Competition of Different Aspergillus flavus VCGs in the Culture Medium and Soil

Hajar Azmoun (MSc) [1], Seyed Reza Fani (PhD)[2*], Hamid Reza Zamanizadeh (PhD)[1]

[1] *MSc student and Professor, Department of Plant Pathology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Assistant professor, Plant Protection Research Department, Yazd Agricultural and Natural Resources Research and Education Center, AREEO, Yazd, Iran*

Information

Article Type:
Original Article

Article History:

Received: 16.02.2022
Accepted: 18.03.2022

Doi: 10.22123/PHJ.2022.330074.1123

Keywords:
Food safety,
Mycotoxin
Biocontrol
Atoxigenic

Corresponding Author:
Seyed Reza Fani

Email: rezafani52@gmail.com

Tel: +98-9132507302

Abstract

Background: The most effective aflatoxin prevention strategy is limiting populations of toxigenic *Aspergillus flavus* strains using non-toxigenic strains, as a biological method.

Materials and methods: Ten different vegetative compatibility groups (VCGs) of *A. flavus* strains (5 toxigenic and 5 non-toxigenic) isolated from the fruit and soil of pistachio orchards were used for the purpose of this study. Next, an equal population of spores of each isolate was mixed together and cultured on a coconut agar medium (CAM) and incubated in the dark at 30 °C for 3 days. On the other side, 20 gr of a mixture containing soil, peat, and pistachio powder was inoculated with the same spore mixture in the flask for 10 days. In addition, 10 colonies were selected randomly from each treatment and cultured on the coconut agar medium for three days. Besides, ammonium hydroxide vapor was used as an aflatoxin marker.

Results: Simultaneous inoculation of the isolates in the culture medium showed 4 different types of color spectra after contacting ammonia vapor. Non-toxigenic and toxigenic isolates were predominant in 60 and 20% of the colonies, respectively. Besides, there was an intermediate situation in the remaining 20% of the culture media. The results showed that toxigenic and non-toxigenic populations were equal in 36% of the treatments. In addition, toxigenic and non-toxigenic isolates were dominant in 40 and 24% of the treatments, respectively. Non-toxigenic isolates with a high prevalence seemed to be qualified volunteers for supplementary biocontrol assays.

Conclusion: Non-toxigenic isolates with a high prevalence seemed to be qualified candidates for supplementary biocontrol assays.

►Please cite this article as follows:
Azmoun F, Fani SR, Zamanizadeh HR. Competition of Different Aspergillus flavus VCGs in the Culture Medium and Soil. Pistachio and Health Journal. 2022; 5 (1): 11-21 .

1. Introduction

A domestic pistachio tree is a dicotyledonous and deciduous plant that is mainly cultivated in subtropical regions. Central Asia is the origin of pistachios, and the main habitats of wild pistachio trees today are countries in this region, including Iran, Turkmenistan, and Afghanistan. According to the available historical documents, the origin of pistachios and their primary habitat in Iran is the northeastern region, which is the land that was later named Parth and then Khorasan [1]. Pistachios, being a strategic product, have the highest rank among other agricultural export products, having a special place among Iranian agricultural products. Aflatoxin contamination is the major export problem of pistachios. Since aflatoxins are toxic and carcinogenic, their levels in foods are carefully monitored, with their limit being 5-15 ng/g in most countries [2, 3]. About 20% of the world food products is annually contaminated with mycotoxins, of which aflatoxin contamination is a great concern. More than 13 *Aspergillus* species are able to produce aflatoxins, and *A. flavus* is the most important species in the pistachio growing regions [4]. More than 100 countries have been proposed to place a limit on aflatoxin levels in different food products [5]. In fact, different methods have been recommended to reduce or prevent the production of carcinogenic fungal toxins [6]. Aflatoxins can be controlled using plant extracts. In this regard, the effects of pistachio kernel coating, with a mixture of whey protein concentrates and selected plant extracts (sage, cumin, and thyme), on inhibiting aflatoxins in storage were investigated. Accordingly, the results showed that with an increase in the concentration of Shirazi thyme in this type of coating, aflatoxin production decreased. Besides, the sage extract had the highest potential for preventing aflatoxin production compared to the thyme extract and cumin [7]. The use of non-toxigenic strains, as a biological control method, is the most effective strategies for eliminating toxigenic *Aspergillus flavus* strains. In this method, the non-toxigenic strain, having been artificially spread in the field or in the orchard environment, overcame toxigenic strains in the environment in a competition for space and food, which reduced aflatoxin production in agricultural crops [8-12]. Interactions among these strains and the mechanism of action were studied in several studies, and the efficacy of non-toxigenic strains was measured by the aflatoxin reduction rate under laboratory and field conditions [9]. The use of biological control in California pistachio orchards yielded good results and significantly reduced aflatoxin levels in the crops [9]. Based on the area cultivated, the large production area of pistachios, and the importance of aflatoxins in the Iranian production, this strategy can be successfully used to increase the quality and health of pistachios and raise their economic rank in international markets [2]. *A. flavus* is a species with varied toxigenic and non-toxigenic strains, strains with different responses to light, strains present in different vegetative compatible groups (VCGs), and strains with diverse capabilities for colonizing living plant tissues [9].

Although no frequent genetic exchanges have been observed among different VCGs, genetic recombination is likely to occur in populations [13]. Genetic recombination has been observed between toxigenic and non-toxigenic strains of *A. flavus*, with some of these recombinants regaining the ability to produce aflatoxins [14, 15]. It has been shown that the non-toxigenic population of *A. flavus* may be

higher than the toxigenic population in some agricultural environments [16]. This could be due to the loss of the aflatoxin production capacity in toxigenic *A. flavus* strains as a result of adaptation to a carbon-rich environment, which makes aflatoxin clusters more genetically instable [16]. Toxigenic strains may have a relative long-term advantage over non-toxigenic strains (biocontrol agents) for surviving in the soil due to their ability to produce aflatoxins, which may be offset in agricultural environments. The dominance of the effective population leads to an increase in the mean mutation and recombination rate of the population [17]. As a result of further evolutions of new VCGs, some of them lost the ability to produce aflatoxins due to mutations in the biosynthetic cluster genes or due to large chromosomal deletions, leading to the loss of entire telomere areas [18].

This study aimed to investigate population changes of different toxigenic and non-toxigenic VCG isolates obtained from different agroecological zones in the culture medium and the soil environment during a short period of time.

2. Materials and methods

Aspergillus flavus isolates

A total of five toxigenic *Aspergillus flavus* isolates and five non-toxigenic *Aspergillus flavus* isolates were obtained from the soil and pistachio nuts of orchards in Kerman, Khorasan Razavi, and Isfahan provinces from the Plant Protection Laboratory of the Yazd Agricultural and Natural Resources Research and Education Center, which were used after purification by the single spore method [19]. The isolates used were different VCGs that were grouped with a microsatellite marker [20] (Table 1).

Table 1. Characteristics of *Aspergillus flavus* isolates.

No.	Code	Location	Source	Cultivar	Toxigenicity	VCG group
1	16453	Kerman	Nut	Badami	Non-toxigenic	1
2	16498	Kerman	Nut	Badami	Non-toxigenic	1
3	16481	Khorasan Razavi	Nut	Fandoghi	Non-toxigenic	2
4	16491	Khorasan Razavi	Nut	Kalleghouchi	Non-toxigenic	2
5	16464	Isfahan	Nut	Ohadi	Non-toxigenic	3
6	T99	Kerman	Soil	Sarakhs	Toxigenic	4
7	T106-3	Khorasan Razavi	Nut	Kalleghouchi	Toxigenic	4
8	T106-6	Khorasan Razavi	Nut	Kalleghouchi	Toxigenic	5
9	T1806	Kerman	Soil	Sarakhs	Toxigenic	5
10	T31	Isfahan	Nut	Ohadi	Toxigenic	6

Medium composition

Coconut agar medium (CAM): One liter of boiling water was added to 200 g of shredded coconut. After 5 min of stirring, the liquid was filtered through 4 layers of cheese cloth. Next, 20 g of agar powder (Himedia, India) was added to it and transferred to an autoclave for 20 min at 1.5 atmospheric pressure and 120 °C for sterilization. In addition, the medium was poured into 9 cm petri plates [21].

5/2 medium: A total of 50 ml of V8 juice and 20 g of agar were added to 800 liters of distilled water and sterilized in an autoclave for 20 min [22].

Simultaneous culture of toxigenic and non-toxigenic isolates in CAM

A. flavus spore production fungal spores were cultured on the V8 medium and incubated at 30 °C in the dark for three days. The collected spores were counted using a turbidimeter (Hach, Germany) and calculated using the nephelometric turbidity unit (NTU) versus the CFU curve of Y = 49,937X, with X = NTU and Y = conidia/ml. The conidial concentration of each isolate was adjusted at 4×10^5 conidia/ml. Next, the spore suspensions of toxigenic and non-toxigenic isolates were mixed together and cultured as a point on CAM and incubated at 30 °C in the dark for three days [23]. To determine the growth status of the isolates in the co-inoculation, the petri dishes were inverted, and 200 µl of 25% ammonium hydroxide was added to the lid of each petri dish. Next, discoloration of the back of the colony to red was considered a measure of toxigenicity of the isolates, with the results recorded accordingly [24].

Co-inoculation of *A. flavus* isolates in the soil mixture

A mixture of 20 g containing equal amounts of soil, peat, and ground pistachios was poured into an Erlenmeyer flask and sterilized three times by an autoclave at 120 °C and at 1 atmospheric pressure. After adjusting humidity at 25% by sterile distilled water, the soil mixture was inoculated with an equal number of different toxigenic and non-toxigenic *A. flavus* spores (4×10^5 spores) and incubated at 30 °C in the dark for 10 days. The fungal population was recovered after preparing the dilution series and culturing it on the 5/2 medium. After the growth of the recovered colonies, 10 colonies were selected randomly from each treatment, cultured on CAM, and incubated for 3 days at 30 °C in the dark. Next, toxigenic and non-toxigenic isolates were screened using the ammonia vapor method as described earlier.

3. Results

Discoloration of the back of the colony in co-culture of isolates

Simultaneous inoculation of the isolates showed 4 different types of color spectra of the fungal colony after contacting ammonia vapor. Discoloration of the back of the colony to red in toxigenic isolates cultured alone was considered as group 1. In addition, non-discoloration of the back of the colony in petri dishes, where non-toxigenic isolates were cultured alone, was considered as group 4. A total of 25 treatments in which a combination of two isolates was cultured were divided into two groups or separate groups based on the predominance of discoloration in the back of the colony. According to the results, in 16% of the treatments (4 treatments), non-toxigenic isolates (group 4) were predominant. In addition, in 20% of the treatments (5 treatments), toxigenic isolates (group 1) were completely predominant. Besides, in 20% of the treatments (5 treatments), there was no significant difference in the growth dominance of toxigenic or non-toxigenic isolates (group 2). However, in 44% of the treatments (11 treatments), non-toxigenic isolates showed relative dominance (Table 2, Fig. 1).

Table 2. Colony classification of the co-growth of toxigenic and non-toxigenic spores of *Aspergillus flavus* in the coconut agar medium (CAM)

No.	Treatment	Group*	No.	Treatment	Group
1	16453	4	19	T99+16491	2
2	16464	4	20	T99+16498	4
3	16481	4	21	T106-3+16453	3
4	16491	4	22	T106-3+16464	3
5	16498	4	23	T106-3+16481	2
6	T31	1	24	T106-3+16498	3
7	T106-6	1	25	T106-3+16491	3
8	T99	1	26	T31+16453	3
9	T106-3	1	27	T31+16464	3
10	T1806	1	28	T31+16481	2
11	T1806+16453	1	29	T31+16491	2
12	T1806+16464	1	30	T31+16498	3
13	T1806+16481	1	31	T106-6+16453	4
14	T1806+16491	1	32	T106-6+16464	4
15	T1806+16498	1	33	T106-6+16481	3
16	T99+16453	3	34	T106-6+16491	2
17	T99+16464	3	35	T106-6+16498	4
18	T99+16481	3			

* Group 1: toxigenic being completely dominant; Group 2: equal dominance; Group 3: non-toxigenic being dominant; Group 4: non-toxigenic being fully dominant

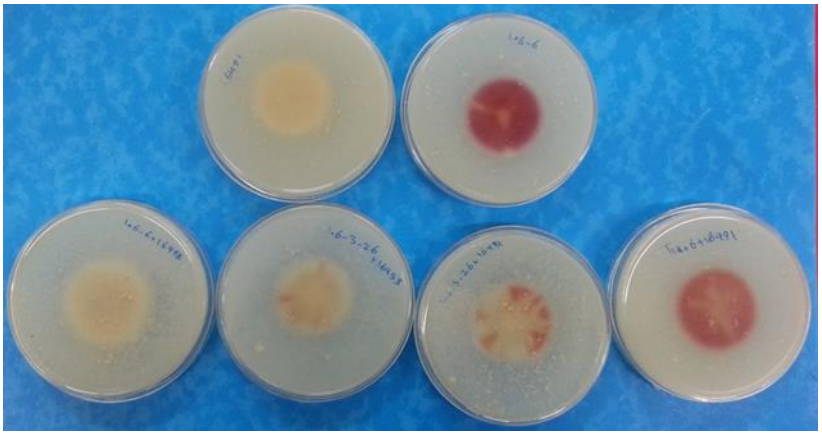

Fig. 1. Discoloration of the back of the *Aspergillus flavus* colony in CAM after reaction with ammonia vapor. Higher row: non-toxigenic isolate on the left and toxigenic isolate on the right; Lower row from left to right: non-toxigenic is completely dominant, non-toxigenic is dominant, there is equal dominance, and toxigenic is dominant, respectively.

Recovery of *A. flavus* isolates from the soil mixture

The results of the random recovery of 10 fungal isolates after preparation of dilution series from soil mixtures showed that the populations of toxigenic and non-toxigenic isolates were equal in 36% of the treatments. Besides, the populations of toxigenic and non-toxigenic isolates were dominant in 40 and 24% of the treatments, respectively. In addition, the predominance of colonies in each treatment varied depending on the composition of the isolates used, with the dominance of 20-80% and 10-90% observed in non-toxigenic and toxigenic isolates, respectively. Furthermore, the highest and lowest efficiencies in the soil substrate occupied by non-toxigenic isolates were observed in isolates 16481 and 16498, in contrast to toxigenic isolates T106-6 and T1806, with the prevalence of 90 and 20%, respectively. Additionally, no intermediate phenotype of discoloration was observed in the reaction of the recovered colonies with ammonia vapor (Table 3, Figs. 2, 3).

Table 3. The ratio of the colony number of toxigenic/non-toxigenic *Aspergillus flavus* isolates after simultaneous inoculation of soil with the mixture of isolates

No.	Treatment	Toxigenic/non-toxigenic colony ratio	No.	Treatment	Toxigenic/non-toxigenic colony ratio
1	16453	0/10	**19**	T99+16491	7/3
2	16464	0/10	**20**	T99+16498	6/4
3	16481	0/10	**21**	T106-3+16453	5/5
4	16491	0/10	**22**	T106-3+16464	4/6
5	16498	0/10	**23**	T106-3+16481	5/5
6	T31	10/0	**24**	T106-3+16498	6/4
7	T106-6	10/0	**25**	T106-3+16491	3/7
8	T99	10/0	**26**	T31+16453	5/5
9	T106-3	10/0	**27**	T31+16464	3/7
10	T1806	10/0	**28**	T31+16481	3/7
11	T1806+16453	5/5	**29**	T31+16491	3/7
12	T1806+16464	6/4	**30**	T31+16498	5/5
13	T1806+16481	8/2	**31**	T106-6+16453	8/2
14	T1806+16491	5/5	**32**	T106-6+16464	6/4
15	T1806+16498	8/2	**33**	T106-6+16481	1/9
16	T99+16453	8/2	**34**	T106-6+16491	5/5
17	T99+16464	5/5	**35**	T106-6+16498	5/5
18	T99+16481	7/3			

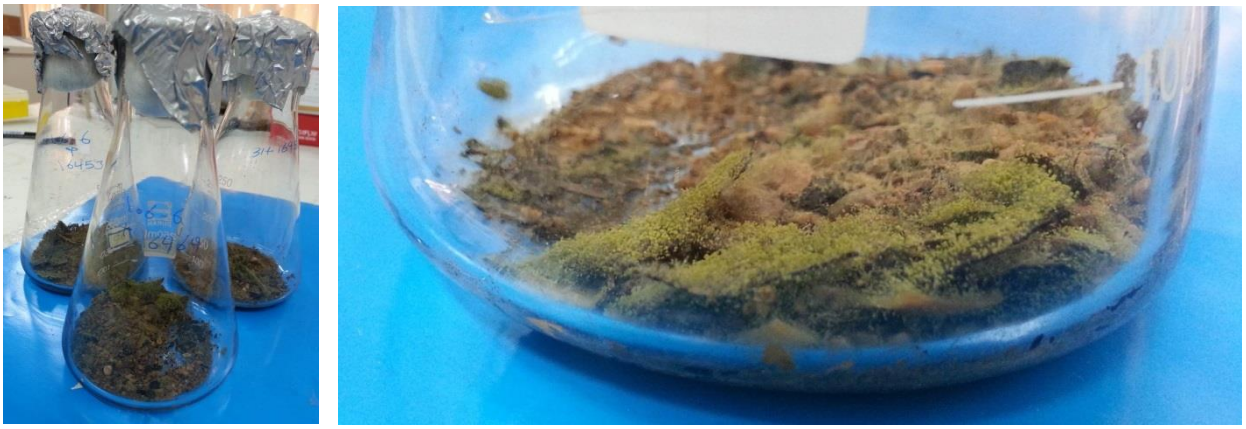

Fig. 2. The soil, peat, and ground pistachio mixture co-inoculated with toxigenic and non-toxigenic *Aspergillus flavus* isolates

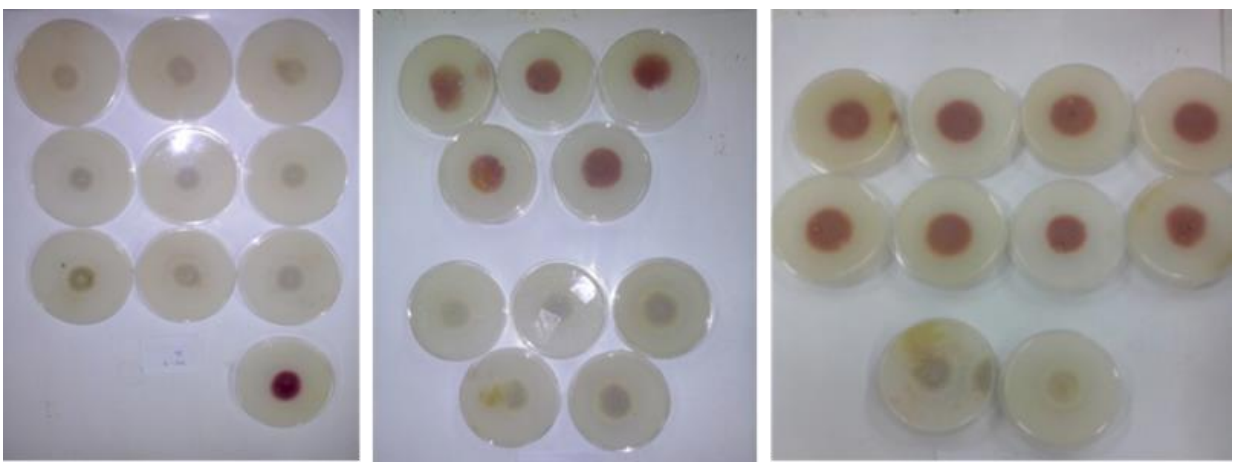

Fig. 3. Different population ratios of toxigenic and non-toxigenic *Aspergillus flavus* in 10 randomly recovered isolates from the soil mixture after 10 days in response to ammonia vapor; left: the non-toxigenic dominant population; middle: the equal population of two isolates; right: the toxigenic dominant population.

4. Discussion

Aflatoxins affect food safety and trades in agricultural and livestock products adversely. In fact, this effect is even greater on an important product such as pistachios, due to their high economic value. Therefore, management of aflatoxin contamination in pistachios is necessary to maintain the health of consumers and international markets. In recent years, public awareness of the dangers of aflatoxins to the health of humans and livestock has increased, and the monitoring of contaminants has become more strict [2, 23]. Different methods have been introduced to reduce aflatoxins in pistachios. These methods include the selection of early splitting-resistant cultivars as primary sources of pistachio contamination as well as other agronomic and physical strategies that can be employed during the growth of pistachio trees, at harvest and post-harvest stages,

transportation, and storage. The use of these methods is not always possible everywhere, being limited in practice [6]. A significant amount of aflatoxins is produced in orchards before pistachio harvest times, so preventing aflatoxin production can be a key factor in controlling aflatoxin contamination [6, 25]. Elimination of *A. flavus* toxigenic strains, using native non-toxigenic strains, is in progress because it is cost-effective and environmentally friendly. Carefully selected native non-toxigenic strains can effectively reduce toxigenic populations before flowering [26]. *A. flavus* populations in Iranian pistachio orchards are the combination of toxigenic and non-toxigenic strains. A study reported that 6.2-25% of the isolates were not able to produce aflatoxins in groups B and G [19]. *A. flavus* is composed of many VCGs, and some VCGs are composed of only non-toxigenic members [27].

Non-toxigenic VCG members are not related to toxigenic VCGs. Thus, they will be considered valuable aflatoxin biocontrol agents if they inhibit the growth and reproduction of toxigenic strains by occupying the substrate.

In this study, five non-toxigenic strains from three major pistachio producing provinces of Iran (Kerman, Khorasan Razavi, and Isfahan) of three different VCGs were evaluated and compared with five toxigenic strains of different VCGs.

The treatments were evaluated qualitatively in both stages of the experiment. In the competition test in the coconut agar medium, 60% of the non-toxigenic strains were more dominant than the toxigenic strains. Among the non-toxigenic strains, isolate 16498 from the Kerman province seemed to be more effective than others. Accordingly, this isolate was quite dominant in competition with two different toxigenic isolates, i.e. T99 and T106-6. However, the efficiency of over 50% of the non-toxigenic isolates can make them be considered as aflatoxin biocontrol agents [23]. It is noteworthy that the toxigenic strains displayed different reactions in the competition test. Strain T1806, for example, was dominant in competition with all non-toxigenic strains. Among non-toxigenic strains, strain 16491 from the Khorasan Razavi province was evaluated as the weakest isolate. Random recovery of strains after simultaneous inoculation of the sterile soil mixture showed that all non-toxigenic isolates were relatively defeated in competition with toxigenic isolates T1806 and T99. The best performance of non-toxigenic strains was related to the competition test with toxigenic isolates T31 and T106-3 that were predominantly non-toxigenic isolates.

Due to genetic differences among the isolates, the possibility of heterokaryon formation and changes in toxicogenic behavior between non-toxigenic isolates was weak. Thus, the dominance of some isolates can be considered a measure of isolate efficiency and its suitability as a candidate for aflatoxin biocontrol in future research. Since there are a variety of microflora in nature and there are complex interactions among different microbial populations, efficiency of the studied strains cannot be considered sufficient. The results of this study showed that there are non-toxogenic strains that are competitive to mitigate aflatoxins. Commercial aflatoxin biocontrol products use a combination of non-toxin producing strains to improve performance [26]. For example, 13 and 12 non-toxigenic strains were used in commercial products in Ghana and Kenya, respectively [26]. Therefore, more studies are needed to identify the best combination of non-toxin isolates to control pistachio aflatoxins in the country.

5. Conclusion

This research can be used as a preliminary study for examining efficacy of non-toxigenic isolates as biocontrol agents of aflatoxins. Some non-toxigenic isolates were successful both in culture and in competition with toxigenic isolates in the soil mixture. Since vegetative compatibilities of the groups of these isolates were different from each other, they can be used as effective volunteers in complementary studies.

Acknowledgments

The authors would like to thank the Yazd Agricultural and Natural Resources Research and Education Center for providing the required materials and isolates and assisting with this project.

Financial support

This research was supported by the Center for Progress and Development (CPDI) of Iran Presidency

.Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

Code of ethics

No living entity was used in this research, and the research stages were passed through in a laboratory.

References

1- Abrishami MH. Iranian Pistachio: Historical Knowledge. Iranian University Publication;**1994**.
2- Moradi M, Fani SR. Review of aflatoxin in pistachio and its control strategies. Plant Pathol. **2018**; 7(2): *22-33*.
3- Mokhtarian M, Tavakolipour H, Bagheri F, Fernandes Oliveira CA, Corassin C.H., Mousavi Khaneghah A. Aflatoxin B1 in the Iranian pistachio nut and decontamination methods: A systematic review. Qual. Assur. Saf. Crop. Foods. **2010**; 12(4): *15-25*.
4- Moradi M, Fani SR, Masoumi H. Population density of *Aspergillus* species belongs to section *Flavi* and *Nigri*on pistachio nut in Kerman Province, Appl. Res. Plant Prot.**2014***;* 3(2): *79-91*.
5- Wu F, Stacy SL, Kensler TW. Global risk assessment of aflatoxins in maize and peanuts: are regulatory standards adequately protective? Toxicol. Sci. **2013**; 135: 251–259.
6- Moradi M, Hokmabadi H. Control of mycotox in bioactives in nuts: farm to fork. In: Tokusoglu Ö. ed. Nut and cereal bioactives sources. Chemistry and applications. CRC Press, **2011**. P. *253-273*.
7- Tavakolipour H, Kalbasi-Ashtari A, Mokhtarian M. Effects of coating pistachio kernels with mixtures of whey protein and selected herbal plant extracts on growth inhibition of *Aspergillus flavus* and prevention of aflatoxin during storage. J. Food Saf. **2020**; 40(1): p.e*12711*.
8- Amaike S, Keller NP. *Aspergillus flavus*. Annu. Rev. Phytopathol. **2011**; 49:107-133.
9- Ehrlich KC. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. Front. Microbiol. **2014**; 5(50):*1-9*.
10- 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*.

11- 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*.
12- 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*.
13- 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: *914–923*.
14- Olarte RA, Horn BW, Dorner JW, Monacell JT, Singh R, Stone EA, et al. Effect of sexual recombination on population diversity in aflatoxin production by *Aspergillus flavus* and evidence for cryptic heterokaryosis. Mol. Ecol. **2012**; 21:*1453–1476*.
15- Horn BW, Sorensen RB, Lamb MC, Sobolev VS, Olarte RA, Worthington CJ, Carbone I. Sexual reproduction in *Aspergillus flavus* sclerotia naturally produced in corn. Phytopathol. **2014**; 104(1): *75-85*.
16- Horn BW, Dorner JW. Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Appl. Environ. Microbiol. **1999**; 65:*1444–1449*.
17- Hartl D, Clark A. Principles of Population Genetics. Sunderland, MA: Sinauer Associates.**1997**.
18- Chang PK, Ehrlich KC, Hua SSH. Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotypes. Int. J. Food Microbiol.**2006**; 108:*172–177*.
19- 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.
20- Fani SR, Azmoun H, Zamanizadeh HR. Detection of genetic diversity in Iranian *Aspergillus flavus* isolates by SSRs. In 1st MYCOKEY International Conference Global Mycotoxin Reduction in the Food and Feed Chain. 2017; 276: 74-75.
21- Davis ND, Iyer SK, Diener UL. Improved method of screening for aflatoxin with a coconut agar medium. Appl. Environ. Microbiol. 1987; 53 (7): 1593-1595.
22- Cotty PJ. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. Phytopathol. **1989**; 79:*808-814*.
23- Probst C, Bandyopadhyay R, Price LE,Cotty PJ. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. Plant Dis. **2011**; 95(2): *212-218*.
24- Saito M, Machida SA. Rapid identification method for aflatoxin-producing strains of *Aspergillus flavus* and *A. parasiticus*. Mycoscience. **1999**; 40: *205-208*.
25- 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*.
26- Peles F, Sipos P, Kovács S, Győri Z, Pócsi I, Pusztahelyi T. Biological control and mitigation of aflatoxin contamination in commodities. Toxins. **2021**; 13(2): *1-19*.
27- Bayman P, Cotty PJ. Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. Can. J. Bot. **1993**; 71:*23-31*.