Effects of Gaseous Ozone (O_3) on Artificial Population and Aflatoxin B_1 Production by *Aspergillus flavus* in Stored Whole Cinnamon in Iraqi Markets

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Abstract

Stored whole cinnamon can become contaminated with aflatoxins.For public health requirements cinnamon should be produced free of AFB_1or bellow EU legislation limit (5µg/kg), soin this study, examined the efficacy of different concentrations (0, 300 and 600 ppm) of ozone on fungal populations (CFU) and AFB_1 production of *A.flavus* on stored whole cinnamon at three a_w levels (0.92, 0.94&0.96 a_w) at 25°C and exposure time (60 min). In general, the results presented in this study showed that the populations AFB_1 produced of *A.flavus*in artificially contaminated whole cinnamon were significantly affected by O_3treatment.

Keywords: *Cinnamon; Ozone; Population; Aflatoxin B_1;Aspergillus flavus.*

Introduction

Cinnamon produced in tropical countries and used as ingredients in traditional foods as food supplements or medicines [3]. Cinnamon can be contaminated with AFB_1 during pre or post-harvest, processing, handling and storage [20]. Aflatoxins (AFs) are mycotoxins produced by many fungal species, especially *Aspergillus flavus, A. nomius* and *A. parasiticus*. These AFs are carcinogenic, teratogenic and mutagenic in human and animals. There are many types of AFs one of them Aflatoxin b_1 (AFB_1), it is the most toxic because classified by International Agency for Research on Cancer (IARC) as class 1a carcinogenic in human and animals[12].

Ozone (O_3) is a strong germicide and has three oxygen atoms. It is used in food industries as an antifungal agent prevents fungal growth in raw materials and used as a preservative in packaging foods [23]. O_3 is produced naturally as a colourless or bluish gas by two methods and artificially by three methods. The most common method for O_3 production is a corona-discharge method (CDM) [16;9]. It will be considered in this study to control fungal population and prevent contamination of cinnamon with AFs.

Natural levels of O_3 are between 0.01-0.15 ppm but exposure to high levels produce some harmful effects in health such as respiratory diseases [11] and cardiovascular diseases [6]. Federal occupational safety and health administration (OSHA) recommended a legislative limit for exposure to O_3 of 0.1 ppm for 8 hr continuously or 0.3 ppm for 15 min [22].

The efficacy of O_3 on fungal species is depending on fungal tolerance to exposure, O_3 (concentration), exposure time, spore morphology and moisture content of substrate [1]. Moreover, O_3 treatment can cause negative impacts in the quality of the food such as oxidation of lipids, change colour, modified of some vitamins and phenolic compounds [18;7].

The effect of O_3 on colonization and AFB_1 production of *A. flavus*in peanuts, wheat, chillies, Barazil naut, corn, maize and red peppers have been studied previously,[21;5;2;19;4;17;10;13]. To my knowledge, there very few studies on cinnamon. The objective of this

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study was to examine (a) the efficacy of O_3 on the total fungal colonization and AFB_1 production of *A. flavus* in artificially contamination on whole dried cinnamon stored at 25°C stored for 20 days.

Material and Methods

Chemicals and reagents

The AflaStar™ – Immunoaffinity Column (AIC) were purchased from Romer Labs PLC (Tulln, Venna, Austria). Aflatoxins Stock was purchased from (Romer Lab UK Ltd, Cheshire, UK). Mealt Extract Agar was purchase from (Oxoid, Basingstoke, Hampshire, UK). Methanol and Acetonitrile were obtained from (Fisher Scientific, UK). All solvents were of HPLC grade.

Collection of cinnamon samples

300 g of whole dried cinnamon were randomly collected from Iraqimarkets and direct plated on MEA and then incubated at 25°C for seven days for fungal isolation.

Preparation of fungal conidial inoculums

Spore inoculums was made from *A. flavus* (DJC_1) isolated from cinnamon, these spores grown on MEA for 7 days and removed by sterile loop and then flooded with 9 ml of sterile water containing 0.025% (w/v) Tween 80.

Media preparation

Malt extract agar (MEA)

This medium was used for culturing, isolating and enumerating fungal species. (15 g MEA powder and 300 ml distilled water (D.W). A small amount of antibiotic agent, chloramphenicol (0.05 g/L) was mixed with the components to prevent bacterial growth. After autoclaved for 1 hr at 121ºC the sterile medium was cooled and poured into 9 cm serial Petri plates and left to cool, and kept at 4ºC in polyethene bags until used.

Preparation of Adsorption curve

A moisture absorption curve was designed to determine the quantities of water required to add to cinnamon samples to modify the moisture contents to the treatment target $a_w$values. This curve was prepared by adding different amounts of distilled water to whole cinnamon and equilibrating at 4ºC for 24 hrs. The a_w was measured with an Aqua Lab 4TE.

Preparation of chilli samples

Thirty grams of whole dried cinnamon were weighed into 9 solid culture vessels and then stoppered with plastic lids with a permeable membrane. The samples divided into three levels of a_w conditions (0.92, 0.94 and 0.96a_w). Each group of chillies adapted by adding the appropriate volume of distilled water based on the moisture absorption curve to obtain the required target levels. All samples were shaken on a (KS 501 digital orbital shaker) for 1hr and then kept at 4ºC for 24 hrs to allow absorption and equilibration. Each vessel was inoculated with a spore suspension of *A. flavus* (10^2 spores/ml) by add 0.25 ml of spore solution to each vessel. All samples mixed well and exposed to a different concentration of O_3 (0, 300 and 600) ppm for 60 min at (6L/min) in 50 ml class tube system. Controls were prepared by exposure to air only. Each treatment divided into three separate replicatesat each a_w levels and stored in plastic chambers at 25ºC for 20 days. Each plastic chamber also contained 500 ml beaker containing a glycerol/water solution of the same a_w to maintain the ERH. The *A. flavus* populations present initially and after 10 and 20 days were quantified at each treatment. The samples were stored at -20°C for AFB_1 analyses at each treatment condition.

Enumeration of *A.flavus* populations on stored whole cinnamon

One g of cinnamon from each treatment and replicate was weighed and mixed with 9 ml of sterile D.W with 0.025% (v/v) Tween 80 to obtain the 10^{-1} dilution. All samples were then serially diluted after vigorous shaking at each dilution using a vortex mixer. 100 µl of each dilution was spread plate onto MEA plates modified a_w at the same a_w of storage a_w. All plates were incubated at 25ºC for seven days the colonies growing were then enumerated.

Extraction of AFB_1 from chillies using HPLC

Weighted three g of cinnamon from each treatment and replicates and placed in 30 ml tube. Added 12 ml of extraction solution (**acetonitrile / water)** (6/4 (**v/v**)) at 15-18°C. Then the samples have been shaken for 1 hr. The extracts were filteredinto contenier using a

funnel containing a Whatman filter paper (12.5 cm). The extracts were diluted with PBS (Phosphate buffered saline) (pH 7.4) until the content of acetonitrile is lower than 5% (**v/v**) and (7 pH).The diluted extracts were transferred to (Romer, IAC) and allowed to pass through the column using a syringe barrel as a reservoir. The flow rate should be not exceeding 1-3 ml/min. The column and the extract must be at 15-18ºC. The IAC should be rinsed with 2 x 10 ml of distilled water. Added 2 ml of methanol directly to the column and calculated the elution in a new Eppendorf tube 2 ml.The elution was evaporated under reduced pressure at 40ºC using a rotary evaporator. Added 1 ml of water: methanol (0.5:0.5) into toxin and filtered the extracts through Nylon 13 mm, 0.2 µm filter directly into amber HPLC vial and then stored at -20ºC until analyses using HPLC.

Statistical Analysis

The data obtained from this study were determined by using the Shapiro Wallis Test for (Non-normality) and one-way ANOVA for (Normality). The significant statistical level was at $P<0.05$ for both factors and their interactions.

Results

The effects of O_3 (ppm) on artificially populations of *A. flavus* on whole cinnamon stored at different a_w levels.

Figure 1 shows the effect of exposure to different doses of O_3 on the colonisation by *A. flavus* on stored whole cinnamon for 20 days at 25ºC and different a_w levels. The results showed that there were clear effects of incubation time and a_w on the populations in stored whole cinnamon and significant effects of O_3 concentrations except on initial colonization at first day of incubation especially, at 0.92 and 0.96 a_w. Statistically, the populations were significantly affected by O_3 concentrations, time, ppm x time, ppm x a_w and a_w but unaffected by time x a_w and ppm x time x a_w (Table 1).

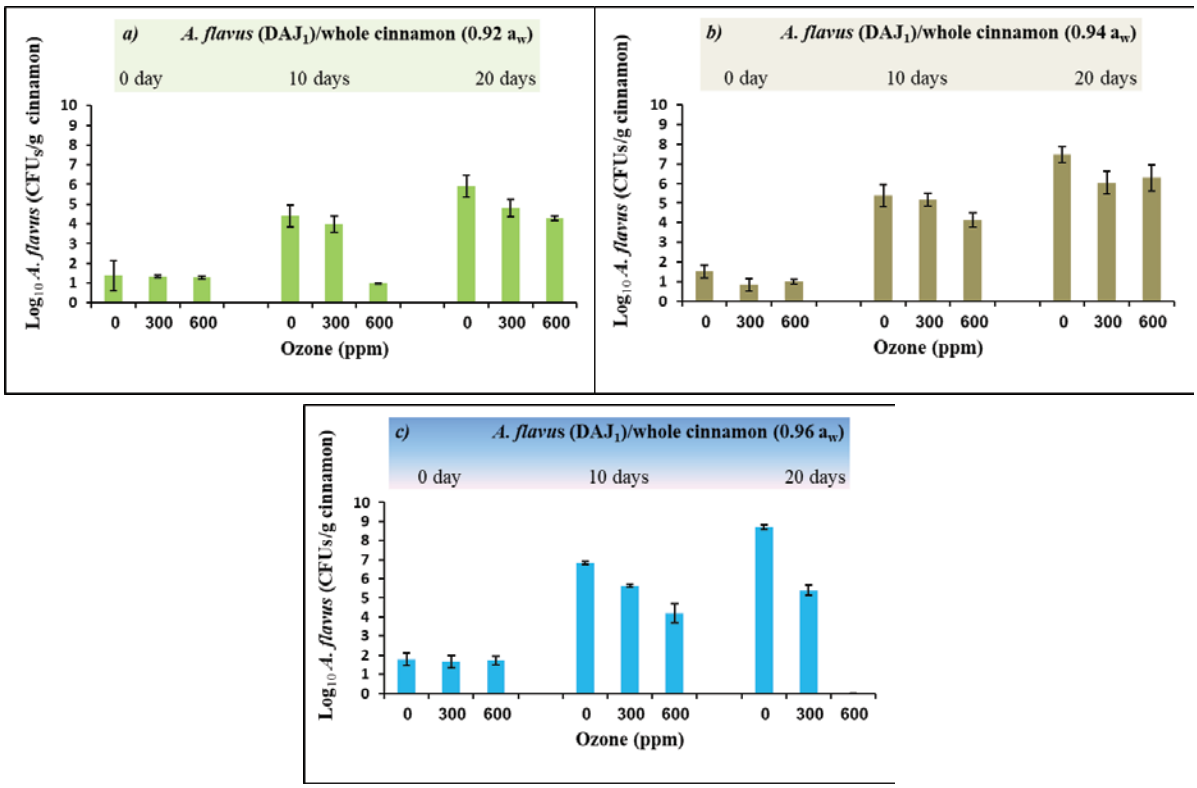

Figure 1 (a,b &c): Effect of O_3 (ppm) on populations of *A. flavus* (DAJ_1) isolated from stored whole cinnamon at 25ºC at (0.92, 0.94 and 0.96a_w) over 20 days. Bars indicate SDM.

The effects of O_3 (ppm) on control the AFB_1 contamination of *A. flavus* in artificially contamination on stored cinnamon at different a_w levels.

Figure 2 showed the efficacy of O_3 concentrations on AFB_1 contamination in stored whole cinnamon for 20 days with four a_w levels at 25°C and exposure time was 60 min. The data of this study reported that AFB_1 of *A. flavus* gradually decreased when increased O_3 concentrations except at 10 days at 0.92 a_w. Also, the AFB_1 production affected by a_w and time. Table 1 shows that there were significant effects of O_3 ppm, a_w, time, ppm x a_w, ppm x time and a_w x time but unaffected by ppm x a_w x time.

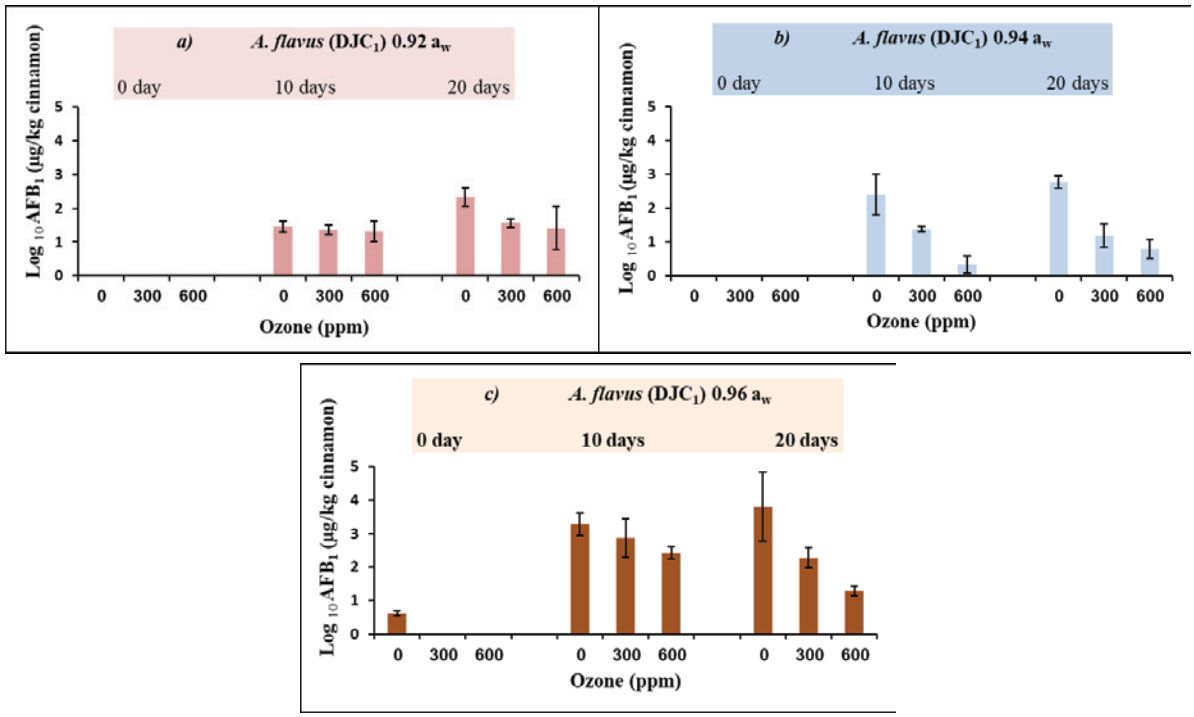

Figure 2 (a,b &c): Efficacy of O_3 (ppm) on AFB_1 contamination of *A. flavus* isolated from stored cinnamon at 25°C at (0.92, 0.94 and 0.96a_w) over 20 days. Bars indicate SDM.

Table 1: Summarise statistical analyses of effects of O_3 (ppm) on total populations and AFB_1 contaminationof *A. flavus* on whole cinnamon at 25°C under three a_w levels (0.92, 0.94 and 0.96a_w) over 20 days storage. ANOVA test (normality data) and Shapiro Wills test (non-normality data).

Factors	Effects	Actions
ppm	*S*[b]	Population
	S	AFB_1 Production
incubation time	*S*[b]	Population
	S	AFB_1 Production
a_w	*S*[b]	Population
	S	AFB_1 Production
ppm x time	*S*[a]	Population
	S	AFB_1 Production
ppm x a_w	*S*[a]	Population
	S	AFB_1 Production
ppm x time x a_w	*NS*[a]	Population
	NS	AFB_1 Production
time x a_w	*S*[a]	Population
	S	AFB_1 Production

NS, not significant *S*, significant *a*) ANOVA test *b*) Shapiro Wills test

Discussion

In this study different gaseous O_3 concentrations (0, 300 and 600 ppm; flow rate of 6 L/min) were used in order to know the effect of O_3 treatment on fungal populations and AFB_1 production in stored cinnamon inoculated with *A. flavus* (DJC_1) 10^2spores/ml at three a_w levels (0.92, 0.94 and 0.96 a_w) for 20 days. The fungal population and AFB_1 production has been measured at (T=0, 10 and 20 days) and compared with control (exposure air only). The results showed that the fungal population affected by gaseous O_3 treatment at all a_w levels andthere clear impact of ozonation on AFB_1 content in whole cinnamon(artificial contamination). To my knowledge, no previous studies conductedon the effects of O_3 exposure on fungal and AFB_1 contamination in stored cinnamon.

However, there are some studies done on the efficacy of O_3 concentrations on fungal populations and mycotoxins production in some products. For example, [1] have shown that the populations of three ochratoxigenic strains were significantly reduced in the coffee beans after exposure to 600 ppm O_3at 0.90 and 0.95 $a_w$compared with control. This data were similar to the findings in the current study. Similar to Akbar[22] presented that the fungal populations of *A. flavus* in peanut samples inoculated with (10^5 spores/g) were reduced when exposed to high concentrations of O_3. [8] found that the fungal contamination in Brazil nuts was completely stopped from the day of the application when exposure to 31 ppm O_3 for 5 hrs. [15] suggested that the fungal contamination of *A. parasiticus* in maize will be inhibition in a 63% after treatment of kernel surface with 50 ppm O_3.

For AFB_1contamination, the findings agree with other studies, forexample, [5] found that AFB_1 content was degraded in artificially contaminated wheat after exposure to 20 and 400 ppm O_3 for 5, 10, 15 and 20 min. [22] found that AFB_1 produced in artificially contaminated peanuts was reduced after exposed to 100, 200 and 400 ppm O_3. Last studies by [1] presented that the OTA content in artificially contaminated coffee stored at 0.75, 0.90 and 0.95 a_w and30^{o}C for 12 days wassignificantly reduced when exposure to 600 ppm O_3 for 1 hr. Recent studies on red pepper by [14] showed that AFB_1 contents in artificially contaminated red pepper treated with O_3 at three doses 20 mg/L, 40 mg/L and80 mg/L for 40 min were reduced depend on O_3 doses.

Conclusion

This study showed that *in situ* the populations and AFB_1production in stored whole cinnamon inoculated with *A. flavus* spores at different a_w levels were relatively affected by O_3 treatment.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

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