*.

28- Savic Z, Dudas T, Loc M, Grahovac M, Budakov D, Jajic I., Krstovic S, Barosevic T, Krska R, Sulyok M, Stojsin V. Biological control of aflatoxin in maize grown in Serbia. Toxins **2020**; 12(3):*162*.

29- Pitt JI, Hocking AD. Mycotoxins in Australia: biocontrol of aflatoxin in peanuts. Mycopathologia **2006**; 162(3): *233-43.*