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The plant growth regulator methyl jasmonate inhibits aflatoxin B_1 production by Aspergillus parasiticus in caper

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Abstract: Aflatoxins, produced by some aflatoxigenic strains of the *Aspergillus* species, are known as potent carcinogenic. Aflatoxin biosynthesis involves lipid peroxidation with the presence of fatty acid hydroperoxides promoting aflatoxin production. Methyl jasmonate (MeJA) derives from α-linolenic acid and is a plant growth regulator, produced as a response to stress, such as by environment or pathogen attack. This study reports on the effect of MeJA added on *A. parasiticus* growth and AFB_1 production in caper, an edible plant of Greek origin used as condiment. AFB1 determination in caper was performed by using HPLC-FD. Five different concentrations of MeJA, 10^{-6} M, 10^{-4} M, 10^{-3}M, 10^{-2} M, and 10^{-1} M, were added in caper samples and the kinetic of the AFB_1 production by *A.parasiticus* was studied for an incubation period of 15 days. Results revealed that MeJA affects AFB_1 production by *A. parasiticus* in a dose-dependent manner. MeJA at a concentration of 10^{-6} M stimulated AFB_1 production after the 9th day of incubation. MeJA at concentrations of 10^{-4} M and 10^{-3}M decreased moderately AFB_1 output. Finally, MeJA added to the caper samples at a concentration of 10^{-2} M and 10^{-1} M inhibited AFB_1 by 97.74% and 98.42% respectively on the 12th day of observation.

Keywords: Aflatoxin B_1, Methyl Jasmonate, *Aspergillus Parasiticus*, Caper, HPLC

1. Introduction

Aflatoxins are polyketide secondary fungal metabolites produced by the toxigenic strains of *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* and they are known as potent carcinogenic, teratogenic as well as genotoxic mycotoxins [1, 2]. Among the four naturally occurring aflatoxins, Aflatoxin B_1 (AFB_1) is the most potent one [3].

Aflatoxin contamination of food and feeds infected with Aspergillus spp. poses a worldwide food safety problem [4]. Seeds in particular, are susceptible and the lipid content of seeds plays a part in determining the severity of aflatoxin contamination [e.g. 5]. Recent reports support an aflatoxin/lipid relationship. It was shown by [6] that cottonseed meal sustained 1000-fold less aflatoxin production by *A. flavus* after lipid extraction.

The plant hormones jasmonic acid and methyl jasmonate, along with their intermediate compounds, produced via the octadecanoid pathway, are important signaling molecules called jasmonates. These are widespread in the plant kingdom and play crucial roles in biotic/abiotic stress responses, as well as in processes related to plant growth and development. In plants, jasmonates are synthesized as a response to systemic or localized signals similar to oligosaccharides released from fungi or plant cell walls during plant-pathogen interactions [7]. According to [8], phytopathogenic bacterial species have developed specific methods in order to attack plant cells and to use plant substances for their own growth. [9] have reported that jasmonic acid levels increased rapidly in response to biotic and abiotic stress such as mechanical stress. Lately, natural elicitors were combined with methyl jasmonate (MeJA) to evaluate its effects on phytoalexin and AFB_1 production in cotton plants [10]. According to [11], the levels of endogenous hormones decreased in seeds of *Zea mays* treated with AFB_1, while [12] reported that substances such as aflatoxins inhibit

the growth and development process in plants.

As far as *Aspergilli* are concerned, interactions between jasmonates, *Aspergillus* growth and aflatoxin production are likely. These interactions are of great interest as they suggest that there is a mechanism involving plant LOX pathways that affects aflatoxin biosynthesis. Once the fungus has invaded the seed, it first destroys the lipid bodies, which are primarily composed of palmitic, oleic, and linolenic acid [13, 14]. Many in vitro studies have shown that lipid oxidation affects aflatoxin biosynthesis [5, 15, 16]. Linoleic as well as linolenic acid can undergo a regio- and stereospecific oxygenation catalyzed by the widely distributed plant stress response enzyme lipoxygenase (LOX) [17] to yield 13S-hydroperoxy-cis-9-trans-11-octadecadienoicacid (13S-HPODE) and 13S-hydroperoxycis-9, trans-11, cis-15-octadecatrienoic acid (13S-HPOTE), respectively [18]. These fatty acid hydroperoxides are further converted through the octadecanoid pathway to jasmonates [19].

Capparis sicula Veill. in Duham, *C. orientalis* Veill in Duham and *C. spinosa* L. are small shrubs widely distributed in Mediterranean countries. Their beneficial properties, including antirheumatic, tonic as well as expectorant activities, have been known since antiquity from numerous populations of different countries around the Mediterranean Sea [20]. The flower buds and the fruits of *C. spinosa* and *C. sicula* have been used as a condiment in Greece since ancient times. Capers are usually commercialized in some Mediterranean countries and they are exported mainly to central European countries, the USA and the UK as a delicatessen product. From a dietary point of view, the flavonoid content and the antioxidant (free radical scavenging) activity of these compounds give a special value to this product [21]. A great deal of attention has been given to Capparis spp. buds, fruits, young shoots and seed oil. [22] reported on caper seeds growing in Turkey with oleic and linoleic acid being the major unsaturated fatty acids, while palmitic acid was the predominant saturated one.

With an oil content of approximately 30%, seeds of *C. ovata* and *C. spinosa* could be interesting sources for the production of vegetable oil. The results indicate that the oil contains linoleic acid as the major fatty acid accompanied by oleic acid and its isomer vaccenic acid. The content and composition of tocopherols are comparable to those of other sources such as rapeseed and sunflower oil. The relatively high content of Δ5-avenasterol is interesting, as it has been suggested as an antioxidant and antipolymerization agent in frying oils [23].

In literature there is no information concerning the AFB_1 production in capers, a plant with a high lipid content, as mentioned above. Therefore, interactions between MeJA concentrations and AFB_1 production, using a natural substrate such as capers inoculated with *A. parasiticus*, are investigated in the present work. More precisely it was examined which MeJA concentrations inhibit the AFB_1 production. Additionally, in the present work the development and validation of a method determining AFB_1 in capers is evaluated.

2. Materials and Methods

2.1. Apparatus

A laminar flow (Telstar Bio IIA, Madrid, Spain); an autoclave, Selecta Autester-E Dry (PBI Milano, Italy), an incubator WTB Binder (Tuttingen, Germany) and a centrifuge Sorvall RC-5B (HS-4) (Norwalk, USA) were used during this study. HPLC was performed using a Hewlett-Packard 1050 (Waldborn, Germany) liquid chromatograph equipped with a JASCO FP-920 (Japan) fluorescence detector and an HP integrator 3395. The HPLC column used was a C18 Nova-Pak (60 Å, 4 μm, 4.6 x 250 mm).

The mobile phase for AFB1 determination [water + acetonitrile + methanol (20+4+3)] was filtered through Millipore HVLP filters (0.45 μm) before use. Detection of the AFB1 hemiacetal derivative (AFB2a) was carried out at λex = 365 nm and λem = 425 nm. The flow rate was 1 ml min^{-1} while the retention time was 12.60 (±0.2) min for AFB_1 in caper.

2.2. Reagents

The AFB_1 standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). The filters and the C_{18} Nova-Pak HPLC column were from Waters (Millipore, Milford, MA, USA). The Aflatest immunoaffinity columns were from Vicam and HPLC solvents were of HPLC grade (LABSCAN, Dublin, Ireland). Trifluoroacetic acid was purchased from Merck (Darmstadt, Germany). The purity of MeJA used was tested by GC analysis using a Hewlett-Packard GC (flame ionization detector) on a BPX70-coated fused-silica capillary column [24].

2.3. Media

Aspergillus flavus parasiticus agar (AFPA) was prepared by dissolving 4 g of yeast extract (Oxoid) (Basingstoke, Hampshire, England), 2 g of bacteriological peptone (Oxoid), 0.1 g of ferric ammonium citrate, 0.2 ml of Dichloran (0.2% in ethanol, Fluka, Neu-Ulm, Switzerland), 0.2 g of chloramphenicol (Oxoid) and 3 g agar (Oxoid) in 200 ml of distilled water, final pH 0.0-0.5 [25]. Czapek Dox agar (CZA) waspreparedby dissolving 0.4 g of sodium nitrite, 0.1 g of potassium chloride, 0.1 g of magnesium sulfate, 0.002 g of ferric sulfate, 0.2 g of dipotassium phosphate, 6 g of sucrose, 3 g of agar, 0.002 g of zinc sulfate, and 0.001 g of copper sulfate in 200 ml of distilled water, final pH 6.0-6.5 [26].

2.4. Preparation of Spore Inocolum

The aflatoxigenic strain *A. parasiticus* sp (IMI 283883) utilized throughout this study was obtained from the International Mycological Institute (Engham, Surrey, UK). An inoculum was obtained by growing the mold on a slant of stock cultures of CZA, which were maintained at 5° C [27,28]. Spore inoculum was prepared by growing *A.parasiticus* on CZA for 7 days at 30°C and spores were harvested aseptically using 10 ml of sterile 0.01 % v/v Tween 80 solution [29]. AFB_1 transfer from the initial culture was minimized by

centrifuging the spore suspension (1000 g for 1 min) and resuspending the spore in 10 ml of sterile Tween 80 solution twice. Dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) from the initial spore suspension were prepared in sterile tubes containing 10 ml of 0.05% Tween 80 (v/v) suspension. The spore concentration was determined by the spread plate surface count technique, using 0.1 ml of each dilution on four AFPA plates [25, 30] after incubation at 30° C for 2 days. The population size was estimated by the reverse intense yellow/orange coloration of the colonies. In order to obtain an inoculum containing 10^2 conidia, plates with 10-100 colony forming units (cfu) were selected and the desired 10^2 spore quantity used in the present study was estimated. The quantity of 10^2 spores / flask was chosen as it was the minimum concentration found in the literature producing detectable amounts of AFB_1 by *Aspergillus* [31].

2.5. Inoculation

Three flasks, containing 15 g of capers, were used for each case and for each day of observation as shown in Table 1. MeJA in ethanol at final concentrations of 10^{-1}, 10^{-2} 10^{-3} 10^{-4} and 10^{-6} M $flask^{-1}$ was added into each of the three flasks containing caper (non inoculated) for each day of observation. All flasks, including control samples (both inoculated or not) as well as samples both treated with MeJA or not and samples inoculated with *A. parasiticus* and non-inoculated, were incubated under stationary conditions at 30° C. Immediately after autoclaving for 30 minutes at 115° C, as it is suggested for safety reasons [32], AFB_1 was determined on days 0, 3, 7, 9, 12 and 15 of incubation. The experiment was repeated in triplicate

Table 1. *Inoculated samples of caper*

Samples of caper (15 g) inoculated with *A.parasiticus* with MEJA addition at different concentrations
Caper without MeJA addition , non- inoculated + ethanol
Caper + 100 conidia of *A.parasiticus* + ethanol
Caper + 100 conidia of *A.parasiticus* + (10^{-6} M) corresponding to 0.0022mg MEJA in ethanol
Caper + 100 conidia of *A.parasiticus* + (10^{-4} M) corresponding to 0.227mg MEJA in ethanol
Caper + 100 conidia of *A.parasiticus* + (10^{-3} M) corresponding to 2.267mg MEJA in ethanol
Caper + 100 conidia of *A.parasiticus* + (10^{-2} M) corresponding to 22.667 mg MEJA in ethanol
Caper + 100 conidia of *A.parasiticus* + (10^{-1} M) corresponding to 226.667mg MEJA in ethanol

Three flasks were used for each case and for each day of observation. All samples were incubated during 15 days and AFB1 was determined on days 0, 3, 7, 9, 12, 15.

2.6. Sampling and Treatment

An initial amount of 10 kilograms of capers originated from Cyclades, which are the most popular in the market, were purchased from the market of Syros in the summer of 2011.

The capers were washed with water, cut into pieces and, in order to eliminate their natural microbiota, they were treated before inoculation with *A. parasiticus* as follows: dipped in 70% ethanol and shaken for 1 min. Following that, ethanol is rejected. Then washed with 1.25% NaOCl for three minutes and rinsed with sterile distilled water twice. After treatment, representative samples of 15 gr from the damaged capers (in order to obtain samples with similar natural microbiota) were aseptically employed into sterile flasks forming a solid mass [33].

2.7. AFB_1 Determination Derivatization and HPLC Analysis

The content of each flask containing caper was mixed with 30 ml of methanol water (80-20) and well-shaken for 10 min. After filtration, an aliquot of 1 ml from each flask was used for AFB_1 analysis. The 1 ml-aliquot from the filtrate was mixed with 10 ml of distilled water. The mixture was transferred onto an Aflatest immunoaffinity column (flow rate 6 ml min^{-1}) and washed twice with 10 ml of distilled water. The column was then allowed to dry by passing air through it. AFB_1 was eluted with 2 ml of acetonitrile (flow rate: 0.3 ml min^{-1}). Before derivatization, the eluate was evaporated to dryness on a water bath under a gentle steam of nitrogen [34].

A derivative of AFB_1 (AFB_{2a}, hemiacetal of AFB_1) was prepared by adding 200 μl of hexane and 200 μl of trifluoroacetic acid to the evaporated solution of AFB_1 eluate, heating for 10 min at 40°C in a water bath, evaporating to dryness under nitrogen, redissolving in an appropriate volume of water-acetonitrile (9:1) to give a concentration of <10 ng ml^{-1} and analyzing by HPLC (volume injected: 40 μl). AFB_{2a} shows enhanced fluorescence compared to AFB_1 [35]. In addition to that, AFB_{2a} is less toxic compared to AFB_1 because of its protein binding properties [36].

2.8. Statistical Analysis

Data were analyzed by one-way ANOVA and t-test. The mean differences, which are significantly different, were examined by using the Tukey's test [37].

3. Results and Discussion

3.1. Characterization of the AFB_1 Determination in Capers

In the present study, the analytical protocol for the determination of AFB_1 in capers was in-house characterized regarding the following criteria: linearity, accuracy, repeatability, internal reproducibility, limits of detection (DL) and quantification (QL). Additionally, the limits of repeatability (r) and reproducibility(R) were calculated as following: r = 2.8 x SDr and R = 2.8 x SDR, respectively. As far as the limits of detection (DL) and quantification (QL) are concerned, they were calculated using two different ways: (1) according to the following formulas $DL = [b_0 + 3S\ (b_0)]/\ b1$, $QL = [b_0 + 10S\ (b_0)]/b_1$, where b_0 is the response of the blank (intercept of the calibration model), S (b_0) is the standard deviation of the blank and b_1 is the sensitivity (calibration model slope) and (2) as the signal-to-noise ratio 3:1. The accuracy was estimated by analyzing samples (15 g) of caper

spiked with different quantities of AFB_1. Furthermore, a Fisher test was applied to confirm the acceptability of the linear regression. If the F1 ratio is greater than the critical value F [1, p (n- 1), 1- a] corresponding to a Fisher variable with a risk a = 0.1% for 1 and p (n - 1) degrees of freedom, the regression model can be considered as acceptable.

Table 2. *Accuracy, recovery, detection limit of the AFB1 determination in caper*

	AFB_1 (ng g^{-1}) added					Recovery of the method%	RSD%	Detection Limit ng g^{-1} caper
Samples	**2**	**5**	**10**	**15**	**20**			
	AFB_1 (ng g^{-1}) recovered							
1	1.82	4.45	8.14	12.91	16.90	79.00	6.53	0.06
2	1.87	4.48	7.90	12.98	14.96			
3	1.88	4.50	7.75	12.47	16.70			
4	1.81	4.52	8.25	12.24	15.44			
Average	1.85	4.49	8.01	12.65	16.00			
SD	0.04	0.03	0.23	0.35	0.95	y = 0.36 (±0.27) + 0.79 (±0.02) x		
RSD%	1.90	0.66	2.83	2.80	5.92	R = 0.9984		

Thereupon, the hypothesis must be checked if the lack of fit of the model is negligible and that it is a straight line throughout the chosen field. If the F1 ratio is less or equal to the critical value F[p - 2, p (n- 1), 1 -a] corresponding to a Fisher variable with a risk of 0.1% for p -2 and p (n -1) degrees of freedom, the field of linearity chosen can then be approved. For the determination, p = 5 levels and n = 4 repetitions were used (Table 2). Regarding the linearity, an AFB_1 standard solution (5 μg ml^{-1} in methanol) was prepared by diluting 10 μl from an AFB_1 stock solution (0.5 mg ml^{-1} in methanol) measuring its absorbance at 335 nm (A = 0.385). Thereupon, solutions of AFB_1 in mobile phase were prepared at concentrations of 10, 5, 2.5, 2 and 1 ng ml^{-1}. The volume injected into the column was 40 μl. Linear regression was used to prepare standard curves by using the peak areas mean values of five injections for each one of the five solutions. The equation of the standard curve was y= - 2.57(± 0.69) 10^5 +9.6(± 0.13) 10^5x, where y is the peak area of AFB_1 injected (40 μl) and x is the amount in ng of standard AFB_1 injected (40 μl).

As far as the repeatability is concerned, this parameter was estimated by analyzing five subsamples (15 g) of capers spiked with 100 ng of AFB_1 under repetitive conditions (mean recovered = 97.36, RSD = 6.31%). The internal reproducibility was also estimated by determining five samples (15 g) of capers at time intervals (at five different days of the month). The samples were spiked with 100 ng AFB_1 (mean recovered = 95.54, RSD = 5.76%). In the present study the limits of repeatability (r) and reproducibility (R) were found to be: r = 17.19 and R = 15.40, respectively (SDr = 6.14 and SDR= 5.50).

The accuracy of the method applied in capers was studied by analyzing samples (15 g) of capers spiked with AFB_1 at different concentrations corresponding to AFB_1 ng g^{-1} being shown in Table 2.

The regression coefficient r of the curve was found to be r = 0.9984 [y = 0.36(±0.27) + 0.79 (±0.02)], with y being the concentration of AFB_1 (ng g^{-1}) of capers recovered and x the concentration of AFB_1 (ng g^{-1}) of capers spiked.

Moreover, a Fisher test was applied to confirm the acceptability of the linear regression. The F(1,15) ratio found (1258.38) was greater than the critical Fisher value of 16.58 at a risk 0.1% for 1 and 15 degrees of freedom. Subsequently, the regression model can be considered acceptable. Finally, the lack of fit of the model as the experimental F ratio (1.97) is lower than the critical value F(3,15) = 9.335 with a risk a = 0.1% for 3 and 15 degrees of freedom, thus the field of linearity chosen can be approved. The mean recovery factor taken from the above equation was found to be 79% (RSD% = 6.53). The AFB_1 detection limit (DL) was determined at 0.08 ng g^{-1} of capers and the quantification limit (QL) at 0.21 ng g^{-1} of capers. Both DL and QL have been defined by using the signal-to-noise ratio 3:1 and 6:1, as 0.06 ng g^{-1} and 0.12 ng g^{-1}, respectively. In the present study the lower DL = 0.06 ng g^{-1} was used throughout this work.

3.2. Aflatoxin B_1 Production in Capers

In the present study, *A. parasiticus* was used since AFB_1 production is a more constant trait in this fungus than *in A. flavus* [38]. Moreover, AFB_1 was studied throughout this research as it is the most potent mycotoxin and usually the one produced at the highest levels by toxigenic strains [39].

To our knowledge, there is no information in the literature concerning the AFB_1 occurrence and production in capers. Hence, caper was selected as the natural product to be used as a substrate in order to study the effect of different MeJA concentrations on AFB_1 production. Moreover, capers were damaged in order to facilitate fungus penetration and achieve results in a short time.

Previously, [40] showed that MeJA at concentrations of 10^{-4} M and 10^{-6} M had no significant effect on the mycelial growth in yeast extract sucrose medium (YES) but did affect AFB_1 production. On the contrary, treatment of *A. parasiticus* cultures with 10^{-2} M MeJA inhibited mycelial growth and AFB_1 production as well. It should be noted that a preliminary study of different MeJA concentrations on the *A.parasiticus* growth in AFPA medium was applied (Markaki unpublished data Fig.1), which revealed that MeJA concentrations below 10^{-3} M (corresponding to 2.267 mg MeJA) did not affect the mycelial growth of *A. parasiticus*.

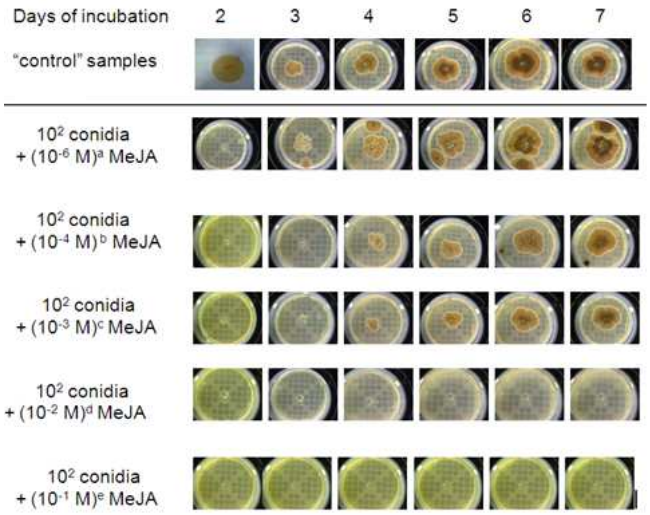

Figure 1. *Macroscopic observation of the inhibition effect of MeJA on A.parasiticus in AFPA. The MeJA concentrations used, correspond to the MeJA concentrations added to the caper samples (ranging from 10^{-6} M to 10^{-1} M); (a: 0.0022 mg, b: 0.227 mg, c: 2.267 mg, d: 22.667 mg, e: 226.667 mg). Concentrations above 10^{-3} M MeJA significantly inhibited mycelial growth. Mycelial growth in control samples (without MeJA addition) was not affected.*

Therefore, we were interested in adding MeJA at higher concentrations, such as 10^{-3}, 10^{-2} and 10^{-1} M that could inhibit AFB_1 production. The MeJA concentrations of 10^{-6} M and 10^{-4} M were additionally used in order to have comparable results of the AFB_1 production in capers against the AFB_1 production in YES medium as reported from our laboratory by [40].

The MeJA concentrations used in this work were chosen to be both above as well as below the concentration of 10^{-4} M. The latter was found to be effective as a post-harvest treatment for suppressing the decay caused by *Botrytis cinerea* on strawberries, for reducing the decay by *Penicillium digitarum* in grape fruit as well as for reducing microbial contamination in celery and peppers [41].

3.3. MeJA Effect on AFB_1 Production in Capers

In the present study, AFB_1 production was measurable from the day 0 of the incubation due to the inoculation of capers with 10^{-2} conidia of *A.parasiticus*.

Maximum AFB_1 production in samples treated with MeJA at concentrations of 10^{-6} M, 10^{-4} M and 10^{-3} M amounted to 6.33, 2.90 and 1.45 μg $flask^{-1}$ (15g) on the 12th day of observation, respectively. Furthermore, maximum AFB_1 output in samples treated with MeJA at higher concentrations, namely 10^{-2} M and 10^{-1} M, was 0.32 and 0.22 μg $flask^{-1}$ on the 7th day of observation, respectively. AFB_1 maximum production in control samples (without MeJA addition) inoculated with *A.parasiticus*, was 4.43 μg $flask^{-1}$ on the 12th day of observation. The AFB_1 amounts produced in samples treated with 10^{-2} M and 10^{-1} M MeJA are negligible compared to the AFB_1 production in samples with MeJA added at concentrations of 10^{-6} M, 10^{-4} M, 10^{-3} M and in control samples as well, during the entire incubation period (Fig. 2 Table 3). It must be mentioned that AFB_1 in not inoculated samples of caper either with or without MeJA addition was not detectable.

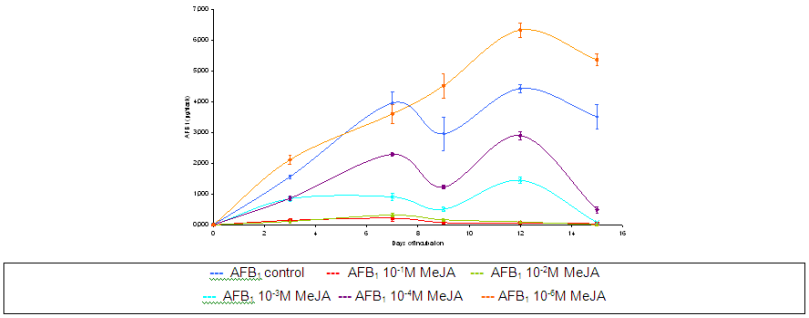

Figure 2*. Aflatoxin B_1 production in capers by A.parasiticus. Caper samples with MeJA concentrations 10^{-1} M, 10^{-2} M, 10^{-3} M, significantly reduced AFB_1 production against control, while concentrations above 10^{-4} M stimulated AFB_1 production by A.parasiticus in capers.*

Table 3. *AFB_1 production by A.parasiticus in caper after MeJA addition at different concentrations*

	Cultures					
	caper +10^2 conidia (control)[a]	**caper + 10^2 conidia + MeJA (10^{-1} M) [b]**	**caper + 10^2 conidia + MeJA (10^{-2} M)[c]**	**caper + 10^2 conidia + MeJA (10^{-3} M)[d]**	**caper + 10^2 conidia + MeJA (10^{-4} M)[e]**	**caper + 10^2 conidia + MeJA (10^{-6} M)[f]**
	AFB_1 production					
Days	μg $flask^{-1}$ [h] (±SD)	μg $flask^{-1}$ [h] (±SD)	μg $flask^{-1}$ [h] (±SD)	μg $flask^{-1}$ [h] (±SD)	μg $flask^{-1}$ [h] (±SD)	μg $flask^{-1}$ [h] (±SD)
0	N.D.[g]	N.D.[g]	N.D.[g]	N.D.[g]	N.D.[g]	N.D.[g]
3	1.57 (±0.06)	0.15 (±0.03)	0.12 (±0.024)	0.85 (±0.10)	0.87 (±0.08)	2.12 (±0.15)
7	3.96 (±0.35)	0.22 (±0.11)	0.32 (±0.076)	0.91 (±0.11)	2.29 (±0.06)	3.61 (±0.31)
9	2.96 (±0.55)	0.077 (±0.005)	0.17 (±0.036)	0.51 (±0.08)	1.23 (±0.07)	4.52 (±0.39)
12	4.43 (±0.13)	0.070 (±0.016)	0.10 (±0.021)	1.45 (±0.10)	2.90 (±0.13)	6.33 (±0.23)
15	3.51 (±0.40)	0.042 (±0.010)	0.008 (±0.002)	0.08 (±0.02)	0.49 (±0.10)	5.36 (±0.20)

[a] caper samples inoculated with *A.parasiticus*
[b] 226.667 mg MeJA 15g $caper^{-1}$
[c] 22.667 mg MeJA 15g $caper^{-1}$
[d] 2.267 mg MeJA 15g $caper^{-1}$
[e] 0.227 mg MeJA 15g $caper^{-1}$
[f] 0.0022 mg MeJA 15g $caper^{-1}$
[g] Non-Detectable
[h] Corresponds to 15g of capers $flask^{-1}$

The statistical analysis using one-way ANOVA, showed that the F_{exp} = 8.110 was higher than the F_{theor} = 2.533 for df 5, 30. Thus, the AFB_1 difference between the six groups is statistically significant ($p<0.05$).

AFB_1 production after MeJA addition at a concentration of 10^{-6} M was higher compared to control, particularly after the 9th day of observation. The t-test applied on the AFB_1 production between control samples and samples treated with 10^{-6} M MeJA showed that t_{exp} = -2.288 < t_{theor} = 2.571 (5 df, p=0.05); Following this, the observed difference is not statistically significant for the entire incubation period. After the 9th day (t_{exp} = 16.70 > t_{theor} = 4.303 (2 df, p=0.05) the AFB_1 output is significantly higher compared to control. On the other hand, the AFB_1 production after MeJA addition at a concentration of 10^{-4} M was lower compared to the control samples.

The t-test t_{exp} = 3.442 > t_{theor} 2.571 (5 df) showed that this reduction is statistically significant. Moreover, the AFB_1 production after addition of MeJA at concentrations of 10^{-4} M, 10^{-3} M, 10^{-2} M and 10^{-1} M was significantly lower compared to control with t_{exp} = 3.442, t_{exp} = 3.667, t_{exp} = 3.977 and t_{exp} = 3.962, respectively > t_{theor} = 2.571 (5 df , p=0.05) for the whole period of observation.

In the samples treated with MeJA at the lowest concentrations, AFB_1 production was stimulated after the 9th day of incubation. Table 4 shows that on the 12th day of observation, AFB_1 production was increased by 42.89% in samples treated with MeJA at a concentration of 10^{-6} M, while MeJA added at a concentration of 10^{-4} M inhibited AFB_1 production by 34.54%. This is in agreement with the report on the AFB_1 production in *A.flavus* cultures treated with 10^{-4} M and 10^{-3} M MeJA by [42]. On the other hand, the highest concentrations of MeJA, 10^{-2} M and 10^{-1} M, inhibited AFB_1 by 97.74% and 98.42% on the 12th day of observation, respectively. Following that, this work concludes that the higher the MeJA concentration, the higher is the inhibition effect.

Table 4. *AFB_1 Stimulation and inhibition induced in capers by different MeJA concentrationscompared to control samples (without MeJA addition) on the 12th day of incubation*

MeJA addition mg flask^{-1} [a]	AFB_1 µg flask^{-1} [a]	% inhibition	% stimulation
0	4.43	_	
0.0022 (10^{-6} M)	6.33	_	42.89
0.227 (10^{-4} M)	2.90	34.54	-
2.267 (10^{-3} M)	1.45	67.27	_
22.667 (10^{-2} M)	0.10	97.74	-
226.667 (10^{-1} M)	0.07	98.42	_

[a] corresponds to 15 g of capers flask^{-1}

In the case of *Aspergilli*, interactions between jasmonates, mycelial growth and aflatoxin production have been reported by several authors. These interactions are of great interest as they suggest that there is a mechanism involving plant LOX pathways that affect aflatoxin biosynthesis. It is interesting that both inhibition and stimulation of aflatoxin production by various LOX metabolites have been reported. Furthermore, some hydroperoxy fatty acids may exert a stronger signaling influence on aflatoxin/sterigmatocystin (AF/ST) biosynthesis than on others. For example, aflatoxin biosynthesis by *A. parasiticus* was stimulated in a synthetic medium containing a mixture of 30% 13S-HPODE and ~70% 13-HPODE although 13-HPODE had an inhibitory effect when tested alone. In addition MeJA treatment at concentrations ranging from 10^{-6} M to 10^{-3} M reduced AFB_1 production by *A. flavus* grown on either Czapek Yeast Extract Agar (CYA) medium or pistachios in storage [42].

On the contrary, [43] reported that treatment with MeJA at a concentration of 10^{-4} M stimulated AFB_1 production by *A. parasiticus* grown on YES medium. [44] underlined that lipoxygenase generated signals, such as jasmonates, have both inhibitory and promoting effects on the AFB_1 production. Moreover, [40] reported that either treatment of *A. parasiticus* cultures with 10^{-2} M MeJA or with 10^{-4} M and 10^{-6} M MeJA either inhibited or stimulated AFB_1 production, respectively.

By studying the MeJA concentrations added in caper samples used in the present study, the median inhibitory concentration (IC_{50}) was calculated at 0.433 mg MeJA flask^{-1}. This is an intermediate MeJA concentration found to be between 10^{-3} M and 10^{-4} M.

Previously, [45] showed that when olives were treated with MeJA at different concentrations, AFB_1 production was concentration-dependent in both inoculated olives with *A.parasiticus* as well as in non-inoculated olives. Meanwhile, [42], based on their observations with pistachios (a more favorable substrate for AFB_1 biosynthesis by aflatoxigenic fungi compared to olives), exhibited reduced aflatoxin contamination when exposed to MeJA.

AFB_1 biosynthesis by *A.parasiticus* is a natural fungal reaction that occurs when the death phase has approached and the secondary metabolism begins. However, when MeJA is added, differences occur in the mycotoxin biosynthesis in both a synthetic medium (YES) [40] as well as in capers. Furthermore as the mode of action of MeJA is still unclear the so called "host-fungus interaction" theory, can be used in order to explain to a certain extent our experimental results.

According to this theory, since both plant (Ja's, MeJA) as well as fungal (psi factors) oxylipins resemble each other in terms of structure and biosynthesis, it is likely that fungi may use endogenous metabolic lipid enzymes as well as endogenous oxylipins in order to initially colonize the host and subsequently to multiply and produce their toxins. Besides, recent evidence has shown that members of different fungi have evolved, in order to use the plants defense response deriving from the plant oxylipins for their own population [46,47].

In this case, the observed AFB_1 stimulation at the lowest MeJA concentration is likely to be induced by the "manipulation" of the plant regulator - which is actually the

plant's defense response - in favor of the fungus.

In the case of the AFB_1 inhibition, it is possible that the presence of high MeJA concentrations affects the physiological fungal activities by suppressing the mycelial growth in capers (Markaki unpublished data) as well as the AFB_1 biosynthesis, since according to the "reciprocal crosstalk" theory between host and fungus, plant oxylipins interact with the fungal system [48].

Furthermore, MeJA may act at a genetic level, by inducing partial or complete AFB_1 inhibition. Thus, it is essential to further study the genetic profile of the fungus with or without MeJA addition.

4. Conclusions

The results of the present study revealed the possibility of the existence of different mechanisms, by which MeJA might influence AFB_1 biosynthesis when different concentrations are used. Moreover, as it was shown in the present study, the effectiveness of MeJA produced by plants depends, in addition to other factors, on its concentration levels and therefore AFB_1 production could be predictable only under defined conditions. However, in this work, it was revealed that MeJA concentrations above 10^{-4} M inhibited AFB_1 production.

Thus, the use of MeJA in crop storage areas could be an effective, simple, and safe way for reducing post-harvesting aflatoxin production. This suggestion should be applied with thoughtfulness since the presence of mycotoxins in food products and the amounts produced, entirely depend on the ecological and processing parameters of the foodstuff [49].

Nomenclature

AFB_1, Aflatoxin B_1; AFB_{2a}, Aflatoxin B_1 hemiacetal derivative; A., *Aspergillu*s; AFPA, Aspergillus flavus parasiticus agar; CFU, Colony forming units; CZA, Czapek Dox agar; HPLC, High performance liquid chromatography; SD, Standard deviation; YES, Yeast Extract Sucrose; LOX, lipoxygenase; 13S-HPODE, 13S-hydroperoxy-cis-9-trans-11-octadecadienoic acid; 13S-HPOTE, 13S-hydroperoxycis- 9, trans-11, cis-15-octadecatrienoic acid; DL, limit of detection; QL, limit of quantification; MeJA methyl jasmonate; RSD, relative standard deviation.

Conflict of Interest

We hereby confirm that the authors have no conflict of interest regarding the present work.

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