

Evaluation of ozone gas as an anti-aflatoxin B1 in wheat grains during storage

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Abstract

Wheat (*Triticum aestivum*) is one of the most important agricultural crops. Egypt has one of the highest wheat per capita consumption levels in the world (108 kg/person/year). Wheat must be produced free of hazardous contaminants. However, previous investigations showed that wheat could be contaminated by aflatoxins above the limits that may be critical for health. In post-harvest situations, crop spoilage, fungal growth, and mycotoxin formation result from the interaction of several factors in the storage environment.

In this study, use of ozone gas achieved as an anti-aflatoxin B1 in wheat grains during storage. Wheat samples were artificially infected with spores count of *Aspergillus flavus* ATCC 28542 strain (105, 104, 103, 102 and 101 spores/kg) and ozonation at 20 and 40 ppm ozone gas for 5, 10, 15 and 20 min at room temperature.

We not observed any amount of AFB1 in wheat samples were ozonation for 10, 15 and 20 min with spores count of *Aspergillus flavus* strain 105 to 101 (spores/kg), But AFB1 was formed in samples treated for 5 min at 20 and 40 ppm ozone gas with artificially infected spores count 105 and 104 (spores/kg) solely. While 5 min of exposure to ozone gas were sufficient inhibition production of AFB1 with the number of spores fewer than 104 spores/kg.

Keywords: Aflatoxin B1; inhibition; Ozone; *Aspergillus flavus*; storage; wheat

1. Introduction

Aflatoxins (AFs) are a group of highly carcinogenic mycotoxins produced primarily by the fungus *Aspergillus flavus*. Within the group of AFs, Aflatoxin B₁ (AFB₁) is the most toxic, and it is known for its harmful effects on humans and animals. AFs contamination is a worldwide problem, especially in warmer climates, and the toxin can enter the food chain through contaminated food and feed products. They are considered an unavoidable food and feed contaminant [7].

A variety of moulds routinely infect the world's cereal crops. Under certain field or storage conditions, some moulds can produce toxin metabolites "mycotoxins". (AFs) are a group of highly carcinogenic mycotoxins produced primarily by the fungus *Aspergillus flavus*. Within the group of AFs, Aflatoxin B₁ (AFB₁) have been reported to be carcinogenic, teratogenic and tremorgenic to a wide range of organisms, and known to cause hepatic carcinoma in humans [19].

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Toxigenic fungi infect agricultural crops both in the field and in storage. Conditions favoring the development of mycotoxins in cereals before and after harvest are important to grain exporting countries concerned with marketing high-quality products. In post-harvest situations, crop spoilage, fungal growth, and mycotoxin formation result from the interaction of several factors in the storage environment [24,9,1].

AFs production is the consequence of a combination of species, substrate and environment. The factors affecting AFs production can be divided into three categories: physical, nutritional and biological factors. Physical factors include temperature, pH, relative humidity of the atmosphere, water activity, moisture, light, aeration and level of atmospheric gases. AFs production in the substrate can happen in the field and in storage conditions between 20 and 40 °C with a 10- 20% of moisture and 70-90% of relative humidity in the air [27].

AFs are produced at the end of the exponential phase or at the beginning of the stationary phase of the mould growth [6]. The development of the fungus is favored if the grains are damaged by insects or rodents. Some spores of the substrate bud and grow as mycelia generators of AFs because, when breathing, they produce water increasing the humidity of the grains. AFs producing by *Aspergillus* its maximum production rate at the fifth day, that is, when the mould comes up to the stationary growth phase [33]. Fungal growth on stored grain causes significant reductions in both the quantity and the quality of the grain. For example, in the United States, more than 400 million tones of grain are stored every year and the annual storage losses caused by fungi and insects are estimated at more than \$500 million [15]. Most of the problems of post harvest decay are associated to fungi, mainly to *Aspergillus* and *Penicillium* species, development during storage [29].

A variety of chemical, physical, and biological treatments have been tested for their ability to reduce or eliminate the AFs in contaminated feeds and foods [14].

Ozone gas is a powerful oxidant capable of reaction with numerous chemical groups. Ozone is a powerful oxidising agent with a demonstrated ability to reduce populations of bacteria and fungi in a diversity of use situations [17]. This powerful oxidant makes it possible to destroy many micro-organisms and organic molecules. The generally

recognized as safe (GRAS) in 1997, this reagent is more and more used for food treatment as grain [12]. It decomposes to diatomic oxygen rapidly due to its short half-life, which is about 20–50 min in the atmosphere and 1–10min in water [11]

Ozone treatment of grain is generally applied in silos or vessels. Prior to ozone application, it is necessary to characterise the dynamics of ozone movement through the various grain types to optimise ozone generators for use on large commercial storage bins [31]. There are many methods for the production of ozone, such as electrical discharge in oxygen, electrolysis of water, or thermal, photochemical or radiochemical methods. For industrial use ozone is generated mainly from pure oxygen or atmospheric oxygen in a corona discharge process [20,25]. In corona discharge, air or pure oxygen is fed into a unit that converts the oxygen to ozone using high voltage. The attractive aspect of ozone is that it decomposes rapidly (half-life of 20–50 min) to molecular oxygen without leaving a residue [15]. Fungal inactivation and subsequent decontamination of toxins depends upon several factors including ozone concentration, exposure time, pH and moisture content of the grain mass [22]. Numerous studies have been focused on the use of ozone in filamentous fungi inactivation [3,32,27,10,2]. This study was carried out to investigate the effect of ozone gas on the growth of *A.flavus* and accumulation of AFB₁ during storage wheat grains.

2. Material and Methods

2.1. Chemicals and reagents. Aflatoxin B₁ from *Aspergillus flavus* was purchased from Sigma Chemical Co. (St. Luis, MO 63118, U.S.A.). The immunoaffinity column AflaTes® HPLC were obtained from VICAM (Watertown, MA, USA). Methanol, trifluoroacetic acid, and sodium chloride, were purchased from Sigma chemical Co. (St. Louis, MO, U.S.A.). All solvents were of HPLC grade. The water was double distilled with Millipore water purification system (Bedford, M A, USA). Potato dextrose agar (PDA) and Yeast extract and peptone were purchased from Sigma-Aldrich, France.

2.2. Fungal strain: Toxigenic strain of *Aspergillus flavus* (ATCC 28542) was purchased from MIRCEN, (Microbial Research Center, Faculty of Agriculture, Ain Shams University, Cairo, Egypt).

2.3. *Wheat samples*: Wheat samples were obtained from the South Cairo Mills Company, Cairo, Egypt.

2.4. *Ozone generator*: Ozone gas was produced from air using ozone generator Model OZO 6 VTTL OZO Max Ltd, Shefford, Quebec Canada (<http://www.ozomax.com>).

3. Methods:

3.1. *Preparation of inocula*: Preparation of spore suspensions: The fungal culture was grown on (PDA) slants at 28 °C for about 14 days or until good sporulation was observed. Spores were harvested by adding 10 ml of sterilized aqueous solution of Tween 80 (0.05% v/v) to cultures [28]. Spore suspensions were then centrifuged at 20,000 rcf for 5 min and the Supernatants discarded. The spore concentrations were adjusted to yield a final count of 10^5 spores/ml and the ensuing preparations were used as spore inoculum.

3.2. *Artificially infection wheat grain with different spores count*: Sterile wheat samples grains with moisture content 14 % were artificially infected with spores count of *Aspergillus flavus* ATCC 28542 (10^5 , 10^4 , 10^3 , 10^2 and 10 spores/kg) and incubated. Samples were ozonation for 5, 10, 15 and 20 min at room temperature with two different ozone concentrations (20 and 40 ppm) and as same samples with spores count were untreated by ozone. Unsterile wheat samples without artificially infected were treated with ozone as a same concentration and exposure time and other unsterile wheat samples without ozone treated and without artificially infected were used as a control samples. Samples were taken for analysis at 15, 30, 45, 60, 75 and 90 day.

3.4. *Spore count*: The spore suspension was filtered and pooled in a sterile bottle and numbers of spores were counted using a haemocytometer.

3.5. *Preparation samples treatment*: Five hundreds grams of each artificially contaminated whole wheat grain with *Aspergillus flavus* strain were transferred into a 2000ml flask and the sample flask was plugged with a silicone stopper with 2 holes in it. One hole was for the ozone line and the other was for tubing connected to the ozone destruct unit. The wheat was treated for 5, 10, 15 and 20 min at room temperature with two different ozone concentrations (20 and 40 ppm).

3.6. Extraction of AFB₁:

3.6.1. *Sample extraction*: Weigh 50g sample with 10g salt sodium chloride and place in blender jar. Add to jar 200 ml methanol: water (80:20). Cover blender jar and blend at high speed for 1 minute. Remove cover from jar and pour extract into fluted filter paper. Collect filtrate in a clean vessel.

3.6.2. *Extract dilution*: Pipet or pour 10 ml filtered extract into a clean vessel. Dilute extract with 40 mL of purified water. Mix well. Filters dilute extract through glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4 ml.

3.6.3. *Immunoaffinity chromatography*: Pass 4 ml filtered diluted extract (4 ml = 0.2g sample equivalent) completely through AflaTest ®-P affinity column at a rate of about 1-2 drops/second until air comes through column. Pass 5 ml of purified water through the column at a rate of about 2 drops/second. Elute affinity column by passing 1.0 ml HPLC grade methanol through column at a rate of 1-2 drops/second and collecting all of the sample eluate (1ml) in a glass vial. Evaporated to dryness under stream of nitrogen and was determination of HPLC.

3.6.4. Determination of AFB₁ by HPLC

Derivatization: The derivatives of samples and standard were done as follow: 100 µl of trifluoroacetic acid (TFA) was added to samples and mixed well for 30 s and the mixture stand for 15 min. 900 µl of water: acetonitrile (9:1 v/v) were added and mixed well by vortex for 30 s. and the mixture was used for HPLC analysis.

The HPLC system consisted of Waters Binary Pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi- Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C18 (250 x 4.6 mm i.d.), 5 µm from Waters corporation (USA). An isocratic system with water: methanol: acetonitrile 240:120:40. The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20 µl for both standard solutions and sample extracts.

The fluorescence detector was operated at wavelength of 360 nm for excision and 440 nm for emission.

4. Results and discussion

The results of our study show that ozone gas could inhibit production of AFB₁ in wheat grain during storage when it ozonation at 20 and 40 ppm more than 5 min ozone exposure.

We observed amount off AFB₁ in control wheat sample was without ozone treatment and without artificially infected as **Fig (1)**.The presence of large quantities of toxin after 45 days of storage at 28°C, this is probably due to the presence of some strains capable of producing toxin that are provided with appropriate conditions of temperature, moisture and time of incubation.

The lower amount of AFB₁ production could be due to higher microbial competition and could also be due to the capacity of certain competing fungi to degrade AFB₁ [21].

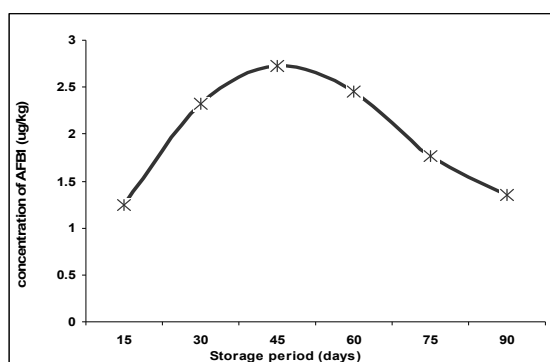


Figure 1. AFB₁ in control wheat grain sample during storage.

The same sample was ozonation at 20 and 40 ppm ozone not observed any amount of AFB₁ during storage period. This means that ozone has provided adequate protection of stored wheat grain when compared to the control. Due to ozonation is able to reduce the viable airborne moulds (*Aspergillus spp.*, *Cladosporium spp* and *penicillium spp.*) [30].

Wheat samples artificially infected with spores count of *Aspergillus flavus* ATCC 28542 strain (10^5 , 10^4 , 10^3 , 10^2 and 10^1 spores/kg) were incubated at 28°C and ozonation at 20 and 40 ppm ozone for 5, 10, 15 and 20 min at room temperature.

We not observed any amount of AFB₁ in wheat samples were ozonation for 10, 15 and 20 min with spores count of *Aspergillus flavus* strain 10^5 to 10^1 (spores/kg).

But AFB₁ was formed in samples treated for 5 min at 20 and 40 ppm ozone gas with artificially infected spores count 10^5 and 10^4 (spores/kg) solely. While 5 min of exposure to ozone gas were sufficient inhibition production of AFB₁ with the number of spores fewer than 10^4 spores/kg.

We observed the amount of AFB₁ was decreased with over a longer storage period **Fig (2)**. The change in amount of AFB₁ during storage was caused by decomposition of chemical structure of toxin, increase in free fatty acids, the lack of nutritional compounds for mould growth, the dominance of other indigenous mould strains [8].

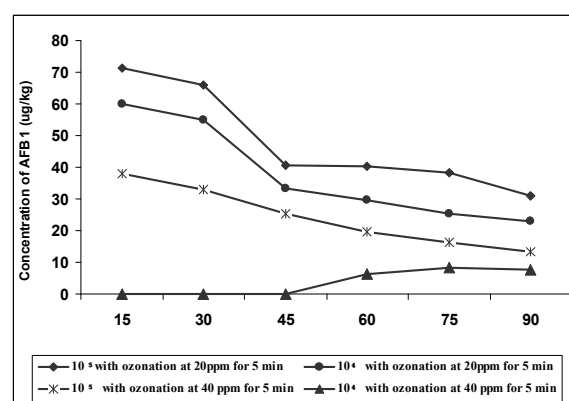


Figure 2. Change in concentration of AFB₁ during storage wheat grain after ozonation at 20 and 40 ppm for 5 min.

Ozone gas is known to possess sporicidal activity [16] and thereby at higher concentrations could be used to reduce the initial load of contaminants on produce (i.e. treatment of raw materials) and to disinfect storage and processing areas [23,18]. Furthermore, a twin approach using high and low level ozone gas could be put into practice where higher concentrations can impart surface sanitation of storage facilities and food handling equipment and sustained low levels could be used for storage and preservation.

Thus, fumigation with ozone gas can be a good method for achieving sanitation and decreasing initial microbial load in food storage facilities and aid in curbing spoilage on a long term. However, it is clear from this work that ozone gas exposure time higher than 5 min would be required to achieve complete spore kills. These results were in agreement with other studies [34,5,10,2].

Fig (3) shown concentrations of AFB₁ in wheat samples were without ozonation but artificially infected with spores count of *Aspergillus flavus* ATCC 28542 (10^5 , 10^4 , 10^3 , 10^2 and 10^1 spores/kg) incubated at 28 °C. All samples appeared the toxin with differences in amounts according to number of spores. We observed the number of spores used in artificially infected was significantly in amount of AFB₁ produced in wheat grain. This result was in agreement with Antony and Singleton, (2011) [4].

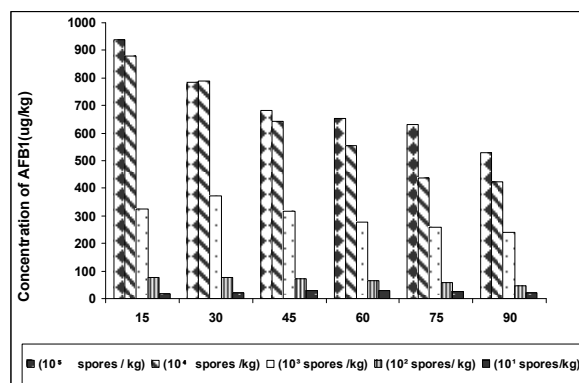


Figure 3. concentration of AFB₁ in wheat samples artificially infected with *A. flavus* and ozone untreated.

Stored wheat grain contains fungi that can come from the plant itself, i.e., fungi come from the grain during harvest and transportation, and also fungi that develop during wheat grain storage. If moisture, temperature and aeration rise, fungi can develop fast and lower the quality of stored material. Furthermore, the stored wheat grain contains a large quantity of fungi which find a suitable nutrition medium, and with other favorable conditions, some of the fungi can synthesize toxic metabolites [13].

During food storage, spores are the major source of inoculums. Hence reduction or inhibition of spore production is very advantageous in food storage facilities. Such inhibition/reduction of spore production has been previously observed in fungi cultured under ozone rich environment [5].

One part of stored wheat is manufactured either for human food (flour, grits), or for animal feed (flour, bran). If wheat is contaminated with AFs to a great extent, AFs can contaminate the above-mentioned products during the process of wheat grain grinding. If the wheat grain is greatly contaminated with AFs-producing fungi, this contamination can be transmitted to those products due to intermediate products of wheat grain being milled to flour.

If these products are stored for a longer period and if conditions are favourable for development of moulds and production of AFs, further contamination with AFs will occur. AFs can be transmitted to people and animals by food products such as bread, rolls, pasta, cakes, and fodder [13].

The use of ozone gas to control of growth of *A. flavus* and AFB₁ produced in wheat grain offers a promising tool, considering the advantages of this gas. Ozone gas has advantages over traditional fumigants: Ozone decomposes rapidly (half-life 20–50 min) to molecular oxygen; it leaves no residue; it can be generated on site and it requires no storage and subsequent disposal of chemical containers. Current use of ozone by the food industry includes the storage preservation of vegetables and fruits, surface decontamination of perishable foods, and disinfection of manufacturing equipment, water and packaging materials. It can be produced in situ and it has low cost [11]. Therefore we work out this study useful in Application of ozone gas in controlled storage wheat grain.

5. Conclusion

Ozone gas can inhibit growth of degraded AFB₁ in wheat grains, and generally there was significant variation between ozonation time on reduction of AFB₁ and all one concentration of used ozone. Consequently, the treatment with ozone at 40 ppm for 20 min could be an effective method for the degradation of AFB₁ in wheat grain.

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