Methyl Jasmonate Stimulates Aflatoxin B_1 Biosynthesis by *Aspergillus parasiticus*

Sophia Vergopoulou, Dia Galanopoulou, and Panagiota Markaki*

Department of Food Chemistry, School of Chemistry, University of Athens, Athens, Greece

Aflatoxin B_1 (AFB_1) is a highly toxic and carcinogenic metabolite produced by certain *Aspergillus* species on agricultural commodities. One factor promoting the production of aflatoxin is the presence of high levels of fatty acid hydroperoxides often found in plant material under stress. Jasmonic acid (JA) and its methyl ester (MeJA) are derived from linolenic acid, and their biosyntheses involve the production of lipid hydroperoxides. Exposure of aflatoxigenic mold to jasmonates is likely because the mold attacks plant material and possibly initiates the production of jasmonates. In this study the effect of MeJA on the growth of *Aspergillus parasiticus* and AFB_1 biosynthesis is reported. MeJA, at a final concentration of 10^{-4} M in yeast extract sucrose medium, did not have any apparent effect on mycelial growth during the 16 days of observation but did increase significantly the levels of AFB_1 after the seventh day of growth. After the ninth day, AFB_1 production was decreased in contrast to the control cultures, where the production was constantly increasing. AFB_1 determination was performed by immunoaffinity and HPLC after derivatization to AFB_{2a}.

Keywords: *Aflatoxin B_1; methyl jasmonate, A. parasiticus; HPLC*

INTRODUCTION

Aflatoxins (AF) are highly toxic and carcinogenic secondary metabolites produced by the molds *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*. AF contamination of agricultural commodities and their products occurs following *Aspergillus* spp. infection, and therefore there is an international public health concern. Aspergilli typically gain access to plant seeds by environmental stress (heat and daught) or via insect damage (*1*). *Aspergillus* lipases have been reported to be involved in the primary events after fungal invasion, because *A. flavus* first destroys the lipid bodies and not the starch granules of maize kernels (*2*). On the other hand, the fatty acid content of seed lipid bodies, composed mainly of palmitic, oleic, and linoleic acids, supports growth of *Aspergillus* spp. in vitro (*3*). Considering that many studies have shown that lipid oxidation also affects AF biosynthesis (*4*−*7*), it is important to determine how linoleic as well as linolenic acids and their oxidation metabolites are taking part in the lipid−*Aspergillus*−mycotoxin interactions.

Linoleic and linolenic acids can be oxidized by plant stress response enzyme lipoxygenase (LOX) (*8*). LOX isozymes catalyze the formation of hydroperoxy fatty acids such as 13*S*-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13S-HPODE) or 13*S*-hydroperoxy-*cis*-9,*trans*-11,*cis*-15 octadecatrienoic acid (13S-HPOTE) from linoleic and linolenic acid, respectively (*9*). 13S-HPODE and 13S-HPOTE can be further metabolized by plant enzymes to give jasmonates, which induce the expression of genes encoding defensive proteins or enzymes involved in the biosynthesis of defensive secondary metabolites (*9*, *10*).

* Address correspondence to this author at the Department of Food Chemistry, Panepistimiopolis Zografou, University of Athens, GR 157 71 Athens, Greece (telephone 0030-1-7274489; fax 0030-1-7274476; e-mail markaki@chem.uoa.gr).

Methyl jasmonate (MeJA) is one of the jasmonic acid metabolites found in plants. These compounds are synthesized in plants as a response to systemic or localized signals such as oligosaccharides released from fungal or plant cell walls during plant−pathogen interactions (*11*). MeJA has been shown to inhibit aflatoxin B_1 (AFB_1) production by *A. flavus* grown on agar medium but not fungal growth (*12*). This inhibition was also observed in the production of AFB_1 by field strains in the case of pistachios (*12*). Aldehyde metabolites derived from 13S-HPODE and 13S-HPOTE also inhibited the germination of *A. flavus* spores and/or the production of AF (*13*, *14*). In addition, Burow et al. showed that 13*S*-hydroperoxy fatty acids at concentrations of 1, 10, and 100 μM repressed AF pathway gene expression and significantly reduced AF production by *A. parasiticus* (*15*). In the present study the effects of MeJA on *A. parasiticus* growth and AFB_1 production have been examined on a liquid medium. Results are discussed having in view the treatment of edible material of plant origin with MeJA.

EXPERIMENTAL PROCEDURES

Precautions. AFB_1 is a very toxic and carcinogenic substance. We used a fume hood for the preparation of standards. Areas where analyses were carried out were protected from daylight to prevent the decomposition of AFB_1. Also, to avoid loss of AFB_1 by absorption, all pieces of glassware used were soaked in a dilute acid solution for several hours and then were washed and dried in the normal manner (*16*).

Apparatus. An autoclave, Selecta Autester-E Dry, an incubator WTB binder, and a centrifuge Sorvall RC-5B (HS-4) were used during this study. HPLC was performed on a Hewlett-Packard 105 liquid chromatograph equipped with a JASCO FP-920 fluorescence detector and an HP integrator 3395. The HPLC column was a C_{18} Nova-Pak (60 Å, 4 μm, 4.6 × 250 mm). The mobile phase, water + acetonitrile + methanol (360 + 72 + 54), was filtered through 0.45 μm filters before use. Detection of the hemiacetal derivative (AFB_{2a}) was carried

10.1021/jf010074+ CCC: $20.00
Published on Web 06/14/2001

out at λ_{ex} = 365 nm and λ_{em} = 425 nm. The flow rate was 1 mL min^{-1}, and the retention time was 15 min.

Reagents. AFB_1 standard was purchased from Sigma. The filters and the C_{18} Nova-Pak HPLC column were from Waters (Millipore, Milford, MA). The Aflaprep immunoaffinity columns were from Rhone Diagnostics. All other reagents used were of analytical grade. 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 (*17*).

Media. *Aspergillus flavus parasiticus* agar (AFPA) was prepared by dissolving 4 g of yeast extract (Oxoid), 2 g of bacteriological peptone (Oxoid), 0.1 g of ferric ammonium citrate, 0.2 mL of Dichloran (0.2% in ethanol, Fluka), 0.02 g of chloramphenicol (Oxoid), and 3 g of agar (Oxoid) in 200 mL of distilled water, final pH 6.0−6.5 (*18*). Gzapek Dox agar (GZA) was prepared by 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 (*19*). Yeast extract sucrose (YES) broth was prepared by dissolving 2 g of yeast extract and 15 g of sucrose in 100 mL of distilled water, final pH 6.0−6.5 (*18*).

Preparation of Spore Inoculum. The aflatoxigenic strain *A. parasiticus* speare (IMI 283883) used throughout this study was obtained from the International Mycological Institute, New England. An inoculum was obtained by growing the mold on a slant of stock cultures of GZA, which were maintained at 5 °C (*20*, *21*). Spore inoculum was prepared by growing *A. parasiticus* on GZA for 7 days at 30 °C, and spores were harvested aseptically using 10 mL of sterile 0.01% v/v Tween 80 solution (*22*). AFB_1 carried over from the initial growth was minimized by centrifuging the spore suspension (1000*g* for 10 min) and resuspending the biomass in 10 mL of sterile Tween 80 solution twice. Dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) from the initial spore in sterile tubes containing 10 mL of Tween 80 0.05% v/v suspension were prepared (*23*). The spore concentration was determined by the spread plate surface count technique using 0.1 mL of each dilution on four AFPA plates (*18*, *24*) after incubation at 30 °C for 2 days. The population size was estimated by the reverse intense yellow/orange coloration of the colonies. For obtaining 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 a detectable amount of AFB_1 by Aspergilli (*25*).

Inoculation. Six flasks for each day of observation containing 10 mL of YES medium were inoculated with 10^2 spores/flask of *A. parasiticus* in the appropriate volume from the selected dilution. MeJA in ethanol at a final concentration of 10^{-4} M/flask was added into each of the three flasks for each day of observation. All flasks, control (ethanol alone) and treated with MeJA, were incubated under stationary conditions at 30 °C (*26*). Immediately after 30 min of autoclaving at 115 °C as is suggested for safety reasons (*27*−*29*) the mycelial growth was determined and AFB_1 was assayed on days 0−9, 11, 14, and 16 of incubation. The experiment was repeated in triplicate.

Determination of Mycelial Mass. After cooling, mycelia were filtered through filters that were dried (24 h at 80 °C) and weighed. The mycelium was washed with distilled water and allowed to dry for 24 h at 80 °C. The dry weight of the mycelium was then measured (*23*, *30*).

AFB_1 Determination. The content of each flask was mixed with 30 mL of methanol and shaken well for 10 min. After filtration, an aliquot of 1 mL from each flask was used for AFB_1 analysis. This aliquot could be stored at −20 °C until determination. The aliquot of 1 mL from the filtrate was mixed with 10 mL of distilled water. The mixture was transferred onto an Aflaprep immunoaffinity column (flow rate = 6 mL min^{-1}) and washed twice with 10 mL of distilled water. The column was then allowed once more to dry by passing air through it. AFB_1 was eluted very slowly with 2 mL of acetonitrile (flow rate = 0.3 mL min^{-1}). The eluate could be stored at −20 °C until derivatization (maximum of 24 h). Before derivatization, the eluate was evaporated to dryness on a water bath under a gentle steam of nitrogen (*31*).

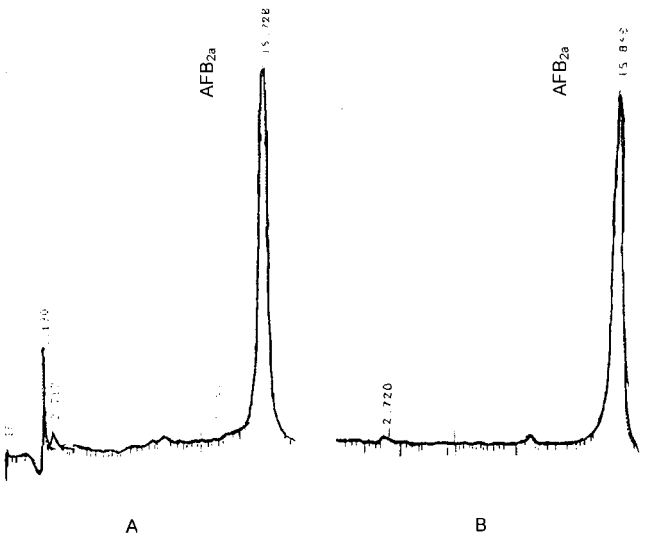

Figure 1. HPLC analysis of (A) standard AFB_1 (0.01 ng) and (B) AFB_1 produced by *A. parasiticus* IMI 283883 (dilution 1:360000), both derivatized to AFB_{2a}. Analysis was carried out in a C_{18} Nova-Pak (60 Å, 4 μm, 4.6 × 250 mm) Waters column with water + acetonitrile + methanol (300 + 72 + 54) at a flow rate of 1 mL min^{-1}. Retention time was 15 min. Injection volume was 20 μL.

Derivatization and HPLC Analysis. 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 at 40 °C in a water bath for 10 min, evaporating to dryness under nitrogen, redissolving in an appropriate volume with water + acetonitrile (9 + 1) to give concentration of <10 ng mL^{-1}, and analyzing by HPLC (volume injected = 20 μL). The AFB_{2a} shows enhanced fluorescence compared to AFB_1 (*32*).

RESULTS AND DISCUSSION

Characterization of AFB_1 Determination in YES Medium. In the present study, the first step was the establishment of *A. parasiticus* culture in YES medium and the determination of AFB_1 levels under standard growth conditions. AFB_1 was determined by HPLC after derivatization to AFB_{2a} as described under Experimental Procedures.

The analytical protocol for the AFB_1 determination in YES medium was in-house characterized: AFB_1 standard stock solution was stored in benzene + acetonitrile (9 + 1) at −20 °C. The calibration curve of standard AFB_1 derivatized to AFB_{2a} was established in the range of 0.5, 1.0, 2.5, 5.0, and 10 ng mL^{-1} in water + acetonitrile (9 + 1). Linear regression was used to prepare standard curves by using the mean values of peak areas of three injections from the five solutions. The mean values of the three experiments showed a satisfactory linearity of the standard curve (A = 967168.9a − 30390.8, r = 0.9999, where A = peak area of derivatized AFB_1 and a = ng of derivatized standard AFB_1; the injection volume was 20 μL). Furthermore, the precision, recovery, and detection limit of the method in YES medium were studied. A satisfactory linear relationship was established between different quantities of AFB_1 (1, 2.5, 5, and 10 μg spiked in 10 mL of YES medium) and quantities recovered (r = 0.0998).

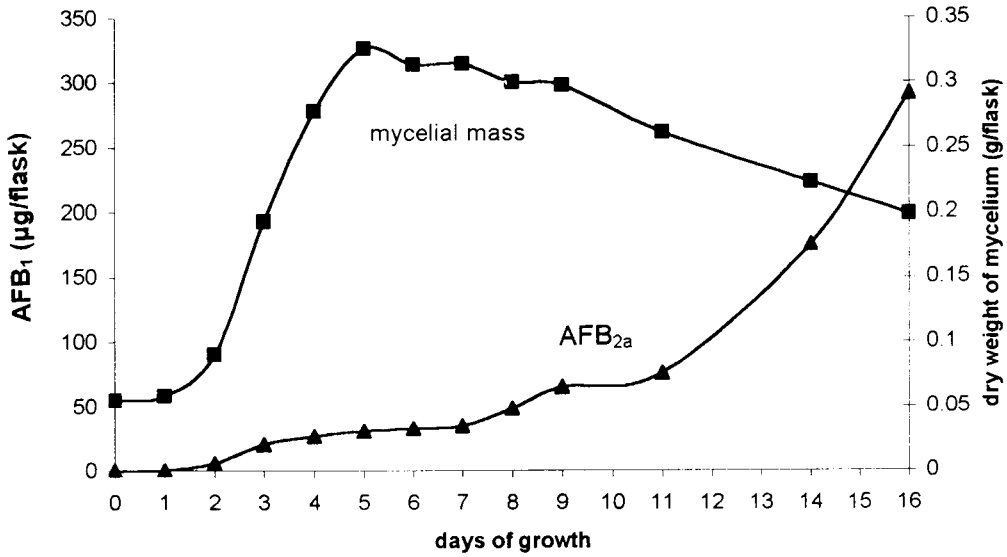

Figure 2. Mycelial growth and AFB_1 production by *A. parasiticus*. Results are from three separate experiments with triplicate cultures (flasks). AFB_1 levels were increased during the death phase of mold growth. AFB_1 was determined after derivatization to AFB_{2a}.

In addition, the mean recovery was found to be 95.3% (CV% = 9.6). The detection limit, based on a signal-to-noise ratio of 3:1 at the retention time (15 min, Figure 1) of the derivatized AFB_1 (AFB_{2a}), was found to be 0.2 ng/flask.

The hypothesis that the regression between the variables (quantities of AFB_1 spiked in YES medium and quantities recovered) is acceptable was also checked by using the Fisher test. The experimental F ratio was 1077.505. This is greater than the critical value F (1,12,1−a) = 9.646, and it corresponds to a Fisher variable with a risk a = 1% for 1 and 12 degrees of freedom. Therefore, the regression model can be considered to be acceptable.

Finally, the lack of fit of the model was found to be negligible, as the experimental F ratio (1.688) is less than the critical value F (2,12,1−a) = 6.2, corresponding to a Fisher variable with a risk a = 1% for 2 and 12 degrees of freedom. Therefore, the field of linearity chosen can then be approved.

***A. parasiticus* Growth and AFB_1 Production in YES Medium.** Yeast extract is a rich source of B complex vitamins, which have been associated with the stimulation of AFB_1 production by *A. parasiticus* (*33*). The presence of magnesium and sucrose in YES medium also enhances AFB_1 production by *A. flavus* and *A. parasiticus* (*25*). Additionally, YES medium is easy to prepare, relatively inexpensive, and very suitable for production of higher levels of aflatoxin than those reported for other media (*23*). Therefore, YES medium was chosen for *A. parasiticus* growth.

The common practice of measuring AFB_1 rather than all four AFs (AFB_1, AFG_1, AFB_2, and AFG_2) was followed throughout this study. AFB_1 is the most important as it is the most potent, and it is usually produced at the highest levels by toxigenic strains (*1*, *15*).

Mycelial growth and AFB_1 production by *A. parasiticus* are shown in Figure 2. The maximum growth of the mold was observed on the fifth day after inoculation. The decrease of mycelial growth that followed was less rapid, and it was clear only after the ninth day of observation.

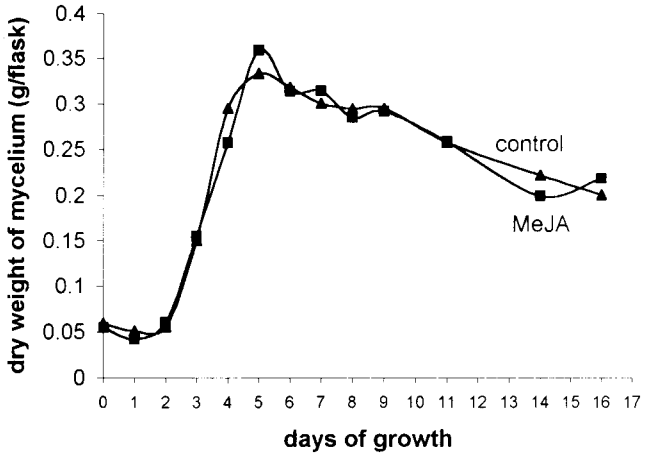

Figure 3. Effect of MeJA on mycelial growth of *A. parasiticus*. The effect of MeJA (10^{-4} M in ethanol) was studied in three separate experiments with triplicate cultures (flasks) against control (addition of ethanol alone). MeJA had no effect on mycelial growth during the 16 days of observation.

The production of AFB_1 was moderate until the seventh day after inoculation. After this period, the levels of AFB_1 were increased dramatically. This is in contrast to a previous study reporting a simultaneous decrease in *A. parasiticus* growth and AFB_1 production (*34*). Although primary and secondary metabolisms can occur simultaneously under certain conditions, nutritional factors may be growth limiting, thus favoring production of AFB_1 while slow growth still occurs (*33*). As described under Experimental Procedures, the initial content of AFB_1 was minimized by centrifuging the spore suspension. However, even at day 0, low amounts of AFB_1 were determined (0.9−1.1 ng/flask). On the first day the production was 6.4 ng/flask.

Effect of MeJA on Mycelial Growth and AFB_1 Production. In the present study, the effect of MeJA at a final concentration of 10^{-4} M on both mycelial growth of *A. parasiticus* in YES medium (Figure 3) and AFB_1 production (Figure 4) is studied. The concentration of MeJA chosen corresponds to the higher levels of MeJA used by Goodrich-Tanrikulu et al. (*12*), who reported that MeJA inhibited the production of AFB_1

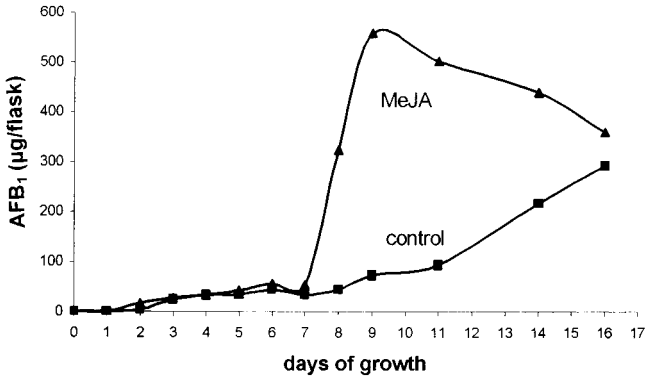

Figure 4. Effect of MeJA on AFB_1 production by *A. parasiticus*. The effect was studied in three separate experiments with triplicate cultures (flasks) against control. MeJA addition increased AFB_1 production only after the seventh day of mold growth, and AFB_1 production was decreased after the ninth day. AFB_1 was determined after derivatization to AFB_{2a}.

by a different *Aspergillus* strain, *A. flavus*. In this work, the effect of MeJA in *A. parasiticus* is in contrast with the effect reported by Goodrich-Tanrikulu et al., increasing the production of AFB_1 after the seventh day of growth as shown in Figure 4. This is also the time in which AFB_1 production in the control cultures occurs at higher levels when compared with those at days 0 and 1. In the case of MeJA-treated cultures, the increase is more rapid and the maximum production is observed on the ninth day. After the ninth day, AFB_1 production decreases, in contrast to the control cultures in which the production is correlatively increasing. At the 16th day, the levels of AFB_1 in cultures with and without MeJA were not significantly different.

A two-tailed Student *t* test for the production of AFB_1 from cultures with and without MeJA was employed. The $t_{exptl} = 2.4$ was found to be higher than the $t_{crit} = 2.1$ (12 df and $p = 0.05$) for the whole period of observation. Therefore, there is a significant difference between the two variables, and therefore the MeJA increases the production of AFB_1.

The decrease of AFB_1 production observed in the MeJA-treated cultures after the ninth day (Figure 4) may be due to AF degradation. The ability of Aspergilli to degrade aflatoxins depends on the age of mycelials, mycelial integrity, the type of substrate (substrates that support growth yield mycelial having the greatest ability to degrade AFB_1), and the amount of AFB_1 present. It is interesting that, as the amount of aflatoxin AFB_1 increases, the rate of degradation also increases (*35*), and this is in good agreement with our results (Figure 4).

The enhancement of AF production by fatty acid oxidation metabolites has already been reported. A mixture of 1 mM linoleic acid and soybean LOX-1 (producing a 7:3 ratio of 13S-HPODE and 9S-HPODE) increased AF levels 50−200 times in *A. parasiticus* cultures (*4*, *7*, *36*). MeJA also did not suppress AFB_1 levels or the levels of the mRNA of a gene encoding an enzyme necessary for AF biosynthesis in *A. parasiticus* and *Aspergillus nidulans* (*15*).

In the present study, MeJA had no effect on mycelial growth during the 16 days of observation (Figure 3). As Passi et al. (*36*) have also reported, lipoperoxides enhanced AFB_1 production without interfering significantly with fungal growth. Zaika and Buchanan (*37*) and Rodriquez and Mohoney (*38*) have reported also that MeJA had no apparent effect on mycelial growth or colony appearance in the case of *A. flavus*.

A. flavus and *A. parasiticus* are strains known to occur generally in agricultural commodities. On the other hand, MeJA is a compound produced by plants, although it can be produced by plant pathogens as well (*39*, *40*). Therefore, mold exposure to MeJA is possible. In addition, MeJA is a metabolite that can influence AFB_1 biosynthesis by Aspergilli, although different studies have given controversial results concerning this influence. Therefore, before MeJA is considered as an inhibitor or a promoter of AFB_1 biosynthesis, further studies are necessary to elucidate the MeJA effect on *A. parasiticus* and *A. flavus* when these molds occur either separately or simultaneously on cultures, plant material, and, furthermore, postharvest crops. Understanding the mechanisms and expressing the AF properties in plant species may lead to AF control strategies. It has to be noted that conflicting results in the literature concerning AF content in agricultural commodities could be explained by the fact that AF production depends on the exact parameters of the system under investigation.

ACKNOWLEDGMENT

We thank Dr. V. Dourtoglou (Vioryl, SA) for supplying methyl jasmonate.

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Received for review January 18, 2001. Revised manuscript received May 1, 2001. Accepted May 7, 2001. This work was partially supported by a grant (70/4/2508) from the University of Athens.

JF010074+