Potential of Cyclopiazonic Acid Production in Non-Aflatoxinogenic Strains of *Aspergillus flavus* of Iranian Pistachio

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Abstract

Introduction: Biocontrol of toxigenic populations with nontoxigenic strains has long been introduced as an effective method of reducing aflatoxins in crops such as corn, cottonseed, oilseeds, and pistachios. But if these nontoxigenic strains produce cyclopiazonic acid, being a less important fungal secondary metabolite, they may have unwanted negative consequences.

Materials and Methods: In this study, it has been attempted to investigate the production of cyclopiazonic acid in 58 non-aflatoxigenic strains and one aflatoxigenic strain of *Aspergillus flavus* obtained from pistachio soil and nuts of orchards in Kerman, Yazd, Khorasan Razavi, Esfahan, Qom, Semnan and Markazi provinces. For evaluating the production of cyclopiazonic acid, the isolates were first cultured in CYA medium, and for each isolate, three inoculations were kept in a dark incubator at 25°C for 14 days. Then, the ability to produce cyclopiazonic acid was assayed by high performance liquid chromatography (HPLC).

Results: The production of cyclopiazone in different isolates was 6.951-357.6 mg/l. The results showed that out of 59 *A. flavus* isolates, as many as 44 isolates were not able to produce cyclopiazonic acid. Also, the percentage of non-aflatoxinogenic isolates that were not able to produce cyclopiazonic acid has been estimated to be 76%.

Conclusion: The results of this study can be useful in selecting suitable and efficient isolates for biological control of aflatoxin in orchards.

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1. Introduction

Pistachio is one of the most important horticultural products in Iran, and Kerman Province has the largest area under cultivation and production of pistachio (1). Most of Iran's non-oil exports belong to pistachio accounting for significant foreign exchange earnings (2). However, contamination of pistachios with *Aspergillus* fungi and the mycotoxins arising from them has faced pistachio exports with serious problems (1, 3, 4). Damage to food and agricultural products destroyed by this toxin in the United States has been estimated to be more than 100 million dollars per year (5). In Africa, more than 750 million dollars is the annual cost of aflatoxin contamination of crops (6). Biological control of aflatoxin using non-toxic isolates of *Aspergillus flavus* is being used worldwide in crops such as cottonseed, peanuts, corn and pistachios.

In the *A. flavus* gene clustering, in addition to the genes responsible for the production of aflatoxins B1 and B2 (7), other secondary metabolites including cyclopiazonic acid, aflaterm, kojic acid, sterigmatocystin, versicolorin ochratoxin, gliotoxin, citrinin, penicillic acid, citreoviridin, and xanthomogenin (8).

Different methods have been recommended to manage the contamination of different products with *Aspergillus* or Aflatoxin, such as cultural, mechanical, physical and biological controls. Each method has its own advantages and disadvantages depending on the place, time, type of product, applicability, and efficiency (9, 10, 11, 12). Sanitary restrictions and international laws and regulations do not allow for the application of many common methods on pistachios (13). Microorganisms including bacteria, yeasts, Actinomycetes and nontoxigenic strains of *A. flavus* are capable of the biological control of its toxigenic strains (14). The usage of nontoxigenic strains of *A. flavus* fungus is being widely used or developed on a number of products including cotton, pistachios, peanuts, almonds and figs. As an effective method, it has reduced 70 to 90% of the population of toxigenic strains in farms and orchards (15).

In 1968, cyclopiazonic acid (CPA) was first discovered and described as a chemical. Cyclopiazonic acid is produced by several species of *Penicillium* (*P. griseofulvum*, *P. camemberti*, *P. commune*, and *P. dipodomyicola*) and *Aspergillus* species (*A. flavus*, *A. oryzae* and *A. tamarii*). CPA has been reported to exist in foods (e.g. oilseeds, nuts, cereals, dried figs, milk, cheese, and meat products), and it is of significant toxicological importance. Moreover, it has been frequently detected in peanuts and corn. The presence of CPA and aflatoxin in corn and peanuts infected with *A. flavus* indicates that synergies are likely to occur.

This mycotoxin is toxic to several animal species including mice, pigs, guinea pigs, chickens and dogs. Tested animals have indicated serious gastrointestinal system problems and neurological disorders after taking CPA-contaminated foods. The affected organs include liver, kidney, heart and digestive system showing degenerative changes and necrosis. Biologically speaking, CPA is a specific inhibitor of the sarco/endo*plasmic reticulum* of Ca2+ -ATPase. The data obtained from the toxicological evaluation of aflatoxin and CPA in broilers show that both aflatoxin and CPA alone and the aflatoxin-CPA combination can adversely affect the of broilers. The effects of aflatoxin and CPA combination have been exacerbated in most cases (16).

Cyclopiazonic acid is an indole tetraminic acid having no strong acute toxicity, and its oral LD_{50} in rodents is 30-70 mg/kg (17). The main target of this toxin is the nervous system; it is therefore considered a neurotoxin. The symptoms associated with CPA include ataxia (lack of muscle control or coordination of voluntary movements) and in severe cases, death caused by spastic paralysis. In addition, it may cause lesions in the digestive system. Cellular effects of being exposed to CPA are associated with inhibition of calcium flux in cells. This toxin strongly and selectively inhibits calcium ATPase activity. To date, no rule exists on CPA levels in food products in any country (17). Several non-aflatoxin strains of *A. flavus* have been recorded in the United States to reduce aflatoxins in corn and other crops. However, if these strains produce CPA, they are likely to bring about unwanted negative consequences. It has been reported that AF36, being a non- *aflatoxinogenic* and CPA-producing strain, produce CPA in treated corn and peanuts. Alternative strains, including Afla-Guard® and K49 biocontrol agents do not produce CPA, and they are also likely to reduce aflatoxin and CPA in treated products. The chronic toxicity of CPA has not yet been investigated. Animal studies show significant detrimental effects of short-term exposure to CPA at low doses. For manufacturers and industry owners, the assurance of this method has to be maintained through transparency (18).

It should be noted that the existing methods for quantitative measurement of cyclopiazonic acid are less sensitive to aflatoxin. Different methods may be used for both quantitative or qualitative measurement of cyclopiazonic acid; one can mention instrumental chemistry (TLC, HPLC, HPLC\MASS, UPLC, GC) of ELISA. In this study, it has been attempted to investigate the ability to produce cyclopiazonic acid in non-*aflatoxigenic* isolates of *A. flavus* using high performance liquid chromatography as well as the morphological characteristics of these isolates.

2. Materials and Methods

2.1. Aspergillus flavus isolates

The isolates used in the present study include 58 non-*aflatoxigenic* strains and one *aflatoxigenic* strain of *A. flavus* obtained from pistachio soil and fruit of the orchards of Kerman, Yazd, Khorasan Razavi, Isfahan, Qom, Semnan and Markazi from Technology and Production Management Pistachio Research Center as well as the Plant Protection Department of Yazd Agricultural and Natural Resources Research and Education Center (3,19). The investigated isolates were cultured on PDA medium for conducting the experiments. Then, they were kept for a short time on sloping PDA medium at 4°C, and the tubes containing agar and glycerol and spore suspension were kept at -20°C.

2.2. Capability or incapability of producing cyclopiazonic acid in *Aspergillus flavus* isolates

Using high performance liquid chromatography (HPLC): The isolates were cultured on CYA medium for toxicity experiments. They were kept in the incubator at 25°C for 14 days in the dark (20). For conducting purification stages as well as purification methods, three blocks of agar were primarily removed from each colony and transferred to 4-ml vials. Then, 1 ml of methanol was added. After 60 minutes, it was passed through 0.45 µm syringe filters (RC 45µm, Altech, Deerfield, IL). The extracted isolates were then evaporated by air pumps. Finally, the isolates were dissolved in 1 ml of mobile phase and the isolates were kept at minus 20 for analysis (21). The mobile phase consisted of a mixture of methanol/acetonitrile (70:30, volumetric/volumetric) that was washed at a flow rate of 0.8 ml/min. 100 µl of the final extract was injected into the HPLC machine (Agilent 1100 Series, Agilent Technology, Santa Clara, CA). After extraction and refining of unknown samples and injection into HPLC, the amount of the produced toxin was measured after being compared with calibration curves. By comparing the peak areas with the calibration curves obtained from standard cyclopiazonic acid solutions, the amount of cyclopiazonic acid was measured in unknown samples. The *fluorometric* detector was set at wavelength, ex= 450 nm and em=285 nm. The materials were prepared including acetonitrile Gold for HPLC (ultragradient grade) and methanol (HPLC grade). Ultrapure water was prepared by Milli-Q system (Millipore, Bradford, MA, USA). Cyclopiasonic acid standards were purchased from Sigma-Aldrich (Milan, Italy). The detection limit of cyclopiazonic acid was 1.2 ng/g.

3. Results and Discussion

3.1. Cyclopiazonic production in the investigated isolates by using HPLC

What is important for the producers of agricultural and horticultural crops sensitive to aflatoxin is that the fungal populations in the microflora of the environment do not produce aflatoxins or help the limitation of *toxigenic populations* (22). Since non- *toxigenic isolates of the* fungi are able to compete with *toxigenic and* substrate occupation isolates, they play an important role in reducing the population of *toxigenic* isolates and the amount of aflatoxin produced in food products (19).

In the present study, it has been attempted to investigate the capability of producing cyclopiazonic acid in 58 non-*toxigenic* isolates taken from different pistachio orchards in Iran. The isolates were from Kerman (14), Yazd (3), Khorasan Razavi (26), Semnan (1), Isfahan (11), Qom (3) and Markazi (1) provinces.

The results of high performance liquid chromatography (HPLC) indicated that out of 59 non-aflatoxigenic isolates cultured in PDA medium, as many 15 isolates have been able to produce cyclopiazonic acid. CPA production range has been 6.951-357.6 mg/l.

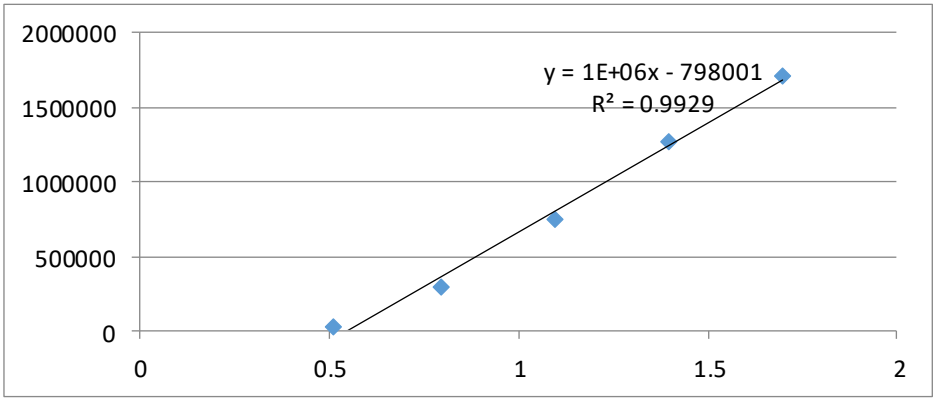

Fig. 1- The standard HPLC calibration curves of acid

Table 3- The evaluation of cyclopyazonic production in non- aflatoxigenic isolates of *Aspergillus flavus* by using HPLC method

No.	ITEM code	Location	Source	Cyclopiazonic Acid (mg/ml)
1	16441	Khorasan Razavi	Nut	N.D
2	16442	Khorasan Razavi	Nut	N.D
3	16443	Kerman	Nut	8.1
4	16444	Kerman	Nut	13.2
5	16445	Kerman	Nut	N.D
6	16446	Kerman	Nut	N.D
7	16447	Kerman	Nut	N.D
8	16448	Yazd	Nut	N.D
9	16449	Yazd	Soil	N.D
10	16450	Yazd	Soil	11.8
11	16451	Kerman	Soil	N.D
12	16452	Kerman	Nut	N.D
13	16453	Kerman	Nut	N.D
14	16454	Kerman	Nut	7.2
15	16455	Esfahan	Nut	6.9
16	16456	Esfahan	Nut	31.0
17	16457	Esfahan	Nut	N.D
18	16458	Esfahan	Soil	N.D
19	16459	Esfahan	Soil	N.D
20	16460	Esfahan	Nut	N.D
21	16461	Qom	Nut	N.D
22	16462	Esfahan	Nut	N.D
23	16463	Esfahan	Nut	N.D

24	16464	Esfahan	Nut	N.D
25	16465	Qom	Soil	N.D
26	16466	Khorasan Razavi	Nut	N.D
27	16467	Qom	Nut	7.8
28	16468	Kerman	Soil	12.3
29	16469	Esfahan	Nut	N.D
30	16470	Esfahan	Nut	N.D
31	16471	Khorasan Razavi	Nut	N.D
32	16472	Semnan	Soil	N.D
33	16473	Markazi	Nut	9.6
34	16474	Khorasan Razavi	Nut	N.D
35	16475	Khorasan Razavi	Nut	N.D
36	16476	Khorasan Razavi	Nut	N.D
37	16477	Khorasan Razavi	Soil	N.D
38	16478	Khorasan Razavi	Nut	N.D
39	16479	Khorasan Razavi	Nut	8.9
40	16480	Khorasan Razavi	Nut	N.D
41	16481	Khorasan Razavi	Nut	N.D
42	16482	Khorasan Razavi	Nut	N.D
43	16483	Khorasan Razavi	Nut	N.D
44	16484	Khorasan Razavi	Nut	N.D
45	16485	Khorasan Razavi	Nut	N.D
46	16486	Khorasan Razavi	Nut	N.D
47	16487	Khorasan Razavi	Nut	N.D
48	16488	Khorasan Razavi	Soil	22.2

49	16489	Khorasan Razavi	Nut	N.D
50	16490	Khorasan Razavi	Nut	357.6
51	16491	Khorasan Razavi	Nut	N.D
52	16492	Kerman	Nut	317.4
53	16493	Kerman	Soil	N.D
54	16494	Khorasan Razavi	Nut	N.D
55	16495	Khorasan Razavi	Nut	N.D
56	16496	Khorasan Razavi	Nut	N.D
57	16497	Khorasan Razavi	Nut	N.D
58	16498	Kerman	Nut	7.5
59	16499*	Kerman	Soil	13.9

ND: Not detected

*****Toxigenic strain with mean aflatoxins production levels of 703.68 and 144.78 ng/Kg for aflatoxin B1 and B2 respectively in YES medium (Fani et al. 2014a).

Cyclopiazonic acid is one of the most important natural pollutants in the class of mycotoxins. It is produced by different species of *Aspergillus* and *Penicillium* in various agricultural products including pistachios, peanuts, corn, barley, and millet; it threatens the health of humans and animals by entering their food cycle (16). The investigation of cyclopiazonic acid production capacity by non-aflatoxigenic isolates of *A. flavus* indicated that as many as 44 isolates did not produce cyclopiazonic acid. However, 15 isolates were able to produce cyclopiazonic acid in the range of 6.9 to 357 mg/l. Thus, although the 15 non- aflatoxigenic isolates of *A. flavus* investigated in the present study do not produce aflatoxin, they can produce cyclopiazonic acid in significant amounts; considering them for the biological control bring about numerous problems for aflatoxigenic species. In this regard, by examining the production of aflatoxin and cyclopiazonic acid by *A. flavus* isolates under 28 different nutritional and temperature conditions, Georgina et al. (2010) have stated that the conditions leading to the production of aflatoxin are also desirable for the production of cyclopiazonic acid. However, the conditions resulting in the production of aflatoxin at acceptable levels could not control cyclopiazonic acid levels. Thus, the use of non- aflatoxigenic

strains capable of producing cyclopiazonic acid would be dangerous as a biological control agent for crop protection would be dangerous. Resnik et al. (1996) have investigated 34 isolates of *A. flavus* and observed that only 5 isolates were capable of producing aflatoxins and the remaining isolates were non- aflatoxigenic. However, from the 34 isolates investigated, as many as 33 isolates were capable of producing cyclopiazonic acid. To conclude, it seems that their usage for biological control of aflatoxigenic fungi will not be very desirable. In a similar study, Ghallagher et al. (1978) have reported that of the 54 isolates of *A. flavus*, as many as 18 isolates were aflatoxigenic. However, 28 isolates were able to produce cyclopiazonic acid. Thus, some isolates of *A. flavus*, despite being non- aflatoxigenic, resulted in the production of cyclopiazonic acid. They have suggested that as a fungal metabolite, cyclopiazonic acid is likely to be more widely produced in foods than aflatoxin, and it can bring about serious risks.

Other researchers have observed and reported the ability of producing cyclopiazonic acid in some toxinogenic and non-toxogenic isolates of *A. flavus*. For example, the studies conducted by Sánchez-Hervás et al. (2008) on different fungal species that can produce mycotoxins in cocoa beans showed that the most common known fungal species are belonged to *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri*. From among 214 strains of *Aspergillus* section *Flavi*, as many as 120 belonged to *A. flavus* and 94 belonged to *A. tamari*. They stated that 64.1% of *A. flavus* strains were able to produce aflatoxins in the range of 100 to 1000 ng/g; this caused a moderate toxicity. However, 34.2% of them also produced cyclopiazonic acid at high toxicity levels with an approximate amount of more than 30 micrograms per gram. Razzaqi Abyaneh et al. (2006) have reported that only 28% of *A. flavus* isolates were capable of producing aflatoxin group B and cyclopiazonic acid production could be measured only in toxigenic and non-toxigenic *A. flavus* isolates. Moreover, none of the isolates of *A. parasiticus* and *A. nomius* could produce cyclopiazonic acid. In addition, a positive correlation has been observed between aflatoxin production and cyclopiazonic acid production arising from *A. flavus* isolates. However, Kim et al. (2014) observed that out of 19 isolates of *Aspergillus*, only *A. flavus* produced aflatoxin, and cyclopiazonic acid production was observed only in *A. oryzae*.

In fact, fungi use a lot of energy to produce aflatoxins, and this makes non-toxigenic fungi more successful in growing and occupying ecological environments; and the biological control of toxigenic fungi is thus achieved (23). Some studies have reported a 70-90% reduction in the population of fungal toxigenic fungi such as *A. flavus* in plants

such as cotton, corn, peanuts, pistachios, almonds and figs (24). However, these non-aflatoxinogenic isolates may lead to the production of other mycotoxins such as cyclopiazonic acid in agricultural and horticultural cops (22).

MMy et al. (25) investigated sixty corn samples from Cairo and Giza provinces in summer and winter in terms of fungal type and capability of producing aflatoxin mycotoxins and cyclopiazonic acid by using high performance liquid chromatography (HPLC). Their results indicated that the predominant fungi were *Aspergillus*, *Fusarium*, *Penicillium* and *Alternaria*. Out of 239 isolates of the *Aspergillus* species, *A. flavus* isolate was identified as the dominant isolate being 63.7% (151 isolates); and they were tested to investigate the production of aflatoxin and cyclopiazonic acid. According to their observations, only 63.6% (96 out of 151 cases) of *A. flavus* isolates produced aflatoxin and cyclopiazonic acid that were on average 9.4 and 12.5 mg/l, respectively. The remaining isolates were reported to be non-aflatoxinogenic and did not produce cyclopiazonic acid.

Therefore, considering the importance and position of pistachios in Iranian exports and the negative role of aflatoxins, it seems that the usage of isolates that not only do not produce aflatoxins but also do not have the ability to produce cyclopyazonic acid, can be a suitable solution in the biological control of toxigenic fungi both before and after pistachio harvest.

Some of the non-toxigenic isolates used in this study have been also capable of producing cyclopiazonic acid. Although these metabolites are not as important as aflatoxins in food hygiene, it is still recommended to use isolates capable of not producing cyclopiazonic acid in supplementary experiments for selecting high-performance strains for mass production and use in orchards for biological control of aflatoxins.

Conflict of Interest

The authors of present researches declare that there is no conflict of interest.

Code of Ethics

In this research, no living thing has been used, and all stages of the research have been conducted in a laboratory.

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