# Asian Journal of Crop Science

ISSN 1994-7879

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OPEN ACCESS Asian Journal of Crop Science

ISSN 1994-7879
DOI: 10.3923/ajcs.2023.37.42

# Research Article
# Effects of Oleic Acid and Temperature on Aflatoxin Production in *Aspergillus flavus* and *Aspergillus parasiticus*

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## Abstract

**Background and Objective:** Aflatoxin is a harmful compound released by fungi that contaminates food and animal products and causes various health complications. Aflatoxin, as a secondary metabolite produced by many *Aspergillus* strains, infects about five billion people around the world, through many stored foods, including peanuts, corn, sunflower seeds, nuts, rice, etc. This paper demonstrated the anti-aflatoxigenic effect of oleic acid as a natural compound inhibitor on mycotoxin production in toxigenic isolates of *Aspergillus flavus* and *Aspergillus parasiticus*. **Materials and Methods:** The study was conducted at Azad University of Lahijan and investigated the effect of oleic acid as a natural safe compound with different biological activities on Aflatoxin production in indigenous toxigenic *Aspergillus* species. Concentrations of oleic acid were added to the growth medium of toxigenic *Aspergillus* isolates at different levels. Then, incubated and aflatoxin production was measured using Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** Aflatoxin production was reduced by adding oleic acid to Sabouraud dextrose broth (SDB) medium at 40°C. Current finding showed that oleic acid has more aflatoxin inhibitory properties in higher temperatures. **Conclusion:** It can be concluded that the use of oleic acid as a natural compound inhibits toxin production. The results indicated that this natural safe compound can be used as an effective aflatoxin-production inhibitor in food and feed. It can be considered a new perspective in the application of harmless nutrients in inhibiting the production of aflatoxin in food and animal feed.

Key words: Aflatoxin production, oleic acid, nutrient, temperature, *Aspergillus flavus*, *Aspergillus parasiticus*

Citation: Ahmadian, F., A.C. Nosrati and H. Kioumarsi, 2023. Effects of oleic acid and temperature on aflatoxin production in *Aspergillus flavus* and *Aspergillus parasiticus*. Asian J. Crop Sci., 15: 37-42.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Agricultural science, animal husbandry and food safety continue to be reviewed by many researchers around the world and are becoming economically more important. However, management of agricultural products is a critical issue[1-4].

*Aspergillus flavus* and *Aspergillus parasiticus* are well known for the production of aflatoxin. Aflatoxin production is effected by several factors such as hosts, nutrients and environmental conditions[5,6].

There is a need to examine new approaches for achieving high efficiency in the agriculture and food sector[7]. Physical, chemical and biological methods can also be used to reduce aflatoxins levels in food. However, due to toxin heat-resistant chemical structure, regular heat treatment cannot decompose them after they are produced. It is also should be mentioned that the residues of the toxic compounds and also nutritional loss in processed foods lead to the ineffectiveness of most physical and chemical methods to reduce aflatoxin content in food[8]. In current aflatoxin control ways, emphasis is put on preventing aflatoxin biosynthesis pathways or inhibiting aflatoxin production instead of removing toxins[9].

The role of fatty acids in aflatoxin biosynthesis is not very clear and different results have been reported in other studies[10].

Some previous studies reported a positive correlation between aflatoxin production and lipid compounds and supported that lipid plays a key role in fungal growth and toxin production[11]. Aflatoxin production was reduced more than 800-fold when lipids were removed from the ground whole cottonseed[12]. The effect of different fatty acids on fungal growth, sporulation and aflatoxin biosynthesis in *Aspergillus* is significantly different[13].

However, some research dedicated that unsaturated fatty acids such as oleic acid and linoleic acid tended to inhibit toxin synthesis and have a reducing effect on toxin production in growth medium. From this point of view, it is doubtful that the amount of aflatoxin production may be expected to depend on the ratio of saturated to unsaturated fatty acids[14].

Temperature changes affect the pattern of aflatoxin production by aflatoxin-producing *Aspergillus* species. Some researchers observed limited aflatoxin production at high temperatures (higher than 40°C)[15-16]. This study aimed to identify and determine the role of oleic acid as an unsaturated fatty acid and temperature in reducing aflatoxin production in fungal growth media.

## MATERIALS AND METHODS

**Study area:** The study was conducted at the Department of Microbiology, part of the Faculty of Agriculture of Azad University, Lahijan Branch, Gilan, Iran. The duration of the study was over a period of 2 years (2017-2019).

**Oleic acid:** Oleic acid was purchased from Merck. (Merck, Darmstadt, Germany).

**Fungal isolates:** *Aspergillus* isolates were collected from agroecological zones in Northern Iran. Two isolates of *Aspergillus flavus* (68 and 80) and 2 isolates of *Aspergillus parasiticus* (45 and 94) were used. Identification of *Aspergillus* isolates was based on ICPA. The International Commission on Penicillium and Aspergillus rules and molecular characterization of the isolates was done by PCR amplification of *aflR* primers. High-Performance Liquid Chromatography (HPLC) was used in this study to evaluate the toxin-producing ability of isolates.

**Tools and types of equipment:** Culture Media and oleic acid were purchased from Merck (Merck, Darmstadt, Germany).

The aflatoxin B1 (AFB1) and total aflatoxin was determined using RIDASCREEN aflatoxin B1 30/15 (R1211) and RIDASCREEN aflatoxin total (R4701) test kits. (R-BioPharm, Darmstadt, Germany) Bio Tek 800 TS Absorbance Microplate Reader (Agilent-United States of America) HPLC Alliance 2695 (Waters-United States of America).

**Preparation of spore inoculum:** Sabouraud dextrose agar (SDA) medium was used for subculturing aflatoxigenic isolates. The SDA plates were incubated at 25°C for 7 days for sporulation. A sterile 0.05% Tween 20 saline solution was used to collect mature spores. Then, the concentrations were measured with a hemocytometer. Inoculation was done with spore suspension. Subsequently, oleic acid was added to Sabouraud dextrose broth at different concentration levels (5, 10 and 15 mmol). The Sabouraud dextrose broth without oleic acid was considered the control medium. All samples were incubated in a shaker incubator (150 rpm) at 25 and 40°C[15].

**Extraction aflatoxin:** In this study, after ten days aflatoxin was extracted from the medium. Then, the broth medium was shaken and homogenized (for 5 min and then mixture was passed through filter paper). Subsequently, 500 µL of each

extract was conveyed to a microtube. Then, 200 µL of solvent was added to them. The mixtures were maintained at 4°C overnight. It should be mentioned that solvent evaporation was performed in the water bath (at 90°C)[17].

**ELISA determination:** Aflatoxin in final extracts was measured by using the competitive indirect Enzyme-Linked Immunosorbent Assay (ELISA) method. The ELISA procedure was performed by R-BioPharm, microtiter plates with 96 wells. It contained materials including a microtiter plate coated with capture antibodies, aflatoxin standard solutions, wash buffer salt Tween, conjugate substrate/chromogen, stop solution and antibody.

Samples were added into separate duplicate wells that were contained 50 µL of the standard solution. Then 50 µL of the conjugate and 50 µL of the antibody were added to the wells, respectively. The plates were shaken manually. Incubation was done for 30 min at room temperature (20-25°C) in a dark room. After pouring out the liquid, wells were washed twice with 250 µL wash buffer. Then, 100 µL of substrate /chromogen was put into each well, mixed gently and incubated in the dark and room temperature (15 min). Then, 100 µL of the stop solution was stopped the reaction and absorbance were monitored at 450 nm using an ELISA plate reader within 10 min (EL×800, BioTek Instruments, United States of America).

**Statistical analysis:** The data were analyzed using SPSS 21 and the One-way Analysis of Variance (ANOVA). The significance of the difference between the average data was determined using Tukey's Test at a confidence level of 95%.

## RESULTS AND DISCUSSION

**Effect of oleic acid:** The results driven from the data analysis and its interpretation reveal that adding oleic acid decreases aflatoxin accumulation significantly (Table 1).

Current results indicated that oleic acid inhibited aflatoxin production in *A. flavus* (68) and (80) (Table 1). Different concentration of oleic acid (5, 10 and 15 mmol) was used for each toxigenic *Aspergillus* isolates. Obtained data proved that a concentration of 15 mmol of oleic acid had the most effect on the reduction of toxins.

**Effect of oleic acid on aflatoxin production in *Aspergillus parasiticus* isolates:** Also, the addition of oleic acid to the growth medium decreased aflatoxin production in *Aspergillus parasiticus* (Table 2).

The results indicated that oleic acid inhibited aflatoxin production in *A. parasiticus* (45) and (94) (Table 2). Between different concentrations of oleic acid (5, 10 and 15 mmol) were used for each toxigenic *Aspergillus* isolate, a higher concentration was more effective on toxins production. No statistically significant difference was found between the concentration of 5 mmol of oleic acid and the control ($p>0.05$).

**Comparison of the effect of oleic acid and incubation temperatures:** The effect of concentrations of oleic acid) at higher levels (incubation temperatures of 25 and 40°C) on aflatoxin production was measured and then contrasted with the control group. The results were provided in Table 3. However, the results showed that the higher concentration of oleic acid at a higher temperature level (15 mmol at 40°C) were more successful to inhibit the aflatoxin compare to the lower concentration of oleic acid at a lower temperature (5 mmol at 25°C). It can be related to the combined effects of dose–dependently inhibitory effect of oleic acid and the positive effects of temperature to inhibit the aflatoxin.

**Comparison of the effect of oleic acid and incubation temperatures on aflatoxin production in toxigenic *Aspergillus parasiticus*:** The results for the inhibitory effect of the higher amount of oleic acid and temperature on aflatoxin production by *Aspergillus flavus*, obtained data in Table 4

Table 1: Effect of concentrations of oleic acid at 5, 10 and 15 mmol levels on toxigenic *Aspergillus flavus* isolates and aflatoxin production

| | | | Total aflatoxin ng mL$^{-1}$ | |
|---|---|---|---|---|
| ID | Fungal isolate | Concentration of oleic acid (mmol) | Inhibition (%) | Concentration (ng mL$^{-1}$) |
| 68 | *Aspergillus flavus* | 0 (Control) | - | 2408.25±3.14$^{a}$ |
| | | 5 | 1.53 | 2371.38±2.37$^{a}$ |
| | | 10 | 1.93 | 2361.61±1.64$^{b}$ |
| | | 15 | 65.03 | 842.02±1.1$^{c}$ |
| 80 | *Aspergillus flavus* | 0 (Control) | - | 2402.00±3.05$^{a}$ |
| | | 5 | 0.8 | 2383.02±1.98$^{a}$ |
| | | 10 | 13.01 | 2089.64±2.17$^{b}$ |
| | | 15 | 48.91 | 1175.25±2.89$^{c}$ |

Means± SD were obtained from three separate experiments and $^{a-c}$Different letters represent significantly different ($p<0.05$) values

Table 2: Effect of different concentrations of oleic acid (5, 10 and 15 mmol) on toxigenic *Aspergillus parasiticus* isolates and aflatoxin production at 25°C

| | | | Total aflatoxin ng mL$^{-1}$ | |
|---|---|---|---|---|
| ID | Fungal isolate | Concentration of oleic acid (mmol) | Inhibition (%) | Concentration (ng mL$^{-1}$) |
| 45 | *Aspergillus parasiticus* | 0 (Control) | - | 2400.66±3.14$^{a}$ |
| | | 5 | 0.58 | 2386.56±2.13$^{ab}$ |
| | | 10 | 1.21 | 2371.38±1.54$^{b}$ |
| | | 15 | 62.66 | 896.25±4.14$^{c}$ |
| 94 | *Aspergillus parasiticus* | 0 (Control) | - | 2936.32±3.05$^{a}$ |
| | | 5 | 18.42 | 2395.24±1.98$^{b}$ |
| | | 10 | 19.12 | 2374.63±2.17$^{b}$ |
| | | 15 | 71.32 | 842.02±2.89$^{c}$ |

Means±SD were obtained from three separate experiments and $^{a\text{-}c}$Different letters represent significantly different ($p<0.05$) values

Table 3: Effect of different temperatures and concentrations of oleic acid on aflatoxin

| | | | | Total aflatoxin ng mL$^{-1}$ | |
|---|---|---|---|---|---|
| ID | Fungal isolate | Concentration of oleic acid (mmol) | Incubation temperature (°C) | Inhibition (%) | Concentration (ng mL$^{-1}$) |
| 68 | *Aspergillus flavus* | 0 (Control) | 25 | - | 2408.25±1.70$^{a}$ |
| | | 15 | 25 | 65.03 | 842.02±2.62$^{b}$ |
| | | 15 | 40 | 80.32 | 473.24±1.89$^{c}$ |
| 80 | *Aspergillus flavus* | 0 (Control) | 25 | - | 2402.00±3. 90$^{a}$ |
| | | 15 | 25 | 0.8 | 2381.14±2.00$^{b}$ |
| | | 15 | 40 | 65.28 | 831.17±3.32$^{c}$ |

Means±SD were obtained from 3 separate experiments and $^{a\text{-}c}$Different letters represent significantly different ($p<0.05$) values

Table 4: Effect of different temperatures and concentrations of oleic acid on aflatoxin production by toxigenic *Aspergillus parasiticus*

| | | | | Total aflatoxin ng mL$^{-1}$ | |
|---|---|---|---|---|---|
| ID | Fungal isolate | Concentration of oleic acid (mmol) | Incubation temperature (°C) | Inhibition (%) | Concentration (ng mL$^{-1}$) |
| 45 | *Aspergillus parasiticus* | 0 (Control) | 25 | - | 2400.66±3.14$^{a}$ |
| | | 15 | 25 | 62.66 | 896.25±4.14$^{b}$ |
| | | 15 | 40 | 65.82 | 820.33±2.32$^{c}$ |
| 94 | *Aspergillus parasiticus* | 0 (Control) | 25 | - | 2936.32±3. 05$^{a}$ |
| | | 15 | 25 | 71.32 | 842.02±2.89$^{b}$ |
| | | 15 | 40 | 75.01 | 733.56±1.65$^{c}$ |

Means±SD were obtained from 3 separate experiments and $^{a\text{-}c}$Different letters represent significantly different ($p<0.05$) values

Table 5: Effect of oleic acid on $AFB_1$ production by toxigenic *Aspergillus flavus and Aspergillus parasiticus*

| | | | | $AFB_1$ ng mL$^{-1}$ | |
|---|---|---|---|---|---|
| ID | Fungal isolate | Concentration of oleic acid (mmol) | Incubation temperature (°C) | Inhibition (%) | Concentration (ng mL$^{-1}$) |
| 68 | *Aspergillus flavus* | 0 (Control) | 40 | - | 424.42±5.89$^{a}$ |
| | | Oleic acid (15 mmol) | 40 | 22.99 | 326.81±6.65$^{b}$ |
| 80 | *Aspergillus flavus* | 0 (Control) | 40 | - | 485.05±1.97$^{a}$ |
| | | Oleic acid(15 mmol) | 40 | 89.94 | 48.78±4.15$^{b}$ |
| 45 | *Aspergillus parasiticus* | 0 (Control) | 40 | - | 164.13±5.89$^{a}$ |
| | | Oleic acid(15 mmol) | 40 | 42.34 | 94.63±6.65$^{b}$ |
| 94 | *Aspergillus parasiticus* | 0 (Control) | 40 | - | 433.29±1.97$^{a}$ |
| | | Oleic acid (15 mmol) | 40 | 28.67 | 309.06±4.15$^{b}$ |

Means±SD were obtained from three separate experiments and $^{a\text{-}b}$Different letters represent significantly different ($p<0.05$) values

showed that a higher concentration of oleic acid and elevated temperature (15 mmol at 40°C) had a significant inhibitory effect on toxin production by *Aspergillus parasiticus*.

**Effects of oleic acid on $AFB_1$ production in *Aspergillus flavus and Aspergillus parasiticus*:** Current results indicated that oleic acid in a concentration of 15 mmol and temperature at 40°C had significant aflatoxin inhibition properties. The effect of this concentrate at 40°C on AFB1 production in four isolates of toxigenic *Aspergillus* was evaluated (Table 5).

The AFB1 inhibition property with oleic acid was in agreement with our total aflatoxin inhibition results in all studied isolates. All obtained results confirmed that oleic acid significantly reduces AFB1 production by aflatoxigenic *Aspergillus flavus* and *Aspergillus parasiticus* isolates.

Previous studies indicated that oxidative stress in *Aspergillus flavus* and *Aspergillus parasiticus* caused aflatoxin biosynthesis at metabolic and transcriptional levels. Clear evidence showed that aflatoxin biosynthesis is regulated by oxidative stress. High levels of oxidative stress-inducing factors activated the expression of aflatoxin biosynthesis gene cluster at the transcriptional level[9].

Oxidative stress leads to lipid peroxidation and the generation of free radicals and in consequence aflatoxin production. This evidence proved that oxidative stress is a prerequisite for aflatoxin production in toxigenic strains[18,19]. Therefore, oxidative stress inhibitors can affect the control or reduction of aflatoxin production.

In this study, reactive oxygen species (ROS) removal was suggested as an aflatoxin prevention strategy[19].

Previous reports showed that several fatty acids in certain concentrations have cytoprotective effects against oxidative stress, however, the same results don't be found when cells were treated with high levels of fatty acids[20]. In contrast, oleic acid as an unsaturated fatty acid has cytoprotective effects at any concentration. Moreover, these effects were not observed in all monounsaturated fatty acids.

Cytoprotective effect was shown both in intracellular oleic acid and oleic acid-induced lipid accumulation. Oleic acid promoted tolerance to oxidative stress and increase adaptability against lipid peroxidation stress[21].

In agreement with these results, Yan *et al.*[10] showed that autoxidation linolenic acid promoted mycelial growth, sporulation and kojic acid production, but inhibited the expression of genes in the aflatoxin biosynthetic gene cluster. According to the findings of this study, it can be assumed that lipid peroxidation through oleic acid pretreatment leads to mild stress that promotes the antioxidative defense system in fungal cells and could inhibit the progression of lipid peroxidation cascade and subsequence oxidative stress and toxin production.

Several studies reported that the influence of different temperatures on toxigenic *Aspergillus* isolates is different. The optimum temperature and water activity for their growth and aflatoxin production are between 16 to 31°C and between 0.82 to 0.99, respectively. According to the results, the maximum growth and aflatoxin production observed at temperature = 25°C and $a_w = 0.95$[22].

The current study results were in agreement with Akinola *et al.*[23], who reported aflatoxin production reduced at incubation/storage temperature above 25°C in toxigenic *Aspergillus parasiticus*. Hassane *et al.*[24] showed that at temperatures higher than 36°C, toxin production by *Aspergillus flavus* was decreased. Also, only traces of AFB1 was detected at 40°C. Effective water activity decreased in elevated temperatures and reduced the relative humidity resulting in less toxin production and accumulation[15]. Based on current findings, oleic acid can be used as a new anti-aflatoxigenic agent and can be added to food and animal feed to inhibit aflatoxin production. However, more research is needed to evaluate its use in other aflatoxin-producing species, perform safety tests of the final product and use this method in aflatoxin control programs.

## CONCLUSION

It can be concluded that oleic acid as a natural compound has a positive effect to inhibit aflatoxin which makes it suitable and can be a harmless substitute to be used as synthetic chemical substances. The results of the current study reveal the anti-aflatoxigenic effect of this substitute. It results can be justified by the reduction of oxidative stress and regulation of toxin production in the fungal cell. Based on the results, oleic acid could be a possible and effective anti-aflatoxigenic agent in food and feed. However, additional research is needed to confirm and build on these findings and support the applicability of the method in aflatoxin control plans.

## SIGNIFICANCE STATEMENT

In recent years, endangering the safety of food and animal feed through mycotoxins have increased. During the last three decades, most studies have focused on biological, chemical and physical control methods of aflatoxin production in agricultural products and only a few studies have focused on the effect of different and key nutrient compounds on aflatoxin production. The current study insisted that the food consumers on using natural compounds instead of synthetic chemical inhibitors reduced the production of aflatoxin. Results showed that unsaturated fatty acids such as oleic acid and linoleic acid have a reducing effect on toxin production. Therefore, investigating the possibility of using natural nutrient compounds in the fungal growth media is a new perspective of anti-aflatoxigenic control methods to protect food and animal feed from contamination with aflatoxin.

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