CRANFIELD UNIVERSITY

YOUSEF YASSEEN ABDEL-RAHIM SULTAN

BIODIVERSITY OF MYCOTOXIGENIC *ASPERGILLUS* SPECIES IN EGYPTIAN PEANUTS AND STRATEGIES FOR MINIMIZING AFLATOXIN CONTAMINATION

CRANFIELD HEALTH

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Ph.D. Thesis
Academic Year: 2007 - 2010

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Peanuts are an important crop grown in Egypt for either local consumption or export to European markets. The present study examined the importance of mycotoxigenic *Aspergilli* in Egyptian peanuts from five different regions (Alexandria, El-Beheira, El-Daqahliya, El-Sharqiya, Asyut) in two seasons (2007, 2008). This led to consideration of different potential strategies to control aflatoxigenic *A. flavus* strains and associated aflatoxin contamination of peanuts. The most common species in peanuts were from *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati*. Both qualitative (coconut cream agar) and quantitative analyses (HPLC) were used to analyse the potential mycotoxin production by strains isolated from peanuts. Of a total of 88 *Aspergillus* section *Flavi* strains examined, 90% were aflatoxigenic. Around 95% were *A. flavus,* based on the ability to produce only aflatoxin B_1 and B_2 (AFB_1, B_2) on YES medium and this was confirmed by molecular analyses. Of 64 *Aspergillus* section *Circumdati* strains only 28% produced ochratoxin A (OTA). None of *Aspergillus* section *Nigri* strains were able to produce OTA. A range of preservatives (phenolic antioxidants BHT, BHA, PP and OG; aliphatic salts SMB, PS and CP, and the antibiotic NM) were examined to control *A. flavus* EGP-B07 isolated from Egyptian peanuts. In general, significant inhibition of growth of *A. flavus* on YES media was achieved with all preservatives studied. Where, OG was the best treatment affecting growth with the lowest LD_{50} (54-117 ppm) followed by BHA (113-154 ppm) at different a_w conditions (0.95, 0.92 and 0.89 a_w). However, there was a fluctuation in the amount of AFB_1 produced under different concentrations of preservatives at all water activity levels. Of all the treatments considered, OG was the best for effectively inhibiting AFB_1 production over the a_w range examined (ED_{50}< 80). This suggested the use of this antioxidant at higher concentrations (100, 1000 and 2000 ppm) to control *A. flavus* and AFB_1 accumulation in stored peanuts. Treatments of 1000 and 2000 ppm resulted in a decrease in *A. flavus* populations by > 4 log CFUs on peanuts at both a_w levels (0.90 and 0.93 a_w) over 14 days storage. The same doses significantly reduced AFB_1 accumulation at 0.90 a_w and for one week at 0.93 a_w. The second control strategy examined was the use of gaseous O_3 for 30 min *in vitro* against two *A. flavus* strains

and *in situ* against one strain at different concentrations. Generally, >100 ppm O_3 significantly stopped the spore germination of *A. flavus* EGP-B07 and *A. flavus* SRRC-G 1907 on YES media within 48 hrs. Also, concentrations of 75- 300 ppm significantly decreased the sporulation of both strains with no effect on mycelial growth for 3 days. The same concentration significantly affected AFB_1 production, but only at 0.89 a_w. Spore viability of *A. flavus* EGP-B07 on inoculated peanuts (10^3 and 10^5 spores g^{-1}) at 0.93 a_w was significantly decreased at 100, 200 and 400 ppm O_3. About 5-8 log CFUs reduction was accomplished on treated samples at both inoculation levels over 4 days storage when compared with control (using air). However, a significant reduction of AFB_1 production was only observed at 400 ppm at both levels. The third strategy was the use of biological control or natural metabolites. The actinomycete AS1 was found to have the best antifungal activity against *A. flavus.* This was found to be a *Streptomyces* spp. AS1 completely inhibited growth of *A. flavus* on half-strength nutrient agar (½ NA) using the agar streak method. However, inoculation of the actinomycete onto stored peanuts showed that there was no antagonistic effect on *A. flavus* populations at 0.93 water activity (a_w) over a 14 day storage period. Ethyl acetate was the best solvent for extraction of metabolites from the AS1 isolate. Crude extracts of AS1 at 50 and 100 ppm completely inhibited spore germination of conidia of *A. flavus* on YES media over 48 hrs. Furthermore, >85 % inhibition of mycelial growth was observed using these concentration at 0.95, 0.92 and 0.89 a_w conditions at 25°C. The results also showed that doses 50, 200 and 500 ppm of AS1 metabolite significantly inhibited populations of *A. flavus* on stored peanuts at 0.90 and 0.93 at 25°C over 14 days storage. The amounts of AFB_1 produced by *A. flavus* on peanut samples at 0.90 a_w were significantly decreased by AS1 concentrations (50-500 ppm) after 7 days storage, with no effect after 14 days. However, at 0.93 a_w the effect on AFB_1 contamination of peanuts was different. Doses of 200 and 500 ppm of AS1 metabolite significantly controlled AFB_1 accumulation during 14 days storage of peanuts.

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ABBREVIATIONS

½ NA	Half-strength Nutrient Agar
½ NB	Half-strength Nutrient Broth
AFB_1	Aflatoxin B_1
AFL	Aflatoxicol
AFR_o	Aflatoxin R_o
AFs	Aflatoxins
a_w	Water activity
BC	Before Christ
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CAST	Council for Agricultural Science and Technology
CD	Corona discharge
CFU	Colony Forming Unit
CP	Calcium propionate
DBHQ	Di-butylated hydroxyquinone
DG	Dodecyl gallate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
ED_{50}	Effective dose, 50%
EPRI	Electric Power Research Institute
FAO	Food and Agriculture Organization
FDA	US Food and Drug Administration
GRAS	Generally recognized as safe
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
ICMSF	International Commission on Microbiological Specifications for Foods
ICRISAT	The International Crops Research Institute for the Semi-Arid Tropics
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal dose, 50%
MC	Moisture content
MEA	Malt Extract Agar

MIC	Minimum inhibitory concentration
MS	Malt Salt Agar (10% NaCl)
NA	Nutrient Agar
NB	Nutrient Broth
NM	Natamycin
OG	Octyl gallate
OSHA	Occupational Safety and Health Administration
OTA	Ochratoxin A
OTB	Ochratoxin B
OTC	Ochratoxin C
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PG	Propyl gallate
PHC	Primary hepstocellular carcinoma
PMEA	Peanut Meal Extract Agar
PP	Propyl Paraben
PS	Potasium sorbate
R_f	Retention factor
rpm	Revolutions per minute
SCF	EC's Scientific Committee for Food
SMB	Sodium Metabisulphite
SMKY	Sucrose, Magnesium sulphate, Potassium (K) nitrate, Yeast extract
TDI	Tolerable Daily Intake
TLC	Thin Layer Chromatography
USDA-FAS	United States Department of Agriculture-Foreign Agricultural Service
UV	Ultraviolet
WHO	World Health Organization
YES	Yeast Extract Sucrose Agar

Chapter 1

LITERATURE REVIEW

1 LITERATURE REVIEW

1.1 INTRODUCTION

Peanut (*Arachis hypogaea* L.) is an annual crop grown predominantly in the Mediterranean region (Aydin, 2007). Egypt is considered to be one of the most important countries exporting peanuts into the European market. The annual production of peanuts in Egypt is around 190,000 tonnes and its yield is 3170 kg ha^{-1} (USDA-FAS, 2009).

A variety of oil-rich seeds, particularly peanuts are important substrates for the growth and subsequent aflatoxin production by different members of *Aspergillus* section *Flavi* group: *A. flavus* Link, *A. parasiticus* Speare, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Aflatoxins are potent hepatotoxic carcinogenic metabolites (IARC, 1993). These species can infect cereals before and after harvest (Cotty, 1997; Barros *et al.,* 2003). The aflatoxin-producing fungi in pre-harvest peanuts occur under conditions of heat and drought stress during the latter stages of the growing season (Cole *et al.,* 1982; Hill and Lacey, 1983; Dorner and Cole, 2002).

Pre-harvest peanut seeds can contain mycelia and spores of aflatoxigenic fungi, which can result in a significant decrease in grain quality when they are stored. If the storage conditions are not good, serious damage and aflatoxin accumulation at higher than the internationally accepted levels can occur. Because peanuts are primarily used for food, strict regulatory limits for the amount of aflatoxin in peanut products have been established. Among all classes of aflatoxin, aflatoxin B_1 (AFB_1) is known to be the most significant in terms of animal and human health risk. The International Agency for Research on Cancer of WHO included AFB_1 as a primary group of carcinogenic compounds (IARC, 1993). Therefore, raw peanuts now entering the European Economic Community's border must have < 4 µg kg^{-1} total aflatoxins and not more than 2 µg kg^{-1} AFB_1 (European Commission, 2006).

In addition to aflatoxins, ochratoxin A (OTA) is also an important mycotoxin which can be occasionally produced on peanuts when infected by ochratoxigenic fungal species e.g *A. carbonarius* (Magnoli *et al.,* 2007). Although there are no established

permissible limits of OTA on peanuts, it may be important in certain regions to determine OTA simultaneously with aflatoxins.

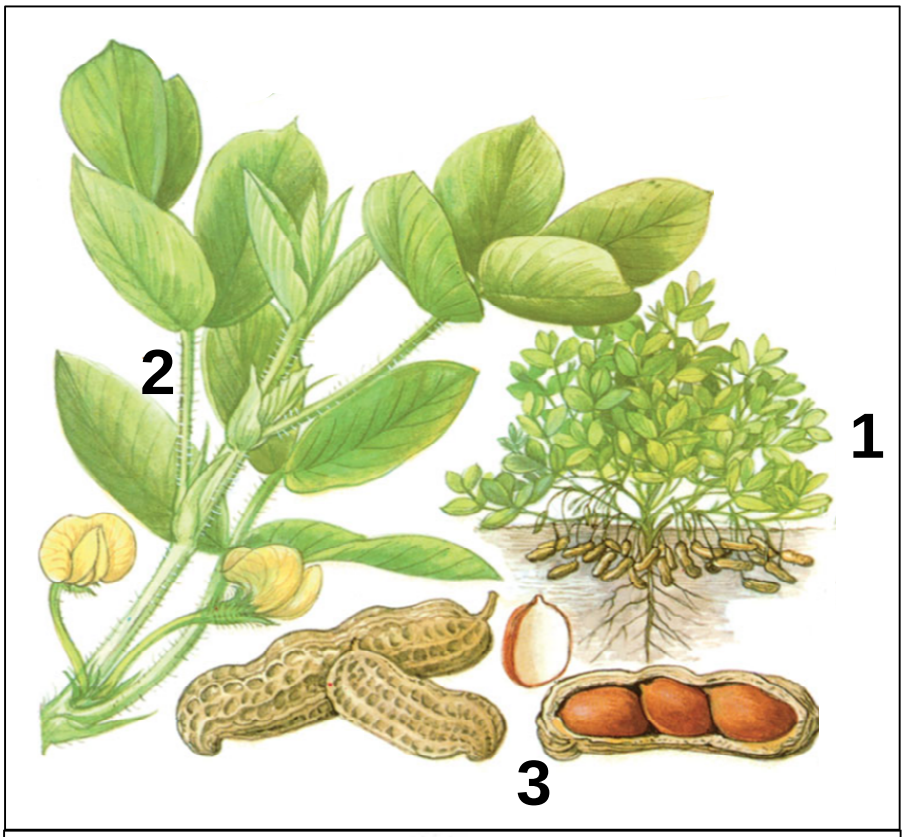
Risks associated with aflatoxin-contaminated foods can be reduced through the use of specific processing and decontamination procedures. Practical decontamination must: (1) inactivate, destroy, or remove the toxin, (2) not produce or leave toxic residues in the food/feed, (3) retain the nutritive value of the food/feed, (4) not change the acceptability or the technological properties of the product, and, if possible, (5) destroy fungal spores (Park, 2002).

Reduction in aflatoxin exposure can be achieved through prevention measures such as improved farming and proper storage practices and/or enforcing standards for food and feed within countries and across borders (CAST, 2003). Because it is impossible to completely avoid some amount of aflatoxin contamination, a variety of strategies for detoxification in foodstuffs have been proposed. These strategies have included physical methods of separation, thermal inactivation, irradiation, solvent extraction, ozonation, adsorption from solution, microbial inactivation and chemical methods of inactivation (Samarajeewa *et al.,* 1990; CAST, 2003).

1.2 ANATOMY OF PEANUT PLANT AND COMPOSITION OF SEEDS

Peanuts or groundnuts (*Arachis hypogaea* L.) belong to the leguminosae or Fabaceae family and supply both oil and nuts. It is of South American origin, being grown there since at least 3000-2000 BC, but it is now cultivated in many tropical and subtropical countries. With an annual global production of about 30 million metric tonnes, groundnuts rank in the top 25 of the world's food crops. Plants of the cultivated peanut species are annuals; either erect (up to 60 cm tall) or prostrate (usually under 30 cm tall). The flowers are self-pollinated and, after pollination, the flower stalk (peg) elongates, forcing the young pod (fruit) into the ground where it matures (Figure 1.1a, b). Harvesting is by hand, particularly of the prostrate forms; erect forms can be harvested by mechanical means. The groundnut pod, with its wrinkled surface network, usually contains two nuts (seeds) in the Spanish or Virginia types, three to four (possibly up to six) in the Valencia types. Nuts are extracted from the pod either by hand or by mechanical

(a)



(b)

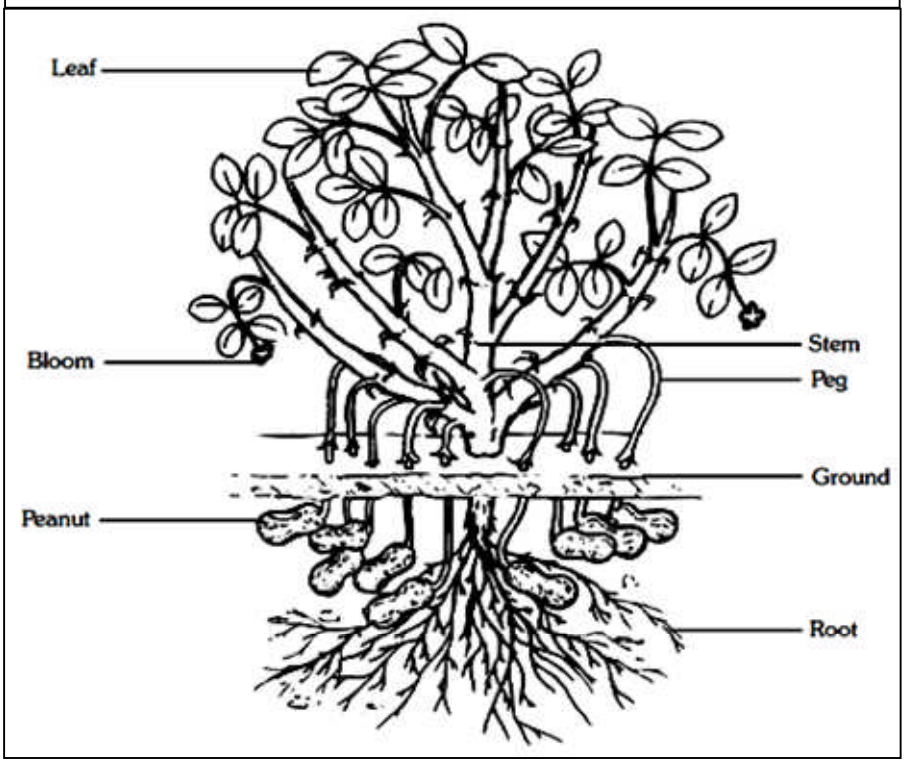


Figure 1.1. Components of peanut plant: (a) 1. whole plant, 2. peanut flowering shoot, 3. pods and nuts and (b) peanut plant in detailed (Vaughan and Geissler, 2009)

means (Burow *et al.,* 2008; Vaughan and Geissler, 2009). These nuts are rich in nutrients. They contain 35-55% oil, with a high proportion of the unsaturated oleic and linoleic acids. The seeds also contain 16-36% protein and are good sources of the essential minerals. The nuts contain vitamins E and the B complex. Oil extracted from the seed is used for cooking, as a salad oil, in margarine, in India as vegetable ghee, and for fish preservation. The seed residue, with 50–55 per cent protein, can, under certain circumstances, be a useful animal feed (Knauft and Ozias-Akins, 1995; Winch, 2006; Vaughan and Geissler, 2009).

1.3 THE AFLATOXIN PROBLEM IN PEANUTS

Aflatoxigenic fungi penetrate the pods during their growth period whilst still in the soil. This takes place in two ways: firstly via Infection through invisible damage to the pods or seeds or invisible infections of the pods. Secondly, the infection occurs after the pegs have been pushed down into the soil (Keenan and Savage, 1994; Anderson *et al.,* 1995; Gianessi, 1997). Yet when the plant enjoys good growing conditions, the fungi may remain inactive and no significant amounts of aflatoxin are produced. This is because peanut plants have a natural protection mechanism. The growing plant produces immune substances (phytoalexins), which have an anti-fungal suppressing effect (arachidin). All cultivation measures that encourage healthy, natural growth in effect support this protection mechanism. The production of phytoalexins decreases towards maturity, as well as due to water deficiency, and ceases altogether if a drought continues. In contrast, the *A. flavus* is still able to produce and create aflatoxins under much drier conditions (Keenan and Savage, 1994).

1.4 MYCOTOXINS

The term “mycotoxin” is derived from the Greek word «mycos» meaning mushroom and the Latin word «toxicum», which means poison. It designates the toxic chemical substances produced by certain forms of mould, which develop on some food products (Goldblatt, 1972; Quillien, 2002). Common moulds produce many different types of mycotoxins, depending on the growing conditions. *Aspergillus flavus,* for example, can produce several different forms of aflatoxin. In general, there are more

than 300 known mycotoxins produced by other moulds and experts estimate that there are many more mycotoxins yet to be discovered (CAST, 2003).

1.4.1 Aflatoxins

Aflatoxins are produced in nature by *Aspergillus flavus*, *Aspergillus parasiticus* and later *A. nomius* described as an aflatoxigenic species. *A. flavus* is ubiquitous (Kurtzman *et al.*, 1987; Payne, 1998; Ito *et al.,* 2001; Moss, 2002). Since the discovery of aflatoxins, it has become the most widely reported food-borne fungus, reflecting its economic and medical importance and ease of recognition, as well as its universal occurrence (Pitt, 2000). The aflatoxins were categorised as a biological hazard, since they are produced as secondary metabolites of certain fungi (Mortimore and Wallace, 1994; Snyder, 1995). On the other hand, Corlett (1998) and Sperber (2001) reported that aflatoxins should be considered as naturally occurring chemical hazards.

Historical background

Historically, in 1960 more than 100,000 young turkeys on poultry farms in England died after a few months from an evidently new disease that was called "Turkey X disease". It was shortly found that the difficulty was not limited to turkeys but it also included ducklings and young pheasants (Blount, 1960; Rodricks *et al.,* 1977). A survey of the early outbreaks showed that they were all associated with feeds, namely Brazilian peanut meal (*Sargeant et al.*, 1961).

An intensive investigation of the suspect peanut meal was undertaken and it was quickly found that this peanut meal was highly toxic to poultry and ducklings with symptoms typical of Turkey X disease. Speculations made during 1960 regarding the nature of the toxin suggested that it might be of fungal origin. In fact, the toxin-producing fungus was identified as *Aspergillus flavus* (Goldblatt, 1969; Goldblatt and Stoloff, 1983; Payne, 1998). In the word aflatoxin, the first syllable “a” was derived from the genes *Aspergillus*, the second one, “fla”, from the species *flavus* and the term, “toxin”, came from the adjective “toxic” (Micco *et al.,* 1987).

Chemical structure

Chemically, aflatoxins are dihydrofuran or tetrahydrofuran moieties fused to coumarin (Hussein and Brasel, 2001, Figure 1.2). There are four main aflatoxins, B_1, B_2, G_1 and G_2. Of these, B_1 and G_1 are produced most frequently by both *A. flavus* and *A. parasiticus* in the largest amounts whereas *A. parasiticus* produces B_1, B_2, G_1 and G_2. Under long wave ultraviolet light aflatoxins B_1 and B_2 fluoresce blue and aflatoxins G_1 and G_2 fluoresce green. The band G designations of the toxins refer to the colour of fluorescence. The subscripts 1 and 2 refer to the separation pattern of these compounds on thin layer chromatography (TLC) plates, with B_1 having the highest R_f value followed by B_2 then G_1 and G_2 in most solvent systems (Bullerman, 1979; Micco *et al.,* 1987; Goto *et al.,* 1996; Hussein and Brasel, 2001).

In addition to these four aflatoxins, two additional toxins are of significance: these are aflatoxins M_1 and M_2. The M toxins were first isolated from the milk of lactating animals fed aflatoxin preparation; hence, the M designation. Aflatoxin M_1 and M_2 are hydroxylated metabolites of aflatoxin B_1 and B_2. The subscripts again refer to separation patterns on TLC plates. The M toxins also fluoresce blue when exposed to long-wave UV light, but separate at a lower R_f value on TLC plates than the B and G toxins (Bullerman, 1979; Frobish *et al.,* 1986).

Regulatory limit for aflatoxins

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) summarized the maximum levels of aflatoxins in imported peanuts for human consumption and livestock feeds (Table 1.1). The aflatoxin B_1 level for foodstuffs varied from 0 ppb (Netherlands) up to 20 ppb in USA. The Egyptian Standard (1990) and Food and Agriculture Organization, FAO (1997) established permissible limits of aflatoxins in food and dairy products and feeds (Table 1.2).

Optimal condition for aflatoxins formation

Undoubtedly, the most important species of toxigenic *Aspergilli* are those producing aflatoxins, namely *A. flavus* and *A. parasiticus*. Both species grow at temperatures ranging from 10 to 43 °C, with an optimum near 32-33 °C, with aflatoxins being

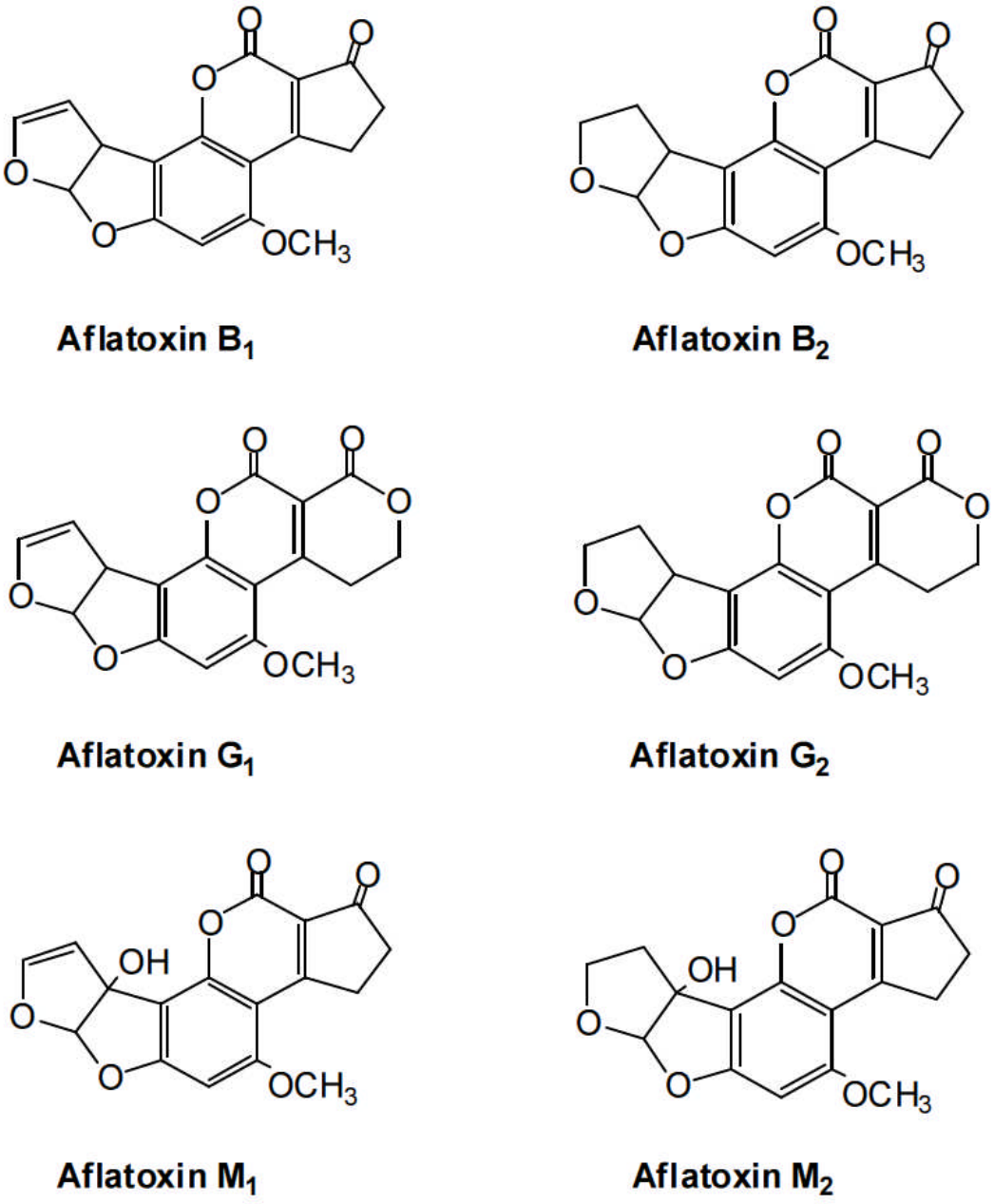


Figure 1.2. Chemical structures of aflatoxins

Table 1.1. Maximum possible level of aflatoxin in imported peanuts (Freeman *et al.,* 1999).

Country	Aflatoxin type	Maximum permissible level (ppb)	
		Foodstuffs	Livestock feed
Belgium	B_1	5	20
France	B_1	1	20
Germany	B_1	2	20
Ireland	B_1	5	20
Italy	B_1	5	20
The Netherlands	B_1	0	20
Sweden	B_1,B_2, G_1, G_2	5	10
UK	B_1,B_2, G_1, G_2	4	20
USA	B_1,B_2, G_1, G_2	20	20

Table 1.2. Maximum tolerated levels of Aflatoxins in foodstuffs, dairy products, and animal feedstuffs in Egypt (C.F. Egyptian standard, 1990 and FAO guidelines, 1997).

Country	Commodity	Aflatoxin(s)	Level (ppb)
Food	Peanut (product)s, oil seed (products)s, cereal (product)s	$B_1B_2G_1G_2$	10
		B_1	5
	Maize	$B_1B_2G_1G_2$	20
		B_1	10
	Starch and its derivatives	$B_1B_2G_1G_2$	0
		B_1	0
Dairy	Milk, dairy products	$G_1G_2M_1M_2$	0
		M_1	0
Feed	Animal and poultry feeders	$B_1B_2G_1G_2$	20
		B_1	10

produced between 12 and 40 °C (ICMSF, 1996). The optimum water activity (a_w) for growth is near 0.99 a_w, with minima reported as 0.80-0.83 a_w. Aflatoxins are produced in greater quantities at higher levels (0.98-0.99 a_w), with toxin production ceasing at or near 0.85 a_w (Magan and Lacey, 1984; Pitt and Miscamble, 1995).

Although both species grow over the pH range of 2-10, aflatoxin production by *A. parasiticus* has been reported at occur between pH 3.0 and 8.0, with an optimum near pH 6 (ICMSF, 1996). Reduction of available oxygen by modified atmosphere packaging of foods in barrier film or with oxygen scavengers can inhibit aflatoxin formation by *A. flavus* and *A. parasiticus*. A number of nutritional factors influence aflatoxin biosynthesis, including the presence of various carbohydrate and nitrogen sources, phosphates, lipoperoxides, and trace metals in the growth medium (Luchese and Harrigton, 1993). However, the role of such factors in aflatoxin biosynthesis is unclear. Many of the effects may in fact be exerted indirectly through interference with primary metabolism (Ominski *et al.,* 1994; Marín *et al.,* 1998; Pitt *et al.,* 2000).

Aflatoxicosis

Aflatoxicosis refers to poisoning from the ingestion of aflatoxins in contaminated food or feed. It can occur from acute exposure of very high doses of contaminated grain over a short period of time, or from the chronic ingestion of low levels of aflatoxin over longer periods of time (Eaton and Groopman, 1994). Aflatoxins cause acute liver damage, liver cirrhosis, induced tumours and teratogenic effects in both animals and humans (Stoloff, 1977; Pitt, 2000). A wide variation in LD_{50} values has been obtained in animal species tested with single doses of aflatoxins. For most species, the LD_{50} values ranges from 0.5 to 10 mg kg^{-1} body weight. Animal species respond differently in their susceptibility to the chronic and acute toxicity of aflatoxins. The toxicity can be influenced by environmental factors such as exposure level, duration of exposure, age, health, and nutritional status of diet (Bullerman, 1979; FDA, 1992).

Aflatoxin B_1 is documented as a hepatic carcinogen in many animal species and is listed as a Group I carcinogen by the International Agency for Research on Cancer, especially implicated in the cause of human primary hepatocellular cacinoma (PHC). Metabolism plays a major role in determination of the toxicity of aflatoxin B_1. Studies

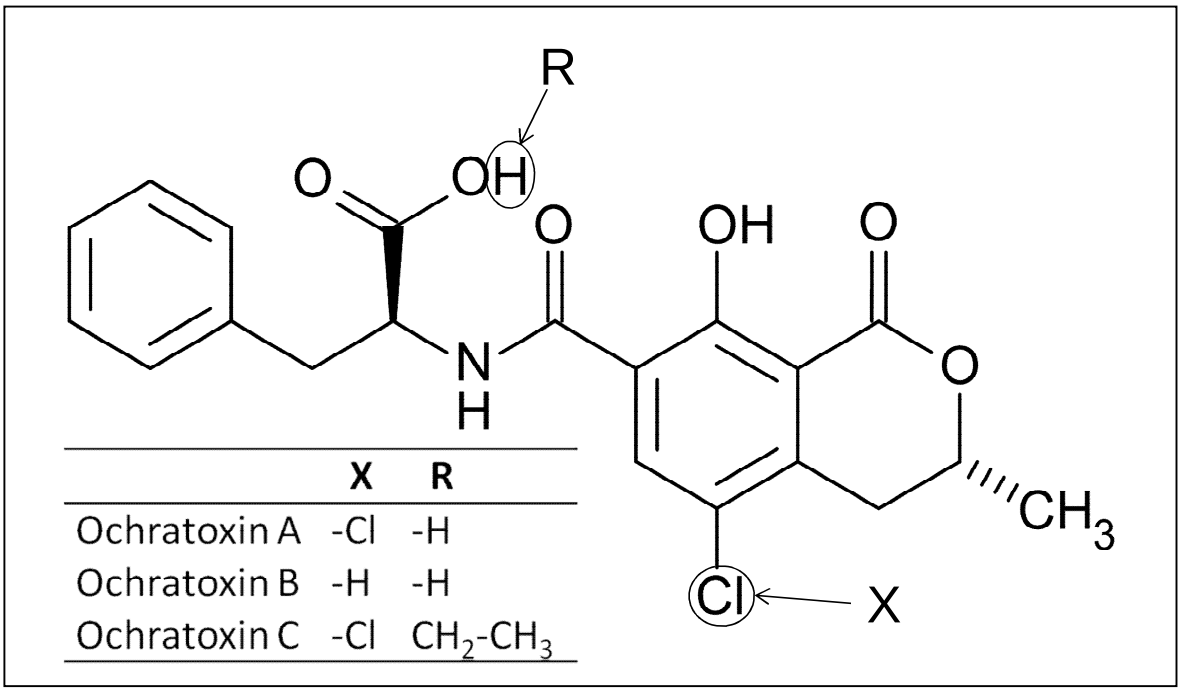
show that this aflatoxin requires metabolic activation to exert its carcinogenic effect. (Pitt *et al.* 2000; Creppy, 2002; Henry *et al.,* 2002; Sun and Chen, 2003).

1.4.2 Ochratoxins

Ochratoxin A, B, and C (OTA, OTB, OTC) are group of secondary metabolites produced by fungi of two genera: *Penicillium* and *Aspergillus* (Figure 1.3). These moulds are able to grow at different conditions and on different commodities. Contamination of food crops can thus occur worldwide (Aish *et al.*, 2004). OTB and OTC are much less toxic than OTA. Consequently, OTA has received the most attention (Zollner and Mayer-Helm, 2006). OTA is a fluorescent compound produced primarily by *Aspergillus ochraceus, Aspergillus* section *Nigri* species and *Penicillium verrucosum* (CAST, 2003).

Kidney is the main organ affecting by expousure to OTA especially at high concentrations, liver can be affected as well (Richard, 2007). OTA is also found to be carcinogenic in experimental animals and suspected as a causitive agent in some human diseases. The Balkan Endemic Nephropathy, associated with upper urinary track urothelial cancer, is one of these diseases which can be caused by ochratoxin (Pfohl-Leszkowicz *et al.*, 2002; Pfohl-Leszkowicz and Manderville, 2007). OTA can weaken the immune system in animals through depression of antibody responses, reduce size of immune organs, changes in immune cell number and function, and malformation of cytokine production. In addition, it can cause immunotoxicity probably caused by cell death following apoptosis and necrosis, in combination with slow replacement of affected immune cells (Al-Anati and Petzinger, 2006).

OTA has been documented as a global contaminant of a diverse range of commodities such as cereals and cereal products, beer, wine, cocoa, coffee, dried fruits, grape juices, and spices in varying amounts but at relatively low levels (Sforza *et al.,* 2006; Zollner and Mayer-Helm, 2006; Richard, 2007). The International Agency for Research on Cancer in 1993 classified OTA as possible carcinogenic in human. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed OTA in 1995 and again in 2001 when they retained the provisional weekly intake of 100 ng kg^{-1} bodyweight (JECFA, 2001). OTA was considered by the EC's Scientific Committee for Food (SCF) in 1998 which concluded that it would be better



	X	R
Ochratoxin A	-Cl	-H
Ochratoxin B	-H	-H
Ochratoxin C	-Cl	CH_2-CH_3

Figure 1.3. Chemical structure of ochtatoxin A (OTA) and its derivatives

to reduce exposure of OTA as much as possible and proposed a tolerable daily intake (TDI) of less than 5 ng kg^{-1} bodyweight (SCF, 1998). In European legislation (EC) No 472/2002 dated 12 March 2002; maximum levels of OTA in some foods were established. These are summarised in Table 1.3.

1.5 MYCOBIOTA AND MYCOTOXINS OF PEANUTS IN EGYPT

Many studies have been done on the mycobiota and mycotoxin content of Egyptian peanuts. For example, Moubasher *et al.* (1979) isolated 20 genera and 66 species, in addition to 2 strains of *Aspergillus nidulans* and one of *Humicola grisea* from 80 peanut seeds(uncovered or covered within shell at the time of sampling) samples and 40 samples of shells collected from different places in Egypt (Cairo, Giza, Bani-Suef, El-Minya, Assiut, Sohag, Qena and Aswan). At 28°C, *A. flavus* was represented in 80%, 60% and 80% of the samples constituting 16.1%, 8.4% and 27.2% of the total count of fungi of the shells, covered and uncovered seeds respectively. In further study, Moubasher *et al.* (1980) studied the behaviour of mesophilic and thermophilic fungi under different levels of moisture content of peanut seeds (8.5%, 13.5%, 17.5% and 21%), period of storage (1,2 and 4 or 6 months) and temperature of storage (5°,15°, 28° and 45°C). They measured the total count of seed-borne fungi under such conditions and found that *A. flavus* population on seeds were highest in 13.5% mc stored at 15°C for one month.

Peanut samples (*Arachis hypogaea L.*), collected from Upper Egypt, were stored at 28°C for 240 days at different levels of relative humidity (40-100%). *Aspergillus* was the main component of fungal flora, arising in stored seeds. *A. fumigatus* was the dominant fungal species in total (40-80%), but at 100% RH was surpassed by *A. flavus. A.terreus* and *A.ochraceus* were also good colonizers of peanut seeds (El-Hissy *et al.,* 1981).

El-Maghraby and El-Maraghy (1987) examined the mycoflora and mycotoxins in 40 peanut samples collected from 40 places in Egypt (from Alexandria in the north to Asswan in the south). They found that the most frequent genus was *Aspergillus*. Forty seven percent of the samples proved to be toxic to brine shrimp larva. Thin layer chromatographic analysis revealed that 11 samples of peanuts were contaminated by aflatoxins.

Table 1.3. European legislation for maximum levels of ochratoxin A (µg kg^{-1}) allowed in various food (European legislation No 472, 2002).

Product	**Maximum levels (µg kg^{-1} or ppb)**
Raw cereal grains	5
All products derived from cereal intended for direct human consumption	3
Dried vine fruit (currants, raisins and sultanas	10

Subsequently, El-Magraby and El-Maraghy (1988) examined the fungal density in these samples. They found 43 species, belonging to 16 genera. They noticed that the most dominant genera were *Aspergillus*, *Penicillium* and *Fusarium* and from the previous genus, *A.fumigatus*, *A. flavus*, *A.niger*, *P. chrysogenum* and *F.oxysporum* were the most frequent species. Forty-nine isolates belonging to 12 species were examined for production of mycotoxins, after growth in liquid medium containing two carbon sources (sucrose or cellulose). Thin layer chromatographic analysis revealed that the quality and quantity of mycotoxins was higher on sucrose than cellulose. Mycotoxins identified were aflatoxins B_1, B_2, G_1 and G_2, citrinin, Fumagillin, diacetoxyscirpenol T-2 toxin, Satratoxin H and zearalenone.

1.6 CONTROL STRATEGIES OF AFLATOXINS

1.6.1 Physical control

For aflatoxins, multiple processing and/or decontamination schemes have been successful in reducing aflatoxin concentrations to acceptable levels. Physical cleaning and separation procedures, where the mould-damaged kernel/seed/nuts are removed from the intact commodity, can result in 40-80% reduction in aflatoxin levels. Processes such as dry and wet milling result in the distribution of aflatoxin residues into less utilized fractions of the commodity (Park, 2002).

Aflatoxin levels in peanuts represent a great deal of public concern, therefore, the minimization of their content is an important target in the peanut industry. Peanut roasting and colour sorting has been shown to decrease aflatoxin levels by 92.5%. It was noted that peanut by-products contained relatively high levels of aflatoxins, whereas the shell, the seed coat and the rejected peanut seeds contained 177.0, 99.6 and 226.8 µg Kg^{-1}, respectively (Ayesh, 2001).

Electronic colour sorting after removing the seed coat is believed to be more efficient for removing damaged seeds than colour sorting seeds prior to blanching because of the increased contrast between the damaged and white seed background (Whitaker, 1997). Peanut processing procedures such as steep tank coat splitting, sorting, and frying can reduce aflatoxins to non-detectable levels in finished products (Anon, 1993).

Solid aflatoxin B_1 is stable to dry heat up to its melting point of 260°C. The thermal decomposition temperature is 269°C with as high as 300°C in certain foods. The presence of moisture in foods may enhance degradation while binding or association of aflatoxins with proteins may protect the toxin (Fischbach and Campbell, 1965; Ciegler and Vesonder, 1983).

Normal food processing and preparation conditions appear to cause, on average, 60% degradation under laboratory conditions. With edible oils (peanut oil, olive oil, and coconut oil) 200°C is required for degradation. Heat treatment such as steam flaking (steam at 25-75 psi for 1-5 min), explosion cooking (dry steam at 33-43psi for 20-25 s), dry heat roasting (heating up to 128-149°C), micronizing (infrared heat at 149°C for 20-50s) and popping (370-427°C for 15-20s) are already applicable to food processing. Microwave treatment at high energy levels shows great potential for aflatoxin degradation (Samarajeewa *et al.,* 1990)

1.6.2 Chemical control

Chemical degradation of aflatoxins currently seems to be more practical. Among many chemicals screened for their ability to detoxify pure aflatoxin B_1, chlorinating agents such as sodium hypochlorite, chlorine dioxide, gaseous chlorine, oxidising agents such as hydrogen peroxide, ozone and sodium bisulphite; and hydrolytic agents such as acids and alkalis appear to be effective (Samarajeewa *et al.,* 1990; Mukendi *et al.,* 1991).

To try and control *A. flavus* and aflatoxin production a range of preservatives have been used. Most of the effective and widely used preservatives are acids. For example, the weak lipophilic organic acid such as sorbate, benzoate, and propionate, or the inorganic ones such as sulfite or nitrite, all of which are most effective at pH values < 5.5. Indeed, with the possible exception of the parabens, for some application, there is still no wide spectrum anti-microbial preservatives that are highly active at pH values close to neutral (Russell and Gould, 2003).

(a) Preservatives

The term "chemical preservative" as defined by the Food and Drug Administration (FDA) in United States means any chemical that, when added to food tends to

prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties (FDA, 2002). A broader definition is used by the commission of the European communities in Directive 95/2/EC on food additive other than colours and sweeteners: “Preservatives are substances which prolong the shelf-life of foodstuff by protecting them against deterioration caused by micro-organisms”. The mechanism of action of preservatives against microorganisms is usually based on either (1) destruction of the cell wall or membrane, (2) inhibition of various enzymes in the microbial cell or (3) destruction of the genetic structure of the protoplasm (Nielsen and De Boer, 2000).

Phenolic antioxidants

Many phenolic compounds are active as antioxidants. Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) and gallates were introduced in the 1940s (Miková, 2001). Their chemical structures are shown in Figure 1.4. Most of the approved antioxidants are phenolic derivatives, usually substituted by more than one hydroxyl or methoxy group. Gallates are esters of gallic acid, which is a natural compound, but propyl, octyl, and dodecyl esters are not found in nature. Synthetic phenolic antioxidants are always substituted by alkyls to improve their solubility in fats and oils, and reduce their toxicity. Mixtures of phenolic antioxidants often show synergistic activities, e.g., BHT and BHA (Omura, 1995). In addition to their antioxidant activity, most phenolic substances possess antimicrobial activity in food (Raccach, 1984). Antimicrobial action of antioxidant including fungi is suggested to be mainly caused by weakening or destroying the permeability barrier of the cytoplasmic membrane (Thompson, 1996).

Many studies of the fungicidal and fungistatic activity of different antioxidants like BHA and BHT have been focused on controlling growth and aflatoxin production by *A. flavus* and *A. parasiticus* (Fung *et al.,* 1977; Beuchat and Jones, 1978; Lin and Fung, 1983). The ester of p-hydroxybenzoic acid (propyl paraben-PP-) has been reported to have a strong fungicidal activity (Chipley and Uraih, 1980).

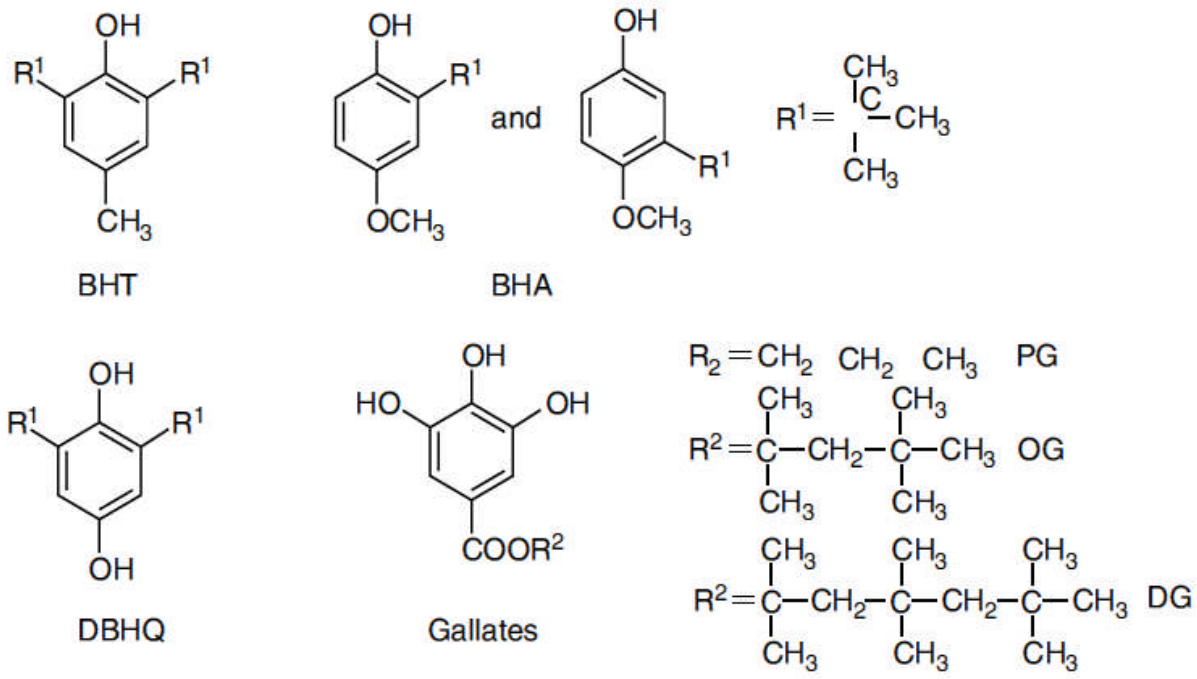


Figure 1.4 Chemical structures of the most important synthetic antioxidants. BHT, di-tert-butylhydroxytoluene; BHA, tert-butylhydroxyanisole; DBHQ, di-tert-butylhydroquinone; PG, propyl gallate; OG, octyl gallate; DG, tert-dodecyl gallate (Pokorny, 2007).

Nesci *et al.* (2003) determined the effect of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trihydroxybutyrophenone (THB) and propyl paraben (PP) (at concentrations of 1, 10 and 20 mmol) on growth and aflatoxin B_1 production by *Aspergillus* section *Flavi* on a maize extract medium under different water availability conditions. They found that the antioxidants most efficient in controlling *A. flavus* germ tube elongation rates were PP, BHT and BHA. All strains were much more sensitive to all antioxidants tested on the growth rate at 0.937 a_w. The antioxidants PP and BHA completely inhibited AFB_1 production by all strains when added at 1 mmol L^{-1}. Decreased AFB_1 levels in comparison with the control were observed with BHT at 1, 10 and 20 mmol at 0.982 a_w.

Likewise, Passone *et al.* (2005) observed an increase in the lag time and a reduction in the growth rate of 100% of the *Aspergillus section Flavi* strains. This was due to the action of BHT at doses of 10 and 20 mmol L^{-1} at 0.982, 0.971 and 0.955 a_w. However, many of these treatments stimulated AFB_1 accumulation in most of the fungi tested. They found that the more effective antioxidants were PP and BHA, which increased the lag phase, reduced the growth rate and AFB_1 production in all of the strains at the four a_w levels assayed (0.982, 0.971, 0.955 and 0.937 a_w). At concentrations of 10 and 20 mmol L^{-1}, these antioxidants totally inhibited fungal development.

More recent *in situ* studies on peanuts by Passone *et al.* (2008) compared the efficacy between analytical and industrial grades of antioxidant (BHA, PP, BHT) at 10 and 20 mmol g^{-1} concentrations on *Aspergillus* section *Flavi* populations, other natural competing mycoflora and AFB_1 accumulation at different a_W levels over 35 days of stored peanuts. Assays were carried out on natural peanuts conditioned at different a_w levels (0.982, 0.955, 0.937). They demonstrated that some combination of treatments of BHA-PP, BHA–PP–BHT mixtures totally inhibited the growth of *Aspergillus* section *Flavi* and peanut mycoflora at all conditions tested. In the same way, these mixtures completely inhibited aflatoxin accumulation. The study showed that both antioxidant grades were effective fungal inhibitors of *Aspergillus* section *Flavi* populations in peanuts over a wide range of a_w levels for 35 days.

Natamycin and potassium sorbate

Natamycin is active against most fungi at low concentrations. The minimum inhibitory concentration (MIC) for natamycin against all food-borne fungi is less than 20 ppm, while the solubility of natamycin in aqueous food systems is around 40 ppm. It has been demonstrated in practice that under acceptable hygiene conditions, this concentration of dissolved natamycin is sufficient to prevent fungal growth (Stark, 2003).

Mahjoub and Bullerman (1986) used *Aspergillus flavus* NRRL 6555 in whole olives and olive paste samples containing variable amounts of either natamycin or potassium sorbate and incubated at 15°, 25°, and 35°C for 7, 14 and 21 days for whole olives and at 15° and 25°C for 8 and 16 days for olive pastes. The initiation time of growth was parallel to the concentrations of either preservative applied. However, at 15°C, natamycin at 160 and 320 µg g^{-1} completely inhibited the growth on whole olives for 21 days and olive paste for 7 and 15 days, respectively. All levels of potassium sorbate inhibited fungal growth at 15°C, but at 25°C, 6000 µg g^{-1} only delayed growth for 15 days. The extent of growth at the end of the incubation periods was parallel to the temperatures of incubation. The analyses for AFB_1 production in all samples at all levels of preservatives and control were negative.

Sodium metabisulphite and calcium propionate

Adegoke *et al.* (1991) found sodium metabisulphite to be effective in degrading AFB_1 in lafun and gari. They observed that using sodium metabisulphite during the production of gari (a fermented cassava product heated to 50-70^{o}C during production) degraded AFB_1 levels by 66, 61, 42 and 37%, respectively, for sodium metabisulphite levels of 1.0%, 0.8%, 0.5% and 0.3%.

Bintvihok and Kositcharoenkul (2006) studied the toxic effects of calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers. They found that levels in the liver and the muscle were highest in the aflatoxin B_1-supplemented groups and lower in the aflatoxin B_1-calcium propionate supplemented groups. Such studies indicated that addition of calcium propionate to diets containing AFB_1 appears to be effective in reducing toxicity.

(b) Ozone treatment

Ozone (O_3) is formed by a high energy input that splits the oxygen (O_2) molecule in the air. Single oxygen (O) molecules rapidly combine with available O_2 to form ozone ($3\ O_2 \leftrightarrow 2\ O_3$ + heat and light). In nature, the source of this high energy is the ultraviolet irradiation from the sun and also lightning discharge. Ozone is also a by-product of various photochemical oxidation processes involving hydrocarbons, oxygen, and nitrogen (Muthukumarappan *et al.*, 2000 and 2009; Suslow, 2004).

Ozone, relatively stable in air but highly unstable in water, decomposes in a very short time. It cannot be stored and must be generated continuously. The only product of ozone, when it decomposes, is oxygen; so, food products treated with ozone are free of disinfectant residue. Ozone exists in the gaseous state at room and refrigeration temperature and it is partially soluble in water. It has a pungent, characteristic odour described as similar to “fresh air after a thunderstorm” (Kim *et al.*, 2003).

Ozone gas can be generated using various methods. The most common methods are corona discharges (CD), photochemical, electrolytic, and radiochemical. Corona discharge in a dry process gas containing oxygen is presently the most widely used method for ozone generation. A schematic diagram of ozone generation by corona discharge method is given in Figure 1.5. There are two electrodes in corona discharge, the high tension and low tension electrodes, separated by a dielectric medium in a narrow discharge gap. When electrons have sufficient energy to dissociate the oxygen molecule, a certain fraction of these collisions occur and a molecule of ozone can be formed from each oxygen atom. The ozone/gas mixture discharged from the CD ozone generator normally contains from 1% to 3% ozone when using dry air, and 3% to 6% ozone when using high purity oxygen as the feed gas (Kogelschatz, 1987; McKenzie *et al.,* 1997; Kim *et al.,* 1999; Mahapatra *et al.,* 2005).

Exposure to high concentrations of ozone can cause some detrimental health effects. In the US, Federal Occupational Safety and Health Administration (OSHA) limits of exposure to ozone specify a 0.1 ppm threshold for continuous exposure during an 8 hrs period and 0.3 ppm for a 15 min period (Suslow, 2004).

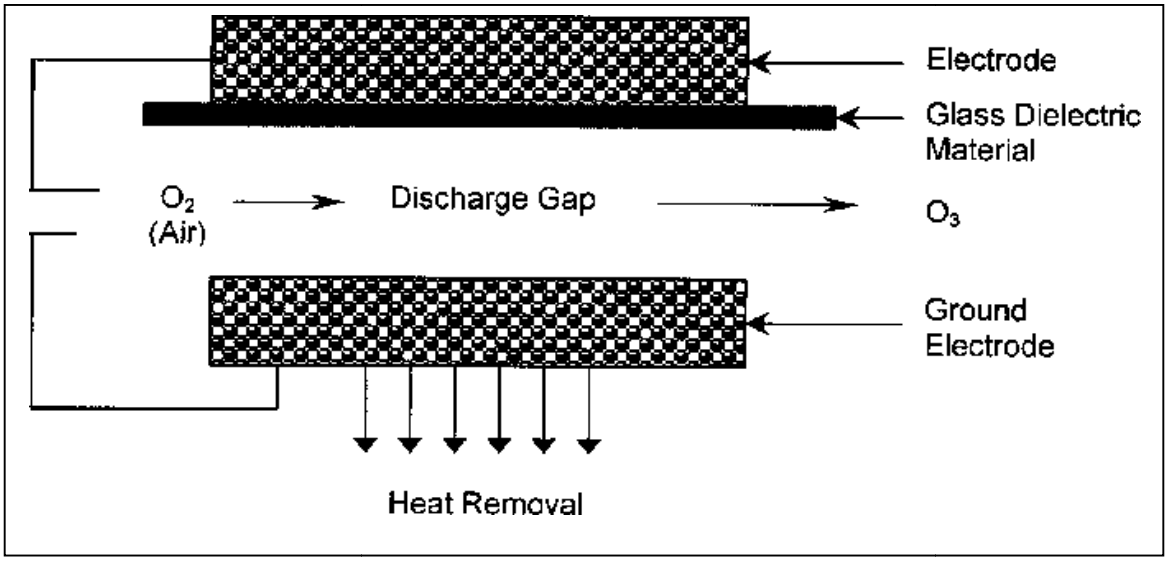


Figure 1.5. Schematic design of Corona Discharge (CD) ozone generation system (Muthukumarappan *et al.,* 2000).

Ozonation is a relatively new method for food processing. It has been used safely and effectively in water treatment for nine decades, at scales from a few gallons per min to millions of gallons per day. It is approved in the US as generally recognized as safe (GRAS) for treatment of bottled water and as a sanitizer for process trains in bottled water plants (FDA, 1995). In June 1997, ozone was deemed the GRAS status as a disinfectant for foods by an independent panel of experts, sponsored by the Electric Power Research Institute (EPRI, 1997).

Disinfecting agents have been extensively used to assure safety and quality in the food industry. However, some of these agents, such as chlorine, can be decrease its activity against some organisms, especially at high pH or against spore-forming microbes.

Furthermore, chlorine can react to form trihalomethanes, which are of concern for both human dietary safety and as environmental pollutants. Therefore, the food industry is searching for applications that are effective against pathogens and toxic contaminants, leading to less loss in product quality, adaptable to food processes and environmental friendly (Karaca and Velioglu, 2007). Ozone treatment has the potential to meet these criteria and gives encouraging results for some problems in the food industry (Graham, 1997). The attractive aspect of ozone is that it decomposes rapidly (half-life of 20-50 min) to molecular oxygen without leaving residues (Kells *et al.*, 2001). As a disinfectant, ozone is 1.5 times stronger than chlorine and is effective over a much wider spectrum of micro-organisms (Xu, 1999).

Efficiency of ozone in microbial inactivation or residue removing strongly depends on environmental factors. An increase in the temperature of an aqueous medium results in decreased solubility and so decreased efficiency of ozone. Decomposition of ozone is also accelerated with increasing temperature. The stability (efficiency) of aqueous ozone increases by decreasing the pH. Increasing humidity is another factor that strengthens ozone efficiency (Kuscu and Pazir, 2004).

In addition to the factors mentioned above, efficiency of ozone is affected by another factor named “ozone demand of the medium.” The stability of ozone under application conditions and the presence of ozone-demanding substances in the

treatment medium greatly affect the level of residual ozone after the application (Kim *et al.,* 2003). If ozone is applied in an environment, such as food systems that are rich in organic matter, it reacts with all compounds and consequently the amount of required ozone will increase (Kim *et al.,* 1999).

Mechanism of action as antimicrobial agent

It is known that ozone could owe its biocidal effectiveness to the direct lysing of cellular walls. Ozone readily oxidizes organic materials in microbial membranes, which weakens the cell wall and leads to cell rupture (Pryor and Rice, 1998). Ozone is a highly unstable molecule with a chemical composition of three oxygen atoms. The third atom is loosely bound and could detach easily from the molecule. The free single oxygen atom readily reacts with other molecules. This process generates a highly reactive oxidant system which affects the structural integrity of the reactant. Such alterations and disruption of cellular components could lead to oxidative damage and lysis of microorganisms (Horváth *et al.,* 1985; Muthukumarappan *et al.,* 2000).

Mechanism of action as an aflatoxin inhibitor

Aflatoxins treated with aqueous ozone can be degraded through oxidative mechanisms at the 8,9 double bond forming the vinyl ether at the terminal furan ring of aflatoxins B_1 and G_1. This involves 1,3-cycloaddition of O_3 at the C8-C9 double bond causing the formation of primary ozonide followed by rearrangement into molozonide derivatives such as aldehydes, ketones and organic acids (Bablon *et al.,* 1991). McKenzie *et al.* (1997) suggested that ozone directly attacks double bonds in aflatoxin, B_2 and G_2 forming organic acids, aldehydes, ketones and carbon dioxide. However, such sites are less reactive than the C8-C9 double bond causing aflatoxins B_2 and G_2 to be more resistant to ozonation than B_1 and G_1.

Zotti *et al.* (2008) demonstrated the mechanism of aflatoxin inactivation synthesized by *A. flavus* colonies using ozonation process. The anthraquinone pigments produced by the fungus may be held in storage for subsequent rapid conversion to aflatoxin. Under this perspective, ozonation could destroy anthraquinonic intermediates, thus preventing the making of aflatoxins.

Application of O_3 technology

In recent years, ozone technology has been applied in agriculture and the food industry. The industrial applications of ozone have been focused on ozone's ability to oxidize and sterilize, without leaving residues. Applications of ozone in the water, wastewater treatment, and food processing appear highly promising. These include preserving raw agricultural commodities during storage and transit (Billion, 1975; Horváth *et al.,* 1985), controlling odour, retarding metabolic processes associated with ripening (Horváth *et al.,* 1985), and sanitizing water utilized for washing food equipment, foods, and packaging materials (Muthukumarappan *et al.,* 2000).

Based on potent disinfection and oxidation properties, various possibilities for use of O_3 in the food industry and agriculture have been developed. There are few published applications of O_3, e.g., cleaning shellfish (Anon, 1972), preserving fish (Haraguchi *et al.*, 1969), aflatoxin reduction in peanuts and cottonseed meals (Dwankanth, 1968), sterilization of bacon, bananas, butter, eggs, mushrooms, cheese, fruits (Gammon and Kerelak, 1973), and beef (Kaess and Weidemann, 1968a; 1968b), disinfection of poultry carcasses and chill water (Sheldon and Brown, 1986; Bolder, 1997).

Studies on the use of ozone in aflatoxin control

The effects of ozone gas in reducing aflatoxin concentration in agricultural products have been investigated and the results of the studies appeared to be promising. Maeba *et al.* (1988) showed that ozone (1.1 mg L^{-1}, 5 min) inactivated pure AFB_1 with no harmful effect of ozone-treated AFB_1 in chicken embryo and rats. McKenzie *et al.* (1997) found that 2% wt ozone for 5 min rapidly degraded AFB_1 and AFG_1 in spiked corn samples with lower effect on AFB_2 and AFG_2. However, higher concentrations (20 wt % ozone) totally degraded all types. In a similar study, McKenzie *et al.* (1998) found that aflatoxins could be reduced by 95% in corn samples treated with 14 wt % ozone for 92 hrs at a flow rate of 200 mg min^{-1}.

Prudente and King (2002) reported that exposing contaminated corn kernels to ozone at 10-12 wt % reduced the level of aflatoxin by about 92%. Proctor *et al.* (2004) combined ozonation with mild heat in breaking down aflatoxins in peanut kernels and flour. They showed that most degradation of AFB_1 and G_1 in all

treatment combinations and all type of aflatoxins were more susceptible to degradation in peanut kernels than in peanut flour. Akbas and Ozdemir (2006) found that the ozonation process (9 mg L^{-1} for 420 min) reduced total aflatoxin and AFB_1 by 24% and 23 %, respectively, for spiked pistachio kernels and only 5% for ground pistachios.

Ozone was used in the detoxification of AFB_1 in red pepper (Inan *et al.*, 2007). In summary, the reductions of AFB_1 content in flaked and chopped red peppers were 80% and 93% after exposures to 33 mg L^{-1} ozone and 66 mg L^{-1} ozone for 60 min, respectively. Giordano *et al.* (2010) reported that ozonation of Brazil nut samples for 5 h at 31 mg L^{-1} completely degraded the aflatoxin content.

Studies on the use of ozone as an antimicrobial agent.

Zhao and Cranston (1995) reported that immersing black peppercorns in water followed by sparging with ozonized air (6.7 mg L^{-1}) for 10 min at an air flow rate of 6 L min^{-1} reduced the microbial population of peppercorn by 3 to 4 log numbers. They also found that treatment ground black pepper with ozone at different moisture levels for 6 h was reduced the microbial population by 3 to 6 log numbers, depending on the moisture content of the spice (higher moisture content led to greater reduction in microbial load). Mason *et al.* (1997) found that exposure cultures of *A. flavus* and *Fusarium verticillioides* to O_3 (5 mg L^{-1}) inhibited the growth, sporulation and mycotoxin production. Kells *et al.* (2001) demonstrated that 50 mg L^{-1} O_3 reduced 63% of the maize kernel contamination level by *A. parasiticus*. Wu *et al.* (2006) found that within 5 min of ozonation, 96.9% of the fungal spores on wheat were inactivated by applying 0.33mg g^{-1} wheat min^{-1}. Finally, Giordano *et al.* (2010) successfully applied ozonation within 5 hrs at 31 mg L^{-1} to inhibit the viability of fungi of the Brazil nut-contaminating microflora and so the toxigenic *Aspergillus species* from the day of application.

Limitations of using ozone

- Applying high doses of ozone for effective decontamination may result in changes in the sensory or nutritional qualities of some food products including; surface oxidation, discoloration and the development of undesirable odours.

- Cells of live microorganisms become more resistant to ozone after ozonation
- The lack of ozone residuals may limit the processor's ability for in-line testing of efficacy.
- According to restrictions relating to human exposure to ozone, plant operators seeking to employ ozone will be faced with system design and process operation challenges.
- The initial cost of ozone generators may be of concern to small-scale food processors but as the technology improves the cost of the generators are coming down (Muthukumarappan *et al.,* 2009)

1.6.3 Biocontrol of aflatoxin

Biological control is the use of living agents to control pests or plant pathogens (Bleve *et al.,* 2006). Microorganisms such as bacteria, yeasts, fungi, protozoa have been tested for their ability in controlling aflatoxin contamination with a variety of methods and efficiencies to less-toxic or non-toxic products. Some aflatoxin-producing fungi from *Aspergillus* species have the capability to degrade their own synthesized mycotoxins (Yin *et al.,* 2008; Wu *et al.,* 2009).

Bacteria

Several bacterial species, such as *Bacillus subtilis* (Kimura and Hirano, 1988), *Bacillus pumilus* (Cho *et al.*, 2009) *Bacillus megaterium* (Kong *et al.*, 2010) *Lactobacilli spp.* (Gourama and Bullerman, 1997), Pseudomonas spp., *Ralstonia spp.*, *Burkholderia spp.* (Palumbo *et al.*, 2006), *Flavobacterium aurantiacum* (Shapira, 2004), and *Rhodococcus erythropolis* (Teniola *et al.*, 2005) have shown the ability to inhibit fungal growth and production of aflatoxins by *Aspergillus spp*. Antagonistic effects of these bacteria could be due to production of antibiotic compounds affecting the growth and the biosynthesis of aflatoxins (Cho *et al.*, 2009; Kong *et al.*, 2010). Also, enzymes produced by some bacteria like *F. aurantiacum* and extracellular extracts from *R. erythropolis* can degrade AFB_1 (Bata and Lásztity, 1999; Alberts *et al.*, 2006). The mechanism of reducing aflatoxins by lactic acid bacteria is due to their adhesion to cell-wall components

(Wu *et al.*, 2009). In most cases, although these strains were highly effective against aflatoxin production and fungal growth under laboratory conditions, they do not give good efficacies in fields because it is difficult to bring the bacterial cells to the *Aspergillus* infection sites on commodities under field conditions (Dorner, 2004).

Yeasts

Similar to bacterial agents, some yeast species (such as *Candida krusei* and *Pichia anomala*) were able to inhibit *Aspergillus flavus* growth in laboratory conditions (Hua *et al.*, 1999; Masoud and Kaltoft, 2006). The mechanism of aflatoxin reduction by yeasts is like that of lactic acid bacteria. The yeast *Saccharomyces cerevisiae* has the capability to bind AFB_1. Further investigation showed that components of the yeast cell wall, called oligomannans after their chemical modification, esterification, were able to bind up to 95% of AFB_1 (Devegowda *et al.,* 1996). The addition of yeast cell walls into feed can lead to a decrease of aflatoxin toxic effect in broilers (Stanley *et al.,* 1993; Santin *et al.,* 2003).

Filamentous fungi

In many field experiments, particularly with peanuts and cotton, significant reductions in aflatoxin contamination in the range of 70 to 90% have been achieved by the use of competitive non-toxigenic *Aspergillus* strains (Dorner, 2004; Pitt and Hocking, 2006; Dorner, 2008). Recently, two non-toxigenic strain based-products have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control aflatoxin contamination in cotton and peanuts in several states of USA (Dorner, 2004). *Trichoderma* spp. are able to produce cell wall degrading enzymes such as β-1,3-glucanases, proteases and chitinases. Since chitin is the major component of most fungal cell walls, a primary role has been attributed to chitinases in the biocontrol activity of *Trichoderma* spp (Calistru *et al.*, 1997)

Some fungal strains (*Aspergillus niger*, *Eurotium herbariorum*, a *Rhizopus sp*., and non-aflatoxin (AF)-producing *A. flavus* are able to degrade aflatoxins by converting AFB_1 to aflatoxicol (AFL). By reducing the cyclopentenone carbonyl of AFB_1, these fungi could convert AFB_1 to aflatoxicol-A (AFL-A), then AFL-A was converted to aflatoxicol-B (AFL-B) by the actions of medium components or organic acids

produced from the fungi (Nakazato *et al.,* 1990). *A. niger* was capable of converting AFL to $AFB_{1,}$ and subsequently AFB_1 could be converted to AFB_{2a}. However, the sum of AFL and AFB_1 suggested that both AFB_1 and AFL were further metabolised to unknown substances by the fungi (Figure 1.6)

Protozoa

Cells of the protozoon *Tetrahymena pyriformis* W are able to degrade pure AFB_1 to another bright-blue fluorescent product and decrease the AFB_1 concentration to 25% in 30 hrs. The action of AFG_1 detoxification by this strain was different. During 10 hrs the concentration decreased to 80% and was constant for the next 20 hrs (Teunisssion and Robertson, 1967). One year later, it was concluded by Robertson *et al.* (1970) that *T. pyriformis* W reduced the carbonyl in the cyclopentane ring of AFB_1 to a hydroxyl group (Figure 1.7). The reduced aflatoxin appears to be identical to aflatoxin R_o (AFR_o).

Actinomycetes

Streptomycetes, the Gram (+) filamentous bacteria, are one of the most investigated groups of actinomycetes for discovery of useful secondary metabolites (Okami and Hotta, 1988, Watve *et al.,* 2001). The results of extensive screening have led to the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary and agriculture. Most of them are produced from *Streptomyces* (Hwang *at al.*, 1994).

It is surprised that no studies have examined the potential of actinomycetes to control aflatoxigenic fungi. However, Sakuda *et al.*, (1999) found that the methanol extract of *Streptomyces* sp. MRI142 (Aflastatin A) inhibited aflatoxin production by *A. parasiticus*. They suggested that aflastatin A may inhibit production of secondary metabolites biosynthesized by fungi via polyketide pathway.

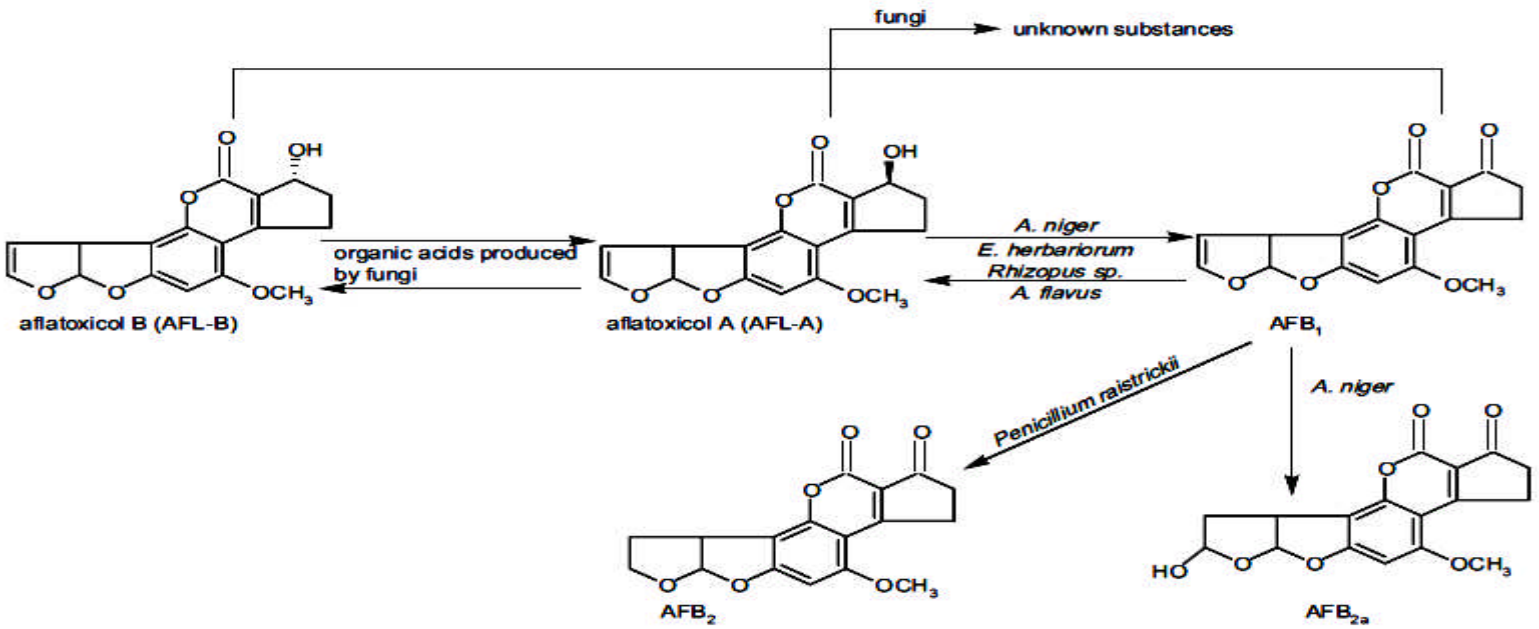


Figure1.6. Metabolic pathways of aflatoxin B_1 by fungi

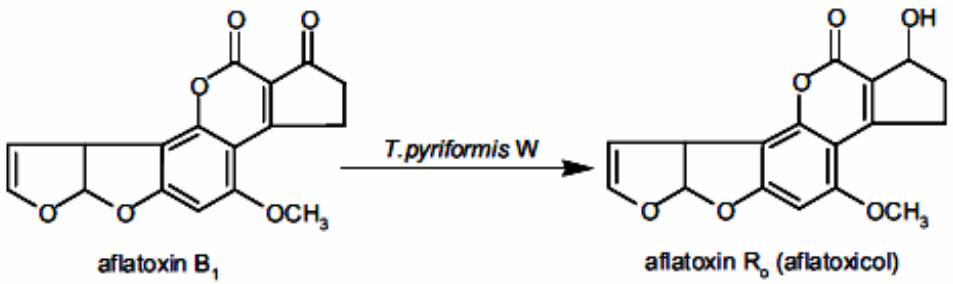


Figure1.7. Degradation of AFB_1 by protozoa

1.7 AIMS AND OBJECTIVES

The main aims of this study are to identify the dominant mycotoxigenic *Aspergillus* species in Egyptian peanuts, to evaluate the toxigenic potential of isolated strains and to develop control strategies for *in vitro* minimizing aflatoxin contamination and then *in situ* at the post-harvest level using preservatives, gaseous ozone and biological agents.

The studies carried out to address these objectives were:

A. Biodiversity of mycotoxigenic *Aspergillus* species in Egyptian peanuts

(i) To isolate the potential mycotoxigenic fungi from peanuts (in shell and seeds) from different regions of Egypt in two seasons.

(ii) To quantify the diversity of aflatoxigenic and ochratoxigenic fungi and their contribution to the total population.

(iii) To evaluate the percentage of species from *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* which were able to produce AFs and OTA.

(iv) To discriminate between *A. flavus* and *A. parasiticus* within *A.* section *Flavi*.

B. Control of aflatoxin using preservatives

(i) To evaluate the potential for *in vitro* effect of selected antioxidants (Propyl paraben, butryl hydroxymethyl toluene, butyl hydroxymethyl anisole, octyl gallate and sodium metabisulphite) and other preservatives (potassium sorbate, calcium propionate and natamycin) on *Aspergillus flavus* and consequently AFB_1 production.

(ii) To identify LD_{50} and ED_{50} of the tested preservatives that inhibited the growth of *A. flavus* and AFB_1` production respectively.

(iii) To apply the best preservative at LD_{50} and ED_{50} concentrations *in situ* on stored peanuts and evaluate its effect on populations of *A. flavus* and AFB_1 production.

C. Control of aflatoxin using ozone treatment

(i) To examine *in vitro* effect of gaseous O_3 on germination, sporulation and AFB_1 production of two strains of *A. flavus* at different a_w levels.

(ii) To evaluate the effect of treating peanuts with ozone on the spore viability of *A. flavus* over storage at different a_w levels and on AFB_1 production as well.

(iii) To find a relationship between the populations of *A. flavus* on peanuts and AFB_1 production after storage.

D. Control of aflatoxin using biological agent

(i) To isolate and screen actinomycetes from peanuts which could be antagonistic to *A. flavus*.

(ii) To examine the selected strain (*Streptomyces* AS1) for competitive and antagonistic effect against *A. flavus in vitro* (media) and *in situ* (peanuts) under different water availability conditions.

(iii) To study the antifungal activity of the ethyl acetate extract from AS1culture broth against *A. flavus* and AFB_1 production *in vitro* and *in situ* on stored peanuts.

The overview of the thesis is schematically represented in Figure 1.8. The work is presented as a series of Chapters (2 to 6) with integrated Materials and Methods, Results and Discussion sections. Chapter 2 shows the importance of studying the biodiversity of mycotoxigenic *Aspergillus* species in Egyptian peanuts and to show that *A. flavus* is the dominant species producing aflatoxin. Chapters 3 to 5 deal with the potential control strategies to inhibit *A. flavus* on peanuts and consequent aflatoxin production. The final chapter (6) includes Conclusions and Future work.

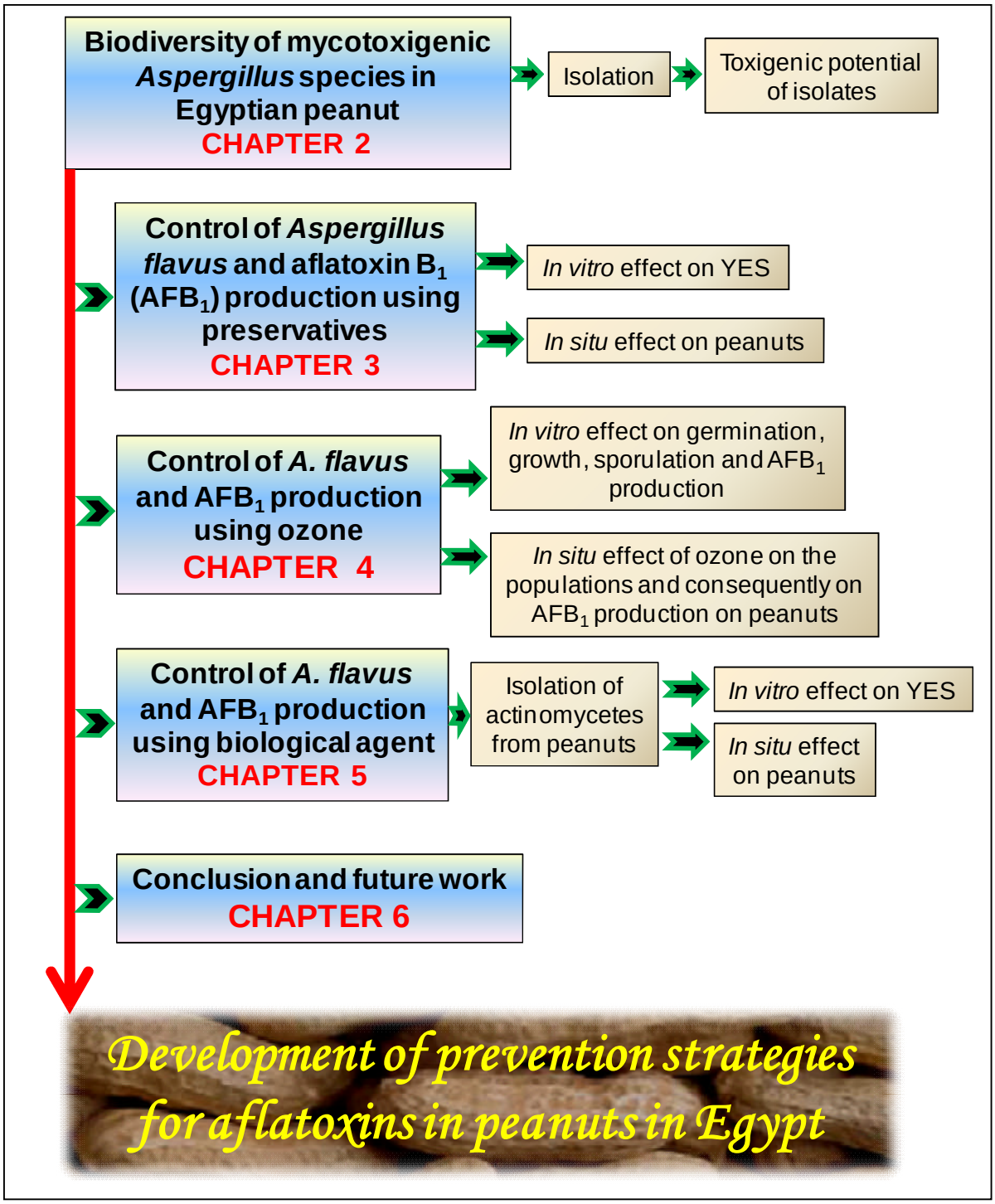


Figure 1.8. Flow diagram of the experimental work carried out in this thesis.

Chapter 2

BIODIVERSITY OF MYCOTOXIGENIC *ASPERGILLUS* SPECIES IN EGYPTIAN PEANUTS

2 BIODIVERSITY OF MYCOTOXIGENIC *ASPERGILLUS* SPECIES IN EGYPTIAN PEANUTS

2.1 INTRODUCTION

The mycotoxins produced by *Aspergillus* spp. which are of greatest significance in peanuts (*Arachis hypogaea* L.) and peanut products include aflatoxins and ochratoxin A (OTA) (Pittet, 1998). Peanuts are important substrates for the growth and subsequent aflatoxin production by different members of *Aspergillus* section *Flavi*: *A. flavus* Link, *A. parasiticus* Speare, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Recently, additional new aflatoxin producing species have been isolated from peanuts in Argentina (*A.arachidicola* sp. nov. and *A.minisclerotigenes* sp. nov (Pildain *et al.*, 2008). The commonest species can infect peanuts and maize both pre- and post-harvest (Cotty, 1997; Barros *et al.*, 2003). Usually, aflatoxin contamination pre-harvest occurs under conditions of heat and drought stress during the latter stages of the growing season (Hill and Lacey, 1983; Blankenship *et al.*, 1984; Dorner and Cole, 2002). Because peanuts are primarily used for food, strict regulatory limits for the amount of aflatoxin in peanut products have been established. For raw peanuts entering the EU, the level must be <4 µg kg^{-1} total aflatoxins and have not more than 2 µg kg^{-1} AFB_1 (European Commission, 2006).

Egypt is a major peanut exporting country and the European markets account for 68% of its peanut exports. Its production has increased steadily, as a result of increased growing areas as well as significant increases in yields during the last two decades (1990 to 2005) from 2.1 to 3.1 t ha^{-1} (Diaz Rios and Jafee, 2008). Peanuts are grown mostly in the north of the country. There are a large number of peanut producers, with many small holders resulting in an overall peanut cultivation area covering 61,000 ha (FAO, 2003). Although some studies, especially in South America, have isolated ochratoxigenic fungi from peanuts, there are no legislative limits for this mycotoxin in peanuts or derived products although these are in place for cereals (Van Egmond, 2004).

In May, 1999 the European Commission suspended the import of peanuts from Egypt due to the presence of aflatoxins in concentrations in excess of the maximum

levels specified in the EU regulations. Since then the Egyptian Government has been actively trying to reduce the aflatoxin content in peanuts. Since 2003, the EU only required random sampling in 20% of peanut consignments imported from Egypt for AFB_1 and total aflatoxins. Subsequently, Egypt has increased its market share in 2005 by >60 million US$ (Diaz Rios and Jafee, 2008).

There have been previous studies on the mycobiota contamination of Egyptian peanuts (Moubasher *et al.*, 1979; Moubasher *et al.*, 1980; El-Hissy *et al.*, 1981; El-Maghraby and El-Maraghy, 1987; El-Magraby and El-Maraghy, 1988; Youssef *et al.*, 2008). However, none of these studies compared the toxigenic potential of the strains in peanut samples from different regions of Egypt. Furthermore, where mycotoxin analyses were carried out this was only qualitative, predominantly using TLC, with very limited quantification of aflatoxins, or other mycotoxigenic fungi present. For example, recent studies in Argentina have found that *Aspergillus carbonarius* was commonly isolated from peanuts under South American cultivation conditions and that many of the strains isolated could produce ochratoxin A (OTA) (Magnoli *et al.*, 2007). No information is available on the isolation of *Aspergillus* section *Circumdati* species, or *Aspergillus* section *Nigri* and whether these may produce OTA in Egypt.

Thus the objectives of this study were to (a) identify the dominant mycotoxigenic fungi in Egyptian peanuts (in shell, and seeds) in two seasons (2007, 2008) in five different regions of Egypt and (b) to examine and quantify the contributions of the mycotoxgenic fungi to the total populations, and (c) to examine the relative ratios of species from *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* which were able to produce aflatoxins and OTA.

2.2 MATERIALS AND METHODS

2.2.1 Collection of peanut samples

Triplicate raw peanut in shell samples (each 500 g) were collected after harvest in two seasons (2007, 2008) from 5 growing regions in Egypt (Alexandria, El-Beheira, El-Daqahliya, El-Sharqiya, all from northern Egypt; and Asyut from southern Egypt) (Figure 2.1). Half of each sample was shelled in a sterile flow bench to obtain the

Fig. 2.1. Peanuts sampling regions in Egypt are marked by red circles (1.Alexandria, 2.El-Beheira, 3. El-Daqahliya, 4. El-Sharqiya, 5. Asyut)

peanut seeds for mycobiota analyses. Samples were kept at 4°C, until fungal enumeration.

2.2.2 Isolation media

Malt extract agar (MEA): Commercial MEA (Oxoid, Basingstoke, Hampshire, UK) was made using the supplier's recommendation. A small amount of chloramphenicol (250 µg ml^{-1}) was added to inhibit bacterial growth and once the components were mixed, the substrate was autoclaved at 121°C and 1 atm for 15 min. The autoclaved medium was allowed to cool to approximately 50°C before being aseptically poured into sterile 90mm diameter Petri dishes. Cooled solidified plates were kept at 4°C in sealed polyethylene bags for a maximum period of 21 days.

Malt salt agar (MS): The same procedures as used for MEA, except that 10% NaCl was added before autoclaving to modify the water activity to 0.95 a_w.

Coconut cream agar medium: 50% coconut butter and 2% agar were heated in distilled water. The mixture was autoclaved and poured into Petri plates and kept at 4°C until used (Dyer and McCammon, 1994).

Yeast extract sucrose agar (YES): YES was prepared by using 20 g of each yeast extract and agar, 150 g sucrose and 0.5 g $MgSO_4.7H_2O$ in 885 ml distilled water. Once the components were mixed, the substrates were autoclaved and poured as described previously. Petri plates were kept at 4°C for a maximum period of 21 days.

2.2.3 Determination of water activity and moisture content of peanuts

The water activity (a_w) of the in shell peanuts and peanut seed samples was determined using the AquaLab 3TE (Decagon Devices, Inc., Pullman, Washington, USA). The moisture content was determined by weighing 10 grams sub-sample of each replicate and oven draying at 105°C for 24 hrs, and then sample was cooled in desiccators and re-weighed to a constant weight. The moisture content (MC) was calculated as the percentage of the wet weight (Deshpande *et al.* 1993).

2.2.4 Mycotoxigenic fungi isolation from samples

Direct plating

A total of 45 peanut seeds (5 x 3 x 3 replicates) were direct plated onto Petri plates of Malt Extract Agar (MEA, Oxoid, Basingstoke, Hampshire, U.K.) and MEA + 10% NaCl (0.95 a_w, MS; Oxoid) from each region. After incubation at 25°C for 7 days, the frequency of fungi was recorded. The isolates of *Aspergillus* section *Flavi, Aspergillus* section *Nigri and Aspergillus* section *Circumdati* were recovered from the peanut seeds and maintained on slants of MEA at 4°C, until examination for their toxigenic potential.

Serial dilutions

The colonisation was assessed as CFUs g^{-1} of dry weight in-shell peanut samples. Triplicate 10g sub-samples from each region were weighed in stomacher bags containing 90 ml of distilled water and left for 10 min to soak. A total of four samples were homogenised for 15 min in the Colworth Stomacher 400. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) were done and 200 µl from each dilution spread plated on MS agar in triplicate. The plates were incubated at 25°C for 7 days and examined periodically. A colony counter was used for total counts and microscopic examinations were made for fungal genera classification. Individual colonies were sub-cultured for detailed examination.

For identification of species, comparisons were made with type strains from culture collections and reference to Pitt and Hocking (2009). Type strains in our laboratories included *Aspergillus flavus* (SRRC-G1907; USDA, USA); *A. parasiticus* (SSWT 2999, USA), *Aspergillus carbonarius* (IMI 388653), *Aspergillus niger*. var *niger* (biseriate; IMI 388550); *A. niger* var. *niger* (uniseriate, IMI 387209), *Aspergillus westerdijkiae* (IBT 21991), *A. steynii* (IBT 22339), and *A. ochraceus* (IBT 11952).

2.2.5 Evaluation of toxigenic potential of isolated strains

Two methods were used to assess the toxigenic potential: a medium-based qualitative system and HPLC analyses for quantification of aflatoxins and ochratoxin A production.

(a) Qualitative assay

Species from the *Aspergillus* section *Flavi*, *Aspergillus* section *Nigiri* and *Aspergillus* section *Circumdati* isolated from peanut seeds were tested, respectively, for their ability to produce aflatoxins (B_1, B_2, G_1, G_2; AFs) or OTA in coconut cream agar 50% (Dyer and McCammon, 1994). The reverse side of the colony was observed daily at 25°C for 6 days under ultraviolet light at 365 nm in a dark cabinet to verify the presence of a blue fluorescent ring, an indication of the presence of AFs or OTA, thus characterizing it as a toxigenic strain.

(b) Quantitative analyses of aflatoxin and OTA

From agar media: For the strains isolated from peanuts five plugs were extracted from each colony (Figure 2.2) after 10 days incubation at 25°C on Yeast Extract Sucrose Agar (YES, Fisher, Loughborough, Leicestershire, U.K.) , transferred to a 2ml Eppendorf tube and weighed (Filtenborg and Frisvad 1980). Strains of *A.* section *flavi* were analysed for aflatoxin contents. A 800 µl chloroform was added to each Eppendorf and shaken for 1 hr using KS 501 digital orbital shaker (IKA $^{(R)}$ Werke GmbH & Co. KG, Germany). The chloroform extract was transferred to a new vial and dried gently under air for derivatization using the method of AOAC (2005) and then analysed quantitatively using HPLC. A 200 µl stock solution of Aflatoxin mix standard in methanol (Supelco, Bellefonte, PA, USA), containing 200 ng B_1, 60 ng B_2, 200 ng G_1 and 60 ng G_1, was dried under nitrogen gas and derivatized as for samples. Four concentrations were prepared for HPLC injection to make standard curve. For OTA extraction, 750 µl methanol was added, the sample shaken for 30 min and centrifuged for 10 min at 15000xg. After filtration of the supernatant, the samples were analysed by HPLC. OTA 50 µg ml^{-1} benzene: acetic acid (99:1, Supelco, Bellefonte, PA., USA) was used. Fifty microlitres of the stock was evaporated under nitrogen and dissolved with methanol to inject 10 µl from four different concentration (125, 250, 375, 500 ng ml^{-1}). Detection limit of AFB_1 using HPLC was 0.8 ng g^{-1} media whereas detection limit of OTA was 0.4 ng g^{-1} media.

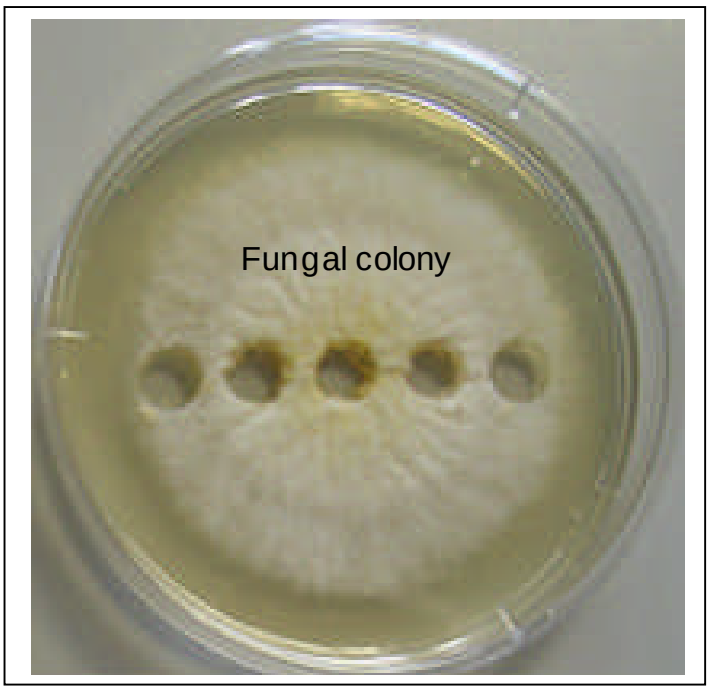


Figure 2.2 Position of plugs taken from *A. flavus* colonies for aflatoxin analysis by HPLC.

Analysis of aflatoxin in peanuts: Three replicates of the peanut samples from each treatment were analysed for AFB_1 using an aflaprep column (Neogene Europe, wide bore). Five grams of ground sample was blended (1 min at high speed) with 1 g salt (NaCl) and 25 ml of 60% methanol. This was then filtered through Whatman No.1 filter paper and the filtrate was collected in a clean vessel. Ten millilitres of filtered extract was diluted 1:1 with phosphate buffered saline (PBS). The diluted extract (20ml=2g sample) was passed through an aflaprep column without drying via a 50 ml glass syringe reservoir attached to the column through an adaptor at a rate of about 1.5 to 2.0 ml per min. By removing the 50 ml glass syringe and the adaptor, the column was filled completely with 25% methanol then the reservoir was reattached to the adaptor. Twenty millilitres of 25% methanol was passed through the column at a rate of 2 ml per min (until air comes through the column). Two millilitres of methanol was pipetted into the column and carefully pushed through the column and collected in a 2 ml Eppendorf tube. The elution was dried gently under nitrogen for derivatization and then HPLC analysis. Detection limit of AFB_1 was 0.012 ng g^{-1} peanut seeds.

Derivatization of aflatoxins

Two hundred microlitre hexane and 50 µl trifluoroacetic acid (TFA) were added to the vial containing the sample or standard. The vial was capped, its contents vigorously mixed (30s) and the mixture was left to stand for 5 min. To the mixture, 950 ml H_2O: acetonitrile (9: 1) was then added and shaken vigorously for 30s and left for 10 min to form two separate layers. The lower aqueous layer was filtered and used for analyses (AOAC, 2005).

HPLC conditions

The HPLC system used for AFs and OTA analyses was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 x 4.6 mm) column joined to a pre- column (security guard, 4x3mm cartridge, Phenomenex Luna). The mobile phase was methanol:water:acetonitrile (30:60:10, v/v/v) using an isocratic flow rate of 1ml min^{-1} at 360 nm excitation and 440 nm emission wavelengths and a 25 min

run time for AFs analyses. For OTA analyses, acetonitrile (57%): water (41%): acetic acid (2%) were isocratically used at the same flow rate at 333 nm excitation, 460 nm emission wavelengths. The run time for samples was 15 min with OTA being detected at 5.75 min.

2.2.6 Molecular discrimination of *A. flavus* and *A. parasiticus*

DNA extraction

Aspergillus section *Flavi* strains was inoculated on the surface of sterile cellophane discs overlaying YES medium. Plates were incubation at 25°C for 6 days. Mycelium of each strain were harvested by removing the cellophane discs and stored at -80 ºC until further use. Genomic DNA of the strains was obtained using the DNAeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions with little modification in buffer's volumes. Mycelium (fresh weigh) was placed in a mortar pre-cooled at -80ºC and ground to a fine powder. One hundred milligram of the powder was suspended in 600 µl of lysis buffer (AP1) and 6 µl RNase of (100 mg/ml) in a 2 ml micro tube. The mixture was vigorously shaken and heated at 65ºC 10 min (overturning 2 or 3 times during incubation). One hundred and ninety five microlitre of Buffer AP2 were added in the tube and gently mixed. The contents were incubated on ice for 5 min and then centrifuged at 14,500 rpm (20,000xg) for 5 min at room temperature. The supernatant was transferred into the QIAsherdder Mini spin column associated with new micro tube. It was centrifuged at 14,500 rpm (20,000xg) at room temperature for 2 min. Filtrate fraction (450 µl) passed through the column was poured into a new 2-ml micro tube and then 675 µl of AP3/E was added and mixed by a pipette. From the mixture, 650 ul was added into the DNeasy Mini spin column attached with new micro tube and Centrifuged at 8,000 rpm (6,000xg) at room temperature for 1 min. Filtrate was discarded and this step was repeated with the remaining mixture. A 500 µl of buffer AW was added in the DNeasy Mini spin column and centrifuged at 8,000 rpm (6,000xg) at room temperature for 1 min. Flow-through was discarded and this step was repeated by adding 500 µl and the centrifuged at 14,500 rpm (20,000xg) at room temperature for 2 min to dry the filter. DNeasy Mini spin column was placed in a new 1.5-ml micro tube and 100 µl of Buffer AE was added in the column. It was incubated at room temperature for 5 min and then centrifuged at 8,000 rpm (6,000xg) for 1 min at room

temperature. DNeasy Mini spin column was removed from the tube. The eluted genomic DNA solution was preserved in the tube at -80°C.

PCR amplification

Differentiation between *A. flavus* and *A. parasiticus* strains isolated from Egyptian peanut samples was carried out according to González-Salgado *et al.* (2008). A pair of primers (Invetrogen), FLA1 (5´-GTAGGGTTCCTAGCGAGCC-3´) and FLA2 (5´-GGAAAAAGATTGATTTGCGTTC-3´) specific to *A. flavus* was used for that differentiation. PCR reactions were performed in 0.2 ml PCR Eppendorf (Starlab Ltd, Milton Keynes, UK) using an Alpha Unit ™ Block Assembly for PTC DNA Engine ™ Systems (MJ Research Inc., Waltham, USA).

The PCR amplification protocol was as follows: 1 cycle of 5 min at 95°C, 26 cycles of 30s at 95°C (denaturation), 30 s at 58°C (annealing), 45 s at 72°C (extension) and, finally, 1 cycle of 5 min at 72°C. Amplification reactions were carried out in volumes of 25 µl containing 1µl of template DNA, 0.25 µl of each primer (100 µM), 2.5 µl of 10xPCR buffer, 2 µl of $MgCl_2$ (25 mM), 0.625 µl of dNTPs (40mM) and 0.2 µl of Taq DNA polymerase ($5U\mu l^{-1}$) supplied by Qiagen (Hilden, Germany). PCR products were detected on 2% agarose ethidiumbromide gels in TAE 1xbuffer (Tris–acetate 40mM and EDTA 1.0 mM). The 100-bp DNA ladder (Qiagen, Hilden, Germany) was used as the molecular size marker. Genomic DNA samples used in this work were tested using the transcription of the housekeeping gene (β-tubulin), Tub1-F (5´-GTCCGGTGCTGGTAACAACT-3´) and Tub1-R (5´-GGAGGTGGAGTTTCCAATGA-3´), obtained from IDT (Integrated DNA Technology Inc. Iowa, USA) as a control. The PCR amplification protocol was as previously detailed except for the changing of the annealing temperature to 65°C and the number of denaturation cycles to 35.

The references strains used in this work were *A. flavus* SRRC-G1907, *A. flavus* A 2092, *A. parasiticus* SSWT BN009-E, and *A. parasiticus* SSWT 2999.

2.2.7 Statistical analysis

Data on populations of mycotoxigenic fungi and total counts (CFUs g^{-1} of dry weight in-shell peanut) on MS medium were logarithmically transformed before statistical analysis. This was required because of the wide range of variability (from single-digit numbers to numbers in hundreds/thousands) (Clewer and Scarisbrick 2001). Statistical significance was determined using Statistica version 9 (StatSoft Inc., Tulsa, OK, USA). Means of $\log_{10}$ CFUs unit were determined by analysis of variance (ANOVA, two and three ways). Numbers which are red in the ANOVA tables indicate a significant difference at $p<0.05$. Fisher's LSD Method ($\alpha=0.05$) was applied to compare significant differences between regions and seasons.

2.3 RESULTS

2.3.1 Water activity and moisture content of peanut samples

Water activity levels (a_w) and moisture contents (MC%) were measured in an attempt to find any correlation with the fungal density of peanut samples from different regions of Egypt in 2007 and 2008 (Table 2.1). Values of either a_w or MC% of in-shell peanuts and seeds from each region were similar. Most of the samples had <0.59 a_w and 9.3% moisture content respectively. However, samples from Alexandria and El-Sharqiya in 2008 had the highest values (0.89-0.91 a_w, 23.4-26.1% moisture content). The background AFB_1 concentrations found in the peanuts in 2007 and 2008 were however very low with the range being 0.8-0.41 and 0.09-0.23 µg kg^{-1} respectively.

2.3.2 Frequency of isolation and populations of mycotoxigenic *Aspergillus* spp. in peanuts from different geographic regions of Egypt in two seasons

Table 2.2 shows the populations ($\log_{10}$ CFUs g^{-1} of in-shell peanut sample) of mycotoxigenic *Aspergillus spp.* and the total fungal populations from the five Egyptian regions in 2007 and 2008. Overall, the total fungal populations of samples collected in 2008, in particular from Alexandria and El-Sharqiya, was significantly higher when compared with 2007. High populations of *Cladosporium* and *Penicillium*

Table 2.1. Water activity levels (a_w) and moisture content (MC%) of in-shell peanuts and seeds collected from different Egyptian regions.

		2007		2008	
Regions	**Type of sample**	**aw**	**MC%**	**aw**	**MC%**
Alexandria	In-shell peanuts	0.58	8.5	0.91	24.1
	Peanut seeds	0.56	7.0	0.91	26.1
El-Behiera	In-shell peanuts	0.50	8.3	0.54	7.9
	Peanut seeds	0.54	7.6	0.52	6.9
El-Sharqiya	In-shell peanuts	0.48	9.3	0.89	23.4
	Peanut seeds	0.51	8.4	0.92	24.4
El-Daqahliya	In-shell peanuts	0.54	8.8	0.56	8.9
	Peanut seeds	0.56	7.6	0.59	7.5
Asyut	In-shell peanuts	0.56	8.3	0.54	7.3
	Peanut seeds	0.53	6.1	0.50	5.8

Table 2.2. The toxigenic mycobiota populations (± standard error) which were isolated from in-shell peanuts (log_{10} CFUs g^{-1} dry weight) in two seasons based on serial dilution.

Season	**2007**				**2008**			
Regions	*Aspergillus* section *Flavi*	*A.* section *Nigri*	*A. sec*tion *Circumdati*	Total fungal counts	*Aspergillus* section *Flavi*	*A.* section *Nigri*	*A.*section *Circumdati*	Total fungal counts
Alexandria	4.24^{a}±0.22	3.96^{a}±0.15	ND	4.45^{a}±0.17	5.57^{a}±0.09	4.71^{a}±0.11	4.84^{a}±0.02	5.97^{a}±0.06
El-Behiera	3.00^{b}±0.21	3.84^{ab}±0.06	ND	3.95^{b}±0.07	2.91^{b}±0.10	3.51^{b}±0.06	0.72^{b}±0.37	3.68^{b}±0.06
El-Sharqiya	3.68^{c}±0.30	3.54^{ab}±0.22	ND	4.10^{ab}±0.12	5.56^{a}±0.01	4.79^{a}±0.17	4.27^{c}±0.14	5.93^{a}±0.03
El-Daqahilya	3.78^{c}±0.17	3.47^{bc}±0.16	ND	4.06^{ab}±0.12	3.25^{b}±0.07	3.38^{b}±0.04	2.26^{d}±0.32	4.37^{c}±0.17
Asyut	3.21^{b}±0.18	3.74^{ac}±0.16	ND	3.90^{b}±0.10	3.11^{b}±0.05	2.55^{c}±0.19	2.51^{d}±0.05	3.86^{b}±0.31

Means followed by different letters (within columns) for each section are significantly different (P=0.05).

ND: non detected, below the detectable levels.

species were present in samples from Alexandria and El-Sharqiya in 2008 (4-5 log units g^{-1}). However, *Wallemia sebi*, yeasts, *Acremonium* species were present in samples from the other regions in both seasons. *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* groups were the most prevalent mycotoxigenic fungi in both seasons. Samples from Alexandria and El-Sharqiya collected in 2008 had the highest total populations among the regions examined, and subsequently the highest populations of mycotoxigenic fungi. The *Aspergillus* section *Circumdati* were only found in samples from the 2008 season (0.7- 4.8 log CFUs g^{-1}).

Aspergillus section *Circumdati* was observed from all regions in 2008, but none in 2007. However, the other two mycotoxigenic groups were isolated in 2.9-5.6 and 2.6-4.8 log CFUs g^{-1} respectively for the two seasons.

Table 2.3 shows the statistical analyses of the single and interaction between single factors of mycotoxigenic groups in relation to region and season. This shows that these were statistically significant effects of region and season on *Aspergillus* section *Flavi and Aspergillus* section *Circumdati,* while for *Aspergillus* section *Nigri* season had no effect.

Figure 2.3 shows the frequency of isolation of the mycotoxigenic fungal groups from peanut seeds in the different regions in 2007 and 2008. The frequency of these fungal groups ranged from 0 to 78.3 and from 4.7 to 52.6% collected in 2007 and 2008 respectively. Generally, the percentage of *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* groups in 2007 was higher than that isolated in 2008. In 2007, *Aspergillus* section *Flavi* group represented 78, 44, 27, and 25% of the total count in samples from El-Daqahelaya, Alexandria, El-Beheira, El-Sharqiya regions respectively. While, there was none found in samples from the southern region (Asyut). However, they were contaminated with *Aspergillus* section *Circumdati* (33%) in addition to *Eurotium* and *Alternaria* as the dominant other genera (33 and 25% respectively). The second most important group was *Aspergillus* section *Nigri* which can produce OTA. Peanut seeds from El-Beheira contained the highest percentage frequency of *Aspergillus* section *Nigri* (45%) when compared with the other regions having less in 2008. The frequency of isolation of *Eurotium* and *Acremonium* species from all regions was high when compared to that of the *Aspergillus* section *Flavi* group (4.7-10.2%), *Aspergillus* section *Circumdati*

Table 2.3. Statistical analyses of significance of *Aspergillus* spps. (log_{10} CFUs g^{-1} in-shell peanuts) in relation to regions, season and their interaction.

Effect	SS	DF	MS	F	P
Aspergillus* section *Flavi					
Intercept	440.3368	1	440.3368	5525.136	0.000000
Regions	18.5865	4	4.6466	58.304	0.000000
Season	1.8346	1	1.8346	23.019	0.000110
Regions x season	6.5233	4	1.6308	20.463	0.000001
Error	1.5939	20	0.0797		
Aspergillus* section *Nigri					
Intercept	419.3993	1	419.3993	6745.462	0.000000
Regions	5.6958	4	1.4239	22.902	0.000000
Season	0.0281	1	0.0281	0.451	0.509343
Regions x season	5.1241	4	1.2810	20.604	0.000001
Error	1.2435	20	0.0622		
Aspergillus* section *Circumdati					
Intercept	63.92400	1	63.92400	833.1498	0.000000
Regions	16.41431	4	4.10358	53.4837	0.000000
Season	63.92400	1	63.92400	833.1498	0.000000
Regions x season	16.41431	4	4.10358	53.4837	0.000000
Error	1.53451	20	0.07673		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at P=0.05.

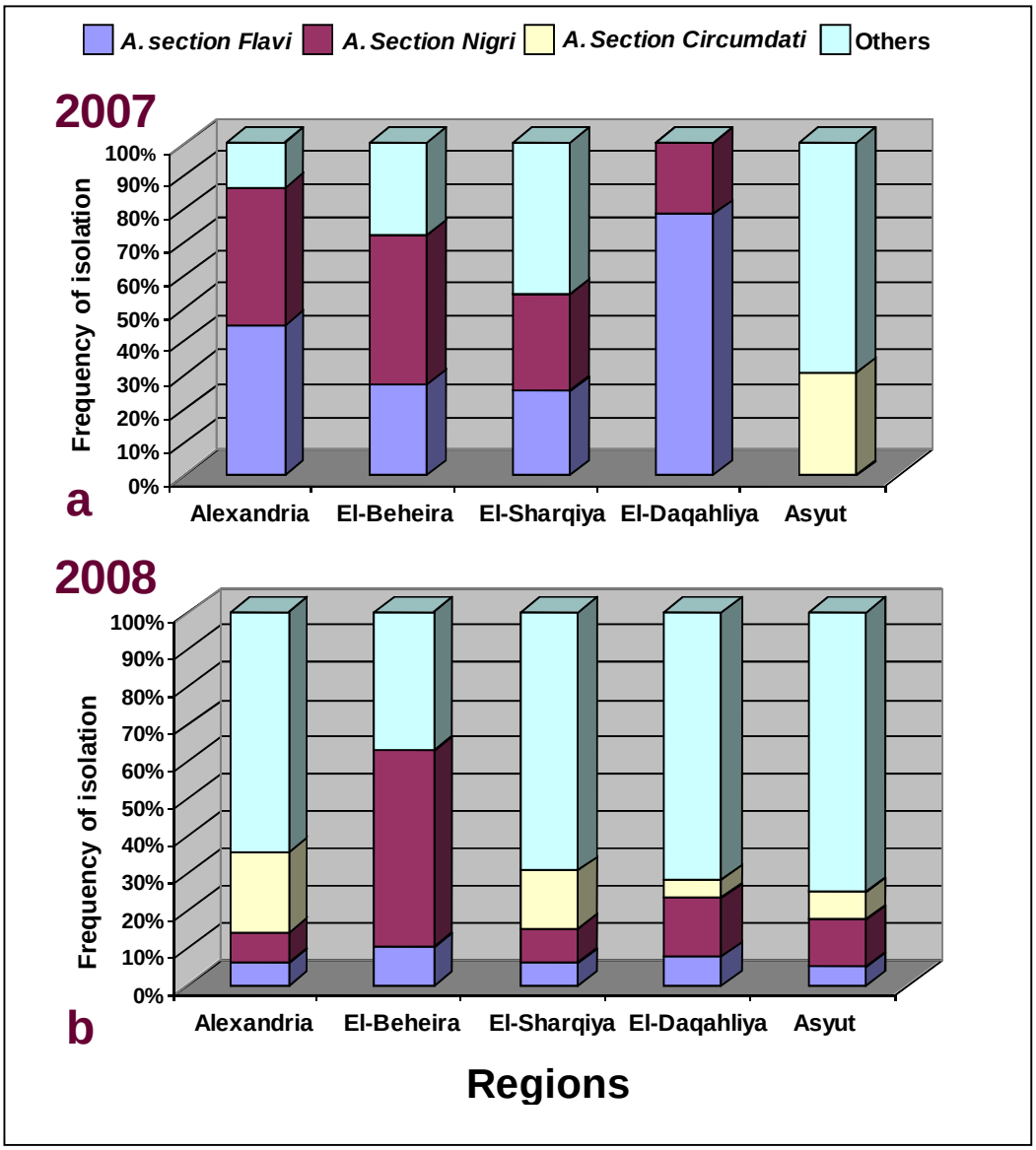


Figure 2.3. Frequency of isolation of dominant mycotoxigenic fungal Sections from peanut seeds from different regions in (a) 2007 and (b) 2008 based on direct plating on MS medium at 25°C for 7 days.

group (5.1-21.2%) and *Aspergillus* section *Nigri* groups (9.4-52.6%). In 2007, Xerophilic *Eurotium* species represented 26%, 47% and 70% of the total fungal count in samples from El-Beheira, El-Daqahliya and Asyut region, respectively. However, *Acremonium* was dominant in Alexandria and El-Sharqiya samples. In 2008, *Penicillium* and *Acremonium* were found in samples of Alexandria and El-Sharqiya. However, samples from these areas were contaminated with *Eurotium* species as a dominant in 2007.

2.3.3 Toxigenic potential of isolated strains of mycotoxigenic genera

Screening of fungi isolated from peanut seeds for aflatoxin production by the fluorescence method on coconut cream agar medium revealed that among 56 and 32 *Aspergillus* section *Flavi* isolates in 2007 and 2008 almost all except for 6 and 8 strains respectively, produced a blue fluorescence (Table 2.4). The same medium was also used to examine *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* isolates for ochratoxin A (OTA) production. No blue fluorescence was observed in any of *Aspergillus* section *Nigri* isolates tested (28 and 50 isolates in 2007 and 2008). Of the *Aspergillus* section *Circumdati* isolates in 2007 and 2008 (12 and 52 respectively) 42 and 25 %, were ochratoxigenic.

A total of 88 *Aspergillus* section *Flavi* isolates were tested by HPLC for quantification of the ability to produce aflatoxins (AFAs; B_1, B_2, G_1, G_2). A further 64 *Aspergillus* section *Circumdati and* 78 *Aspergillus* section *Nigri* were screened for production of OTA on a conducive YES medium. Table 4 also shows that there were differences between the qualitative and quantitative methods. For example, of 88 *Aspergillus* section *Flavi* isolates only 5 gave false negative results. For *Aspergillus* section *Circumdati* of 64 isolates, only 4 gave a false positive in the two seasons when compared with HPLC results. The mean aflatoxigenic *Aspergillus* section *Flavi* isolates for both seasons was 90% based on HPLC analysis. The concentrations of AFB_1 produced ranged from 10 to 100,000 ng g^{-1}. However, 2 (2.3%) isolates were able to produce AFB_1 >100,000 ng g^{-1}. The mean percentage ochratoxigenic *Aspergillus* section *Circumdati* isolates was 28% and the concentration range of OTA was from 1-3000 ng g^{-1}. None of the 78 *Aspergillus* section *Nigri* isolates produced OTA. This suggests that most of the isolates from these groups were, respectively, *A. flavus*, *A. westerdijkiae* and *A. niger* (uniseriate).

Table 2.4. AF- and OTA-producing ability of *Aspergillus* section *Flavi, Aspergillus* section *Nigri and Aspergillus* section *Circumdati* isolated from peanut seeds [*ND* not detected (below the detection limit using HPLC)].

Year	Group name	No. strains	Mycotoxin type	Positive producing strains		Conc. (ng g^{-1} medium
				Medium	HPLC	
2007	*Aspergillus* section *Flavi*	56	AFB_1	50 (89%)[a]	51 (91%)[a]	10.9 to 248,460.5
	Aspergillus section *Circumdati*	12	OTA	6 (50%)[b]	5 (42%)[b]	96.6 to 2,871.8
	Aspergillus section *Nigri*	28	OTA	0 (0%)[c]	0 (0%)[c]	ND
2008	*Aspergillus* section *Flavi*	32	AFB_1	24 (75%)[a]	28 (87%)[a]	12.8 to 75,849.3
	Aspergillus section *Circumdati*	52	OTA	16 (31%)[b]	13 (25%)[b]	0.8 to 766.9
	Aspergillus section *Nigri*	50	OTA	0 (0%)[c]	0 (0%)[c]	ND

[a] *Aspergillus flavus* strains

[b] *Aspergillus westerdijkiae strains*

[c] *Aspergillus niger* strains

Molecular analyses of *Aspergillus* section *Flavi* strains isolated from Egyptian peanuts were further tested using the primer pair FLA1 and FLA2. A single fragment of about 500 base pair (bp) was only amplified when genomic DNA from *A. flavus* strains was used, but not from *A. parasiticus* strains where no product was observed.

More than 95% of the tested *Aspergillus* section *Flavi* were *A. flavus* and this is shown in Figure 2.4 as an example of the results. Isolate in lane 16 was *A. parasiticus* and non-aflatoxigenic as well. These result confirmed those obtained from analysis of isolates on YES medium for AFs. This showed that all the aflatoxigenic isolates produced AFB_1 and B_2 and none produced AFG_1 and G_2.

There was approximately 90% compatibility of the results between coconut agar method and HPLC. For both seasons, the mean *Aspergillus* section *Flavi* isolates producing aflatoxins was 90%, and for ochratoxin A, 28% of *Aspergillus* section *Circumdati* isolates. However, no *Aspergillus* section *Nigri* group produced OTA.

2.4. DISCUSSION

2.4.1. Frequency of isolation and populations of mycotoxigenic *Aspergillus spp.* from Egyptian peanuts

Peanuts are an important crop grown in Egypt for either local consumption or exporting to European markets with an annual production around 190,000 tons (USDA-FAS, 2009). Because of the EU strict regulatory limits for the aflatoxins in peanuts (<4 mg kg^{-1} for total AFs and 2 for AFB_1), it was very important to evaluate the mycotoxigenic fungi in Egyptian peanuts. Samples from four regions in the north (Alexandria, El-Beheira, El-Sharqiya and El-Daqahelaya), which are important areas growing peanuts (FAO, 2003), and from one region in the south (Asyut) were collected. Two seasons (2007 and 2008) were chosen to represent this study.

In this study, there was a positive relationship between a_w levels and MC of peanut samples (Dorner, 2008). This suggests that either value could be a good indicator of quality of storage. Values of water activities observed in this study (0.48-0.59 a_w) except those for samples from Alexandria and El-Sharqiya in 2008 (about 0.90 a_w)

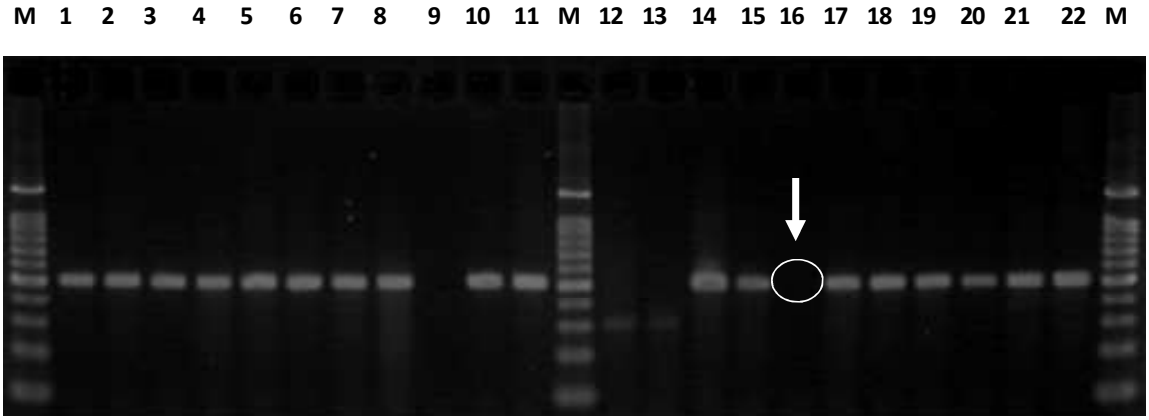


Fig. 2.4. PCR-based detection of *A. flavus* using FLA1/FLA2 primers. Lanes 1–8: tested strains. Lane 9: non-template control. Lane 10: *A. flavus* 2092. Lane 11: *A. flavus* SRRC- G1907. Lane 12: *A. parasiticus* SSWT BN009-E. Lane 13: *A. parasiticus* SSWT 2999. Lanes 14–22: tested strains. M: DNA molecular size marker. White arrow indicates *A. parasiticus* strain in lane 16 where no confirmatory bands were present.

were below the minimum range of 0.78-0.80 a_w established for the growth of *A. flavus* (Lacey *et al.*, 1991). As expected, high fungal populations were observed in in-shell peanut samples from these two areas (4-5 $\log_{10}$ CFUs g^{-1}) which had the highest a_w and MC levels. *Aspergillus* was consistently the most frequent genus in seeds and in-shell peanuts and was the basic component of the fungal populations. The increase in the frequency of *Aspergillus spp.* can be explained by the fact that these fungi occur both pre- and post-harvest in tropical and sub-tropical regions (Anderson, *et al.,* 1995; Gianessi, 1997). That result is in agreement with the findings of previous studies examined peanut samples from different regions in Egypt (Moubasher *et al.,* 1979; El-Maghraby and El-Maraghy, 1987, 1988; Youssef *et al.,* 2008).

Significantly, the population of *Aspergillus* section *Flavi* and *Aspergillus* section *Circumdati* in in-shell samples changed with the year whereas that of *Aspergillus* section *Nigri* was unaffected. The populations *of Aspergillus* section *Circumdati* ranged from 0.72 to 4.84 $\log_{10}$ CFUs g^{-1} in samples from all regions in 2008 and none in 2007. Whereas, *Aspergillus* section *Flavi* (2.9-5.6 $\log_{10}$ CFUs g^{-1}) and *Aspergillus* section *Nigri* (2.6-4.6 CFUs g^{-1}) were found in samples in both seasons. A similar frequency was also found by Gonçalez *et al.* (2008) for *A. flavus* in full pod maturity (33.6%). In Brazil, Nakai *et al.* (2008) reported that in kernels, in addition to *A. flavus* (21.2%), *A. niger* was isolated but at a very low frequency (0.6%). However, they found that among representatives of the genus *Aspergillus*, only *A. flavus* (10.3%) were isolated from shells. Moubasher *et al.* (1979) found *A. flavus* to constitute 16.1, 8.4 and 27.2% of the total count in the shells, in covered and uncovered peanut seeds respectively. They also found that *A. niger* was recorded from 95% of peanut samples tested, constituting 20.7% of the total fungi and 35% of the total *Aspergillus*. Previously, El-Maghraby and El-Maraghy (1987) found *A. flavus* and *A. niger* comprised only 8.1 and 2.2% of the total fungi in Egyptian peanuts. With regard to *Aspergillus* section *Circumdati* group, Tripathi and Kumar (2007) found them to be present in only 12.7% of the total fungi in India. However, none of the studies in Egypt examined potential for mycotoxin production by these genera.

The most predominant species of the total count in seeds from north regions in 2007 were *Aspergillus section Flavi* (25-78%) and *Aspergillus section Nigri* (22-45%) and

only *Aspergillus section Circumdati* (33%) was isolated from samples of the south region. However, the percentage of *Aspergillus section Flavi* in samples of 2008 was lower than of 2007 and ranged from 4.7 to 10.2%. El-Maghraby and El-Maraghy (1988) and Youssef *et al.* (2008) reported that *A. flavus* represented 4 and 4.9% respectively of the total fungi on untreated seeds. In the present study *Aspergillus* section *Circumdati* group represented 5.1-21.2% of mycobiota populations in samples from all regions except from El-Beheira. In contrast, Youssef *et al.* (2008) did not find any *Aspergillus* section *Circumdati* group in peanut samples. In 2008, in the Alexandria and El-Sharqiya samples a higher frequency of *Penicillium* and *Acremonium* species were found in contrast to 2007 where *Eurotium* species were dominant. This may be linked to the higher a_w of these samples. This also suggests that samples from these regions were stored poorly after harvest (Pitt and Hocking, 2009).

2.4.2. Toxigenic potential of isolated strains of fungal species

From all peanut seeds samples collected during 2007 and 2008, 88, 78 and 64 strains of *A.* section *Flavi*, *A.* section *Nigri* and *A.* section *Circumdati* respectively were isolated. Toxigenic potential of the isolates was measured using coconut agar medium (qualitative method) and HPLC (quantitative). There was approximately 90% compatibility of the results between coconut agar method and HPLC. Similar results were obtained by Giorni *et al.* (2007) who found that 73% of *Aspergillus* section *Flavi* strains isolated from maize showed fluorescence when inoculated on Coconut extract agar and 70% of strains were positive when tested by HPLC. For both seasons, the mean *Aspergillus* section *Flavi* isolates producing aflatoxins was 89.3%, and for ochratoxin A, 33.5% of *Aspergillus* section *Circumdati* isolates. However, no *Aspergillus* section *Nigri* group produced OTA. Nakai *et al.* (2008) found in Brazil that 93.8% of *Aspergillus* section *Flavi* isolated from Brazilian peanuts produced aflatoxins. Also, Sánchez-Harvás *et al.* (2008) reported 64.1% of isolates from cocoa beans produced aflatoxins.

Depending on the HPLC analyses of AFs produced by strains of *Aspergillus* section *Flavi* on YES medium, the aflatoxigenic strains produced just AFB_1 and AFB_2 and none were able to produce AFG_1 and AFG_2. This means that strains belonged to the *A. flavus* group. Confirmation by molecular analyses to differentiate between *A.*

flavus and *A. parasiticus* within *Aspergillus* section *Flavi* including non-aflatoxigenic strains indicated that >95% belonged to *A. flavus*. The results for OTA production by *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* group in this study differ from those obtained by Sánchez-Harvás *et al.* (2008) who found that 49.2% of black *Aspergilli* strains were able to produce OTA in cocoa beans and suggested that *Aspergillus* section *Circumdati* is probably a relatively unimportant source of OTA in cocoa production. In contrast, Recently, *Aspergillus* section *Circumdati* species, especially *A. westerdijkiae*, were found to be commonly present on coffee beans (Noonim *et al.* 2008). Magnoli *et al.* (2007) reported that 32% of *Aspergillus* section *Nigri* isolated from Argentinean peanut seeds were OTA producers in culture. Additionally, Bayman *et al.* (2002) demonstrated that none of the isolates of *Aspergillus* section *Circumdati* or *Aspergillus melleus* from tree nuts and fig orchards produced OTA, although all isolates of *Aspergillus alliaceus* did produce the toxin. This is unusual as *Aspergillus* section *Nigri* isolates normally contaminate grapes, grape products and wine with OTA (Mateo *et al.*, 2007).

In conclusion the present study demonstrated the susceptibility of Egyptian peanuts to colonise with *Aspergillus* section *Flavi,* especially *A. flavus* isolates*, Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* groups as dominant fungi from different regions of Egypt. The a_w of the samples, season, and regions were important factors affecting the fungal populations. It may be important to not overlook the presence of ochratoxigenic fungi (28%) in peanuts in addition to aflatoxigenic species (90%) and to perhaps formulate guidelines for OTA as well. *Aspergillus* section *Nigri* group isolates from Egypt were unable to produce OTA in peanuts. This contrasts with results from South America. Poor post-harvest storage of peanuts in Egypt could increase the risk from contamination with aflatoxins above the EU legislative limits.

Chapter 3

CONTROL STRATEGIES USING PRESERVATIVES

3 CONTROL STRATEGIES USING PRESERVATIVES

3.1 INTRODUCTION

Although the use of physical methods used in food hygiene and safety has in general resulted in better control of spoilage microorganisms, care is needed to ensure that the organoleptic quality of the food is not significantly affected. They also need to be technologically and economically feasible. In many cases physical control is not feasible and thus chemical preservation will often be necessary (Nielsen and De Boer, 2000). The use of chemical antimicrobials in foods is a common method of food preservation. A wide range of chemicals or additives are used in food preservation systems to control pH, such as aliphatic acids and antioxidants, to provide food functionality as well as preservation action. Food additives must achieve the desired function in the food to which they are added, and they must be safe to the consumer under the intended conditions of use (Rahman, 2007).

Many chemical products are used to prevent the growth of bacteria and fungi. Antioxidants such as propyl paraben (PP) have been shown to inhibit mycelia growth of *A. flavus*, *P. aurantiogriseum* and *F. graminearum* (Thompson, 1994). Sulfide based compounds such as sodium metabisulphite are also considered as antioxidants. It is widely used as a multi-functional antimicrobial agent. It has been shown to completely inhibit mycelia growth of *A. parasiticus* at concentration as low as 0.1% (Chourasia, 1993). Weak acid preservatives including propionate, sorbate and benzoate were also reported as effective growth inhibitors of several fungi including *A. flavus*, *A. niger* and *P. digitatum* (Chakrabarti and Varma, 2000; Palou *et al.*, 2002). Natamycin (or pimaricin), one of the applicable preservatives in cheese, is an antibiotic agent produced by *Streptomyces natalensis* and *S. chattanoogensis*. It is very active against yeasts and moulds (0.1-50 ppm), but has no activity against bacteria (>10,000 ppm) (Nielsen and De Boer, 2000).

A common strategy to minimize the entry of aflatoxins into the peanut chain is the use of chemical treatments at the post-harvest level to reduce both fungal growth and potential toxin production. Antioxidants such as butylated hydroxyanisole (BHA), propyl paraben (PP) and butylated hydroxytoluene (BHT) are allowable for use as antimicrobial agents by the US Food and Drug Administration (FDA) and are

regarded as safe (GRAS) chemicals. The maximum usage level of single or multiple antioxidants approved by the legislation is 200 μg g^{-1} based on the percentage of fat or oil in food (Codex Alimentarius, 2006). Considering that the oil content of peanuts ranges from 35% to 55% (Knauft and Ozias-Akins, 1995), a dose of 100 μg g^{-1} based on the total weight could be applied. Also, use of salt compounds in food preservation has wide application with advantages of having relatively low mammalian toxicity, a broad spectrum of modes of action and relatively low cost (Olivier *at al.*, 1998).

The objectives of this study were to evaluate the potential for (a) *in vitro* control of aflatoxin production by *Aspergillus flavus* isolates from Egyptian peanuts using phenolic antioxidants (propyl paraben, butylated hydroxy toluene, butylated hydroxy anisole and octyl gallate), aliphatic salts (sodium metabisulphite, potassium sorbate, calcium propionate) and natamycin and (b) *in situ* efficacy of the best preservatives for controlling contamination in stored peanuts.

3.2 MATERIALS AND METHODS

3.2.1 Fungal strain

Aspergillus flavus EGP-B07 isolated from Egyptian peanuts was used for inoculation either in *in vitro* or *in situ* experiments. The fungal strain was sub-cultured before examination on MEA. The spores were gently dislodged from the colony surface into suspensions of 10 ml sterile distilled water containing 0.05% Tween-20 in 25 ml Universal bottles. The resulting spore suspension was filtered though two layers of Miracloth (CALBIOCHEM, Merk, Darmstadt, Germany). Fungal spore concentration was determined using a hemocytometer and adjusted to 10^6 spores ml^{-1}. This was used for inoculation of either media or peanuts.

3.2.2 Media

Malt extract agar (MEA), malt salt agar (MS): The same procedure as the one described in Section 2.2.2 was used for preparation of these media.

Yeast extract sucrose agar (YES): YES was prepared as decribed in Section 2.2.2 but at different water activities. The distilled water was adjusted to 0.982, 0.955 and

0.928 a_w by the addition of glycerol (9.2, 23 and 36.8 g 100 ml^{-1}, respectively) before using in medium. The final a_w levels of YES were 0.95, 0.92 and 0.89 a_w respectively.

3.2.3 Preservatives

The following preservatives were used: phenolic antioxidants [2(3)-tertbutyl-4 hydroxyanisole (BHA); 2,6-di (tert-butyl)-p-cresol (BHT); n-propyl p-hydroxybenzoate (PP) and 3,4,5-Trihydroxybenzoic acid octyl ester (OG)], salts of aliphatic acids (Calcium propionate, potassium sorbate and sodium metabisulphite) and natamycin. All test compounds (Table 3.1) were obtained from Sigma (Dorset, UK) except natamycin. Delvocid, the natamycin commercial product (50% active ingredient), was from DSM Food Specialities (Delft, The Netherlands). The antioxidants were dissolved in methanol and a stock solution containing 20 mg ml^{-1} of each was made up. Different concentrations (50, 100, 200, 500 ppm) were prepared by adding appropriate amounts to sterilized medium (YES) at different a_w levels (0.95, 0.92 and 0.89). Bottles of molten media were thoroughly shaken prior to pouring, to ensure that the antioxidant was dispensed in each treatment. Natamycin was also dissolved in methanol and a stock solution containing 2 mg ml^{-1} of natamycin was made up. This solution was diluted to screen a range of concentrations (1, 5, 10, 25 ppm) in YES medium.

Calcium propionate (500, 1000, 2000 and 3000 ppm), potassium sorbate and sodium metabisulphite (500, 1000, 2000 ppm) were prepared from stock solutions of 200 mg ml-1 distilled water followed by filtration through a 0.22 µ filter. The culture media were prepared and autoclaved. When the molten agar medium had cooled to about 50°C, the required volume of each salt was added from the stock solution to obtain the desired concentration in the medium. The medium was vigorously shaken and poured into 9 cm Petri dishes. Two controls at different aw conditions were prepared: one for antioxidant and natamycin treatments by adding just methanol to media before pouring and the other one for aliphatic salts by using the normal media.

Table 3.1. List of preservatives screened for inhibitory effects against mycelial growth of *Aspergillus flavus* and aflatoxin production *in vitro* and *in situ.*

Compound	Formula	Molecular weight
Butylated hydroxytoluene (BHT)	$C_{15}H_{24}O$	220.35
Butylated hydroxyanisole (BHA)	$C_{11}H_{16}O_2$	180.24
Propyl paraben (PP)	$C_{10}H_{12}O_3$	180.20
Octyl gallate (OG)	$C_{15}H_{22}O_5$	282.33
Sodium metabisulphite (SMB)	$Na_2S_2O_5$	190.10
Potassium sorbate (PS)	$C_6H_7O_2K$	150.22
Calcium propionate (CP)	$C_6H_{10}CaO_4$	186.22
Natamycin (NM)	$C_{33}H_{47}NO_{13}$	665.725

3.2.4 *In vitro* studies on impact of preservatives on growth of *A. flavus*

YES plates at different a_w conditions were centrally inoculated with 5 µl *A. flavus* suspension ($1x10^6$ spores ml^{-1}) after incorporation of treatments. Three replicates of each treatment were used. Petri dishes of the same a_w values were sealed in polythene bags. The inoculated plates were incubated at 25°C. Growth rates of *A. flavus* for treatments and controls were determined by daily measurement of colony diameters in two directions at right-angles to each other over periods of 7-10 days. Linear regression of the colony radius (mm) against time was used to determine the relative growth rates (mm day^{-1}). Also according to the colony area, 1-5 agar plugs (Figure 3.1) were taken from the *A. flavus* colony using a cork borer (0.8 cm), transferred to a 2 ml Eppendorf tube, weighed, and frozen at -20°C until later aflatoxin analysis by HPLC.

3.2.5 *In situ* studies on effect of octyl gallate on colonisation and aflatoxin contamination of stored peanuts

Because of the difficulty to obtain large amounts of peanuts from Egypt in this study, redskin peanuts 500 g (Julian Graves Ltd., Kingswinford, West Midlands, U.K.) imported from China was used in this study. A moisture adsorption curve was prepared in order to accurately determine the amounts of water required to add to peanuts to obtain the target a_w levels. This curve was obtained by adding different quantities of water to the peanuts and equilibrating at 4°C for 24 hrs. The a_w was measured with an AquaLab 3TE (Decagon Devices, Pullman, Wash., USA).

The method used by Giorni *et al.* (2008) for maize was used with some modification for peanut studies. Twenty five grams of raw peanuts was weighed into solid culture vessels (Magenta, Sigma Ltd, U.K.), closed with plastic lids containing a permeable membrane (Figure 3.2), covered with aluminium foil and then autoclaved at 121°C for 15 min (Kimura and Hirano, 1988). Peanuts were allowed to cool at room temperature. The a_w was measured, and then samples were divided into two groups of a_w levels (0.90 and 0.93 a_w). Each group of peanuts was conditioned with the appropriate amount of water using a moisture adsorption curve to get the required a_w levels and kept at 4°C for 48 hrs with periodic shaking to allow absorption and equilibration. Vessels containing peanuts of the same a_w were enclosed together in

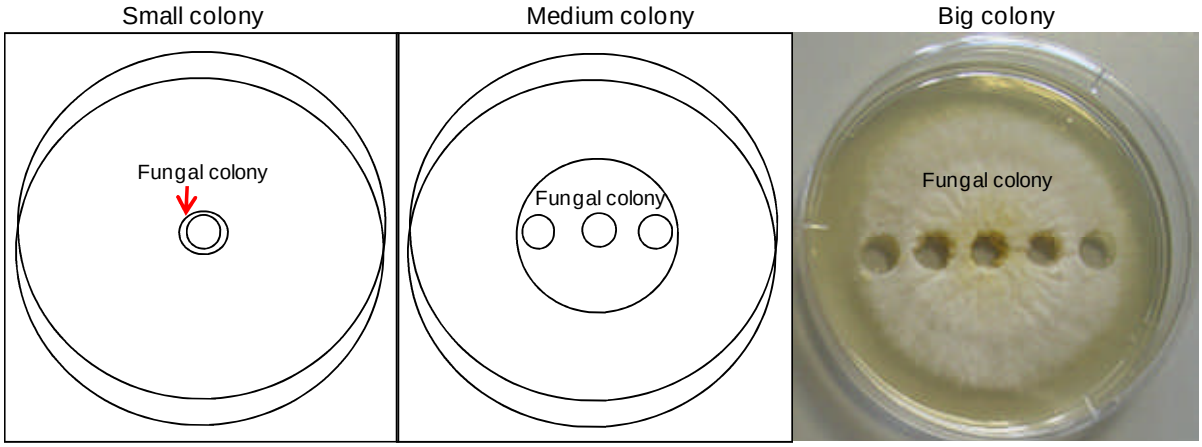


Figure 3.1 Position of plugs from different size of *A. flavus* colonies for aflatoxin analysis by HPLC.

Figure 3.2. Solid culture vessels of peanut samples for *in situ* experiment

plastic chambers. Each chamber had a beaker with glycerol/water solution of the same a_w as the peanut grains, to maintain the equilibrium relative humidity. To study the effect of OG, 3 different concentrations of OG dissolved in ethanol were prepared. From each one in triplicate, 500 µl were added to the vessels to get the final concentrations of 100, 1000 and 2000 ppm. Controls were prepared by addition 500 µl ethanol to each replicate. After 1 hr, treated samples and controls were inoculated with spores of *A. flavus* at room temperature in order to obtain a final concentration of 10^4 spores g^{-1}, by mixing thoroughly. The colonisation by *A. flavus* and aflatoxin production was examined at 0.90 and 0.93 a_w in stored peanuts before storage (Time =0), after 7 and 14 days.

3.2.6 Enumeration of fungi

The spread-plate method on MS medium was used to enumerate the populations of *A. flavus* on peanut samples. Between 2-3 g peanuts from each treatment and replicate was mixed with sterile water-Tween containing 0.1% mycological peptone in a ratio of 1:9 to get 10^{-1} dilution in 60 ml aseptic polypropylene container (Fisher Scientific Ltd., Loughborough, Leicestershire, UK). Samples were kept for 20 min to soak and then vigorously shaken using a vortex mixer. Serial dilutions were made and 200 µl aliquots of each dilution were then transferred aseptically to MS plates and spread uniformly. Three plates were prepared for each dilution and the plates were incubated at 25°C for 7 days before colonies were counted.

3.2.7 Extraction and quantification of AFB_1 form media and peanut samples using HPLC

As described in Section 2.2.5.

3.2.8 Statistical analyses

As described in Section 2.2.7 and because the wide range of variability, the logarithmic values of CFUs of *A. flavus* and AFB_1 levels (ng g^{-1}) were used for analyses. Means of growth rate, $\log_{10}$ CFUs of *A. flavus* and $\log_{10}$ AFB_1 concentrations were determined by analysis of variance (ANOVA, two and three ways analyses) ($p<0.05$). Fisher's LSD Method ($\alpha=0.05$) was applied as well to compare significant differences between treatments and controls.

3.3 RESULTS

3.3.1 *In vitro* effect of preservatives on growth of *A. flavus*

Phenolic antioxidants

The effect of interacting conditions of antioxidant concentrations and a_w levels on efficacy of the antioxidants BHT, BHA, PP and OG on the growth rate (mm day^{-1}) of *A. flavus* EGP-B07 at 25°C for 10 days were studied. Growth was completely inhibited at 500 ppm of these compounds, with the exception of BHT.

Compared with controls at each a_w level mycelial growth was gradually inhibited by increasing the concentration of BHT (50 to 500ppm) (Figure 3.3). The ranges of these reductions were 1.7- 44.2%, 12.6- 40.6% and 6.5- 50% at 0.95, 0.92 and 0.89 a_w respectively. Overall the effects of BHA, a_w and their interaction, had a significant ($P<0.05$) effect on growth of *A. flavus* (Tables 3.2). The a_w was the main factor which affected growth, followed by BHT treatment.

Interestingly, the use of 50 ppm of BHA either had no effect on growth at 0.89 a_w or stimulated it at 0.92 and 0.95 a_w when compared with the controls (Figure 3.4). However, a significant reduction was observed at 100 and 200 ppm. The percentage reductions reached 50, 53 and 100% with 200 ppm at 0.95, 0.92 and 0.89 a_w respectively. All factors were statistically significant (Tables 3.3). In contrast with BHT, this treatment was the major factor affecting growth followed by a_w.

Similar to the pattern of BHT impacts on growth, PP gradually inhibited mycelia extension as concentration was increased (50 to 200ppm) at all a_w levels (Figure 3.5). Around 50% inhibition was obtained using 200 ppm at 0.95 and 0.92 a_w. Moreover, growth was completely inhibited at 0.89 a_w using the same concentration. PP treatment, a_w, and their interaction had a significant effect on growth (Table 3.4). Like BHT, a_w was the major factor, followed by PP treatment.

The antioxidant OG was the best treatment affecting growth of *A. flavus* when compared with BHT, BHA and PP. At 50 ppm this treatment significantly reduced

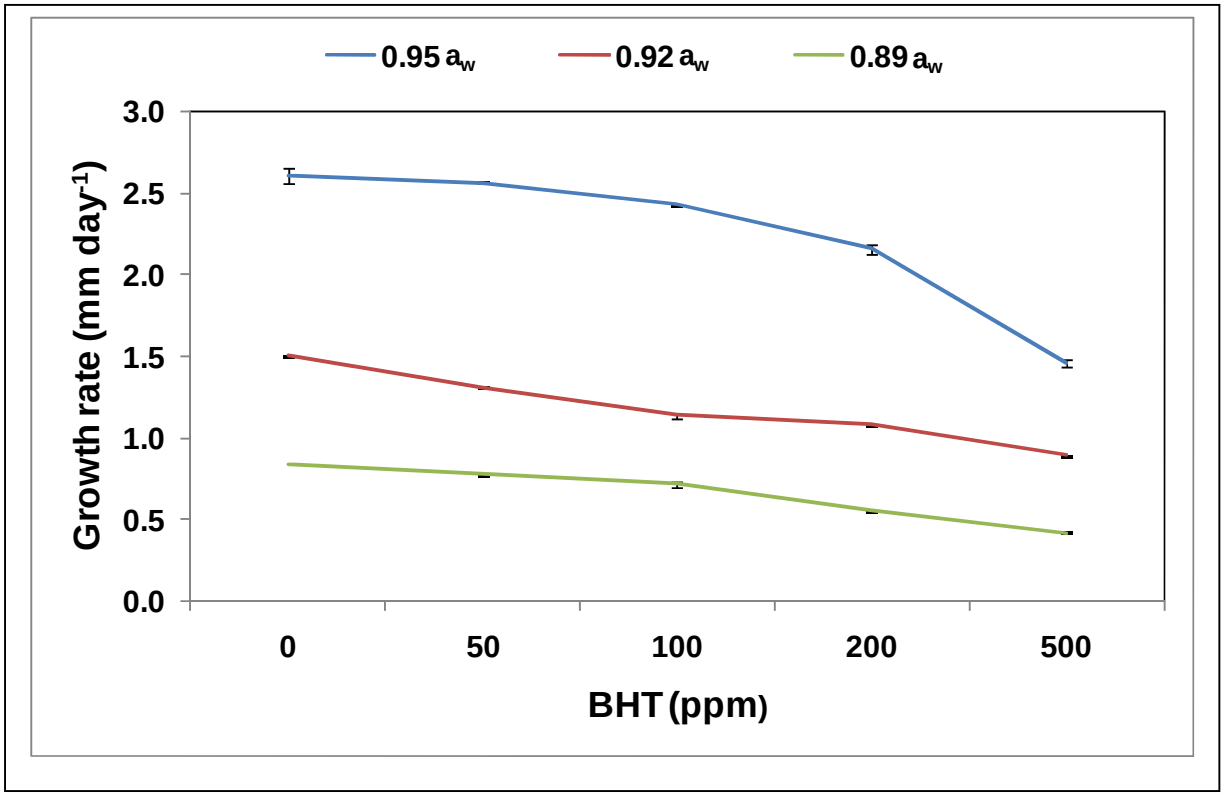


Figure 3.3. Effect of butylated hydroxytoluene (BHT) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.2. Analysis of variance of the effect of the antioxidant BHT, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	83.59192	1	83.59192	73366.09	0.00
BHT (ppm)	2.93194	4	0.73299	643.32	0.00
a_w	19.48297	2	9.74148	8549.81	0.00
BHT x a_w	0.76493	8	0.09562	83.92	0.00
Error	1.0971	30	0.00114		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

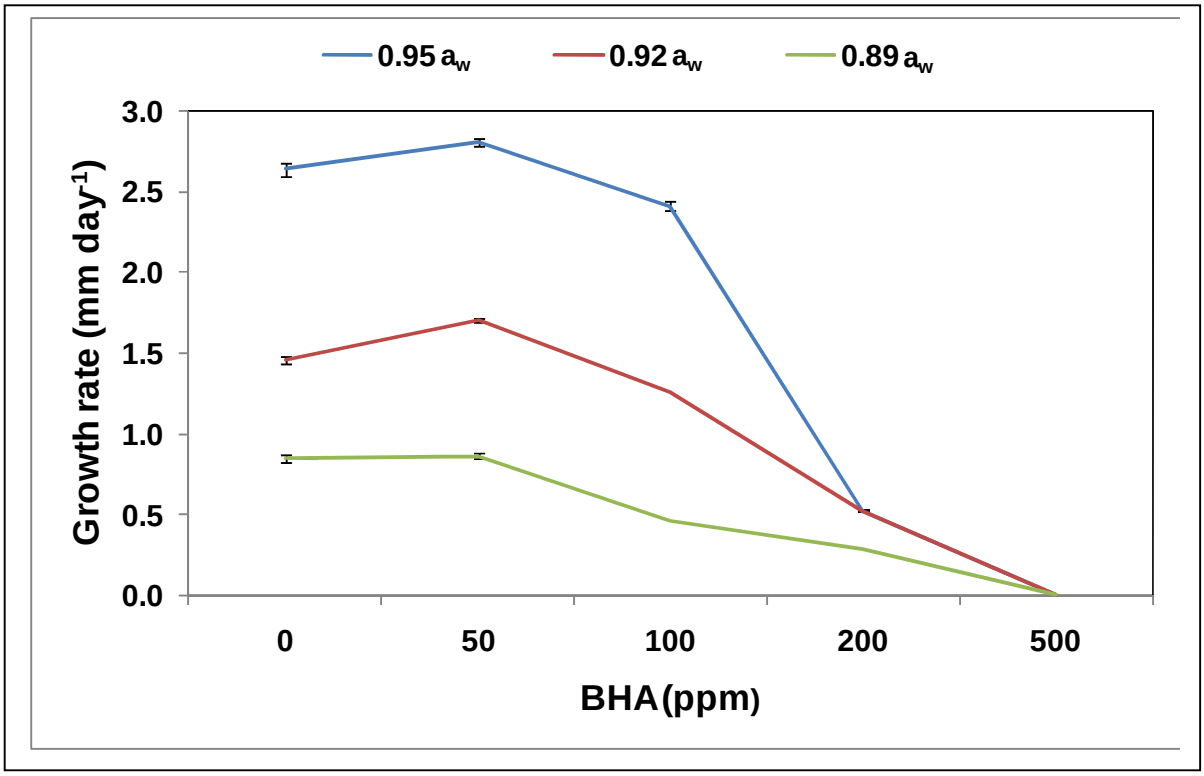


Figure 3.4. Effect of butylated hydroxyanisole (BHA) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.3. Analysis of variance of the effect of BHA, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	49.90145	1	49.90145	32877.34	0.00
BHA (ppm)	22.38077	4	5.59519	3686.37	0.00
a_w	10.58070	2	5.29035	3485.52	0.00
BHA x a_w	5.92419	8	0.74052	487.89	0.00
Error	0.04553	30	0.00152		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

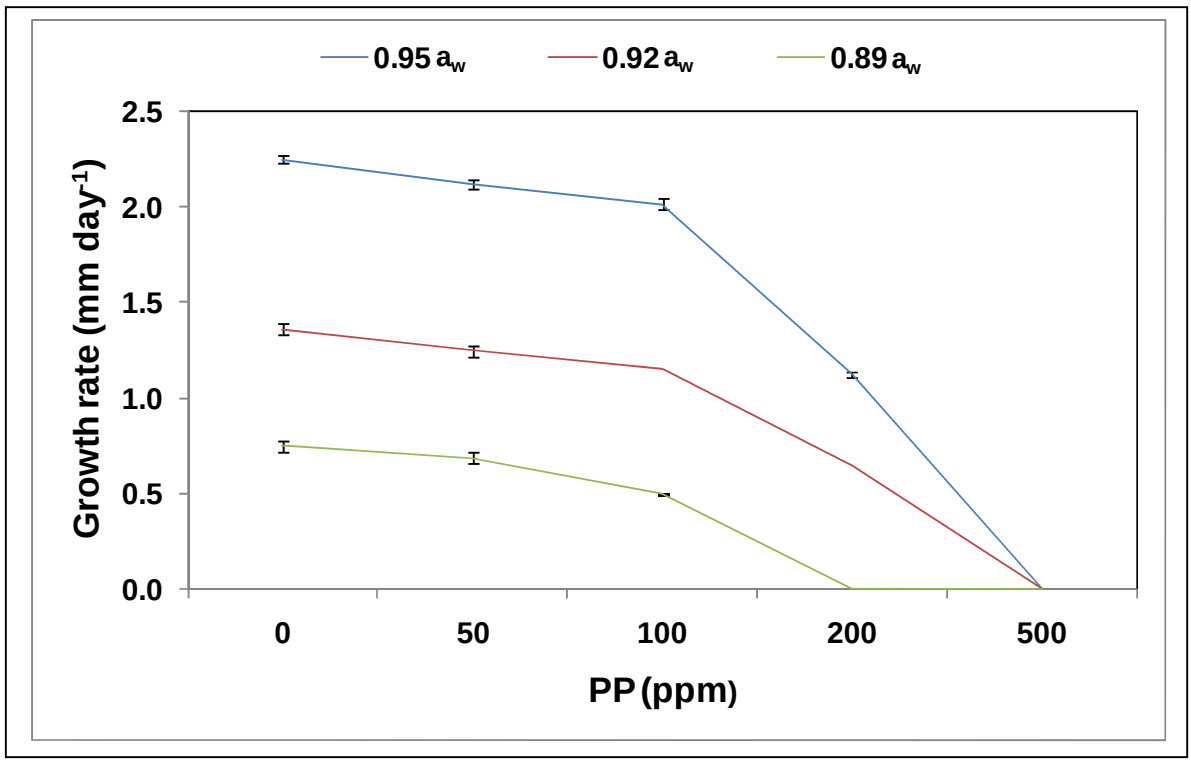


Figure 3.5. Effect of propyl paraben (PP) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.4. Analysis of variance of the effect of the antioxidant PP, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	38.26484	1	38.26484	41703.06	0.00
PP (ppm)	13.63218	4	3.40804	3714.27	0.00
a_w	9.35892	2	4.67946	5099.92	0.00
PP x a_w	2.56353	8	0.32044	349.23	0.00
Error	0.02753	30	0.00092		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

the growth by >30% under all a_w conditions when compared with controls (Figure 3.6). It suppressed mycelial growth of *A. flavus* by 78% at 200 ppm at 0.95 a_w and completely at the lower a_w levels. Similar to other antioxidants, OG treatment, a_w and their interactions significantly affected the growth rate (Table, 3.5). OG treatment was the main factor followed by a_w.

Aliphatic salts and natamycin

Growth of *A. flavus* was stimulated at 500 and 1000 ppm of SMB at each a_w level when compared with control (Figure 3.7). However, considerable inhibitory effect was observed at 2000 ppm with inhibition percentages of 49, 45 and 67% at 0.95, 0.92 and 0.89 a_w respectively. Statistically, growth was significantly affected by SMB, a_w and their interaction ($P<0.05$; Table 3.6). The a_w was the major factor which affected growth followed by the concentration parameter.

Apart from PS concentrations, around 25 and 60% inhibition was obtained at 0.92 and 0.89 a_w respectively when compared with the higher a_w level treatments (Fig. 3.8). A significant reduction was observed at all a_w condition with the highest effect at 2000 ppm when compared with the controls. Effects of PS, a_w and their interaction significantly inhibited the growth of *A. flavus* ($P<0.05$; Table 3.7). Changing a_w condition of the media was the main parameter affecting growth followed by PS treatment.

Regardless of the concentration of CP, a significant decrease in growth of *A. flavus* colonies was observed at the lowest a_w (0.89) tested when compared with those in which more water was available (0.92 and 0.95 a_w) (Fig. 3.9). In general, a dose of 3000 ppm significantly inhibited the growth of *A. flavus* at all a_w levels when compared with the control. This inhibition ranged from 18 to 32%. Statistically, all the factors considered (CP, a_w and their interaction) significantly influenced fungal growth (Table 3.8).

When compared with the controls, significant effects of NM on growth was observed using all concentration (1- 25 ppm) at 0.95 a_w (Fig. 3.10). However, this was evident at 5 ppm at the lower a_w levels. Maximum inhibition was accomplished using 25 ppm (20- 35%) for all a_w conditions. Statistical analyses on the growth of *A. flavus*, concentration of NM, a_w and their interaction were statistically significant (Table 3.9).

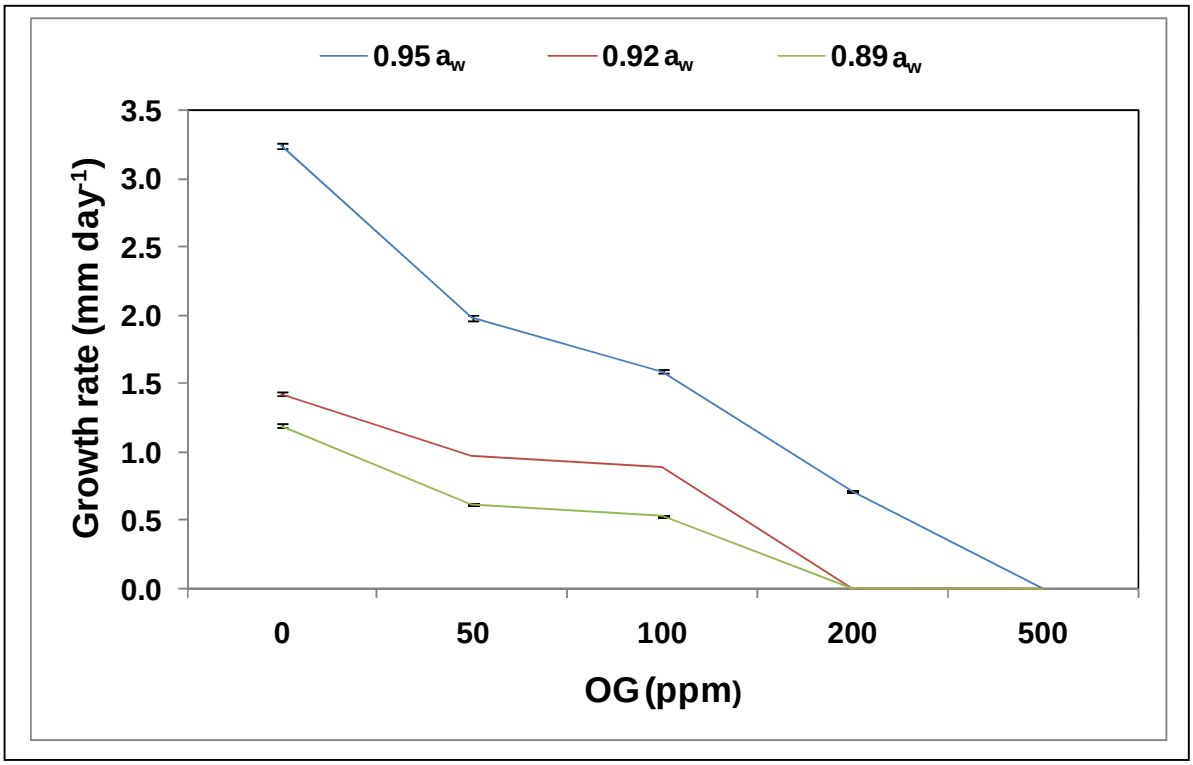


Figure 3.6. Effect of octyl gallate (OG) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.5. Analysis of variance of the effect of antioxidants OG, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	34.66403	1	34.66403	98509.43	0.00
OG (ppm)	22.10388	4	5.52597	15703.89	0.00
a_w	9.11265	2	4.55632	12948.32	0.00
OG x a_w	4.16444	8	0.52055	1479.33	0.00
Error	0.01056	30	0.00035		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

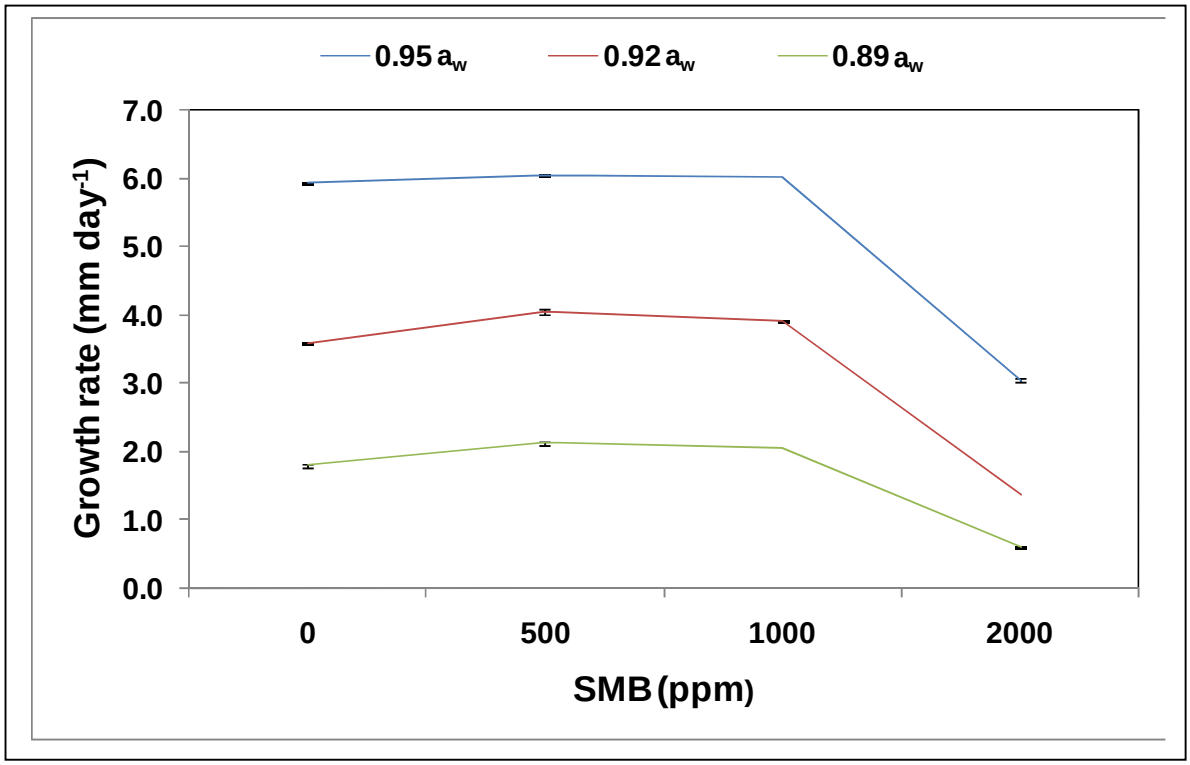


Figure 3.7. Effect of sodium metabisulphite (SMB) on *in vitro* growth of *A. flavus* at different water activity at 25°C for 7 days. Bars indicate standard error of the means.

Table 3.6. Analysis of variance of the effect of SMB, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	409.6576	1	409.6576	237457.3	0.00
SMP (ppm)	35.3448	3	11.7816	6829.2	0.00
a_w	78.8838	2	39.4419	22862.4	0.00
SMP x a_w	2.9991	6	0.4999	289.7	0.00
Error	0.0414	24	0.0017		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

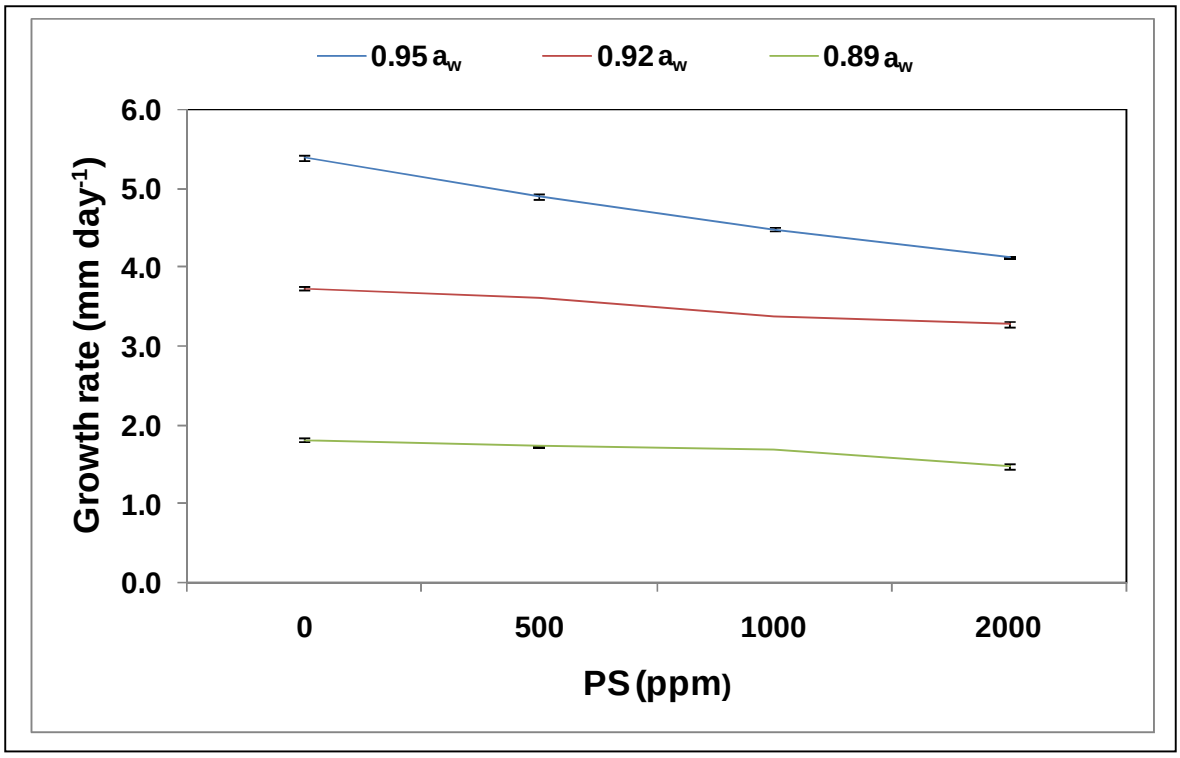


Figure 3.8. Effect of potassium sorbate (PS) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 7 days. Bars indicate standard error of the means.

Table 3.7. Analysis of variance of the effect of PS, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	392.0117	1	392.0117	262329.7	0.000000
PS (ppm)	2.3557	3	0.7852	525.5	0.000000
a_w	56.3706	2	28.1853	18861.3	0.000000
PS x a_w	0.9005	6	0.1501	100.4	0.000000
Error	0.0359	24	0.0015		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

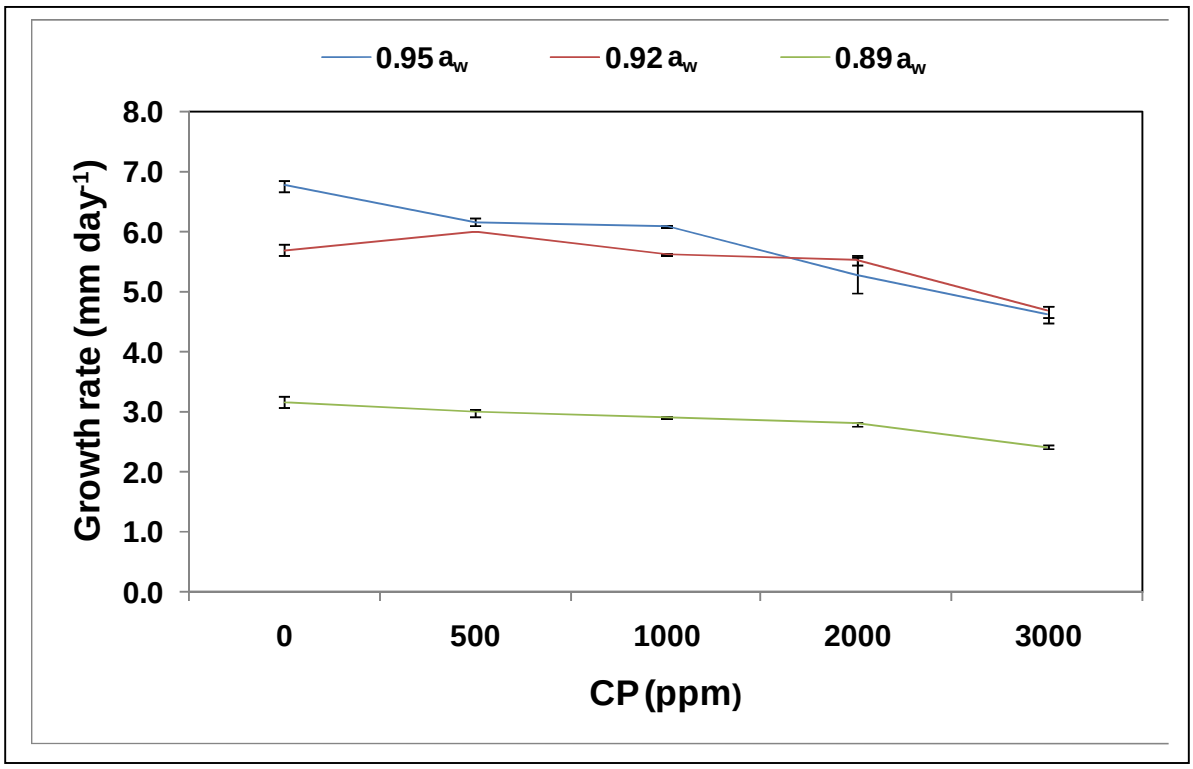


Figure 3.9. Effect of palcium propionate (CP) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 7 days. Bars indicate standard error of the means.

Table 3.8. Analysis of variance of the effect of CP, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	1000.315	1	1000.315	28622.58	0.000000
CP (ppm)	9.644	4	2.411	68.99	0.000000
a_w	78.534	2	39.267	1123.57	0.000000
CP x a_w	2.656	8	0.332	9.50	0.000002
Error	1.048	30	0.035		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

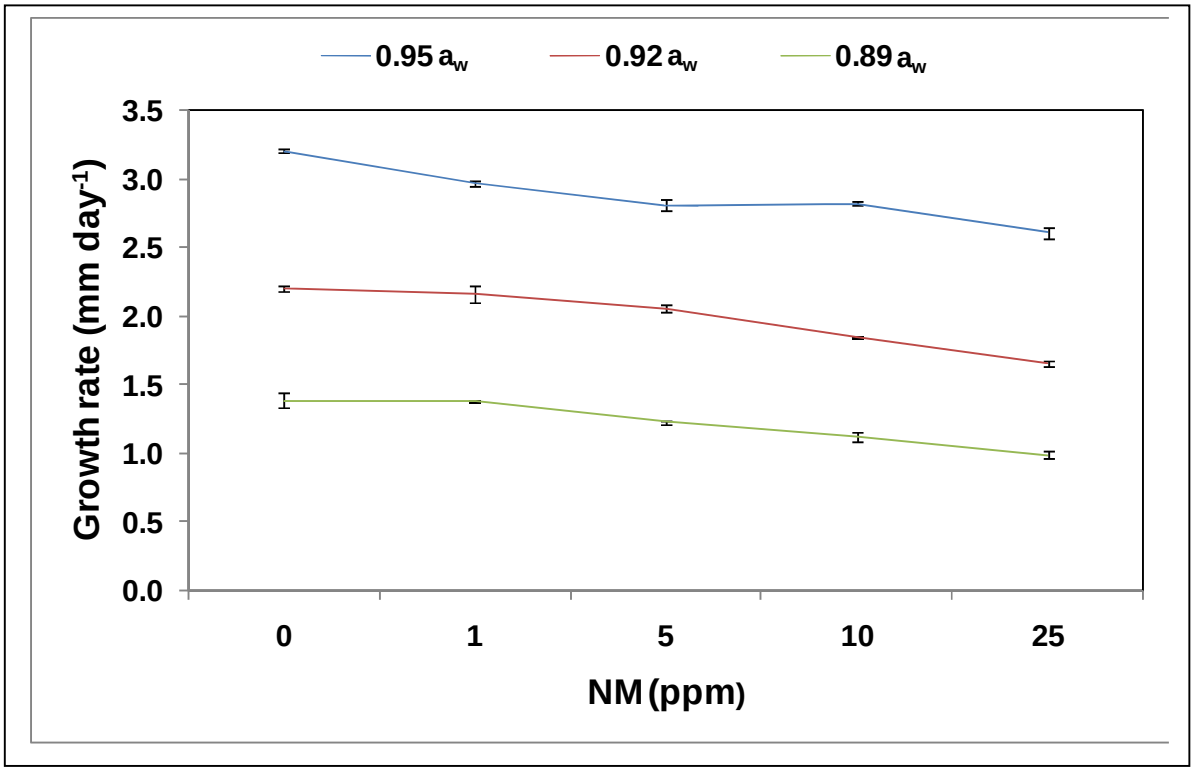


Figure 3.10. Effect of natamycin (NM) on *in vitro* growth of *A. flavus* at different water activity levels at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.9. Analysis of variance of the effect of NM, water activity (a_w) and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	184.9836	1	184.9836	57151.82	0.000000
NM (ppm)	1.4620	4	0.3655	112.92	0.000000
a_w	20.6966	2	10.3483	3197.17	0.000000
NM x a_w	0.1098	8	0.0137	4.24	0.001705
Error	1.0971	30	0.035		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Similar to aliphatic salts, a_w was the major factor followed by NM concentration which affected growth.

Most preservatives inhibited the growth of *A. flavus*, however, the best compound with the lowest LD_{50} value was OG followed by BHA over the water activity range tested (Table 3.10).

3.3.2. *In vitro* effect of preservatives on AFB_1 production by *A. flavus*

Phenolic antioxidants

The impact of antioxidant treatments on AFB_1 (log_{10} ng g^{-1} medium) production by *A. flavus* on YES at three water availability conditions at 25°C for 10 days was studied. Generally, there was a fluctuation in the amount of AFB_1 produced under different concentrations of antioxidants at all water activity levels.

Using BHT treatment resulted in a significant inhibition of AFB_1 at only at 100, 200 and 500 ppm at 0.89 a_w and 500 ppm at 0.92 a_w (Fig. 3.11). Otherwise, AFB_1 production was either unaffected or stimulated using the other concentrations at all a_w levels tested. AFB_1 production was significantly affected by BHT, a_w, and their interaction ($p<0.05$) (Table 3.11). The a_w was the major factor, with BHT the least significant one.

In general, BHA stimulated AFB_1 production by *A. flavus* regardless of a_w treatment. When compared with the controls, the highest stimulation was observed at 50 and 100 ppm (3.1 and 3.0 log unit increase, respectively) at 0.95 a_w followed by 0.92 a_w (0.8 and 1.1 log units increase, respectively). Only at 200 ppm, was AFB1 production significantly reduced at 0.95 and 0.92 a_w (Fig. 3.12). Statistical analyses showed that BHA treatment, a_w and their interaction were statistically significant ($p<0.05$) (Table 3.12). The main effect was produced by a_w followed by BHA treatment effect.

Regardless of the concentrations of PP, AFB_1 levels on YES media at 0.92 a_w were significantly higher than those at 0.95 and 0.89 a_w (Figure 3.13). Interestingly, AFB_1 was found to be unaffected at the highest a_w condition (0.95 a_w) at all concentrations (0, 50, 100 and 200 ppm) when compared with the control. However, it significantly reduced by around one log unit at the lowest a_w level (0.60 log unit) using 100 ppm

Table 3.10. LD_{50} values (ppm) of treatments used for inhibition of growth of *A. flavus* when grown at different water availability conditions at 25°C. OG is highlighted as the best treatment.

	Water activity level		
Treatment	**0.95**	**0.92**	**0.89**
BHA	141	154	113
BHT	564	690	495
OG	85	117	54
PP	200	188	118
SMP	2020	1750	1870
PS	> 2000	> 2000	> 2000
CP	> 3000	> 3000	> 3000
NM	> 25	> 25	> 25

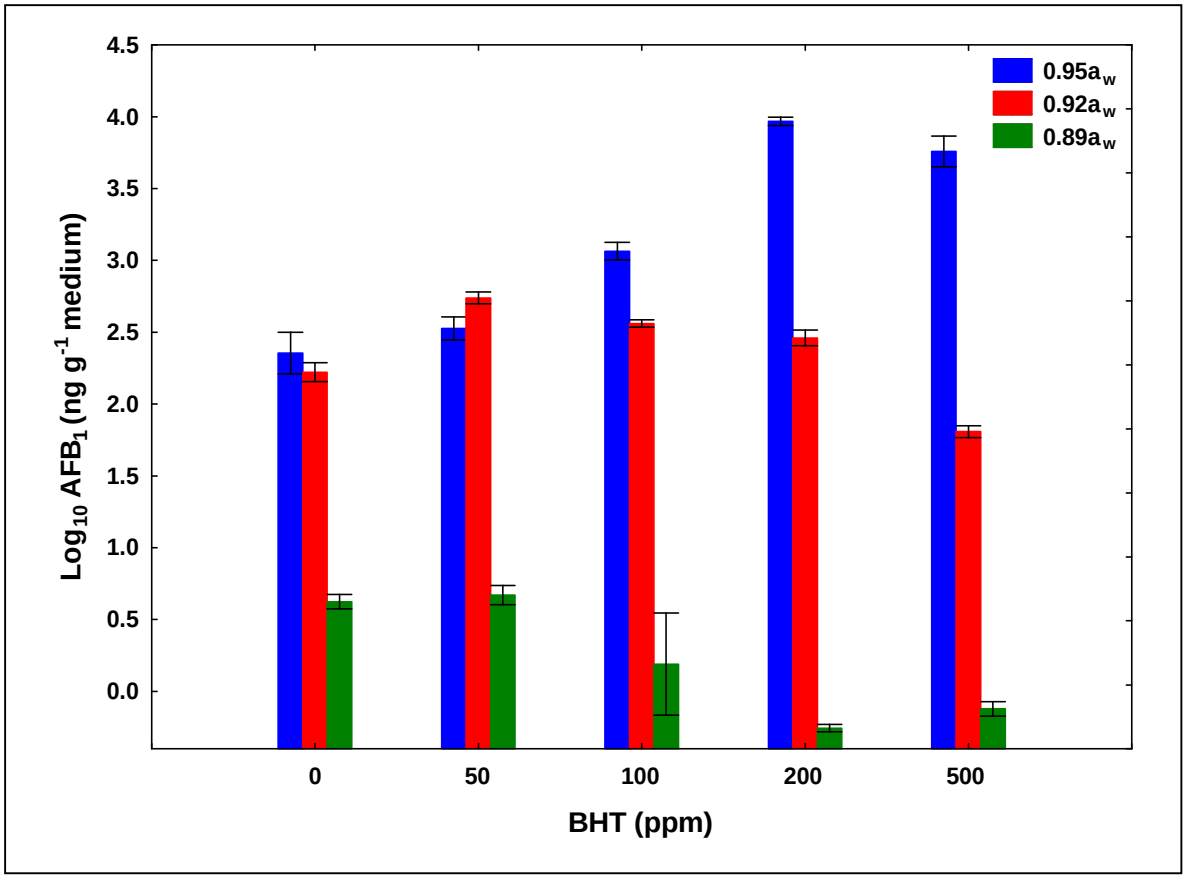


Figure 3.11. Effect of butylated hydroxytoluene (BHT) on AFB_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.11. Analysis of variance of the effect of BHT, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus.*

Effect	SS	DF	MS	F	P
Intercept	163.2078	1	163.2078	4280.473	0.000000
BHT (ppm)	0.6046	4	0.1512	3.964	0.010645
a_w	68.2365	2	34.1182	894.824	0.000000
BHT x a_w	9.2766	8	1.1596	30.412	0.000000
Error	1.1439	30	0.0381		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

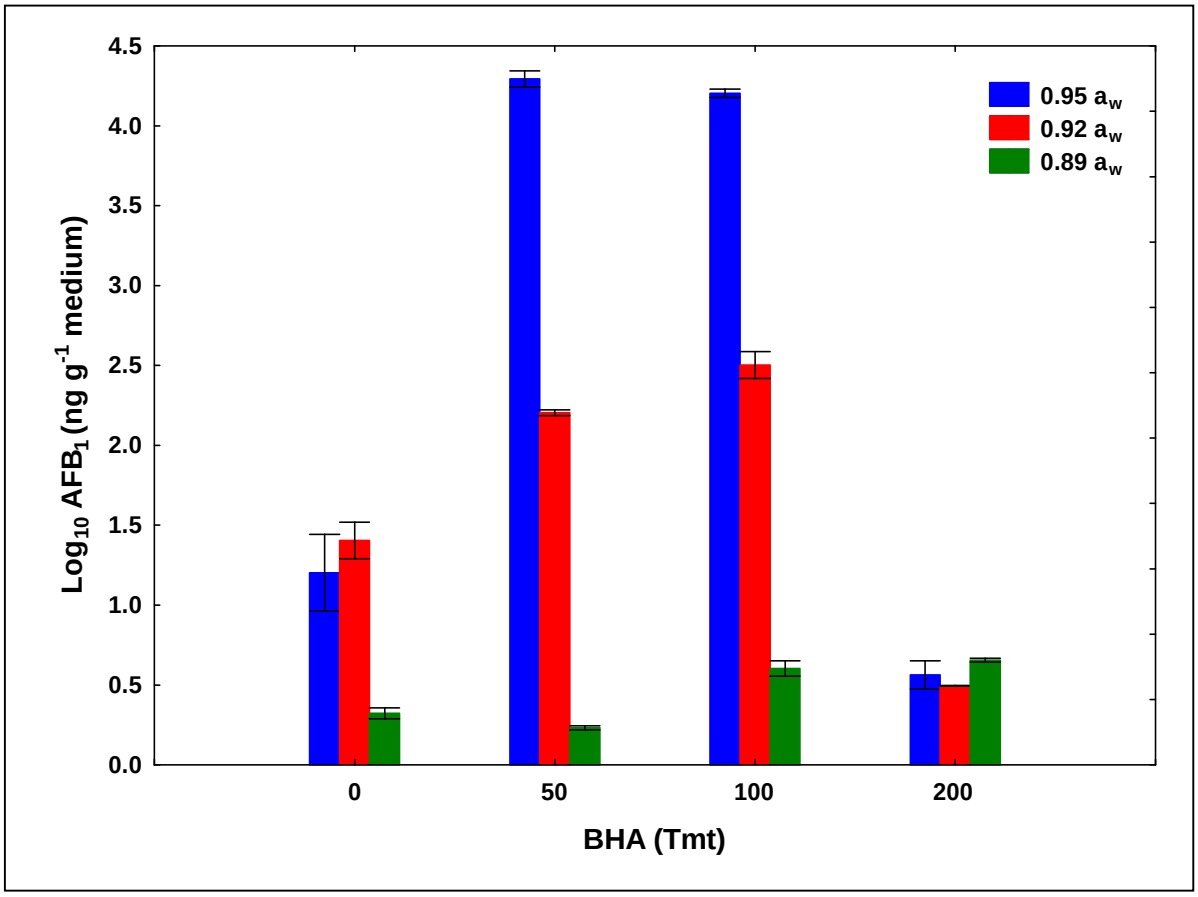


Figure 3.12. Effect of butylated hydroxyanisole (BHA) on AFB_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.12. Analysis of variance of the effect of BHA, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	87.28969	1	87.28969	3762.388	0.00
BHA (ppm)	22.96997	3	7.65666	330.020	0.00
a_w	26.91452	2	13.45726	580.039	0.00
BHA x a_w	19.30313	6	3.21719	138.668	0.00
Error	0.55681	24	0.02320		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

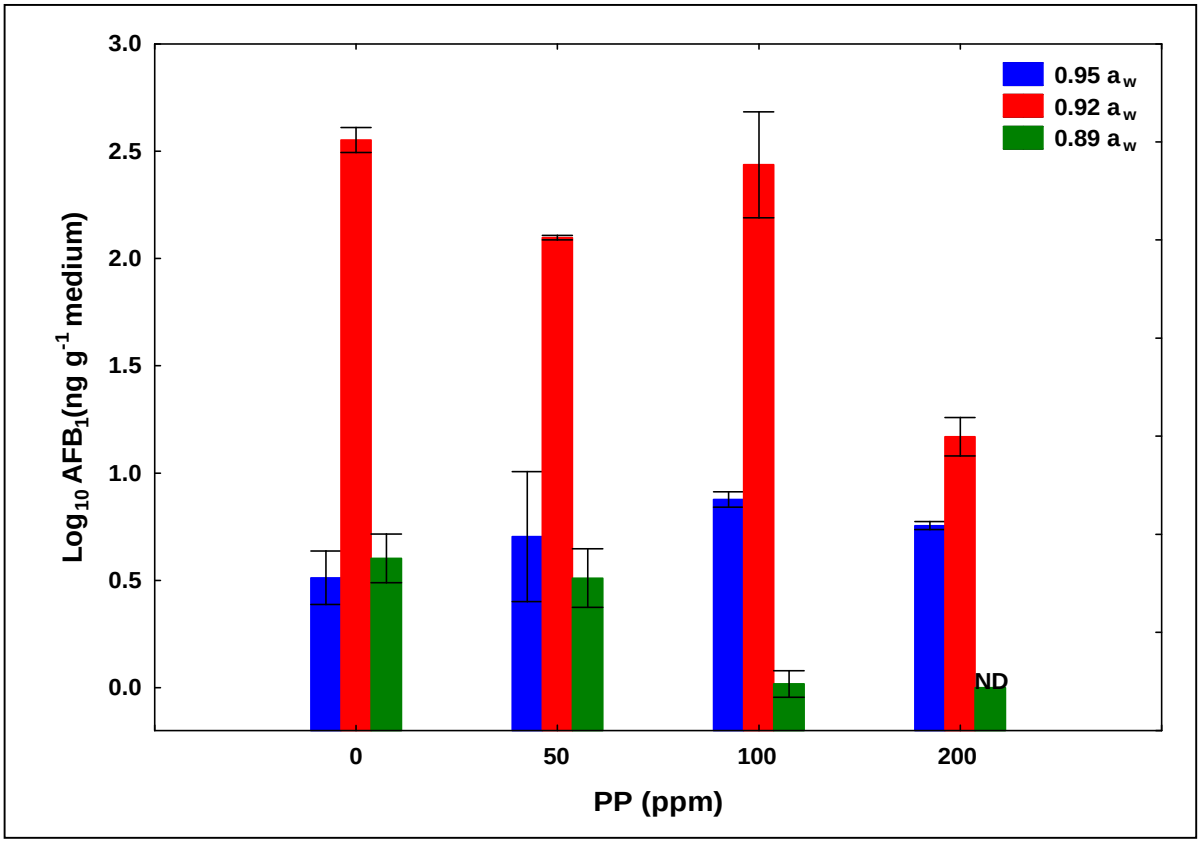


Figure 3.13. Effect of propyl paraben (PP) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days (Bars indicate standard error of the means). ND: Non detected.

and 1.4 log units at 0.92 a_w with a dose of 200 ppm. With the knowledge that there was no growth displayed on plates treated with 200 ppm at 0.92 a_w, AFB_1 was not quantified in this treatment. Similar to general patterns of BHT and BHA impacts on AFB_1 production, PP, a_w, and their interaction ($p<0.05$) showed significant effects (Table 3.13).

Regardless of OG treatment, while mycelial growth was much slower at 0.89 a_w than that of the higher a_w conditions, AFB_1 production was higher at 0 and 50 ppm (Figure 3.14). However, at other a_w levels and treatment concentrations OG was very effective at inhibiting AFB_1 production. Statistically, AFB_1 production was significantly affected by OG, a_w, and their interactions ($p<0.05$) (Table 3.14). In contrast with other antioxidants, OG treatment was the main factor affecting AFB_1 production followed by a_w factor.

Aliphatic salts and Natamycin

The impact of salts of aliphatic acids (SMB, PS and CP) on AFB_1 (log ng g^{-1} medium) produced by *A. flavus* on YES at three water availability conditions for 10 days was studied.

Figure 3.15 shows that SMB had very little effect on AFB_1 production except at 0.89 a_w and the highest concentration tested. SMB, a_w, and their interaction significantly affected AFB_1 production at $P<0.05$ (Table 3.15). SMB treatment was the main factor affecting AFB_1 production followed by a_w.

For PS, intermediate concentrations appeared to have some effect by inhibiting AFB_1 production. However, at the highest concentration tested aflatoxin production was stimulated (Figure 3.16). Statistically, all the factors considered (PS, a_w and their interaction) significantly influenced AFB_1 production by *A. flavus* at $P<0.05$ (Table 3.16). The a_w was the major effect showed significance followed by PS treatment.

Regardless of the CP concentration, the highest levels of AFB_1 were found at 0.95 a_w and decreased at 0.92 and 0.89 a_w (Figure 3.17). In general, AFB_1 production was significantly inhibited at all concentrations and a_w levels when compared with the controls. AFB_1 production was significantly influenced by CP, a_w and their interaction at $P<0.05$ (Tables 3.17). The a_w was the major effect showed significance

Table 3.13. Analysis of variance of the effect of PP, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	35.91852	1	35.91852	667.4665	0.000000
PP (ppm)	1.74107	3	0.58036	10.7846	0.000127
a_w	20.61648	2	10.30824	191.5559	0.000000
PP x a_w	2.66746	6	0.44458	8.2615	0.000077
Error	1.23770	23	0.05381		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

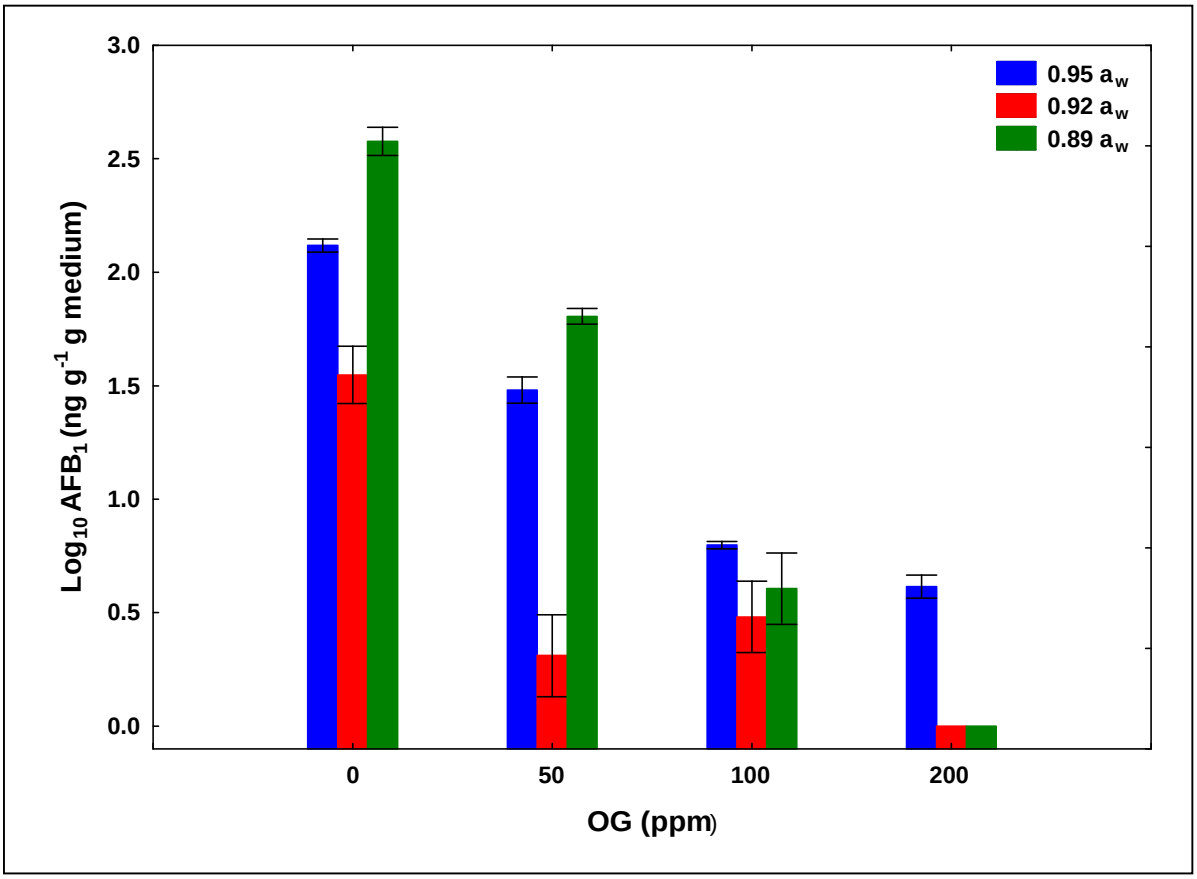


Figure 3.14. Effect of octyl gallate (OG) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.14. Analysis of variance of the effect of OG, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	36.54653	1	36.54653	1587.646	0.000000
OG (ppm)	17.73473	3	5.91158	256.809	0.000000
a_w	3.26657	2	1.63328	70.953	0.000000
OG x a_w	2.43852	6	0.40642	17.656	0.000000
Error	0.52944	23	0.02302		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

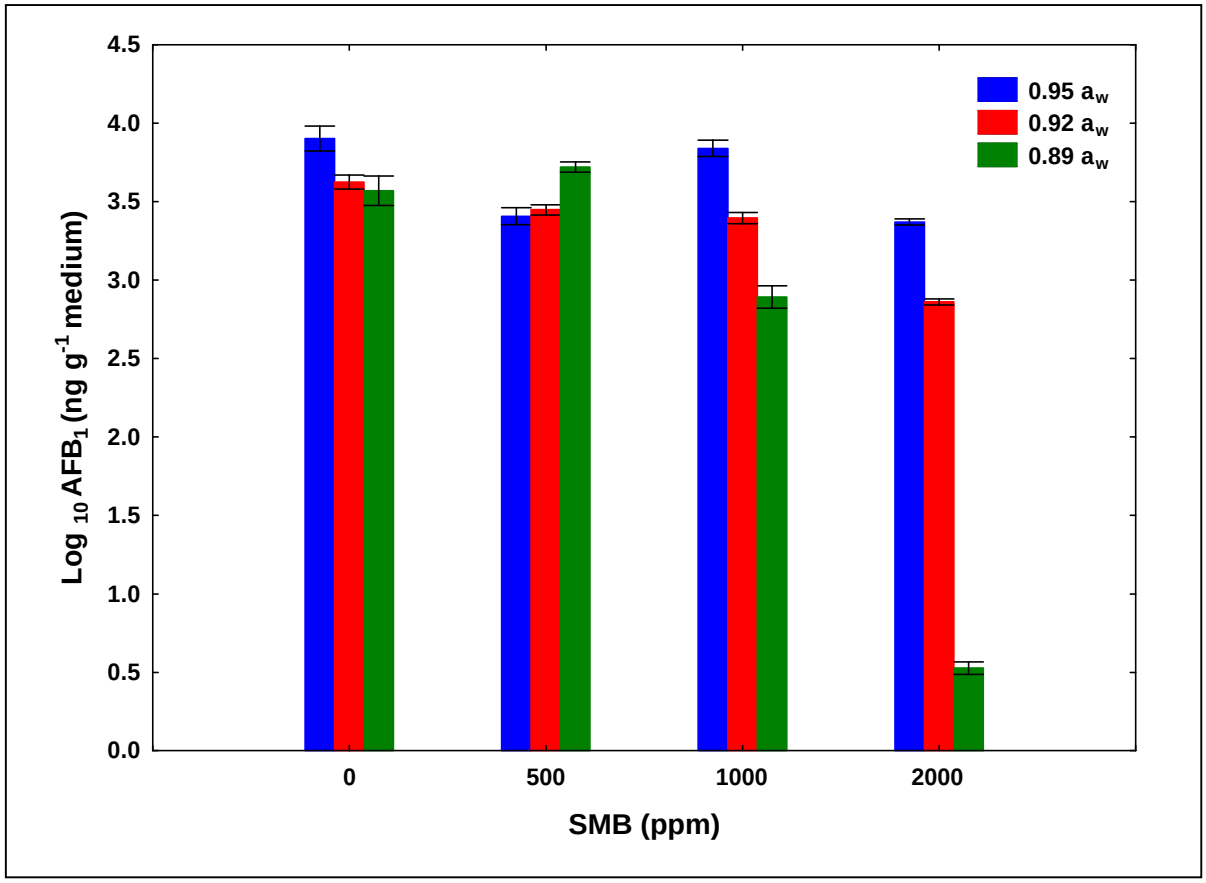


Figure 3.15. Effect of sodium metabisulphite (SMB) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.15. Analysis of variance of the effect of SMB, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	371.7361	1	371.7361	44414.36	0.00
SMB (ppm)	11.5454	3	3.8485	459.81	0.00
a_w	5.6993	2	2.8497	340.47	0.00
SMB x a_w	9.8022	6	1.6337	195.19	0.00
Error	0.2009	24	0.0084		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

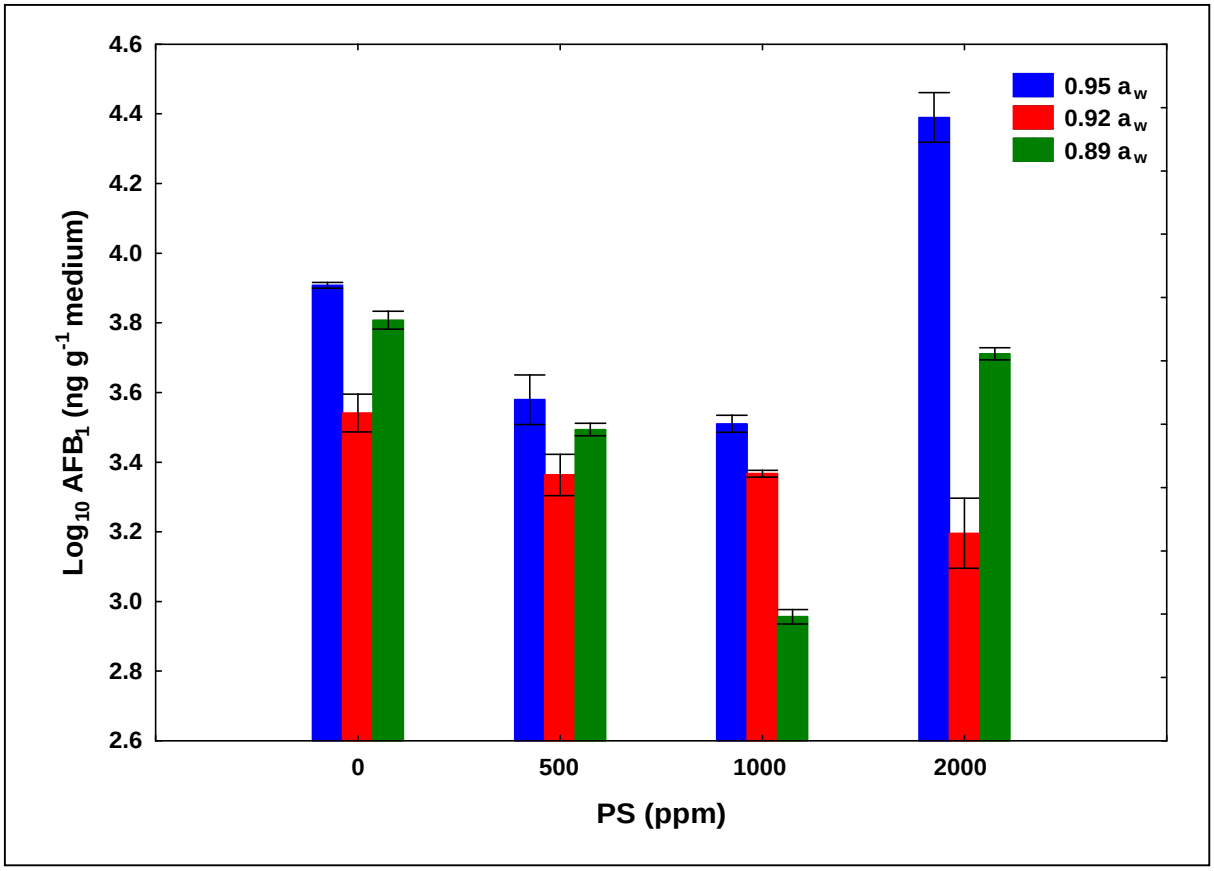


Figure 3.16. Effect of potassium sorbate (PS) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.16. Analysis of variance of the effect of PS, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	458.4968	1	458.4968	62649.80	0.000000
PS (ppm)	1.4859	3	0.4953	67.68	0.000000
a_w	1.4855	2	0.7428	101.49	0.000000
PS x a_w	1.4469	6	0.2412	32.95	0.000000
Error	0.1756	24	0.0073		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

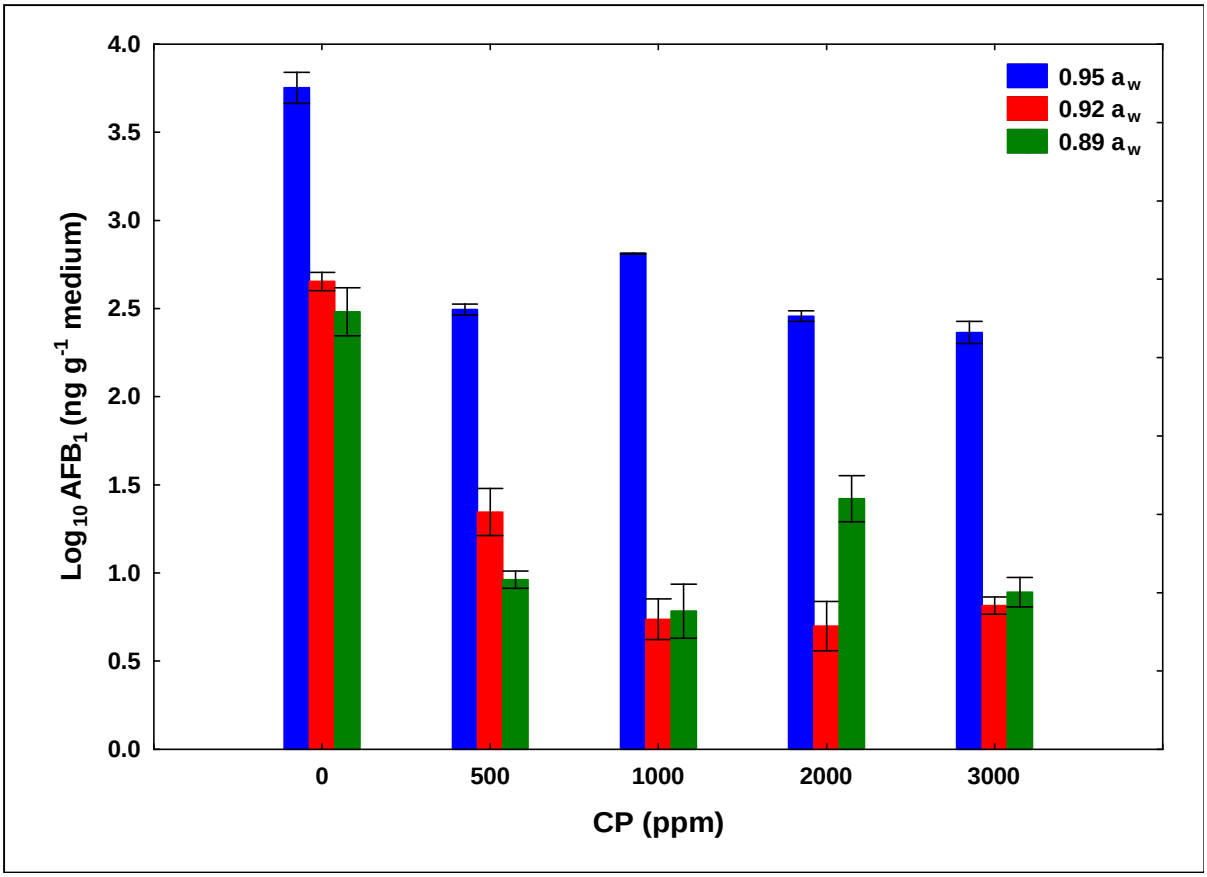


Figure. 3.17. Effect of calcium propionate (CP) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.17. Analysis of variance of the effect of CP, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	142.3406	1	142.3406	5142.227	0.000000
CP (ppm)	16.0815	4	4.0204	145.241	0.000000
aw	22.4393	2	11.2197	405.324	0.000000
CP x aw	1.9152	8	0.2394	8.648	0.000005
Error	0.8304	30	0.0277		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

when combined with PS, CP salt. Similar to PS, a_w was the main factor affecting AFB_1 production.

Natamycin appeared to have very little effect on AFB_1 production except at 0.89 a_w and the highest concentration tested (25 ppm; Figure 3.18). Analysis of variance showed significant effects of NM (1-25 ppm) and a_w as well as their interaction on AFB_1 production by *A. flavus* at $P<0.05$ (Table 3.18). The major effects were produced by a_w followed by NM treatment and by interactions of a_w x NM.

Table 3.19 shows that of all the treatments considered, OG was the best for effectively inhibiting AFB_1 production over the a_w range examined ($ED_{50}< 80$).

3.3.3. *In situ* influence of OG on the populations of *A. flavus* and AFB_1 production in stored peanuts

Figure 3.19 shows the impact of OG treatments (100, 1000 and 2000 ppm) on populations of *A. flavus* isolated from peanuts (log_{10} CFUs g^{-1} peanuts) at 0.90 and 0.93 a_w after 7 and 14 days storage. Regardless of OG treatment, the populations on peanuts adjusted at 0.93 a_w were higher than those of 0.90 a_w. Before peanuts storage (T=0) and when compared with controls, there was no immediate effect of OG at different concentrations on the populations of *A. flavus* at 0.90 and 0.93 a_w. Statistically at 0.90 a_w and after one week storage, no change was observed in populations of the control and treated samples except at the higher dose of OG (2000 ppm) which significantly decreased the populations isolated. This trend was a little different at 0.93 a_w over 7 days storage. At 100 ppm no effect of OG was observed. However at 1000 and 2000 ppm there was some effect on *A. flavus* populations isolated from stored peanuts. This resulted in a decrease in populations by > 4 log CFUs on peanuts at both a_w levels (0.90 and 0.93 a_w).

Table 3.20 shows the ANOVA of the statistical effect of the antioxidant OG, storage time, a_w and their two and three interactions on the populations of *A. flavus* (log_{10} CFUs g^{-1} peanuts). Single treatments, OG x Time, Time x a_w interactions all significantly affected the populations isolated from stored peanuts. However, there was no effect of the interactions of OG x a_w and OG x Time x a_w. Storage time was the main significant effect followed by a_w conditions.

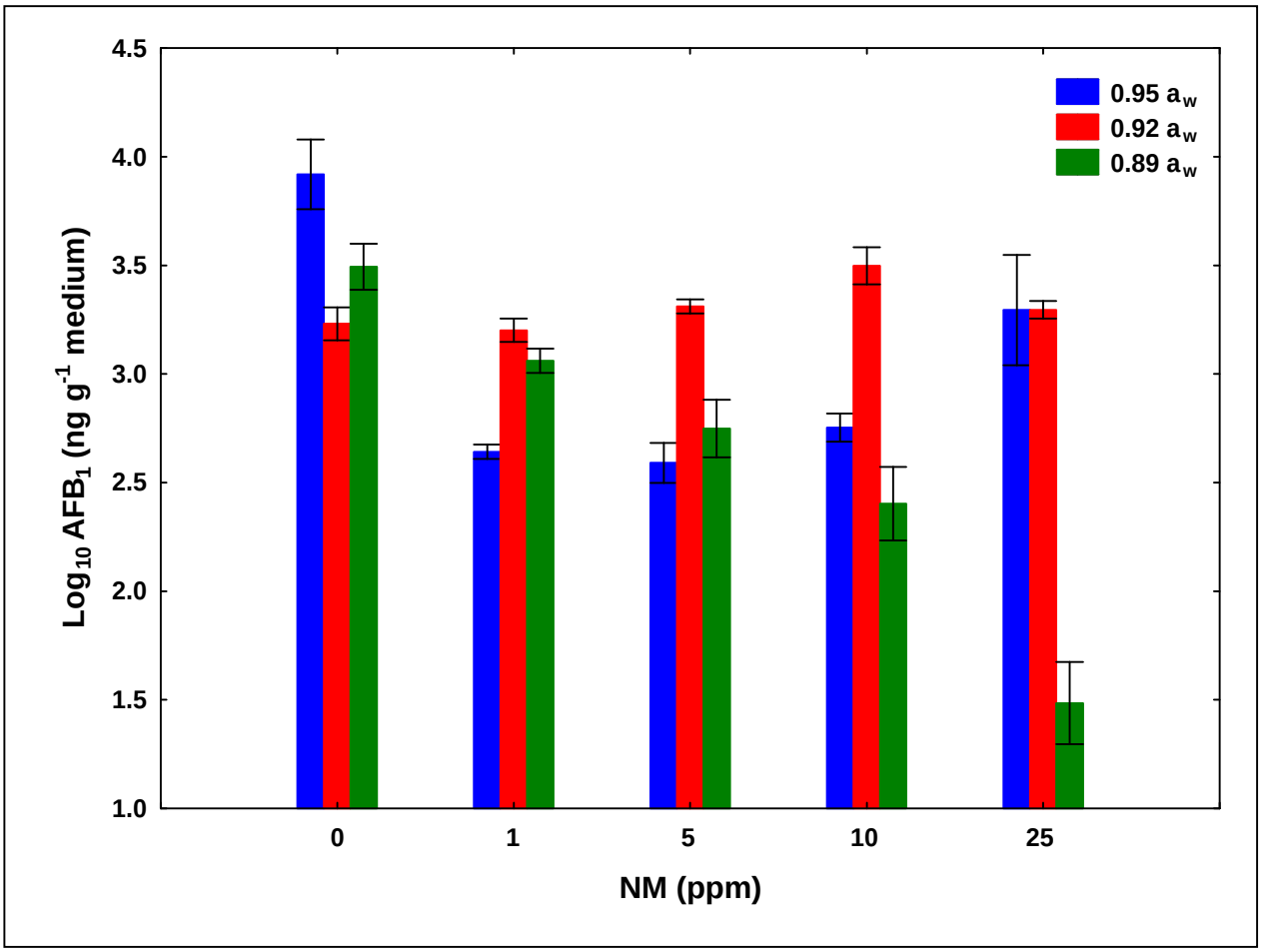


Figure 3.18. Effect of natamycin (NM) on aflatoxin B_1 production by *A. flavus* on YES at 25°C for 10 days. Bars indicate standard error of the means.

Table 3.18. Analysis of variance of the effect of NM, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus*.

Effect	SS	DF	MS	F	P
Intercept	403.7365	1	403.7365	9226.446	0.000000
NM (ppm)	3.8096	4	0.9524	21.765	0.000000
aw	3.4012	2	1.7006	38.863	0.000000
NM x aw	7.1195	8	0.8899	20.337	0.000000
Error	1.3128	30	0.0438		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Table 3.19. ED_{50} values (ppm) of treatments used for inhibition of AFB_1 production by *A. flavus* when grown at different water availability conditions at 25°C for 10 days.

	Water activity level		
Treatment	**0.95**	**0.92**	**0.89**
BHA	190	200	ST
BHT	ST	> 500	80
OG	80	35	80
PP	ST	180	75
SMP	> 2000	> 2000	1600
PS	ST	> 2000	ST
CP	>3000	510	490
NM	ST	ST	22

ST: stimulation

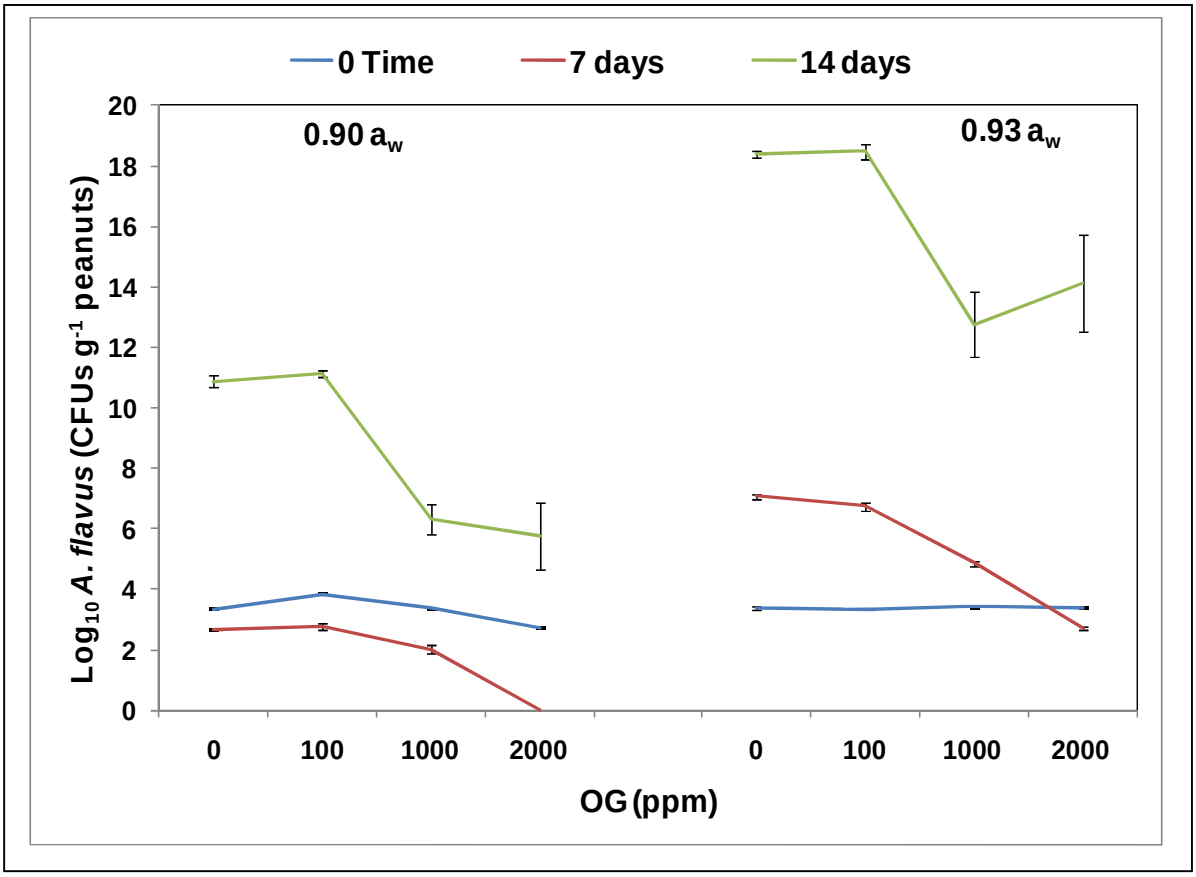


Figure 3.19. Effect of OG on populations of *A. flavus* isolated from stored peanuts at 25°C over 14 days storage. Bars indicate standard error of the means.

Table 3.20. Analysis of variance of the effect of OG (100, 1000, 2000 ppm), time, water activity (a_w) and their interactions on the populations of *A. flavus* on peanuts.

Effect	SS	DF	MS	F	P
Intercept	2671.286	1	2671.286	3532.985	0.000000
OG (ppm)	112.701	3	37.567	49.685	0.000000
Time	1169.946	2	584.973	773.672	0.000000
a_w	217.826	1	217.826	288.092	0.000000
OG x Time	74.369	6	12.395	16.393	0.000000
OG x a_w	1.927	3	0.642	0.850	0.474493
Time x a_w	157.113	2	78.557	103.897	0.000000
OG x time x a_w	4.539	6	0.757	1.001	0.437383
Error	32.512	43	0.756		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

As mentioned before, there was no direct effect of OG after initial addition to the peanuts. Consequently, the small amounts of AFB_1 detected before storage (T=0) were probably the natural background level in the peanuts (Figure 3.20). Apart from OG treatment, no significant difference in AFB_1 contents was observed after one week storage at 0.90 a_w when compared with the controls. However, the production was significantly decreased after 14 days at 1000 and 2000 ppm (1 and 1.2 log units respectively) when compared with controls. Also, the same doses (1000 and 2000 ppm) significantly inhibited AFB_1 production in stored peanut after 7 days storage at 0.93 a_w. At this level and after 14 days storage, *A. flavus* produced similar amounts of AFB_1 at the tested doses of OG when compared with control.

From a statistical point of view, all the factors considered (OG, time, a_w and their interactions) significantly influenced the toxin production except OG x a_w interaction ($P< 0.05$) (Table 3.21). Storage time was the major significant effect followed by a_w conditions as same as those of population influence.

3.4. DISCUSSION

3.4.1 *In vitro* effect of selected preservatives on the growth of *A. flavus*

Based on the studies in Chapter 2, around 90% of the *Aspergillus* section *flavi* strains isolated from peanut seeds from different regions in Egypt were aflatoxigenic. Also, 95% of these strains belonged to *A. flavus*. Since, poor storage conditions may encourage these species to produce aflatoxins in peanuts, control strategies are needed pre- and post-harvest to minimise afaltoxin contamination. The studies in this Chapter were thus conducted to examine the *in vitro* effect of some phenolic antioxidants (BHT, BHA, PP and OG), aliphatic salts (SMB, PS and CP) and NM (antibiotic) on growth of Egyptian strains of *A. flavus* and aflatoxin B_1 production. With an overview of *in vitro* studies, *in situ* studies were done using OG, which had the lowest LD_{50} and ED_{50}, on populations of *A. flavus* and AFB_1 production on peanuts. In general, all preservatives had some inhibitory effects on growth of *A. flavus* on YES medium under the a_w conditions examined. However, both aflatoxin inhibition and stimulation were observed using some of these preservatives.

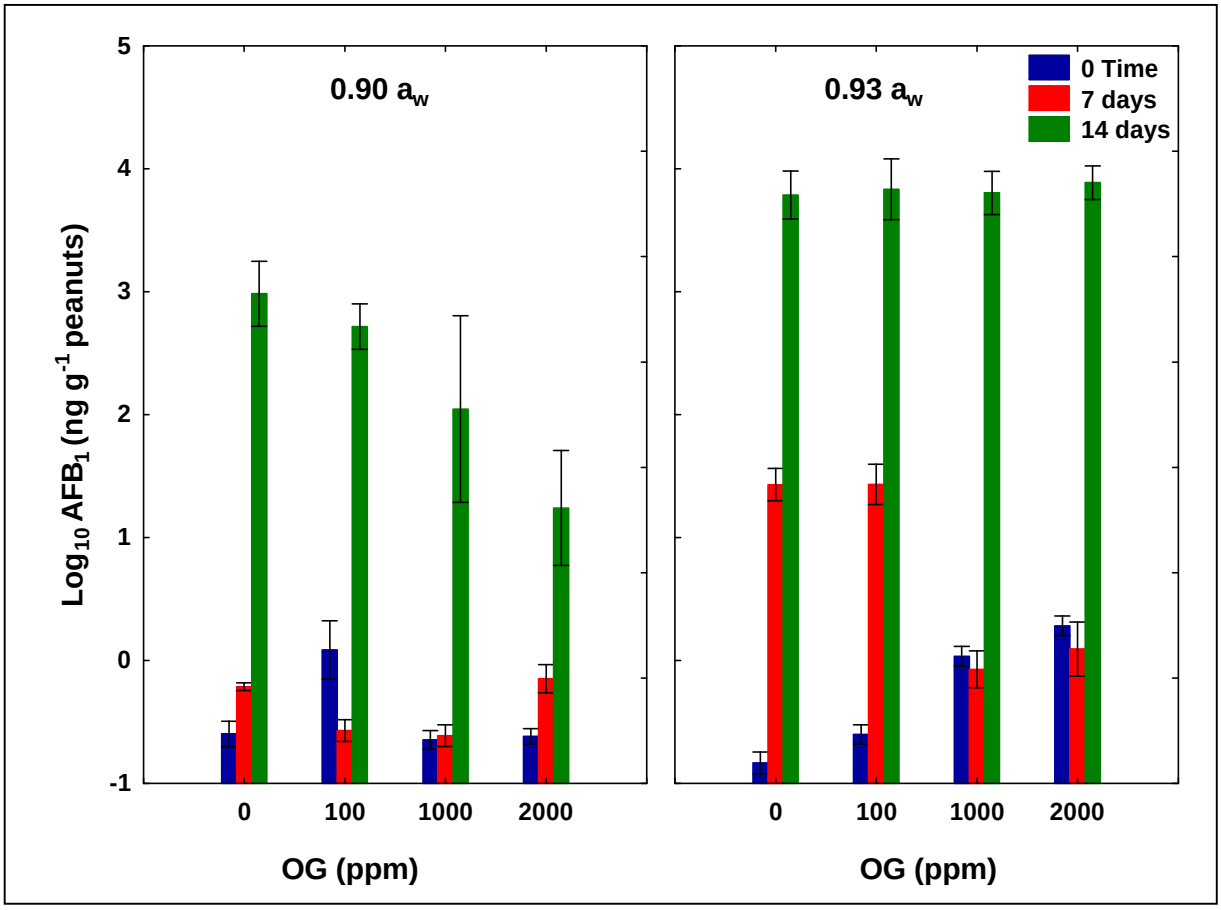


Figure 3.20. Effect of OG on AFB_1 production by *A. flavus* in stored peanuts at 25°C over 14 days storage. Bars indicate standard error of the means.

Table 3.21. Analysis of variance of the effect of OG (100, 1000 and 2000 ppm), time, water activity (a_w) and their interactions on the production of AFB_1 by *A. flavus* on peanuts.

Effect	SS	DF	MS	F	P
Intercept	51.7898	1	51.78979	263.0261	0.000000
OG (ppm)	1.7405	3	0.58016	2.9465	0.045785
Time	140.4503	2	70.22515	356.6542	0.000000
a_w	13.0080	1	13.00805	66.0643	0.000000
OG x Time	3.5148	6	0.58580	2.9751	0.018319
OG x a_w	0.6221	3	0.20736	1.0531	0.381004
Time x a_w	4.8493	2	2.42466	12.3142	0.000084
OG x time x a_w	6.7942	6	1.13237	5.7510	0.000282
Error	7.0884	36	0.19690		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Phenolic Antioxidants

In this study, the growth of *A. flavus* were found to be significantly influenced by antioxidants, a_w, and their interactions ($p<0.05$). It grew faster at the highest a_w level (0.95), with growth inhibited at 0.92 and 0.89 a_w values. Similar results were obtained by Nesci *et al.* (2003) who found that apart from the tested antioxidants (BHT, THB, BHA and PP), changes in a_w alone reduced growth of *A. flavus* and *A. parasiticus* by more than 50% at 0.937 a_w when compared with 0.982a_w. Furthermore, they demonstrated that growth was always inhibited more at lower a_w values, with complete inhibition at 0.809 and 0.747 a_w. Likewise, Passone *et al.* (2005) showed that the lag phase increased by decreasing a_w availability (0.982-0.937 a_w) for both *A. parasiticus* and *A. flavus* isolates. In a recent study, Passone *et al.* (2008) found that the population (CFUs g^{-1}) of *Aspergillus* section *Flavi* from peanuts was influenced by storage, a_w, and showed a decrease of up to 3 log units in the driest treatment (0.937 a_w).

In the present study high concentrations (500 ppm) of BHA, PP and OG completely inhibited the growth of *A. flavus*, at all a_w levels. However, with BHT this concentration reduced growth by > 40% at all a_w levels. Similarly, Nesci *et al.* (2003) found that at all a_w levels, BHA and PP, at 10–20 mmol L^{-1} (≈ 1800-3600 ppm) completely inhibited growth of *A. flavus* and *A. parasiticus* strains. None of the previous *in vitro* studies tested low concentration of antioxidants (<180 ppm) against *A. flavus*. Whereas in this study, the lowest concentration (50 ppm) of BHT, PP and OG significantly reduced growth except for BHA which did not affect growth at 0.95 a_w and stimulated growth at 0.92 and 0.89 a_w levels. Regardless of a_w levels, concentrations of 100 and 200 ppm were more effective in reducing growth using BHA, PP and OG (9-80, 11-100 and 37-100% respectively) than BHT (7.3-34.5%). This is in agreement with Passone *et al.* (2005) who reported that *Aspergillus* section *Flavi* strains showed an increase in their lag phase by between 37 and 90% and 2 and 81% when grown with 1 mmol L^{-1} of PP and BHA, respectively, regardless of the a_w . However, Nesci *et al.* (2003) found that 1 mmol L^{-1} (≈ 180 ppm) BHT and PP increased growth rates at 0.982 a_w when compared with controls. With regard to BHT, Nesci *et al.* (2003) demonstrate that at 10–20 mmol l^{-1} reduced growth by 44-88% at 0.982 a_w. However, Lin and Fung (1983) reported that BHT (0.001, 0.005,

0.01, 0.02 g per plate) did not inhibit growth of six *Aspergilli* strains and species on a solid medium and in salami.

There is no available comparable data for use of OG to control growth and aflatoxin production by *A. flavus*. However, Kubo *et al.* (2001) found that among three tested gallates (C3, propyl, C8, octyl and dodecyl), octyl gallate exhibited the best fungicidal activity with a MIC (minimum inhibitory concentration) of 100 ppm against *A. niger.* Whereas, >3200 and >400 ppm with propyl and dodecyl gallate were required, respectively. Also, Fujita and Kubo (2002) reported that minimal fungicidal concentration of OG was 25 and 50 ppm against *Saccharomyces cervisiae* and *Zygosaccharomyces bailii*, respectively.

At present, information on the mechanism of action of antioxidants on *Aspergillus* species is limited. However, BHA and PP appear to affect the cell membrane, eliminating the pH component of the proton active force and affecting energy transduction and substrate transport processes (Adams and Moss, 1995). Parabens are broad spectrum antimicrobial agents. Because of their high pK values, they are effective at high pH values against bacteria, yeasts and moulds. The antimicrobial action of parabens include several targets in microbial cells: (i) they may inhibited the functions of several enzymes, (ii) they dissolve in membrane lipids and interfere with membrane functions, including transport of nutrients, (iii) they also interfere with the synthesis of protein, RNA and DNA, and in addition (iv), they destroy the membrane potential similar to other weak organic acids (Eklund, 1989). BHT probably acts via a non-specific mechanism involving the perturbation of membrane function (Singer and Wan, 1977). The primary fungicidal activity of octyl gallate comes from its ability to act as a non-ionic surface-active agent (surfactant), affecting the extracytoplasmic region which causes the lethal effect (Fujita and Kubo, 2002).

Aliphatic acids and natamycin

Regardless of SMB concentrations, significant reduction was observed in the growth rate of *A. flavus* at lower a_w levels (0.92 and 0.89). Concentrations of 500 and 1000 ppm stimulated growth at all a_w levels. However, 2000 ppm considerably reduced growth by > 45%. These results are the opposite of those of Chourasia (1993) who indicated that sodium metabisulphite (1000 ppm) did not permit mycelial growth in

SMKY liquid but allowed production of sclerotia on solid media. Mechanism of the fungal inhibition by SMB can be explained through the interaction of sulfite ions with metabolic systems that are cellular intermediary metabolism, energy production, protein biosynthesis, DNA replication and membranes (Gould and Russell, 1991).

A gradual decrease in the growth of *A. flavus* at all a_w levels was observed using PS except the doses 500 and 1000 ppm at 0.95 a_w at which gave the same inhibition effect when compared with controls. At the highest concentration (2000 ppm), 24.1, 10.8 and 16.7% inhibition were achieved. Using higher concentrations to inhibit the growth agrees with previous work by Mahjoub and Bullerman (1986) who demonstrated that only 6000 ppm of PS delayed growth of *A. flavus* on whole olives and olive paste at 25°C for 15 days. Likewise, Panfili *et al.* (1992) found that lower concentrations (500 to 1000 ppm) had no effect on growth of *A. parasiticus* on wheat seeds**.** However, Chakrabarti and Varma (2000) found that *A. flavus* isolated from salted dried fish was most sensitive to PS at low concentrations (200 ppm). Pupovac-Velikonja *et al.* (1986) found that PS stimulated fungal growth of *A. parasiticus* on apple juice culture at 100 ppm.

The highest concentration (3000 ppm) of CP revealed the best inhibition at all a_w levels (18-32%). In contrast, 500 ppm concentration stimulated growth at 0.92 a_w (5.3%) when compared with the controls. Earlier studies by Gowda *et al.* (2004) reported that propionic acid at 1000 - 5000 ppm completely inhibited *A. parasiticus* growth. However, sodium propionate had moderate antifungal properties (36% reduction). It is also known to be metabolised by some *A. flavus* strains.

The various modes of action against fungi using weak acid preservatives like sorbate and propionate, are through membrane disruption (Bracey *et al.*, 1998), inhibition of the functions of essential metabolites (Krebs *et al.*, 1983), stresses on pH homeostasis (Bracey *et al.*, 1998) and through the accumulation of toxic anions within the cell (Eklund, 1985). The principle mode of action of weak acid preservatives, however, is believed to be an increase of pH inside the cell causing an accumulation of protons and anions which cannot re-cross the plasma membrane (Brul and Coote, 1999).

At the lowest concentration of NM tested (1ppm), the growth was only reduced at 0.95 a_w. Whereas, it was significantly inhibited at the higher concentrations (5, 10 and 25 ppm) at all a_w levels when compared with controls. At 25 ppm 19, 25 and 35% inhibition were observed at 0.95, 0.92 and 0.89 respectively. Similar results obtained by Rusul and Marth, (1988) who demonstrated that the presence of 20 ppm NM inhibited growth of *A. parasiticus* after 7 days incubation at 28°C on glucose yeast extract-salt medium. On the other hand Mahjoub and Bullerman (1986) observed that *A. flavus* was completely inhibited at high concentration of Natamycin (160 and 320 ppm) on whole olives for 21 days and olive paste for 7 and 15 days, respectively. Medina *et al.* (2007) found that at 15°C, 5-10 ppm natamycin was effective in reducing growth of *A. carbonarius* almost completely on a fresh red grape extract medium. However, at 20-25°C and all the three a_w (0.98, 0.96, 0.94) levels, growth was only slightly inhibited by 5-10 ppm natamycin. Natamycin, as with other polyene antibiotics, can interact with ergosterol in the plasma membrane of fungi resulting in a loss of membrane function (te Welscher *et al.,* 2008).

3.4.2 *In vitro* effect of selected preservatives on AFB_1 production by *A. flavus*

Antioxidants

Overall, aflatoxin production in the present study fluctuated depending on the different concentrations of antioxidants and a_w levels tested. The inhibitory effect of BHT was clear at the lowest a_w condition (0.89 a_w), thus 100-500 ppm significantly reduced AFB_1 production. Also, this effect was observed at 0.92 a_w at 500 ppm. However at the higher water availability (0.95 a_w) BHA treatment showed a stimulatory effect. This may mean that BHA may only be effective against *A. flavus* at low a_w levels (drier conditions).

AFB_1 production by *A. flavus* was significantly stimulated at 50-100 ppm of BHA, especially at 0.95 and 0.92 a_w (around 3 and one log units, respectively). However, at 200 ppm it was significantly reduced under these a_w conditions. My results contrast with those obtained by Lin and Fung, (1983) who found that BHT (0.001-0.02 g per plate ≈ 50-1000 ppm) did not inhibit toxin production by 3 *Aspergilli* strains. However, aflatoxin production in the presence of BHA was significantly

reduced (P< 0.05). Likewise, Passone *et al.* (2005) observed that AFB_1 levels produced by the *Aspergillus* isolates tested in Argentina were stimulated using BHT, regardless of the a_w levels.

At the highest a_w level (0.95 a_w) the production was either unaffected (50 ppm) or stimulated (100-500 ppm) using PP antioxidant. However, at 0.89 a_w 100 and 200 ppm effectively decreased AFB_1 production at 0.89 a_w and 0.92 a_w respectively. Passone *at al.* (2005) concluded that the *Aspergillus* group isolates tested in their study differed in their sensitivity to the different antioxidants, although BHA and PP were effective in preventing the growth and AFB_1 production at high concentrations of 10 and 20 mmol L^{-1} (≈1800-3600 ppm).

OG treatment was the best antioxidant which significantly reduced AFB_1 production on YES media. The results indicated that the production was significantly controlled at all a_w conditions and all concentrations (50-200 ppm) when compared with controls. There are no available studies on the effect of OG on aflatoxin production by aflatoxigenic fungi. However, Lin and Fung (1983) tested propyl gallate against these fungi. They found that it had no effect on strains of *Aspergilli* at 0.001-0.02 g per plate (≈50-1000 ppm) concentrations.

Most previous studies have shown that using antioxidants reduced the aflatoxin production depending on type of *Aspergillus section flavi* strain used. However, at the same time some of them indicated that the antioxidant stimulated production. Velikonja *et al.* (1987) found that BHA (100-400 ppm) suppressed toxin accumulation by *A. parasiticus* in apple juice (pH 2.5) over a 15 day period. However, earlier peaks of toxin accumulation suggested environmental adaptation of the mould. They demonstrated that BHT concentration of 200 ppm led to a reduction of toxin accumulation by approximately 45%. At 100 ppm, however, BHT stimulated aflatoxin production (1.9 times more G_1 and 7 times more B_1 than the controls).

Aliphatic acids and natamycin

There was no stimulation effect on AFB_1 production using SMB at all concentration. The best results were found at 2000 ppm under all a_w conditions especially at 0.89 a_w which considerably inhibited AFB_1 accumulation (3 log units) when compared with control. Chourasia (1993) indicated that sodium metabisulphite (1000 ppm) did not

permit aflatoxin biosynthesis in SMKY liquid but allowed production of sclerotia and aflatoxin on solid media.

The addition of PS at 500 and 1000 ppm significantly decreased AFB_1 production at all a_w levels, especially at 0.89 a_w using 1000 ppm (0.85 unit). However, at the highest concentration (2000 ppm) AFB_1 production was only decreased at 0.92 a_w. Panfili *et al.* (1992) found that lower concentrations of PS (50 to 1000 ppm) stimulated aflatoxins production by *A. parasiticus* on wheat. However, Pupovac-Velikonja *et al.* (1986) observed that potassium sorbate at 100-400 ppm inhibited toxin production (no detectable amounts of AFB_1 and 3 to 5 times less AFG_1 than in controls).

Generally, CP achieved the best inhibitory effect among the tested aliphatic salts against the accumulation of AFB_1 on media. A significant effect (1.3-1.5 log units reduction) was observed at the lowest concentration (500 ppm) at all a_w levels when compared with the controls. The same pattern was obtained with higher concentrations except that of 2000 ppm at 0.89 a_w which achieved less effect inhibition (1 log unit). Bintvihok and Kositcharoenkul (2006) indicated that addition of calcium propionate to broilers' diets containing AFB_1 appeared to be effective in reducing toxicity. Also, Gowda *et al.* (2004) reported that propionic acid at 500-5000 ppm and sodium propionate at 1000-5000 ppm completely inhibited aflatoxin production on PDA medium.

Natamycin (NM) (1-25 ppm) significantly inhibited the production at 0.95 a_w with less effect at 25 ppm. There were no changes in the amount of AFB_1 produced by *A. flavus* using NM at 0.92 a_w. By increasing the concentration of NM the production gradually decreased at 0.89 a_w reaching the highest reduction at 25 ppm (2 log units). These results agree with those of Rusul and Marth (1988) who demonstrated that increasing the concentration of natamycin (0.5, 7.5, 10, 15 ppm) in glucose yeast extract-salt medium (pH of 5.5) decreased the amounts of AFB_1 and G_1 produced after 3 days incubation at 28°C. However at pH 3.5, no toxin production occurred after 3 days in the medium containing ≥ 7.5 ppm. The presence of 20 ppm natamycin inhibited toxin production after 7 days. Also, Medina *et al.* (2007) found that at 20°C, OTA production by *A. carbonarius* in a fresh red grape medium was

only significantly inhibited by 10 ppm NM at 0.94 a_w, whereas, at 25°C, 5 ppm NM was effective at all a_w levels (0.98, 0.96, 0.94).

Octyl gallate is currently permitted for use in food as an antioxidant additive similar to other phenolic antioxidants (Smith and Hong-Shum, 2003). In the current study, it gave the best effect overall of the tested preservatives where LD_{50} and ED_{50} for both growth of *A. flavus* and AFB_1 production ranged from 35 to 117 ppm in media. This suggested the use of this antioxidant with higher concentrations to control *A. flavus* and AFB_1 accumulation during storage of peanuts.

3.4.3 *In situ* effect of OG on *A. flavus* populations and AFB_1 production on stored peanuts

The populations of *A. flavus* on inoculated peanuts was unaffected by the addition of OG befor storage (T=0) at the two a_w levels used (0.90 and 0.93 a_w). However, after 7 days storage 2000 ppm at 0.90 a_w significantly inhibited the populations. In general, the effect of OG was clear after 14 days storage notably at the higher a_w. At 1000 and 2000 ppm there was a significant inhibition (around 5 log units) of *A. flavus* populations isolated at both levels of a_w by the end of storage when compared with the controls.

Based in *in vitro* results, 100 ppm of OG achieved around 50% growth inhibition overall a_w conditions. However, this concentration had no effect on isolation of populations from stored peanuts at both a_w levels. Passone at al., (2008) examined different combinations of BHT, BHA and PP in concentrations ranging between 10 and 20 mM each (around 2000 and 4000 ppm) at 0.982, 0.955 and 0.937 a_w. They found that most of these combinations completely inhibited the populations of aflatoxigenic *Aspergillus* after 11 and 35 days storage.

In spite of a significant effect of OG treatment (1000 and 2000 ppm) on the populatiosn after 14 days storage at 0.90 and 0.93 a_w, the treatments only significantly decreased AFB_1 production on stored peanuts at 0.90 a_w. This gives a good indicator of the efficiency of OG at the lower water availability to control AFB_1 production by *A. flavus*. What means that the possibility of using OG at an industrial scale to increase the shelf-life of natural contaminated peanuts. A study has been

conducted using a formulation of food-grade antioxidants for postharvest control of peanut *Aspergillus* section *Flavi* populations (Passone *et al.,* 2009). They reported that BHA-PP-BHT mixture (1802 +1802+ 2204 µg g^{-1}) significantly inhibited the populations of *A. flavus* when compared with controls over 5 months storage. They found no aflatoxin contamination in the controls or treated samples during storage. In their study, they determined the antioxidant concentrations after storage to ensure that the residues were under the permissible limit (100 ppm) allowed by Codex Alimentarius (2006). The initial recovery of antioxidants (within 24 hrs) ranged from 3.98-8.53 µg g^{-1}. While by the end of storage time, they ranged from 2.13 to 16.62 µg g^{-1}.

Chapter 4

CONTROL STRATEGIES USING OZONE

4 CONTROL STRATEGIES USING OZONE

4.1 INTRODUCTION

Fungal growth on stored grain causes significant reductions in both the quantity and the quality of the grain. Moreover, many species of fungi produce mycotoxins which are highly toxic to animals and humans (Paster *et al.*, 1995). Aflatoxins are one of important mycotoxins produced, primarily by strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Under poor storage management conditions (temperature and humidity), these fungi can grow on particular foods (e.g. corn products, peanuts and pistachio nuts) and produce aflatoxins. Despite improved food handling and processing, the food industry (especially cereal and oilseed industries) is unable to prevent aflatoxin contamination (Proctor *et al.*, 2004). Aflatoxin contamination of food and feed causes significant economic losses world-wide. The Food and Agriculture Organization estimates that 25% of the world's crops are lost due to aflatoxin contamination (Saad, 2001).

Fumigation is used extensively to preserve stored grains. Loss of fumigants and a trend by consumers to move away from residual chemicals necessitates the development of additional control strategies to inhibit fungal growth in order to reduce post-harvest losses of grains (Kells *et al.*, 2001; Wu *et al.*, 2001)**.** A possible solution could be represented by ozone (O_3), one of the most powerful oxidants. It is effective in oxidizing a wide spectrum of microorganisms and chemicals (Kim *et al.*, 1999). The most important advantage of O_3 over other fungi-inhibiting chemicals used in foodstuff is that it is residue-free. It decomposes to diatomic oxygen rapidly due to its short half-life, which is about 20-50 min in the atmosphere and 1-10 min in water (Mason *et al.*, 1997). O_3 was also found to be effective in the destruction and detoxification of mycotoxins, including aflatoxins (Maeba *et al.*, 1988).

Many uses of O_3 technology have been found in agriculture and the food industry. Applications of O_3 in the water, waste water treatment, and food processing are successfully applied. These include preserving raw agricultural commodities during storage and transit, controlling odours, retarding metabolic processes associated with ripening, and sanitizing water utilized for washing food equipment, foods, and packaging materials (Muthukumarappan *et al.*, 2000 and 2009).

There have been some studies to examine the efficacy of O_3 to control microfungi as part of a hygienic cleaning strategy. For example, Serra *et al.* (2003) highlighted that ozonation is able to reduce the viable airborne moulds (*Aspergillus spp*., *Cladosporium spp*. and *Penicillium spp.*) in a cheese ripening room. Nicoue *et al.* (2004) demonstrated an evident fungicidal and/or fungistatic effect of combined ozone/ions treatments on *Rhizopus stolonifer* and *Botrytis cinerea*, two common postharvest pathogens of strawberries. Vijayanandraj *et al.* (2006) studied the effect on vegetative mycelium and conidia of *Aspergilllus niger*, causing black rot disease in onion. More recently, Zotti *et al.* (2008) found that the growing of *A. flavus* was drastically reduced, and that of *A. niger* completely stopped when treated with O_3. Kells *et al.* (2001) revealed that 50 ppm ozone for 3 d resulted in 63% reduction of *A. parasiticus* on stored maize surfaces.

Several strategies are available for the detoxification of aflatoxins. The use of chemical treatments to decontaminate aflatoxin-containing commodities is being widely used to decompose AFB_1, e.g., ammonia, calcium and sodium hydroxide (Mukendi *et al.,* 1991; Neal *et al.,* 2001; Park, 2001), sodium bisulphite (Yagen, 1989), hydrated sodium calcium aluminosilicates (Phillips, 1999; Huwig *et al.*, 2001). To be effective and adoptable by industry, such methods need to be not only efficacious, but also cost-effective and adaptable to industrial production settings. Ozone gas treatment has the potential to meet these criteria since the product can be treated in a short time and used immediately without need for additional processes (Muthukumarappan *et al.*, 2000).

Therefore, the objectives of this study were (1) to investigate the efficacy of gaseous O_3 on germination, sporulation and aflatoxin production of two strains of *A. flavus* (EGP-B07 isolated from peanuts and SRRC-G 1907 as a reference) on YES media under different a_w conditions, (2) to evaluate the effect of O_3 treatment on the isolation of populations of *A. flavus* from stored peanuts before storage and on its shelf life, (3) to quantify the level of aflatoxin control in comparison with untreated samples and finally (4) to find a relationship between O_3 treatment, populations of *A. flavus* isolated and aflatoxin production after storage.

4.2 MATERIALS AND METHODS

4.2.1 Fungal strains

As described in Section 3.2.1, the concentrations 10^5, 10^6 and 10^7 spores ml^{-1} of *A. flavus* EGP-B07 were prepared to use for inoculation of either media or peanuts.

4.2.2 Media

Malt extract agar (MEA), malt salt agar (MS): The same procedure as described in Section 2.2.2 was used for preparation of these media.

Yeast extract sucrose agar (YES): In addition to the normal YES medium (0.97 a_w) prepared as in Section 2.2.2. The water availability of the media was adjusted using glycerol to obtain 0.95, 0.92 and 0.89 a_w (See Section 3.2.2). Also, the media was poured into 55mm diameter Petri dishes (Fisher Scientific Ltd., Loughborough, Leicestershire, UK) instead of 90mm ones.

4.2.3 Preparation of peanut samples

Peanut samples in culture vessels were autoclaved as described in Section 3.2.5 and then adjusted to 0.93 a_w. Peanuts were inoculated by adding 250 µl from spore suspension 10^5 and 10^7 ml^{-1} at room temperature in order to obtain a final concentration of 10^3 and 10^5 spores g^{-1}, by mixing thoroughly. Then, the inoculated peanuts into vessels were treated with O_3 in aseptic conditions. Controls were treated with air at the same flow rate instead of O_3.

4.2.4 Apparatus for carrying out ozonation

Ozone gas was generated from laboratory corona discharge ozone generator (C-Lasky, Model CL-010-DS, AirTree Ozone Technology Co., Ltd., Sijhih City, Taipei County 22150 Taiwan) using air at a flow rate 6 L $min.^{-1}$ (Figure 4.1). Either YES plates or vessels containing peanuts (without lids) were placed in an O_3 reactor jar (Kilner glass jar, 3200 g) and tightly closed. Ozone gas was applied through the lid of the reactor to get the required concentrations. The concentrations of O_3 in the exit gas from the reactor were measured by the O_3 Analyzer (Model UV 100, Eco Sensors Inc., Santa Fe, New Mexico 87505 USA).

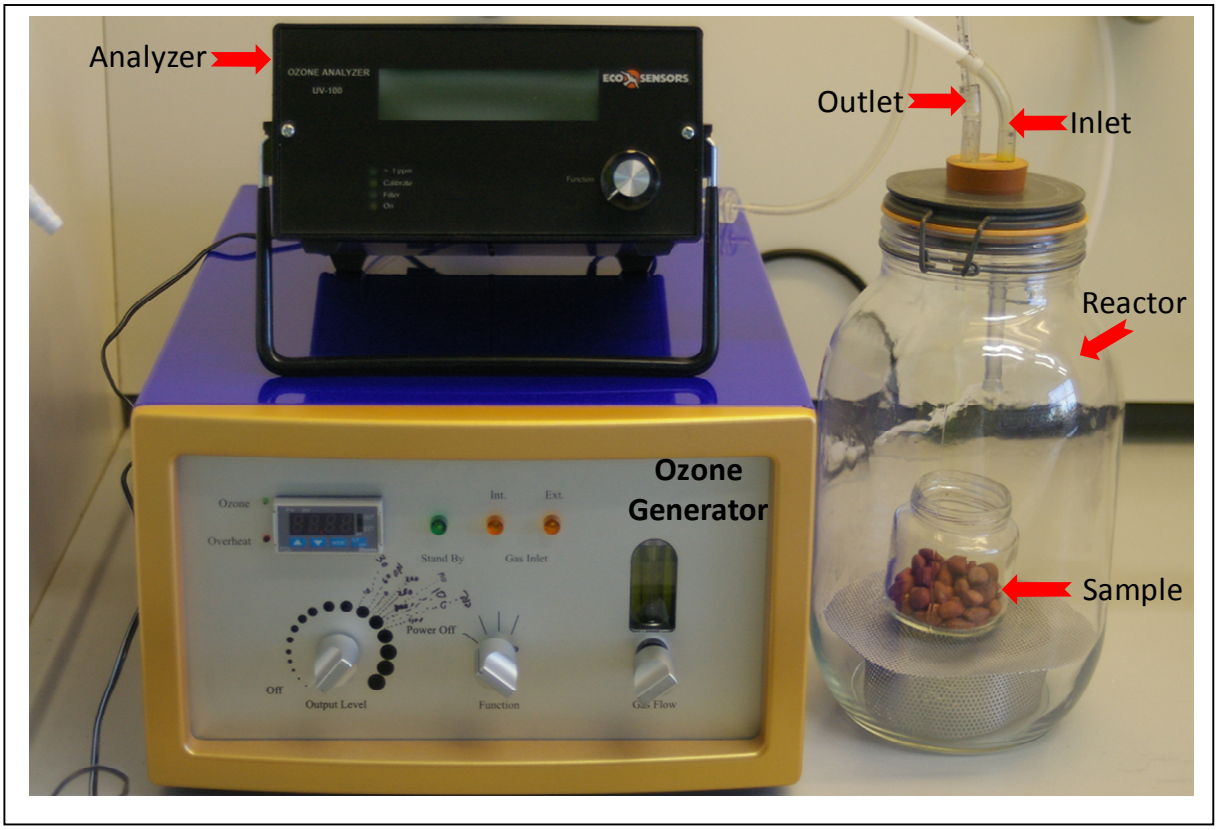


Figure 4.1. Set-up of the ozonation process including the O_3 generator, fumigation vessel and O_3 analyser.

4.2.5 Inactivation of spore germination

In triplicates, 100 µl of *A. flavus* 10^6 spores ml^{-1} was spread plated onto YES medium at different a_w levels (0.89, 0.92, 0.95 and 0.97 a_w) and the plates were aseptically placed in the O_3 reactor without lids. The O_3 generator was adjusted to obtain 20, 40, 100 and 250 ppm in the reactor for 30 min. at 6 L $min.^{-1}$ flow rate. After the treatment, plates were covered and incubated at 25°C for 48 hrs. Periodically every 12 hrs two plugs (0.8 cm each) were removed and microscopic examinations were conducted for determination of spore germination of both strains. After the treatment, plates were covered and incubated at 25°C for 48 hrs. Periodically every 12 hrs two plugs (0.8 cm each) were removed, placed on microscope slides and stained with lactophenol cotton blue. Plugs were covered with square cover slip (22×22 mm) each and four microscopic fields per plug were examined to determine the spore germination of both strains. Pictures of spores and germ tubes were photographed under 40X magnification using a Zeiss Axioskop light microscope equipped with a Zeiss AxioCam digital camera and Axiovision 3.1 software (Zeiss, Göttingen, Germany). Spores were considered to have germinated when the germ tube length was equal to or greater than one-half the spore diameter (Bosch *et al.*, 1995). Germinated spores were counted and recorded as a percentage of the total spore number.

4.2.6 Mycelial growth, sporulation and AFB_1 measurements

Duplicate Petri plates of YES medium at different a_w levels were centrally inoculated with 5 µl of the spore suspension 10^6 ml^{-1} of each strain and incubated at 25°C. At 0.6 and 0.8 cm colony diameter for *A. flavus* EGP-B07 and *A. flavus* SRRC-G 1907 respectively, plates were exposed to O_3 at 75 and 150 ppm. Treated plates were incubated at 25°C for 36 hrs. The colony diameters of replicates were measured in two directions at right angles every 12 hrs. The growth against time and the slope of the linear growth phase was used to obtain the radial growth rate mm/12 hrs. For sporulation measurement, spores were gently dislodged from the colony surface into water-Tween suspensions in 25 ml Universal bottles. One millilitre for 0.89 and 0.92 a_w and 10 ml for the higher a_w were used for collecting spores. Fungal spore concentration was determined using a haemocytometer (Marienfeld, Germany) and dilution was done if required. Sporulation was counted as spores per cm^2 fungal

colony. One to three plugs from each colony were analysed for the AFB_1 content by HPLC.

The same procedures used for growth rate (mm day^{-1} instead of mm 12 hrs^{-1}), sporulation and AFB_1 analyses but with higher concentration of O_3 (100 and 300 ppm) and incubation time (72 hrs) were subsequently carried out. Also, the initial colony diameters for *A. flavus* EGP-B07 and *A. flavus* SRRC-G 1907 were higher (1.2 and 1.4 cm, respectively).

4.2.7 *In situ* effect of O_3 on the population of *A. flavus* EGP-B07 and AFB_1 production on peanuts

The sample vessels were divided into 2 groups depends on the inoculum concentration, one at 10^3 and the second group at 10^5 spores g^{-1} peanuts. In triplicate, samples were aseptically exposed at room temperature to O_3 at 100, 200 and 400 ppm and to air for control samples for 30 min. Vessels containing peanuts were enclosed together in plastic chambers. Each chamber had a beaker with glycerol/water solution of 0.93 a_w the same as that of the peanut treatments, to maintain the equilibrium relative humidity. Populations of *A. flavus* and AFB_1 levels in peanuts were measured before and after treatment and also these parameters were determined again after storage at 25°C for 4 days.

Ozonation was begun when the ozone–air mixture was sparged into 25 g of peanuts sample contained in the reactor. The feed gas was allowed to run continuously under a constant gas flow rate of 6L min^{-1} inside the reactor. The reactor was sealed to prevent leaking. For the control experiments, 25 g of peanuts contained in the reactor was sparged with air instead of the ozone-air mixtures under the same experimental conditions as the ozonation treatment.

4.2.8 Enumeration of fungi

As described in Section 3.2.6.

4.2.9 Extraction and quantification of AFB_1 from media and peanut samples using HPLC

As described in Section 2.2.5.

4.2.10 Statistical analyses

As described in Sections 2.2.7 and 3.2.8.

4.3 RESULTS

4.3.1 Efficacy of ozonation on germination of *A. flavus* spores on media

Figure 4.2, 4.3 and Table 1 (Appendix) show the effect of exposure to different concentrations of O_3 for 30 min prior to incubation for up to 48 hrs. In general, the percentage germination decreased gradually with the increase in O_3 dose and increased with the incubation time except at 0.92 a_w and especially for the *A. flavus* SRRC-G 1907 strain. Within 36 hrs incubation, the germination of this strain at 40 ppm (46-64%) was higher than that at 20 ppm (23-34%). This was confirmed by microscopic examination and plates of the germinating spores of both strains after 24 hrs at 0.92 a_w (Figures 4.4, 4.5). Spore germination of *A. flavus* SRRC-G 1907 was more sensitive to ozonation than *A. flavus* EGP-B07. The spores failed to germinate at 100 and 250 ppm during the incubation time for both strains at all a_w levels except at 0.89 a_w and 100 ppm after 48 hrs.

Table 4.1 shows the ANOVA results for O_3 treatment, incubation time (hrs), a_w, strain type and their two, three and four way interaction on *A. flavus* germination (%). All effects were statistically significant and the ozonation application followed by time and a_w had the main impact on germination rates.

4.3.2 Inhibitory effect of ozone against mycelial growth of *A. flavus*

The effect of O_3 exposure on mycelia of two *A. flavus* strains is shown in Figure 4.6. Generally, growth rate of the mycelia of both strains unaffected by ozonation treatment when incubated for 36 hrs after exposure for 30 min. However, some reduction in growth was significant at 0.92 a_w using O_3 (75 ppm) for both strains.

Table 4.2 shows statistical effects of ozone (75 and 150 ppm), incubation time, a_w, strain type and their two, three way interactions on growth rate of *A. flavus* on YES media. A_w factor had the main significant impact on the growth rate followed by strain. Ozone was the least significant factor.

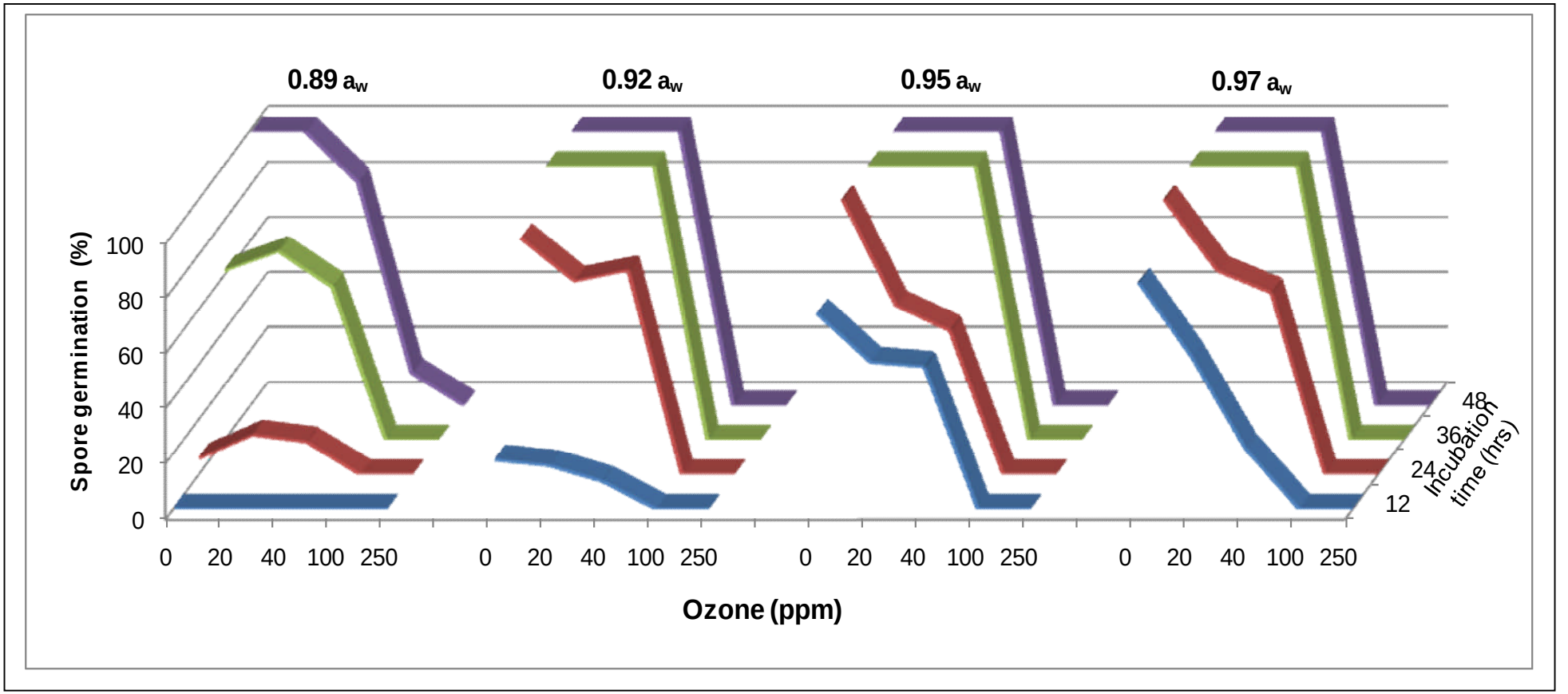


Figure 4.2. Effect of ozone concentration (ppm) on spore germination of *A. flavus* EGP-B07 on YES media over periods of 48 hrs. Ozone exposure was for 30 min at 6 L min^{-1} prior to incubation.

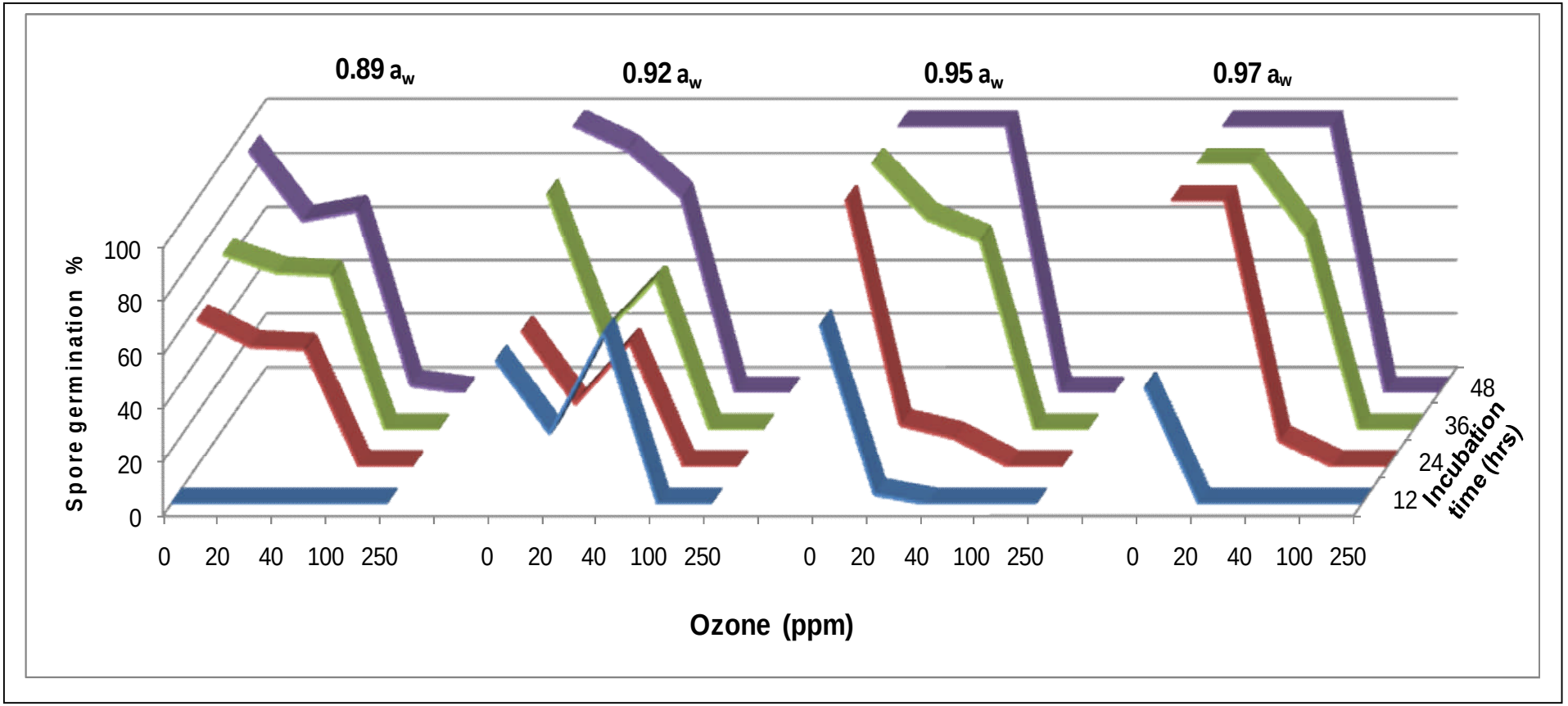


Figure 4.3. Effect of ozone concentration (ppm) on spore germination of *A. flavus* SRRC-G 1907 on YES media for 48 hrs. Ozone treatment was for 30 min at 6 L min^{-1} prior to incubation.

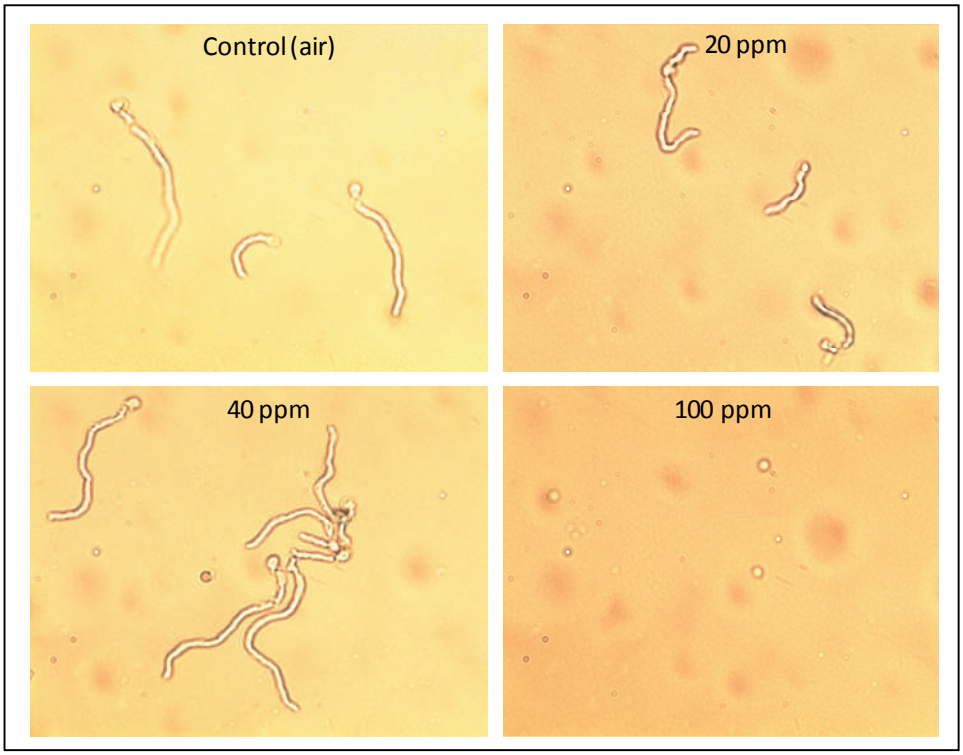


Figure 4.4. Spore germination of *A. flavus* EGP-B07 under different ozone doses for 30 min prior to incubation for 24 hrs.

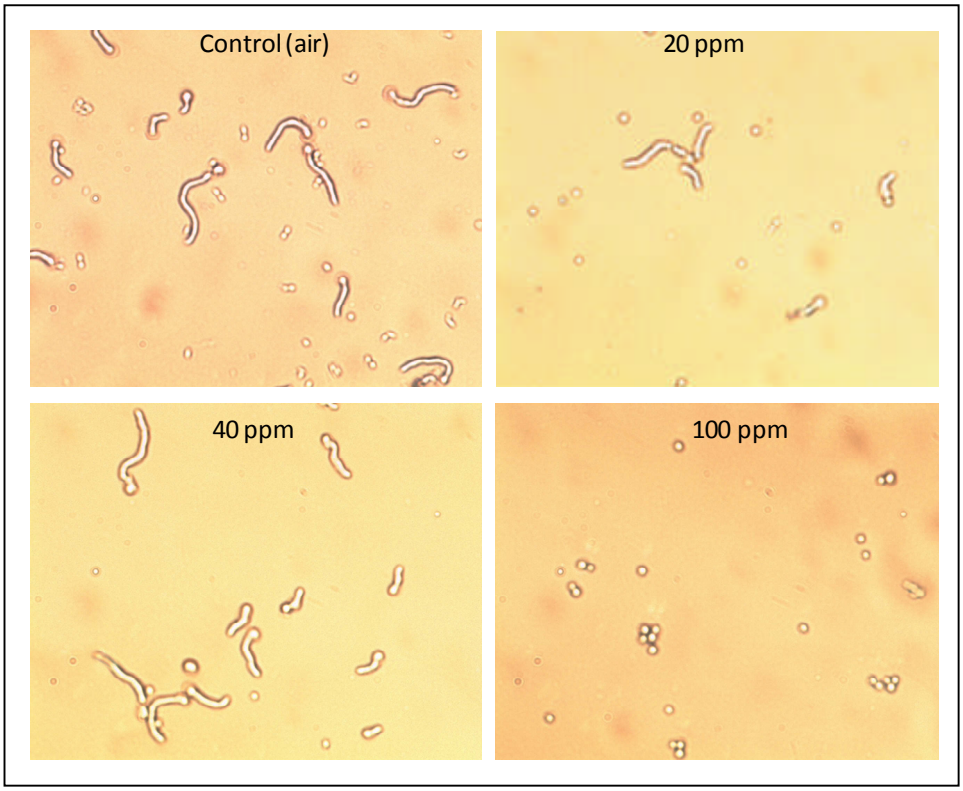


Figure 4.5. Spore germination of *A. flavus* SRRC-G 1907 under different ozone doses for 30 min prior to incubation at 0.92 a_w for 24 hrs.

Table 4.1 Analysis of variance of the effect of ozone, water activity (a_w), strain and their interaction on the growth rate of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	720128.0	1	720128.0	32267.18	0.000000
{1} Ozone	494718.4	4	123679.6	5541.78	0.000000
{2} Time hrs	119536.6	3	39845.5	1785.38	0.000000
{3} a_w	26178.3	3	8726.1	391.00	0.000000
{4} Strain	5470.7	1	5470.7	245.13	0.000000
Ozone xTime (hrs)	83158.1	12	6929.8	310.51	0.000000
Ozone x a_w	29351.0	12	2445.9	109.60	0.000000
Time x a_w	5088.8	9	565.4	25.33	0.000000
Ozone x Strain	7342.7	4	1835.7	82.25	0.000000
Time x Strain	454.3	3	151.4	6.78	0.000190
a_w x Strain	3355.8	3	1118.6	50.12	0.000000
Ozone x Time x a_w	18949.3	36	526.4	23.59	0.000000
Ozone x Time x Strain	2565.0	12	213.8	9.58	0.000000
Ozone x a_wx Strain	5913.4	12	492.8	22.08	0.000000
Time x a_w x Strain	18154.1	9	2017.1	90.38	0.000000
1 x 2 x 3 x 4	19197.5	36	533.3	23.89	0.000000
Error	7119.3	319	22.3		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

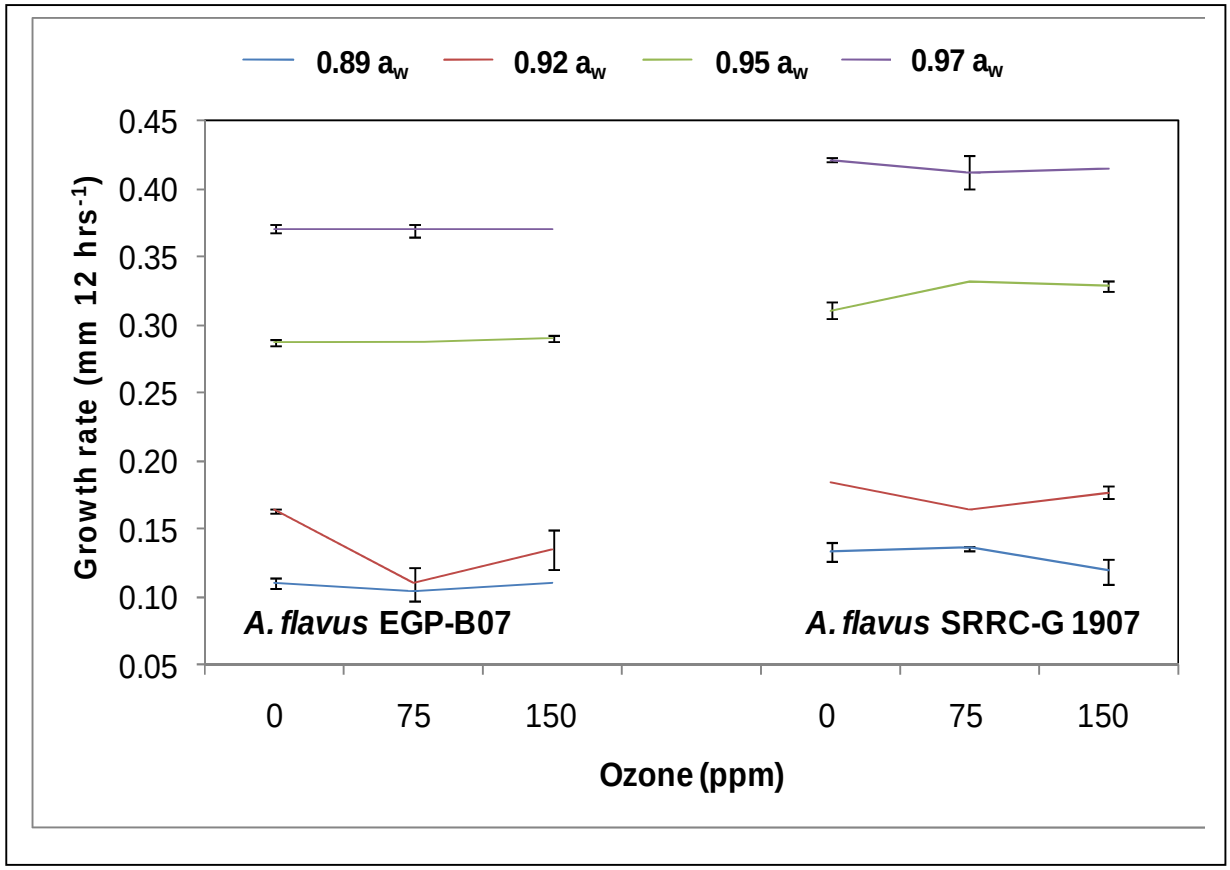


Figure 4.6. Impact of ozone treatment on growth rate of *A. flavus* EGP-B07 and SRRC-G 1907 on YES media at 25°C for 36 hrs. Colonies were exposed for 30 min at 6 L min^{-1} prior to incubation. Bars indicate standard error of the means.

Table 4.2. Analysis of variance of the effect of ozone, water activity (a_w), strain and their interaction on growth of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	2.851875	1	2.851875	41900.83	0.000000
Ozone	0.000564	2	0.000282	4.14	0.028507
a_w	0.593868	3	0.197956	2908.44	0.000000
Strain	0.015230	1	0.015230	223.76	0.000000
Ozone x a_w	0.002631	6	0.000439	6.44	0.000380
Ozone x Strain	0.000406	2	0.000203	2.98	0.069853
a_w x Strain	0.001004	3	0.000335	4.92	0.008373
Ozone x a_w x Strain	0.000688	6	0.000115	1.69	0.167855
Error	0.001633	24	0.000068		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

4.3.3. Effects of ozone on sporulation of *A. flavus* colonies

The effect of exposure to O_3 for 30 min prior to incubation on spore production by vegetative mycelia of two strains is shown in Figure 4.7. This showed that 75 and 150 ppm O_3 significantly inhibited sporulation of both strains at 0.95 a_w and at 0.97 a_w for just *A. flavus* SRRC-G 1907. This represented about 50% and 70% inhibition being achieved using 75 and 150 ppm O_3 doses respectively.

The ANOVA of the statistical effect of ozone (75 and 150 ppm), a_w, type of strain and their two, three interactions on the sporulation of *A. flavus* mycelia (spores cm^2 colony) over 36 hrs is shown in Table 4.3. All factors significantly affected sporulation, except the type of strain and the interaction O_3 x strain.

4.3.4 *In vitro* effect of ozone on AFB_1 production

The amount of AFB_1 produced by *A. flavus* EGP-B07 *in vitro* treated with O_3 at 75 and 150 ppm was significantly decreased when compared with control samples at 0.89 and 0.92 a_w (Figure 4.8). However, the ozonation process significantly inhibited AFB_1 production by *A. flavus* SRRC-G 1907 only at 0.89 a_w. AFB_1 production was significantly affected by ozonation, a_w, type of strain and their two, three way interaction ($P<0.05$) on YES media incubated for 36 hrs (Table 4.4). Similar to growth rate, a_w was the main factor affecting AFB_1 production followed by type of strains and then ozone factors.

Additional experiments were carried out to examine the effect of higher concentrations of O_3 (100, 300 ppm) on growth and afaltoxin production by *these A. flavus* strains (Figure 4.9). The same trend as previously described over 36 hrs incubation was obtained over 3 days. The impact of O_3 was more noticeable at the lower a_w levels (0.89 and 0.92 a_w). Significant reductions in growth rate of *A. flavus* EGP-B07 was observed as a result of O_3 treatment at 0.89 and 0.92 a_w and only at 0.92 a_w for growth of *A. flavus* SRRC-G 1907. Table 4.5 shows the ANOVA results for ozone, a_w, type of strain and their two, three way interaction on the growth rate of *A. flavus*. Ozone treatment was the third factor significantly affecting the growth rate after a_w and strain type factor.

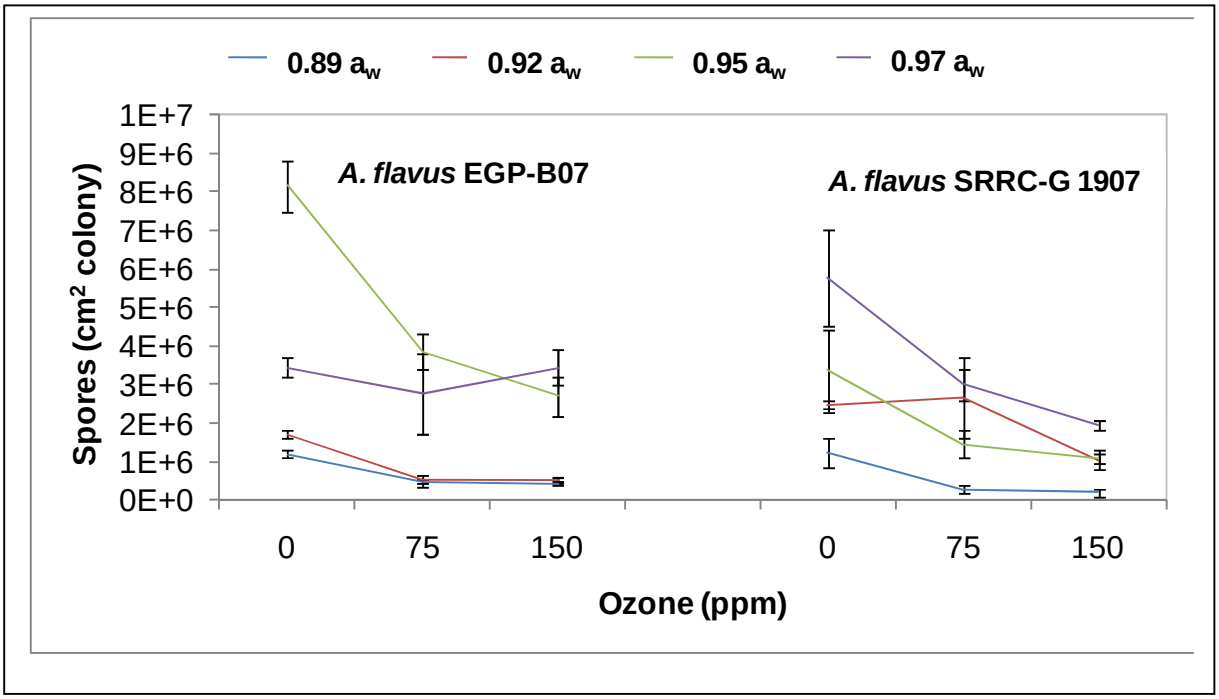


Figure 4.7. Effect of ozonation for 30 min at 6 L min^{-1} prior to determination of spore production by colonies of *A. flavus* at 25°C over 36 hrs. Bars indicate standard error of the means.

Table 4.3 Analysis of variance of the effect of ozone, water activity (a_w), strain type and their interaction on sporulation of *A. flavus* on YES medium for 36 hrs.

Effect	SS	DF	MS	F	P
Intercept	2.401733E+14	1	2.401733E+14	441.3885	0.000000
Ozone	3.485146E+13	2	1.742573E+13	32.0249	0.000000
aw	7.102387E+13	3	2.367462E+13	43.5090	0.000000
Strain	1.876252E+12	1	1.876252E+12	3.4482	0.075638
Ozone x a_w	1.315527E+13	6	2.192545E+12	4.0294	0.006229
Ozone x Strain	8.752042E+11	2	4.376021E+11	0.8042	0.459131
a_w x Strain	2.799966E+13	3	9.333219E+12	17.1525	0.000004
Ozone x a_w x Strain	1.329436E+13	6	2.215727E+12	4.0720	0.005898
Error	1.305915E+13	24	5.441313E+11		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

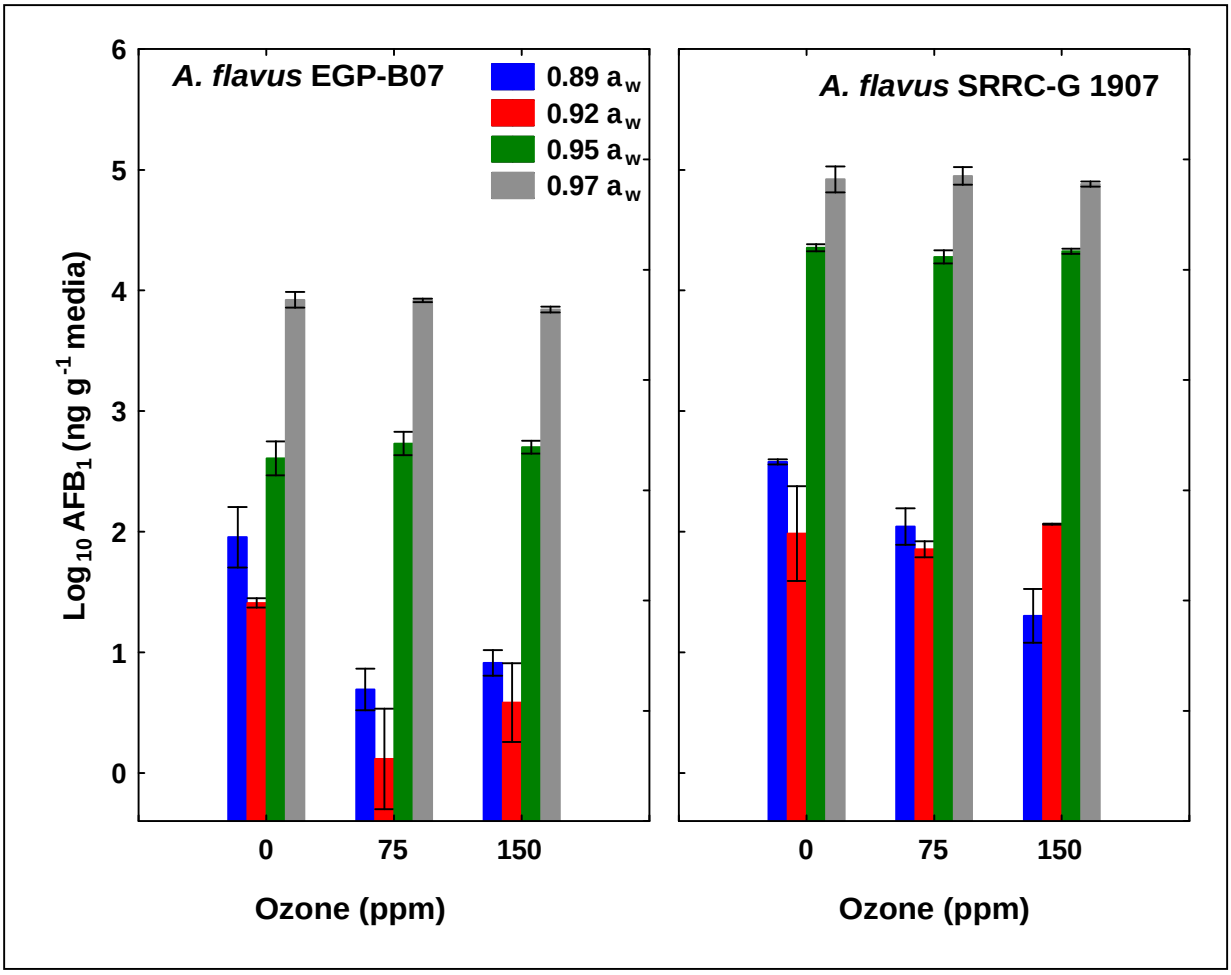


Figure 4.8. Effect of ozone (30 min at 6 L min^{-1}) on AFB_1 production by *A. flavus* EGP-B07 and SRRC-G 1907 on YES media at 25°C for 36 hrs. Bars indicate standard error of the means.

Table 4.4. Analysis of variance of the effect of ozone, water activity (a_w), strain and their interactions on AFB_1 production by of *A. flavus* on YES medium after 36 hrs.

Effect	SS	DF	MS	F	P
Intercept	351.0949	1	351.0949	6231.237	0.000000
Ozone	1.6390	2	0.8195	14.545	0.000073
a_w	79.9980	3	26.6660	473.269	0.000000
Strain	16.6677	1	16.6677	295.819	0.000000
Ozone x a_w	2.3489	6	0.3915	6.948	0.000227
Ozone x Strain	0.3846	2	0.1923	3.413	0.049616
a_w x Strain	1.1858	3	0.3953	7.015	0.001502
Ozone x a_w x Strain	0.8847	6	0.1475	2.617	0.042728
Error	1.3523	24	0.0563		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

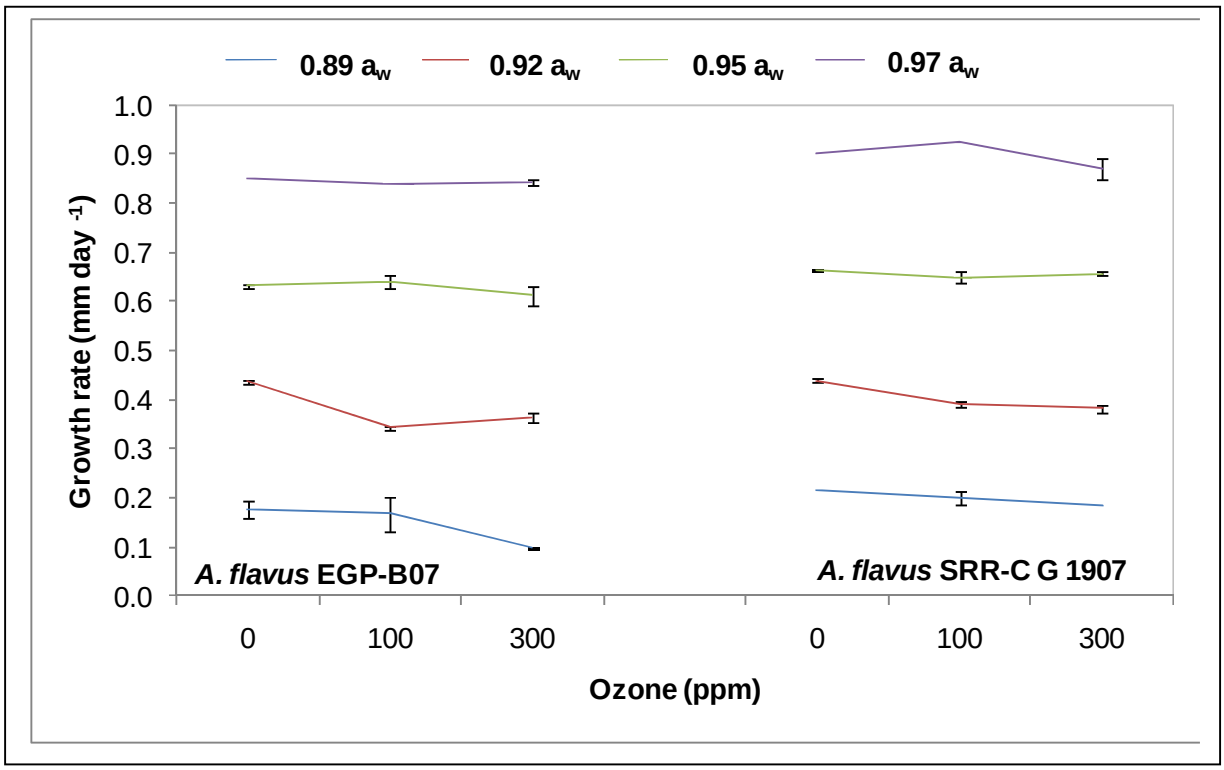


Figure 4.9. Growth rate of *A. flavus* EGP-B07 and SRRC-G 1907 on YES media at 25°C for 3 days. Exposire was for 30 min at 6 L min^{-1}). Bars indicate standard error of the means.

Table 4.5. Analysis of variance of the effect of ozone, water activity (a_w), strain and their interactions on *A. flavus* growth on YES medium at 25°C for 3 days.

Effect	SS	DF	MS	F	P
Intercept	12.98154	1	12.98154	47844.20	0.000000
Ozone	0.01106	2	0.00553	20.39	0.000007
a_w	3.29731	3	1.09910	4050.80	0.000000
Strain	0.01909	1	0.01909	70.36	0.000000
Ozone x a_w	0.00913	6	0.00152	5.61	0.000937
Ozone x Strain	0.00047	2	0.00023	0.86	0.435477
a_w x Strain	0.00236	3	0.00079	2.90	0.055878
Ozone x a_w x Strain	0.00494	6	0.00082	3.03	0.023700
Error	0.00651	24	0.00027		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

At these higher concentrations there was a significant reduction on the ability of colonies to sporulate of the *A. flavus* EGP-B07 (>67% inhibition) and *A. flavus* SRRC-G 1907 (>95% inhibition) at 0.92 a_w when compared with controls (0 ppm) (Figure 4.10). Also, a significant decrease (70% inhibition) was observed by exposing mycelia of *A. flavus* EGP-B07 to 100 ppm O_3 at 0.97 a_w. Analysis of variance of the effect of ozone, a_w, type of strain and their interaction on sporulaton (spores [cm^2 colony]) of *A. flavus* mycelia on YES medium over 3 days is represented in Table 4.6. Single factors and the interaction ozone x a_w were statistically significant however no effect for other interaction two, three factors was noticed.

Figure 4.11 shows the effect of treatment of growing colonies of *A. flavus* EGP-B07 and SRRC-G 1907 with O_3 on AFB_1 production over 3 days incubation under different a_w conditions. When compared with controls (0 ppm), significant reduction of AFB_1 using the dose 300 ppm was observed at 0.89, 0.95 a_w by *A. flavus* EGP-B07 and at 0.89, 0.97 a_w by *A. flavus* SRRC-G 1907. The best reduction of AFB_1 (2.3 $\log_{10}$ ng g^{-1}) was obtained at 0.89 a_w produced by *A. flavus* EGP-B07. Statistical analyses of AFB_1 levels produced by *A. flavus* in YES media showed that ozonation, a_w, type of strain, two and three way interactions were statistically significant except a_w x strain interaction (Table 4.7). The major effects were produced by type of strain whereas *A. flavus* SRRC-G 1907 produced higher amount of AFB_1 than *A. flavus* EGP-B07 followed by a_w and ozone.

4.3.5 *In situ* studies of ozone on peanuts

In situ effect of ozone at 100, 200 and 400 ppm on spore viability of *A. flavus* EGP-B07 inoculated on peanuts was studied. Two concentrations (10^3 and 10^5 spores g^{-1}) were initially prepared for ozonation treatment. The effect of treatment on isolation of viable spores from peanuts is shown in Figure 4.12. Viability of spores was decreased by increasing O_3 concentration with no viable spores present at 200 and 400 ppm treatment of samples inoculated with 10^3 spores g^{-1}. Significant reduction of *A. flavus* populations ranging between 0.8 and 2.2 log CFUs g^{-1} were achieved in peanut samples inoculation with higher concentration of inoculum (10^5 spores g^{-1}) when compared with the population before treatment.

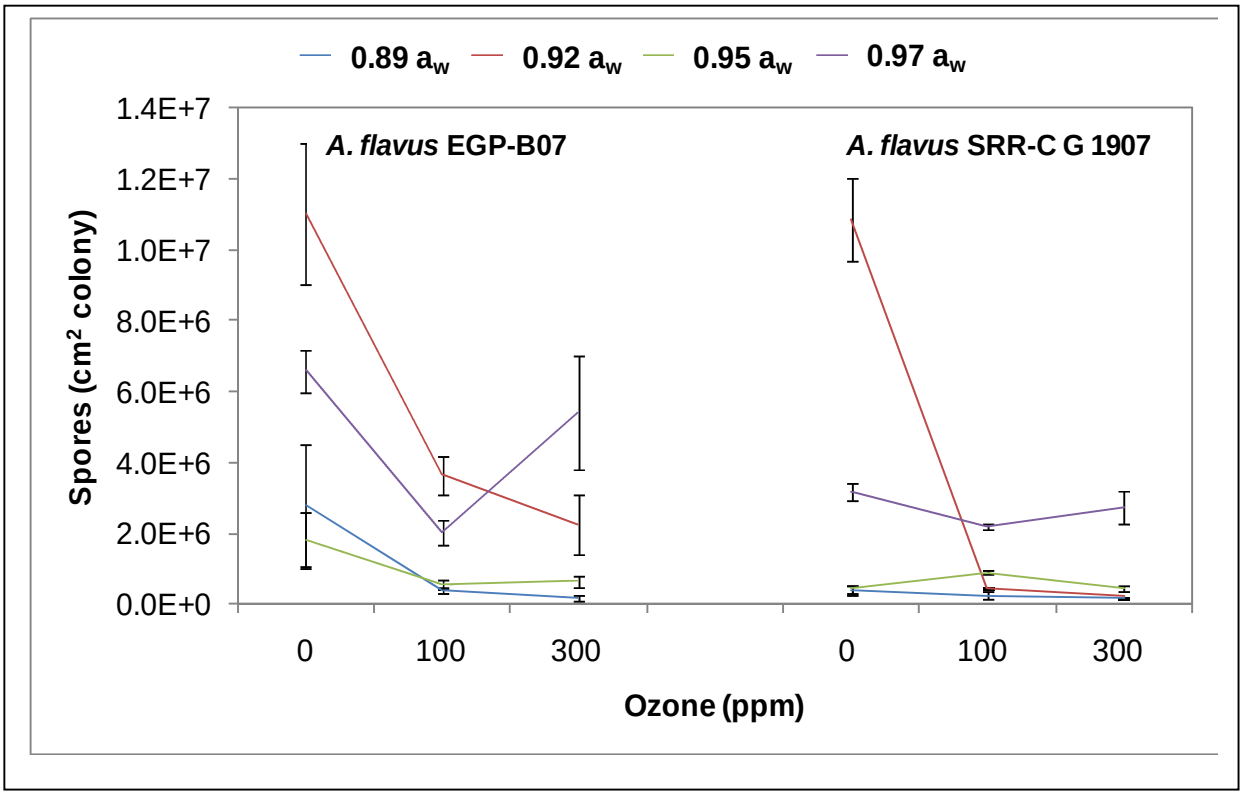


Figure 4.10. Effect of ozonation on the sporulation of colonies of *A. flavus* at 25°C over 3 days. Exposure to ozone was for 30 min at 6 L min^{-1} prior to incubation. Bars indicate standard error of the means.

Table 4.6. Analysis of variance of the effect of ozone, water activity (a_w), strain type and their interactions on sporulation of *A. flavus* on YES medium for 3 days.

Effect	SS	DF	MS	F	P
Intercept	2.950704E+14	1	2.950704E+14	266.1654	0.000000
Ozone	1.112389E+14	2	5.561944E+13	50.1710	0.000000
a_w	1.512270E+14	3	5.040900E+13	45.4709	0.000000
Strain	1.896310E+13	1	1.896310E+13	17.1055	0.000374
Ozone x a_w	1.412917E+14	6	2.354862E+13	21.2418	0.000000
Ozone x Strain	2.568854E+12	2	1.284427E+12	1.1586	0.330870
a_w x Strain	5.105173E+12	3	1.701724E+12	1.5350	0.231059
Ozone x a_w x Strain	1.454820E+13	6	2.424699E+12	2.1872	0.079907
Error	2.660635E+13	24	1.108598E+12		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

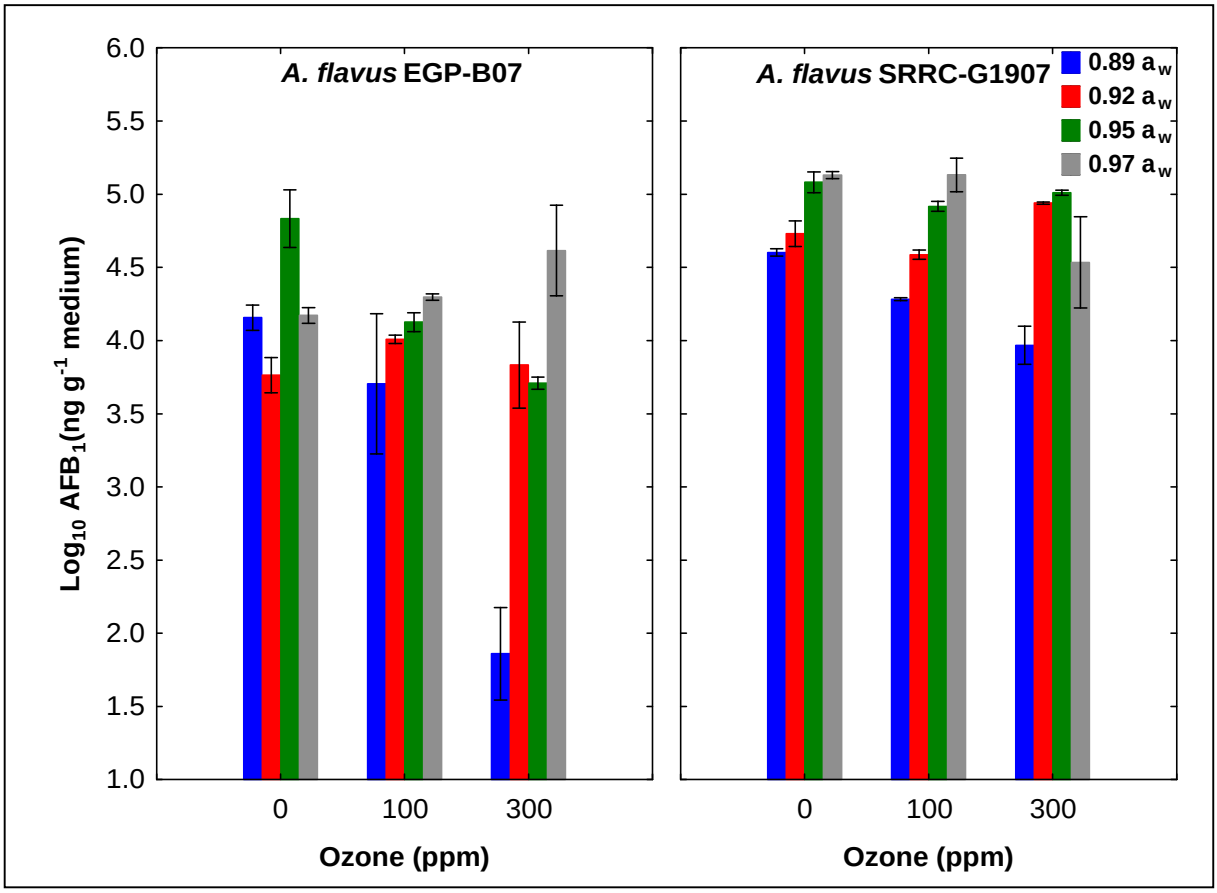


Figure 4.11. Effect of ozone on AFB_1 produced by *A. flavus* EGP-B07 and SRRC-G 1907 on YES media at 25°C for 3 days. Ozone exposure was for 30 min at 6 L min^{-1}. Bars indicate standard error of the means.

Table 4.7. Analysis of variance of the effect of ozone, water activity (a_w), strain and their interactions on AFB_1 production by of *A. flavus* on YES medium at 25°C for 3 days.

Effect	SS	DF	MS	F	P
Intercept	901.2640	1	901.2640	14862.68	0.000000
Ozone	2.0586	2	1.0293	16.97	0.000025
a_w	6.0407	3	2.0136	33.21	0.000000
Strain	8.0649	1	8.0649	133.00	0.000000
Ozone x a_w	3.4018	6	0.5670	9.35	0.000025
Ozone x Strain	0.5027	2	0.2513	4.14	0.028429
a_w x Strain	0.3531	3	0.1177	1.94	0.149910
Ozone x a_w x Strain	2.5513	6	0.4252	7.01	0.000212
Error	1.4553	24	0.0606		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

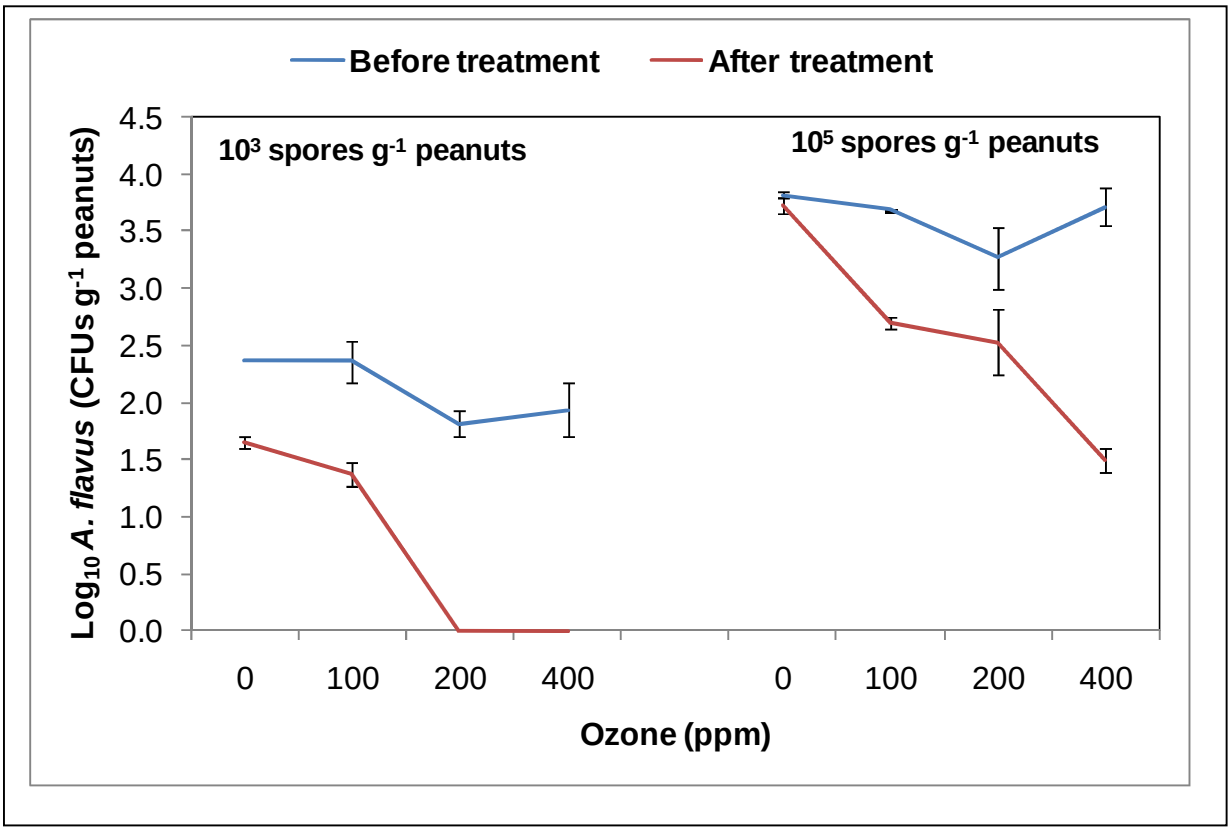


Figure 4.12. Effect of ozone treatment for 30 min at 6 L min $^{-1}$ on isolation of viable populations of *A. flavus* EGP-B07 from peanuts before and after storage. Bars indicate standard error of the means.

Table 4.8 shows the statistical effects of ozone, sample state, initial spores' concentration and their interaction on population (log_{10}CFUs g^{-1}) of *A. flavus* on peanuts before storage. All factors and their interactions showed a significant effect on the populations of *A. flavus* on peanuts. The difference of spore concentration was the main factor affecting the population followed by sample state then ozonation process.

Figure 4.13 shows the populations of *A. flavus* on ozone treated samples before (T=0) and after storage for 4 days. When compared with control samples (using air), population of *A. flavus* on treated samples were significantly lower after storage at all doses and both spore concentrations of inocula. Doses of 100, 200 and 400 ppm before storage gave the same significant reduction of the population (around 5 log CFUs g^{-1}) on samples inoculated with 10^5 spores g^{-1}. However, the population on samples inoculated with lower spores concentration (10^3 g^{-1}) gradually decreased as treatment concentration was increased (5.6-8.3 log CFUs g^{-1}) when compared with control samples. This suggests some effect of O_3 on the shelf life of stored peanuts contaminated with *A. flavus* spores. Significant effects of ozonation process, initial spore concentration, incubation time and their two, three way interaction on the population of *A. flavus* on peanuts 0.93 a_w were observed (Table 4.9). Storage had the main effect followed by ozone treatment.

The effect of O_3 treatment at 100 and 200 ppm, the amounts of AFB_1 oin samples were significantly higher than those before treatment (Figure 4.14). It was very difficult to evaluate the effect of ozone process on the concentration of AFB_1 in samples containing small amounts (0.2-0.4 ppb).

Generally, there was no significant difference in AFB_1 levels in samples before and after ozonation treatment inoculated either with 10^3 or 10^5 spores g^{-1} (Table 4.10).

Figure 4.15 illustrates effect of prestorage ozone treatment on AFB_1 in stored peanuts inoculated with two different concentrations of *A. flavus* spores (10^3 and 10^5 spores g^{-1}). The doses 100 and 400 ppm significantly decreased AFB_1 in samples inoculated with 10^5 spores g^{-1}. However, significant decrease was just notices at 400 ppm in samples inoculated with 10^3 spores g^{-1}. Table 4.11 shows the statistical analysis of the impact of prestorage ozonation, incubation time, initial spore

Table 4.8. Analysis of variance of the effect of ozone treatment, sample status, initial spore concentration and their interactions on viability of *A. flavus* on peanuts.

Effect	SS	DF	MS	F	P
Intercept	194.3189	1	194.3189	5900.954	0.000000
Ozone	7.7403	3	2.5801	78.351	0.000000
Sample status (untreated, treated)	13.2374	1	13.2374	401.984	0.000000
Initial spores conc.	26.4098	1	26.4098	801.996	0.000000
Ozone x sample state	3.4641	3	1.1547	35.065	0.000000
Ozone x initial spore conc.	0.5338	3	0.1779	5.403	0.005801
Sample condition x initial spore conc.	0.2930	1	0.2930	8.896	0.006655
Ozone x sample state x initial spores conc.	0.6474	3	0.2158	6.553	0.002302
Error	0.7574	23	0.0329		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

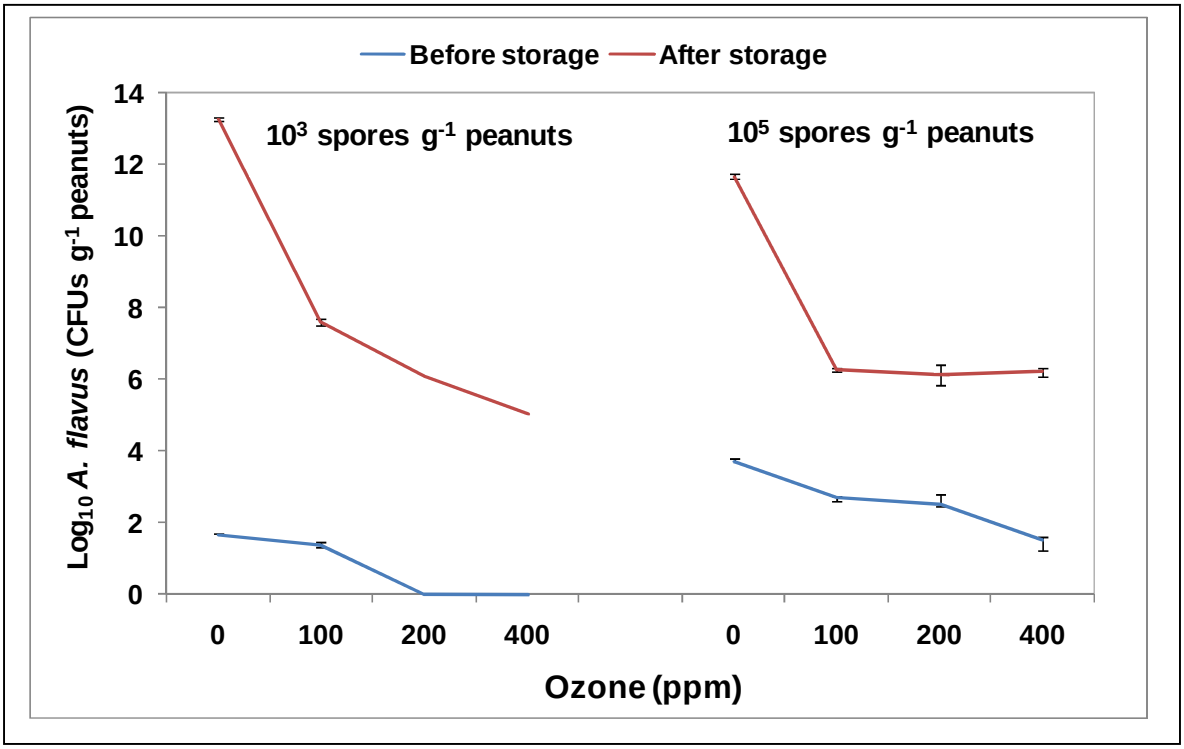


Figure 4.13. Population of *A. flavus* EGP-B07 on ozonated peanut samples before and after storage. Exposure to ozone was for 30 min at 6 L min^{-1} prior to incubation. Bars indicate standard error of the means.

Table 4.9. Analysis of variance of the effect of ozone treatment, incubation time, spore concentration and their interactions on populations of *A. flavus* on peanuts after storage over 4 days.

Effect	SS	DF	MS	F	P
Intercept	1042.483	1	1042.483	11233.76	0.000000
Ozone	137.952	3	45.984	495.52	0.000000
Time	432.849	1	432.849	4664.36	0.000000
Initial spores conc.	6.090	1	6.090	65.62	0.000000
Ozone x time	53.375	3	17.792	191.72	0.000000
Ozone x initial spore conc.	4.328	3	1.443	15.55	0.000002
Time x initial spore conc.	14.928	1	14.928	160.87	0.000000
Ozone x time x initial spores conc.	4.474	3	1.491	16.07	0.000002
Error	2.877	31	0.093		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

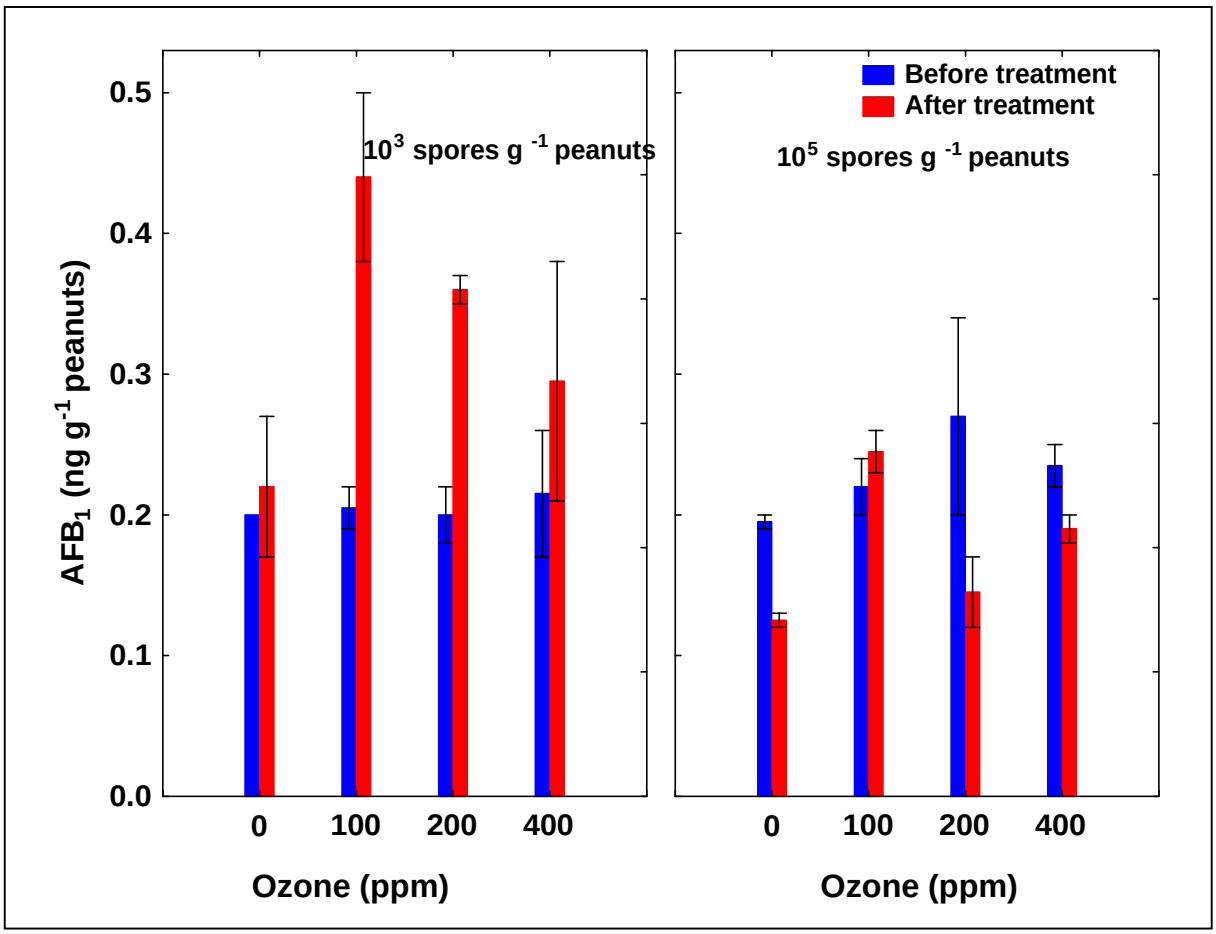


Figure 4.14. Effect of ozone on AFB_1 contents on peanuts inoculated with two different concentrations of *A. flavus*. Ozone exposure was for 30 min at 6 L min^{-1}. Bars indicate standard error of the means

Table 4.10. Analysis of variance of the effect of ozone treatment before storage, sample status, spore concentration and their interaction on AFB_1 levels in peanuts

Effect	SS	DF	MS	F	P
Intercept	1.767200	1	1.767200	625.5575	0.000000
Ozone	0.035075	3	0.011692	4.1386	0.023803
Sample status (untreated, treated)	0.009800	1	0.009800	3.4690	0.080994
Initial spores conc.	0.032512	1	0.032512	11.5088	0.003719
Ozone x sample state	0.026475	3	0.008825	3.1239	0.055226
Ozone x initial spore conc.	0.002813	3	0.000938	0.3319	0.802438
Sample condition x initial spore conc.	0.063013	1	0.063013	22.3053	0.000230
Ozone x sample status x initial spores conc.	0.011513	3	0.003838	1.3584	0.291085
Error	0.045200	16	0.002825		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

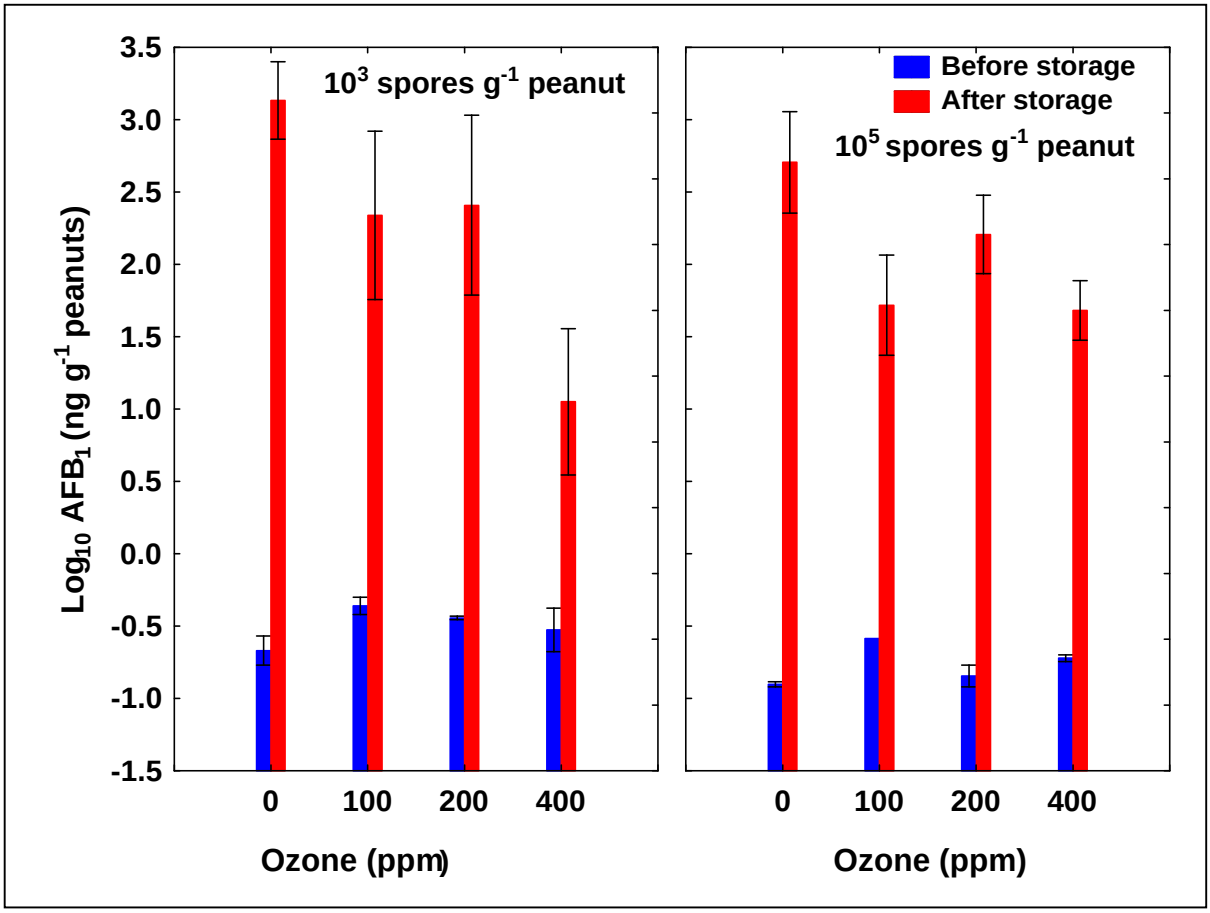


Figure 4.15. Effect of ozone for 30 min at 6 L min^{-1} on AFB_1 production by *A. flavus* on peanuts treated with two different initial inocula of *A. flavus* over 4 days storage. Bars indicate standard error of the means

Table 4.11. Analysis of variance of the effect of preliminary ozoniation, incubation time, spore concentration and their interaction on AFB_1 levels in peanuts after storage over 4 days.

Effect	SS	DF	MS	F	P
Intercept	20.23824	1	20.23824	61.8284	0.000000
Ozone	2.40696	3	0.80232	2.4511	0.090372
Time	67.78314	1	67.78314	207.0795	0.000000
Initial spores conc.	0.38215	1	0.38215	1.1675	0.291622
Ozone x Time	3.74736	3	1.24912	3.8161	0.024249
Ozone x initial spore conc.	0.56993	3	0.18998	0.5804	0.634088
Time x initial spore conc.	0.02583	1	0.02583	0.0789	0.781389
Ozone x time x initial spores conc.	0.47351	3	0.15784	0.4822	0.698031
Error	7.20124	22	0.32733		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

concentration and their two, three way interactions on AFB_1 levels in peanuts accumulated by *A. flavus*. Only incubation time and the interaction ozone x time were the factors significantly affected AFB_1 production.

4.4. DISCUSSION

4.4.1. Germination

Two strains of *A. flavus* were used in this study to evaluate the efficacy of gaseous O_3 on inhibition of spore germination. *A. flavus* EGP-B07 was isolated from Egyptian peanuts and *A. flavus* SRRC-G 1907 was a reference strain. Ozonation inhibited the germination, with concentrations >100 pm completely inhibiting germination over 48 hrs. Generally, the strain *A. flavus* EGP-B07 was more resistant to O_3 exposure at doses < 100 ppm than *A. flavus* SRRC-G 1907.

Very few studies have examined the *in vitro* effect of O_3 on fungi at different a_w levels and rarely on *A. flavus.* Zotti *et al.* (2008) reported that treatment of fungal colonies of *A. flavus* (3 days old) with O_3 for 3 hrs completely inhibited the vitality of the fungal spores. However, the efficacy of O_3 treatment decreased by increasing the colony age (6 and 9 days old). They also found that the species showed a different sensitivity, since *A. flavus* was found to be less sensitive than *A. niger*. Hudson and Sharma (2009) found that exposing 13 different species of environmental fungi including *A. flavus* and *A. niger* applied to sterile surface of a plastic tray lid to ozone 35 ppm for 20 min inactivated 3 log_{10} CFUs of most of the fungi after 48 hrs incubation. Beuchat *et al.* (1999) investigated the inactivation of toxigenic *A. flavus* and *A. parasiticus* in suspension by ozone. They found that D-values (the time required to kill 90% of the conidia) of *A. flavus* were 1.72 ppm and 1.54 min at pH 5.5 and 7.0, respectively; D-values of *A. parasiticus* were 2.08 and 1.71 ppm min, respectively.

4.4.2. Effect of ozone on *in vitro* growth rate, sporulation and AFB_1 production by colonies of *A. flavus*

Since 100 ppm ozone achieved around 90% inhibitions of spore germination of *A. flavus* spores different a_w conditions, two concentrations of O_3 (lower than 100 ppm [75 ppm] and higher [150 ppm]) were used to evaluate the efficacy of ozone on

mycelia growth of *A. flavus* on YES at 0.89, 0.92, 0.95 and 0.97 a_w for 36 hrs. Generally, growth rate of both strains was unaffected. However, significant inhibitory effect was achieved on sporulation of *A. flavus* at 0.95 a_w using both doses of O_3 concentration. There was no relationship between sporulation and AFB_1 production. It was significantly decreased at 0.92 and 0.89 a_w by *A. flavus* EGP-B07 and at 0.89 a_w by *A. flavus* SRRC-G 1907.

Evaluation of the same parameters (growth, sporulation and AFB_1 levels) was conducted but with higher concentration of ozone (100 and 300 ppm) and incubation time (3 days). Similar trends as obtained after 36 hrs incubation were obtained. However, a significant reduction was observed in sporulation of both strains at 0.92 a_w (>67%). AFB_1 production was significantly affected at the higher dose of O_3 (300 ppm) at 0.89, 0.95 a_w by *A. flavus* EGP-B07 and at 0.89, 0.97 a_w by *A. flavus* SRRC-G 1907. Exposing *A. flavus* EGP-B07 on YES at 0.89 a_w to O_3 at 300 ppm gave the best inhibition (2.3 $\log_{10}$ ng g^{-1}). Although a significant decrease was achieved in inhibition of spore production at 0.92 a_w, AFB_1 was unaffected by O3 treatment at these conditions. Studies by Mason *et al.* (1997) applied O_3 (5 days) in agar cultures of *A. flavus* and *Fusarium verticillioides*. They found that sporulation and hyphal growth above the surface of the agar were completely inhibited by the O_3. Vijayanandraj *et al.* (2006) reported that the inhibitory effects of O_3 on *A. niger* was limited to the vegetative mycelium but did not have any effect on spore germination. Also, they found that the O_3 treated spore suspensions with 0.1 % sucrose did not show any changes in the colony morphology, which could be due to the reduced effect of O_3 on spores. There are also reports on reduction in O_3 effectiveness in the presence of dissolved organic and inorganic substances because they have a protective effect to the microorganisms against the action of O_3 (Suslow, 2004). Regarding to effect of O_3 on aflatoxin production, Zotti *et al.* (2008) reported that exposing *A. flavus* colonies to ozone can decrease aflatoxin production indirectly by the bleaching effect on the yellow pigments. This was based on the findings of Shier *et al.*, (2005) that the yellow anthraquinone pigments of the colonies were either biosynthetic intermediates needed to make aflatoxins, or unutilized branch pathway products.

4.4.3. In situ effect of ozone on A. flavus populations and AFB1 production in stored peanuts

Ozone has been affirmed as GRAS for use for food processing (Graham, 1997). Also, the attractive aspect of O_3 is that it decomposes rapidly (half-life of 20-50 min) to molecular oxygen without leaving any residue (Kells *et al.*, 2001). Also, ozone is effective for increasing the storage life (Horváth *et al.*, 1985). In this study, the efficacy of O_3 treatment on spores inhibition before storage depended on the initial concentration of fungal inocula (10^3 and 10^5 spores g^{-1}) and ozone concentration (100, 200 and 400 ppm). In general, the lower the initial spores and the higher the O_3 concentration, the better the inhibitory effect was obtained. The doses of 200 and 400 ppm completely inhibited the viability of fungal spores on peanuts at the lower inoculums level used. At the same time, 400 ppm gave a significant reduction by 2.2 log CFUs g^{-1} in peanuts inoculated with 10^5 spores g^{-1}. Recent studies by Giordano *et al.* (2010) showed that O_3 treatment for 5 hrs at 31 ppm was able to successfully inhibit the viability of aflatoxigenic Aspergillus species on Brazil nuts from the day of application. Also, Kells *et al.* (2001) investigated that treatment of maize with 50 ppm O_3 for 3 days resulted in a 63% reduction in contamination by *A. parasiticus* on the kernel surfaces.

Thus the effect of O_3 treatment on *A. flavus* populations was studied after incubation for 4 days. As a result of decreasing the number of spores using O_3 treatment before incubation, the population of *A. flavus* on treated samples was significantly lower than that on samples treated with air (control) instead of O_3. Regarding the samples inoculated with 10^3 spores g^{-1}, although no spores were viable when treated with 200 and 400 ppm, the populations were 6 and 5 $\log_{10}$ CFUs g^{-1} respectively after storage. This was because the actual viability prior to ozonation (at 200 and 400 ppm) was very small (65 and 85 spores g^{-1} respectively). Regarding to samples inoculated with 10^5 spores g^{-1}, similar significant inhibition in viability of spores obtained as a result of ozonation at 100, 200 and 400 ppm when compared with the controls.

Most previous *in situ* studies were conducted to control post harvest decay of vegetable, fruits and grains, e.g., control of *Botrytis cinerea* and *Sclerotinia seclerotiorum* on carrots (Liew and Prange, 1994), fungi associated with wheat (Wu *et al.*, 2006), fungi on Black peppercorns (Zhao and Cranston, 1995),

Botrytis cinerea on blackberries (Barth *et al.*, 1995), *Rhizopus stolonifer* on table grapes (Sarig *et al.*, 1996), microflora of Dried Figs (Oztekin *et al.*, 2005) and *Rhizopus stolonifer* and *Botrytis cinerea* on strawberries.

Ozone has been found to be effective in reducing aflatoxin levels in different commodities (Prudente and King, 2002, Proctor *et al.*, 2004, Akbas and Ozdemir 2006, Inan *et al.*, 2007). However, few or limited studies have been done on the potential toxicity and possible carcinogenicity of ozone-aflatoxin reaction products. Since ozone did not prevent toxicity of AFB_1 in the adult hydra assay as a result of forming 3-keto derivative of AB_1 (Mckenzie *et al.*, 1997), the concern of this study was to decrease the population of *A. flavus* on peanuts before incubation and consequently decrease AFB_1 production.

Treating raw peanut samples with ozone immediately after inoculation with 10^3 and 10^5 spores g^{-1} unaffected the content of AFB_1 due to its small amount (0.2 to 0.4 ppb) which may be needs more time exposing. However, after storage for 4 days and due to the growth of *A. flavus* and producing AFB_1 with high quantity it was easy to evaluate the effect of preliminary ozonation process on AFB_1 accumulation over storage. The dose 400 ppm of prestorage ozonation significantly inhibited AFB_1 production on both samples with different initial inocula. Also, 100 ppm of ozone was effective in reduction on samples inoculated with 10^5 spores g^{-1}. Similar situation happened but using lower concentration of ozone for long time (Giordano *et al.,* 2010). They found that 14 and 32.5 ppm of ozone completely inhibited AFB_1 production on Brazil nut under ozone for 1 day as a result of completed inhibition of aflatoxigenic fungi.

Chapter 5

BIOLOGICAL CONTROL USING ACTINOMYCETES

5 BIOLOGICAL CONTROL USING ACTINOMYCETES

5.1 INTRODUCTION

Biological control is the use of living agents to control pests or plant pathogens. This approach is being increasingly considered by the scientific community as a reliable alternative to pesticide utilization in field and post-harvest (Bleve *et al.,* 2006). Aflatoxin contamination of crops compromises the safety of food and feed supplies and causes significant economic losses worldwide (Dorner, 2004). Of the many research approaches being studied to reduce and, ultimately, eliminate aflatoxin contamination, biological control is one of the more promising ones, particularly in the medium-term. Numerous organisms have been tested for biological control of aflatoxin contamination including bacteria, yeasts, and non-toxigenic strains of the causal organisms, *Aspergillus flavus* and *A. parasiticus* (Dorner, 2004).

Among microorganisms, actinomycetes are one of the most investigated groups particularly members of the genus *Streptomyces* (Okami and Hotta, 1988). Streptomycetes, are Gram (+) filamentous bacteria, widely distributed in a variety of natural and man-made environments, constituting a significant component of the microbial population in most soils (Watve *et al.*, 2001). Extensive screening of strains has led to the discovery of about 4,000 antibiotic substances from bacteria and fungi, many of which have been applied in human medicine, veterinary science and agriculture. Most of them are produced by *Streptomyces* species (Hwang *at al.*, 1994). Few studies have examined the potential of actinomycetes to control mycotoxigenic fungi *in vitro* and *in situ* especially for control of aflatoxins in peanuts. Bioactive compounds, because of their natural origin, are biodegradable and they do not leave toxic residues or by-products which may contaminate the environment (Aoudou *et al.*, 2010). This suggests that actinomycetes isolated from peanuts could provide antagonistic strains which could control *A. flavus* and aflatoxin production. The objectives of this study were (a) to isolate and screen potential biocontrol strains (actinomycetes) from peanuts, (b) to examine the selected strain (*Streptomyces* AS1) for antagonistic effect against *A. flavus* EGP-B07 *in vitro* (media) and *in situ* (peanuts), (c) to study the antifungal activity of the ethyl acetate extract from the

cultural broth of *Streptomyces* AS1 against *A. flavus* and Aflatoxin B_1 (AFB_1) production *in vitro* and *in situ*.

5.2 MATERIALS AND METHODS

5.2.1 Isolation media

Malt extract agar (MEA), malt salt agar (MS): The same procedure as described in Section 2.2.2 was used for preparation of these media.

Yeast extract sucrose agar (YES): YES was prepared as decribed in Section 3.2.2. The water availability of the media was adjusted using glycerol to obtain 0.95, 0.92 and 0.89 a_w.

Half-strength nutrient agar (1/2 NA): 50% concentration of the supplier's recommendation of commercial NA (Oxoid, Basingstoke, Hampshire, UK) was made up (14 g L^{-1}). To this, an additional technical agar no. 3 (6 g L^{-1}) was added to facilitate solidification. After mixing, the medium was autoclaved and poured into the plates.

Nutrient broth (NB): Nutrient broth (Fluka Chemie Gmbh CH-9471 Buchs, Switzerland) (13 g L^{-1}) was used in 500 ml conical flasks. Flasks were autoclaved and left to cool prior to inoculation.

Half-strength nutrient broth (1/2 NB): 50% Nutrient broth (Fluka Chemie Gmbh CH-9471 Buchs, Switzerland) (6.5g l^{-1}) was used in 500 ml conical flasks. Flasks were autoclaved and left to cool prior to inoculation.

Peanut meal extract agar (PMEA): PMEA was prepared by adding 20g homogenized peanut seeds and 20g agar to 1 litre distilled water. After mixing, the substrates were autoclaved and poured into 9 cm Petri plates as mentioned previously. Petri plates were kept at 4°C for a maximum of 21 days.

5.2.2 Isolation of actinomycetes from peanuts

In-shell peanut samples from three regions in Egypt (one each from: Alexandria, Daqahelaya and Asyut) were used to isolate actinomycetes. Ten grams of each

sample was weighed into Stomacher bags containing 90 ml of distilled water and left for 10 min to soak. The bags were homogenized for 15 min in the Colworth Stomacher 400. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) were made and 200 µl from each dilution plated on ½ NA (containing cyclohexamide, 25mg ml^{-1} media after autoclaving, Kumar and Kannabiran, 2010) in triplicate. The plates were incubated at 25°C for 5 days and examined periodically. Six isolates of actinomycetes (5 from Daqahelaya and one from Asyut samples) were sub-cultured and purified on ½ NA medium. The behaviour of each isolate was tested on different media (½ NA, YES 0.95, MEA, PMEA and MS).

5.2.3 Screening for antifungal efficacy

The isolates of actinomycetes were tested for antagonistic activity against an aflatoxigenic *A. flavus* EGP-B07 strain isolated from Egyptian peanuts. They were inoculated as a single streak with a sterile loop at a point 2.0 cm from the periphery of the Petri plates containing the different media previously mentioned in triplicate. The plates were incubated at 25°C. After two days growth, the plates were inoculated with 5µl of fungal spore suspension (10^6 spores ml^{-1}), placed 4.0 cm from the centre of the actinomycetes streak (Figure 5.1) and incubated at 25°C for 7 days. During the incubation period, the interaction between dual cultures (Index of dominance, I_D) was examined regularly. Each interacting species was given an interaction score based on whether mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (4-0) and dominance at a distance (5-0) occurred (Magan and Lacey, 1984). Also, the antifungal activity was evaluated by measuring the area of fungal colony on YES and comparing with controls (*A. flavus* without the actinomycetes strains).

5.2.4 Effect of incubation time on antifungal efficacy

The isolate obtained from the Asyut region (AS1) was used in these studies. The spores from the pure culture were inoculated into 250 ml of ½ NB medium in 500 ml Erlenmeyer flasks. Two flasks were inoculated and incubated on a rotary shaker (250 rpm) at 25°C; one for 5 days and one for 10 days. After incubation, 50 ml from both cultures were filtered through Whatman No. 1 filter paper. The filtered cultures were examined for efficacy against *A. flavus* on ½ NA and YES 0.95 a_w medium.

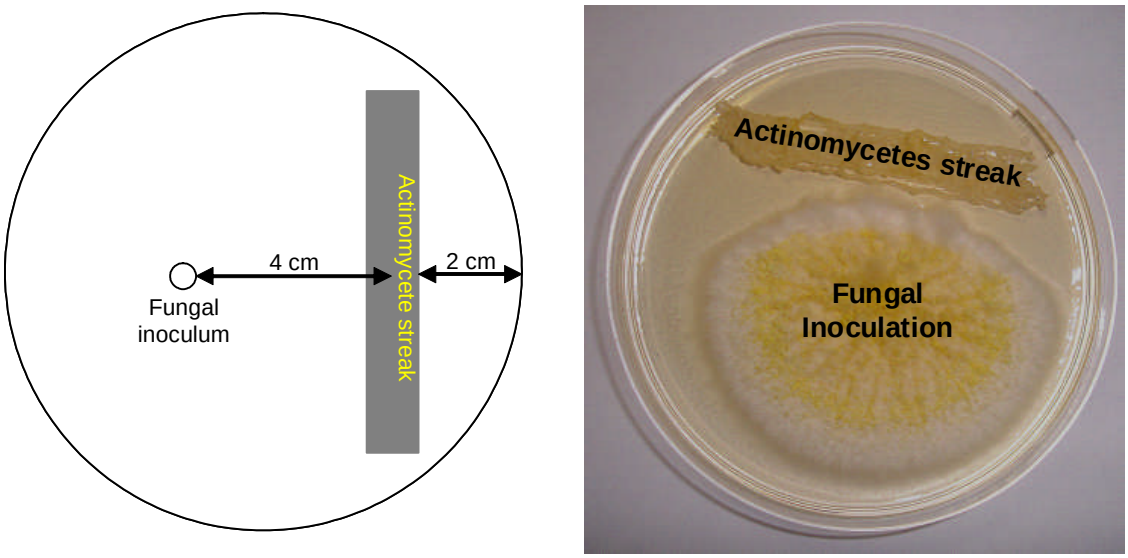


Figure 5.1. Agar streak method (Taechowisan *et al.*, 2005) of screening for antifungal efficacy.

One plug was removed using a cork borer (0.8 cm) from the plates at a point 2.0 cm from the periphery to make a well. In triplicates, 50 µl from each culture (5 and 10 days) were put into the wells. Five µl of the fungal spore suspension was placed 3.0 cm from the centre of the well (Figure 5.2) and treatments incubated at 25°C for 5-7 days.

5.2.5 Extraction of antifungal compounds from broth culture of strain AS1

The broth culture of AS1 after 5 days incubation was used for extraction of metabolites. Chloroform, ethyl acetate, butanol and petroleum ether were examined for efficiency of extraction of antifungal compounds. Two hundred millilitres of AS1 culture was extracted with each solvent. Extraction of antifungal compounds was performed 3 times with an equal volume of each solvent in a 1 Litre separating funnel. The collected solvent extracts were evaporated to dryness using a rotary evaporator at 40°C except for butanol at 90°C. Dry films were dissolved with 5 ml of the same solvent. Broth culture and the solvent extracts were examined for efficacy against aflatoxigenic *A. flavus* on YES medium using the well method described previously. After 5-10 days at 25°C, the diameters of colonies were measured in two diameters at right-angles to each other. Also, 1-5 agar plugs were taken from *A. flavus* colony based on its size using a cork borer (8 mm) and weighed, frozen at -20°C until later for AFB_1 analysis by HPLC.

5.2.6 Effect of AS1 metabolites on spore germination of *A. flavus*

(a) Diffusion assay: The isolate AS1 was inoculated on the surface of sterile cellophane discs overlaying ½ NA medium. Plates were incubated at 25°C for 10 days. Periodically after 2, 5 and10 days, the cellophane discs were removed and 100 µl of *A. flavus* spores ($1x10^6$ spores ml^{-1}) was spread plated onto the media and the plates were incubated at 25°C for 2 days. Microscopic examination was done daily to determine spore germination.

(b) Ethyl acetate extract: Ten litres of AS1 culture in ½ NB (5 days old) were extracted with ethyl acetate and then evaporated. The dry extract was re-dissolved in ethyl acetate at a concentration of 20 mg ml^{-1} as a stock solution. Appropriate

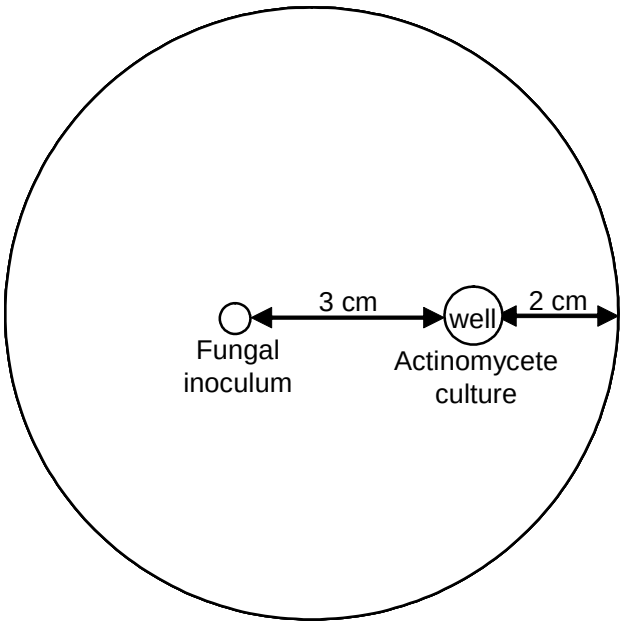


Figure 5.2. Well diffusion method (Dharmaraj and Sumantha, 2009) for antifungal efficacy test

quantities of AS1 extract were transferred aseptically into sterilized medium of YES at different a_w levels (0.95, 0.92 and 0.89) to obtain individual concentrations of 50 and 100 ppm. Bottles of molten media were thoroughly shaken prior to pouring, to ensure that the metabolites were dispensed. A control was prepared by transferring an equivalent amount of ethyl acetate to YES medium before pouring. Fungal inoculation and spore germination were as described previously.

5.2.7 Effect of crude extract of AS1 on the control of *A. flavus* growth and AFB_1 production

YES medium containing 50 and 100 ppm of AS1 crude extract were subsequently inoculated with *A. flavus* in two ways. Firstly, was by central inoculation with a 5 µl of a spore suspension ($1x10^6$ spores ml^{-1}). Secondly, was by placing a 8 mm diameter MEA plug covered with actively growing mycelium (one day old) in the centre. Mycelial growth rates of *A. flavus* for both treatments were determined by daily measurement of colony diameters in two directions at right-angles to each other over periods of 10 days. Linear regression of the colony radius (mm) against time was used to determine the relative growth rates (mm day^{-1}). Also, 1-5 agar plugs were taken from the *A. flavus* colony using a cork borer (0.8 cm), weighed, and frozen at -20°C until later aflatoxin analysis by HPLC.

5.2.8 *In situ* application of strain AS1 and its metabolites in controlling *A. flavus* on stored peanuts.

Peanut samples in cultur vessels were autoclaved as described in 3.2.5 and then adjusted to 0.93 a_w. The effect of strain AS1 on colonisation of *A. flavus* and aflatoxin production was examined. Peanuts were inoculated with *A. flavus* at room temperature in order to obtain a final concentration of 10^4 spores g^{-1}, by mixing thoroughly. Then, the inoculated peanut vessels were treated with strain AS1 (10^5 spores g^{-1}). Controls were inoculated with either *A. flavus* or strain AS1.

The efficiency of AS1 crude extract on inhibition of either the growth of *A. flavus* or aflatoxin production in peanuts was tested. As a preliminary study, at 0.93 a_w, a small quantity of AS1 (20mg ml^{-1} in ethanol) was added to the vessels to reach final concentrations of 1, 5 and 25 mg g^{-1} (ppm) of peanut grains. Treated peanuts and controls (ethanol only) were manually mixed to obtain the required concentration into

vessels and then left for 2 hrs at room temperature. Vessels containing the peanuts were inoculated with *A. flavus* (10^4 spores g^{-1} peanuts).

Higher concentrations of AS1 treatments were also prepared (50, 200 and 500 ppm). Inhibitory effect on *A. flavus* at two a_w levels (0.90 and 0.93 a_w) was studied. All the experiments were carried out with three separate replicates per treatment. The cultures were incubated at 25°C. Peanuts colonisation and AFB_1 measurement was analyzed befor storage, after 7 and 14 days. The spread-plate method was used to enumerate *A. flavus* on MS medium and *Streptomyces* AS1 on ½ NA medium as describes in Section 3.2.6.

5.2.9 Extraction and quantification of AFB_1 in media and peanut samples

AS described in Section 2.2.5.

5.2.10. Cytotoxicity bioassay

Brine shrimp lethality bioassay (Mclaughlin, 1991; Krishnaraju *et al.*, 2005; Al-Bari *et al.*, 2007) was carried out to investigate the cytotoxicity of the ethyl acetate extract of *Streptomyces* strain AS1. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g L^{-1}) under constant aeration at 30°C for 48 hrs. After hatching, active nauplii (larvae, matured shrimp) free from egg shells were collected from the brighter portion of the hatching chamber and used for the assay. The extract was dissolved in DMSO (not more than 50 µl in 5 ml solution) plus sea water (3.8% Nacl in water) to attain concentrations of 0.5, 1, 2.5, 5, 10 µg ml^{-1}. A tube containing 50 µl DMSO diluted in 5 ml was used as a control. Ten nauplii were drawn through a glass capillary and placed in each tube. The number of the nauplii that died after 24 hrs at room temperature was counted. Experiments were conducted together with a control and different concentrations of the test substances in a set of three tubes per dose. The findings are presented graphically by plotting log concentration versus percentage mortality of napplii from which LC_{50} was determined by extrapolation (Goldstein *et al.*, 1974).

5.2.11 Statistical analyses

As described in Sections 2.2.7 and 3.2.8.

5.3 RESULTS

5.3.1 Isolation of actinomycetes and screening for antifungal activity

Six actinomycete isolates were obtained from in-shell peanuts (5 from Daqahelaya, D1-D5, and one from Asyut regions, AS1). The colonies of actinomycetes were recovered from different Petri dishes. Actinomycetes were recognized by their characteristic morphological growth on ½ NA. The colonies appeared dry, rough, sometimes coloured, adhering to the medium with some vegetative mycelium. They were sub-cultured onto ½ NA and incubated at 25 °C for 7 days. Five media (½ NA; YES; MEA; PMEA and MS) were tested to identify the growth behaviour of each isolate at 25°C for 5 days. Figure 5.3 shows that the best media for growth was ½ NA for all isolates followed by YES except for isolate D4 which grew slowly and produced pigments. All strains were unable to grow on MS medium. D3, D5 and AS1 revealed symmetric growth on MEA whereas D1, D2 and D4 showed non-symmetric growth on this medium. White and hard colonies accompanied with pigment production were observed on PMEA medium for D1, D2 and D4. However, isolates D3, D5 and AS1 grew faster on this medium.

To identify the best isolate and medium for efficacy against *A. flavus*, the same media were used for screening all isolates. In general, visual observations indicated that the presence of actinomycete isolates on all media decreased the area of *A. flavus* growth when compared with controls. Whereas, AS1 showed the best inhibition effect (Figure 5.4). Table 5.1 compares the effect of actinomycetes on *A. flavus* and the relative types of interaction observed. The ranges of inhibition were 11-39%, 0-18.5%, 29-100%, 4-55% and 13-38% on YES, PMEA, ½ NA, MEA and MS media respectively. Thus, ½ NA medium was the best medium for producing antifungal compounds against *A. flavus.* Isolate AS1, completely inhibited growth of *A. flavus* on ½ NA medium. Interaction scores between the actinomycetes and *A. flavus* were also measured. There were no clear zones between actinomycetes (D1, D2, D3, D4 and D5) and fungal colonies, but D5 was capable of lysing the test fungal hyphae on YES medium (2/2). However, there was no direct contact between the AS1 colonies and the inhibited fungal colonies with I_D scores of 5/0 on ½ NA and YES, 4/0 on MEA and MS and total of 21/3 on the tested media.

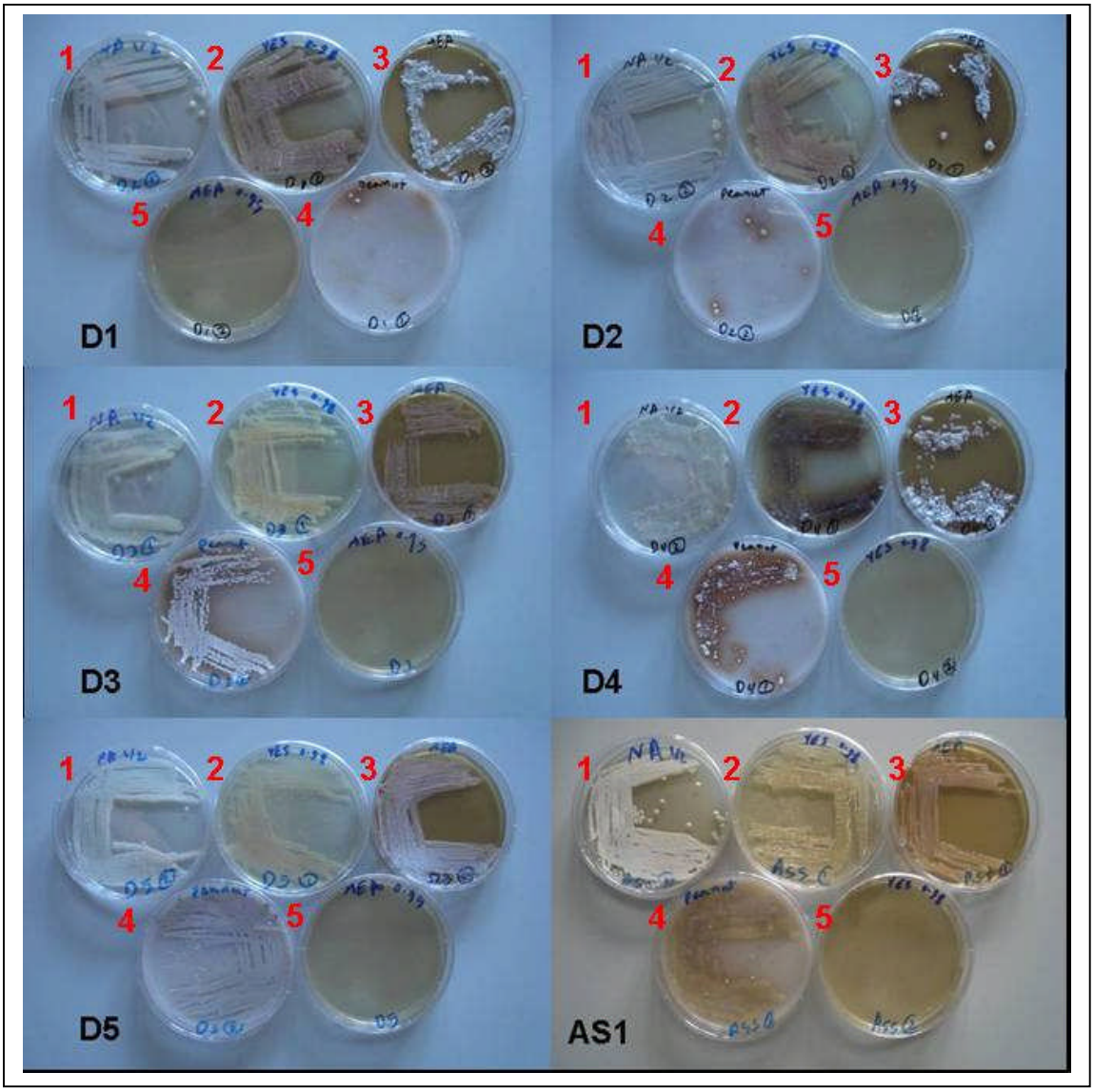


Figure 5.3. The behaviour of the actinomycete isolates from peanuts on different media (1,1/2 NA; 2, YES; 3, MEA; 4, PMEA; 5, MS)

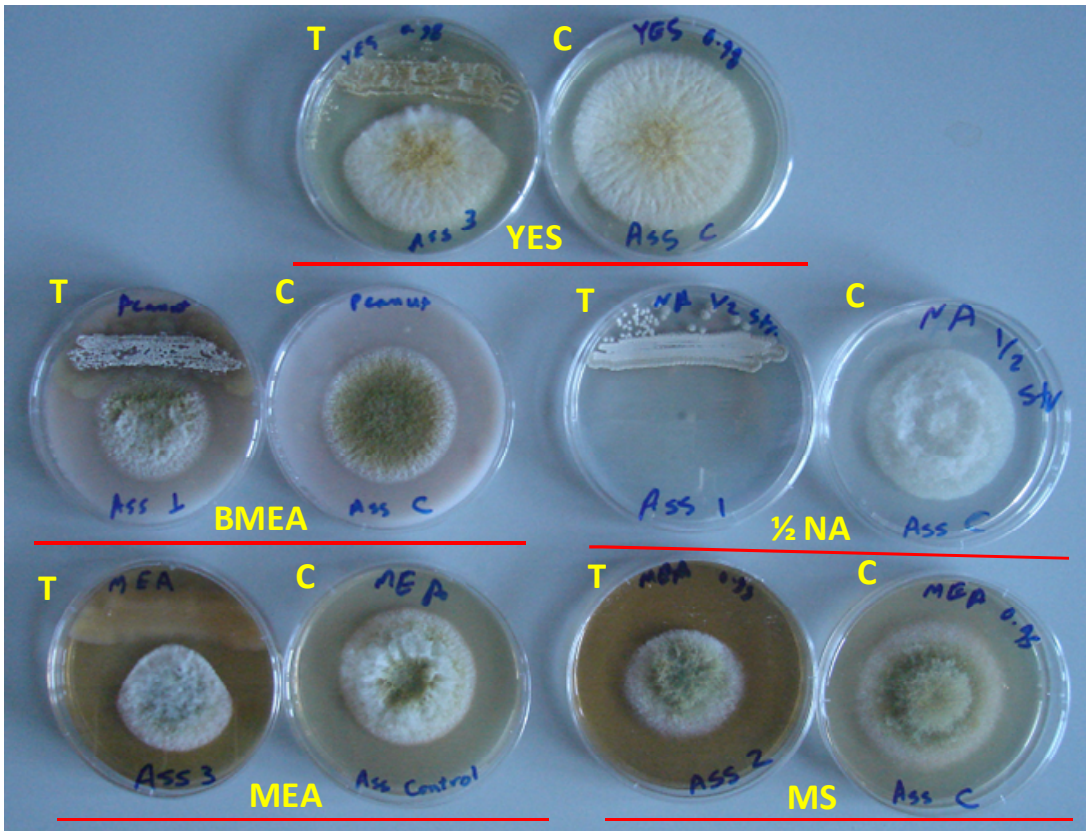


Figure 5.4. The antagonism of actinomycete AS1 isolate against *A. flavus* on different media (T, antagonistic effect of AS1 against *A. flavus*, C, control of *A. flavus*).

Table 5.1. Actinomycetes antagonist against *A. flavus* on different media at 25 °C for 7 days.

Isolates	Medium	Fungal colony area (cm^2)					Interaction score					
		YES	PMEA	½ NA	MEA	MS	YES	PMEA	½ NA	MEA	MS	I_D
D1	Control	39.6	18.9	25.6	22.9	31.2	--	--	--	--	--	--
	Tested	35.3	18.9	14.6	18.1	22.6	1/1	1/1	1/1	1/1	n.g.	4/4
D2	Control	42	18.9	27.4	22.1	25.5	--	--	--	--	--	
	Tested	33.2	15.5	12.9	18.9	22.1	1/1	1/1	1/1	1/1	n.g.	4/4
D3	Control	39.6	18.9	25.6	20.8	25.5	--	--	--	--	--	--
	Tested	24.2	16.3	13.2	20.8	15.9	1/1	1/1	2/2	1/1	n.g.	5/5
D4	Control	43	20.5	29.3	22.1	28.3	--	--	--	--	--	--
	Tested	33.2	16.7	16.3	22.9	21.2	n.g.	1/1	1/1	n.g.	n.g.	2/2
D5	Control	39.6	21.3	27	22.5	27.4	--	--	--	--	--	--
	Tested	28.3	18.9	15.3	20.4	19.6	2/2	1/1	2/2	1/1	n.g.	6/6
AS1	Control	46.6	21.3	25.6	22.5	30.2	--	--	--	--	--	--
	Tested	28.3	17.4	0.0	10.1	18.9	5/0	3/3	5/0	4/0	4/0	21/3

--, Not tested; n.g. not enough growth; Index of Dominance (I_D): 1/1, mutual intermingling; 2/2 mutual inhibition on contact; 3/3, mutual inhibition at a distance; 4/0, dominance on contact; 5/0, dominance at a distance (Magan and Lacey, 1984).

5.3.2 Effect of incubation time on the efficiency of producing antifungal compounds

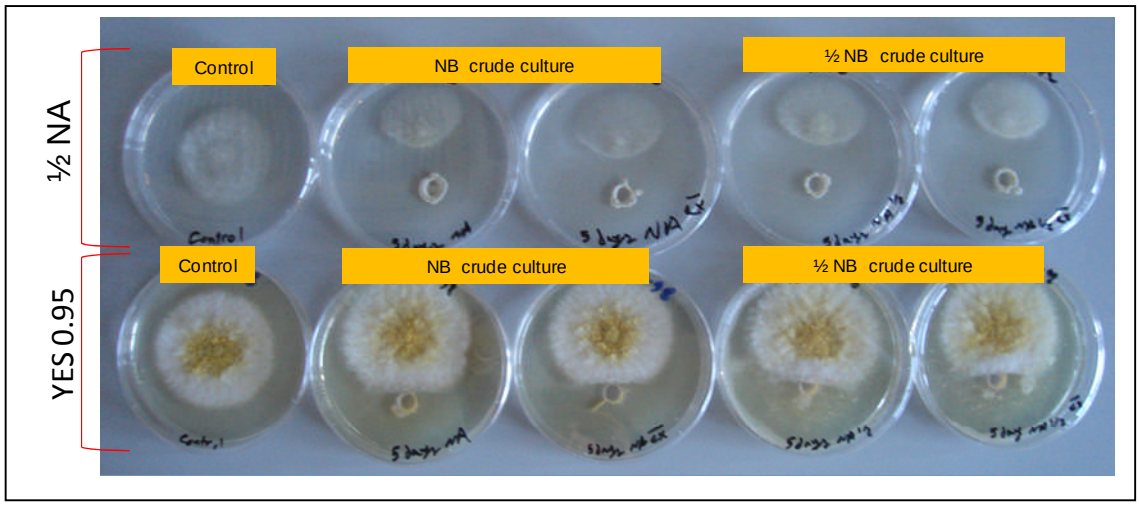
Isolate AS1 was identified as a *Streptomyces spp.* using partial DNA sequencing by CBS (Holland). Two broth media (½ NB medium and NB medium) were examined for producing antifungal compounds by isolate AS1 at 25°C for 5 and 10 days. The antagonistic effect against *A. flavus* by crude cultures at each incubation period were tested on YES 0.95 a_w and ½ NA media using the well diffusion method at 25°C for 5-7 days (Figure 5.5). By visual observation, the inhibition effect on ½ NA was higher than on YES medium. Clear inhibition zones around the wells were noticeable on YES medium using 5 day old AS1 culture. However, *A. flavus* was unaffected when using 10 day old cultures of AS1.

5.3.3 The efficacy of antifungal compounds extracted using different solvents

Antifungal compounds were extracted from five day old broth cultures (½ NB) of isolate AS1. In addition to the filtrate, 4 solvent extracts (chloroform, petroleum ether, ethyl acetate and butanol) were tested for efficacy of extraction using the well diffusion method on YES medium. It is interesting to observe that the biomass of fungal colonies treated with butanol was less and the colonies grew more slowly (10 days) than those treated with the other extracts (5 days). The filtrate and all solvent extracts inhibited fungal growth to different extents. However, butanol, ethyl acetate and chloroform extracts achieved the best inhibition (28.1, 21.6 and 20.1% respectively, Table 5.2).

The effect of crude culture, extracts of fatty chloroform, ethyl acetate and butanol on aflatoxin production were studied (Figure 5.6). The presence of butanol for both control (butanol solvent) and extract completely inhibited AFB_1 production by *A. flavus*. Statistically, the production using crude culture and fatty portion of chloroform extract did not affect AFB_1 production when compared to the controls. However, it was significantly reduced (4 times reduction) using the ethyl acetate extract.

5 days



10 days

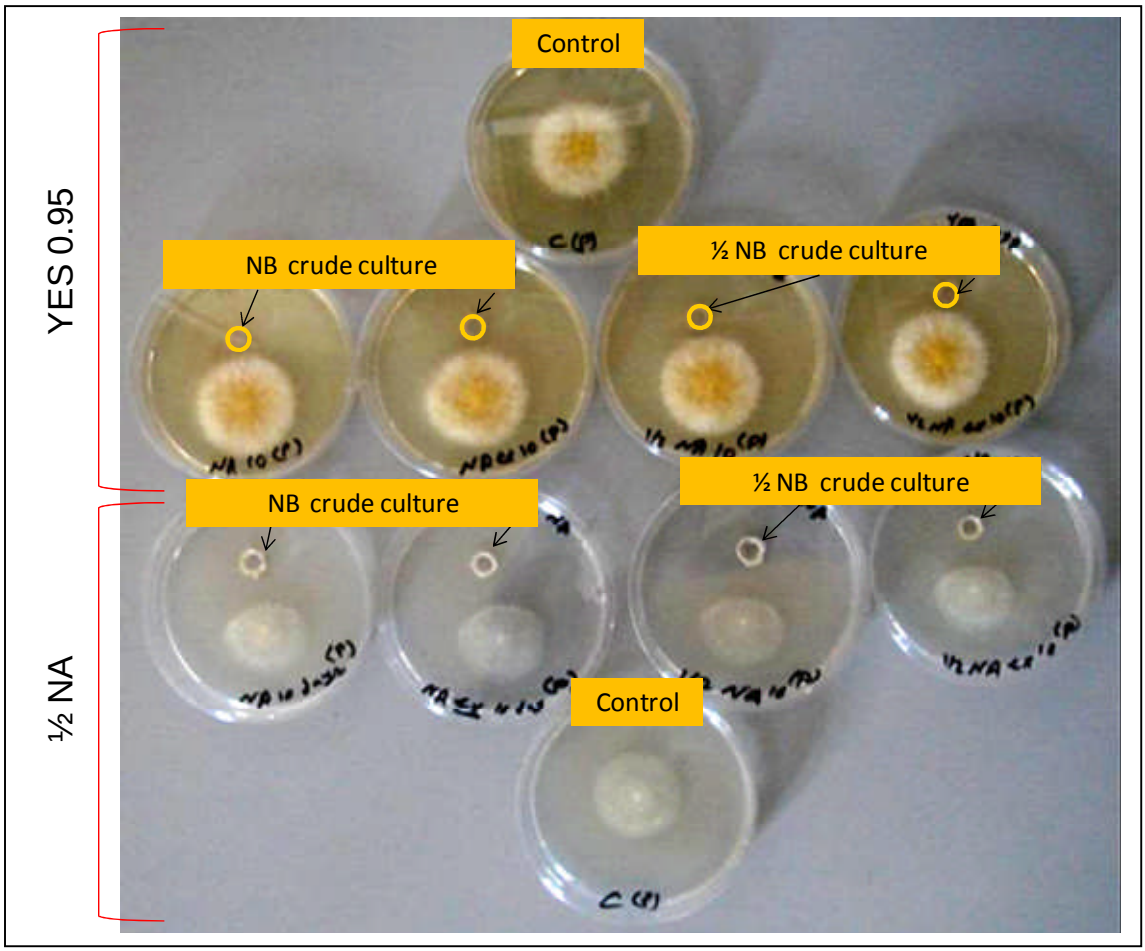


Figure 5.5. Effect of the media type (½ NB and NB) and incubation time (5 and 10 days) on the antagonism of *A. flavus* on ½ NA & YES media by *Streptomyces* AS1.

Table 5.2. Solvent extract antagonism of *A. flavus* (colony area, cm^2) using the well diffusion method on YES at 25°C.

Incubation period	5 days incubation							10 days incubation	
Type of extract	C	CC	CE	PC	PE	EC	EE	BC	BE
Fungal colony area (cm^2)	19.6	20.4	16.3	17.7	16.3	18.5	14.5	16	11.5

C, negative control (without treatment); CC, chloroform control; CE chloroform extract; PC, petroleum ether control; PE, petroleum ether extract; EC, ethyl acetate control; EE, ethyl acetate extract; BC, butanol control; BE, butanol extract)

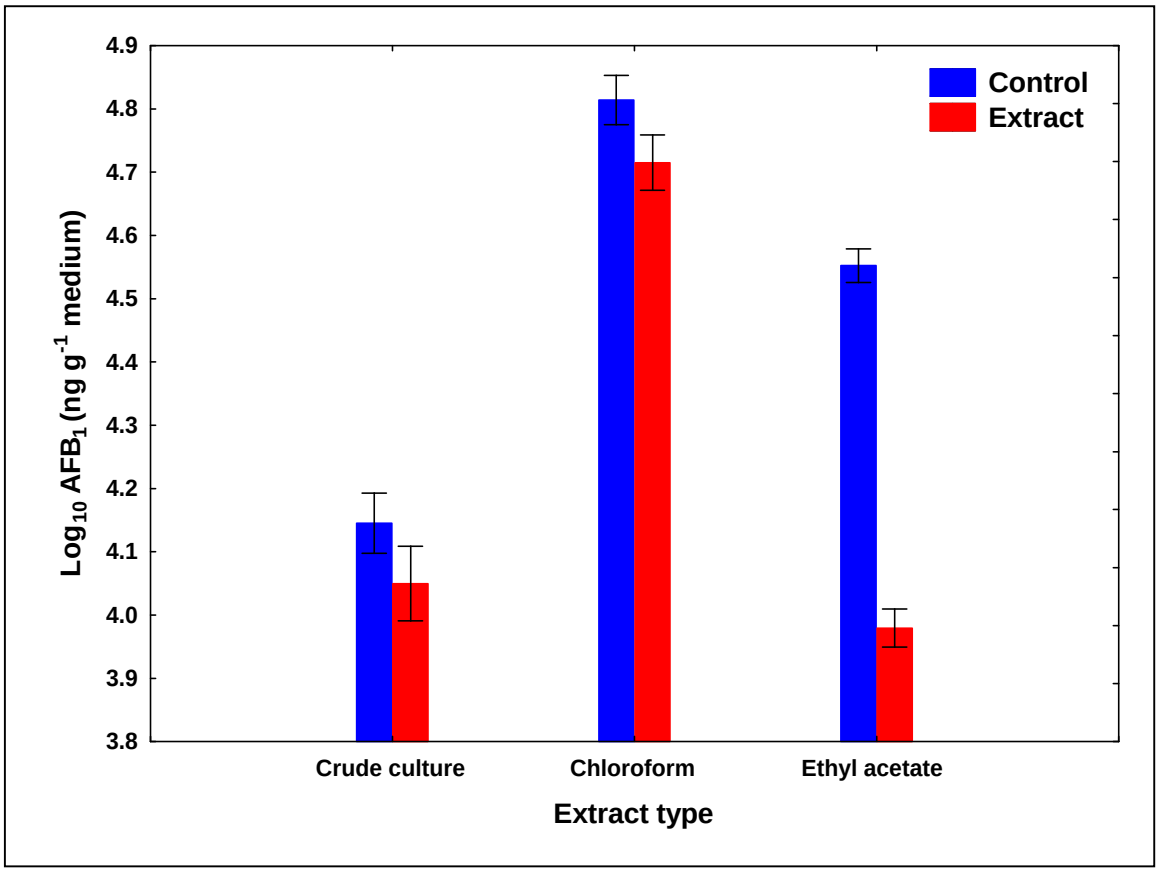


Figure 5.6. Effect of solvent extracts of AS1 culture on AFB_1 production by *A. flavus* on YES medium. Bars indicate standard error of the means.

5.3.4 Effect of metabolites on germination of *A. flavus* spores

(a) Diffusion assay:

Spore germination of *A. flavus* was examined using the metabolites from isolate AS1. The cellophane diffusible metabolites after 2, 5 and 10 days incubation completely inhibited spore germination of *A. flavus* (100%) when compared with controls. Microscopic examination showed that no germination occurred within the 48 hrs test period.

(b) Ethyl acetate extract

The same influence of compound able to diffuse through the cellophane was observed using Ethyl acetate extract. AS1 at 50 and 100 ppm in YES medium at all a_w levels achieved 100% inhibition of *A. flavus* germination compared with controls over 48 hrs.

5.3.5 *In vitro* effect of AS1 crude extract on the growth of *A. flavus* and AFB_1 production

The influence of AS1 at 50 and 100 ppm on the growth of *A. flavus* on YES media at different a_w condition (0.95, 0.92 and 0.89 a_w) is illustrated in Figures 5.7, 5.8. Regardless of AS1, the growth rate was significantly inhibited by decreasing the a_w levels. Compared with control (0 ppm) at each a_w level, more than 85% inhibition of growth was observed using AS1 at both concentrations. AS1 treatmnet was the main parameter showed statistically significant effect on the growth rate (mm day^{-1}) of *A. flavus* on YES medium followed by a_w parameter (Table 5.3).

Similarly, the inhibitory effect of AS1 (50 and 100 ppm) x a_w levels on growth of *A. flavus* mycelia was examined. Growth was completely inhibited at all a_w levels on YES medium (Fig 5.9). However, irregular growth patterns (marked with dashed red border) were observed on the edge of the mycelial plugs placed in the centre of the plates. The growth was decreased regularly by the decreasing a_w level and by the the increase in the concentration of AS1. On the control plates (0 ppm), however, fungal mycelium showed regular, radial growth.

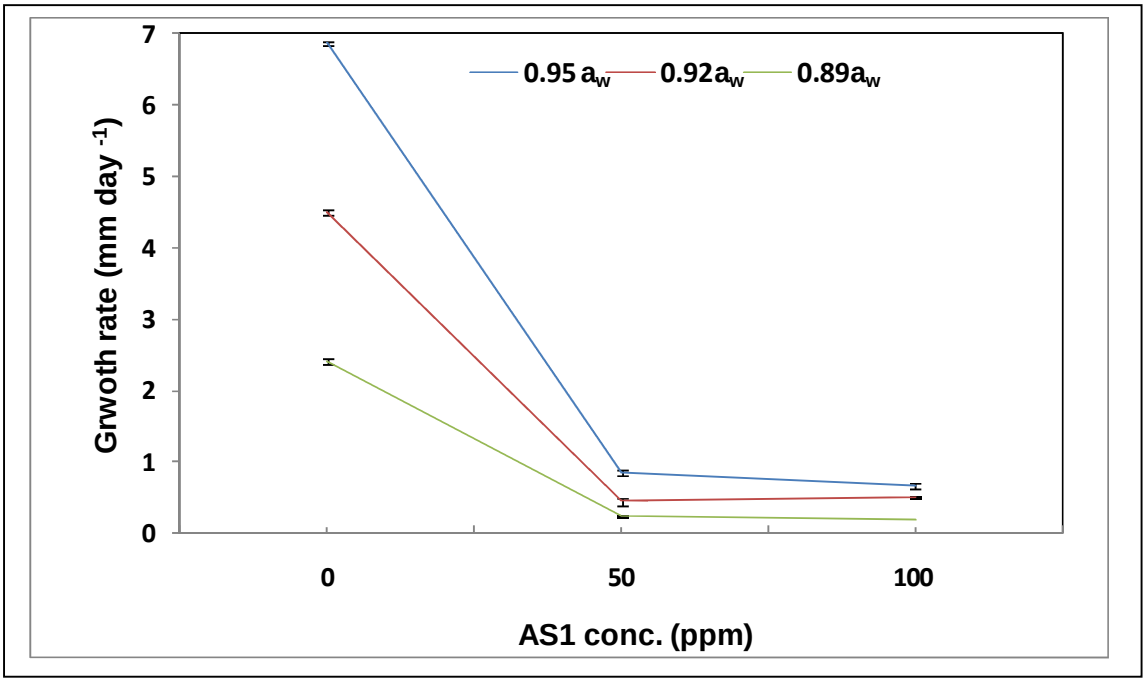


Figure 5.7. Effect of the of AS1 ethyl extract on growth of *A. flavus* on YES medium. Bars indicate standard error of the means.

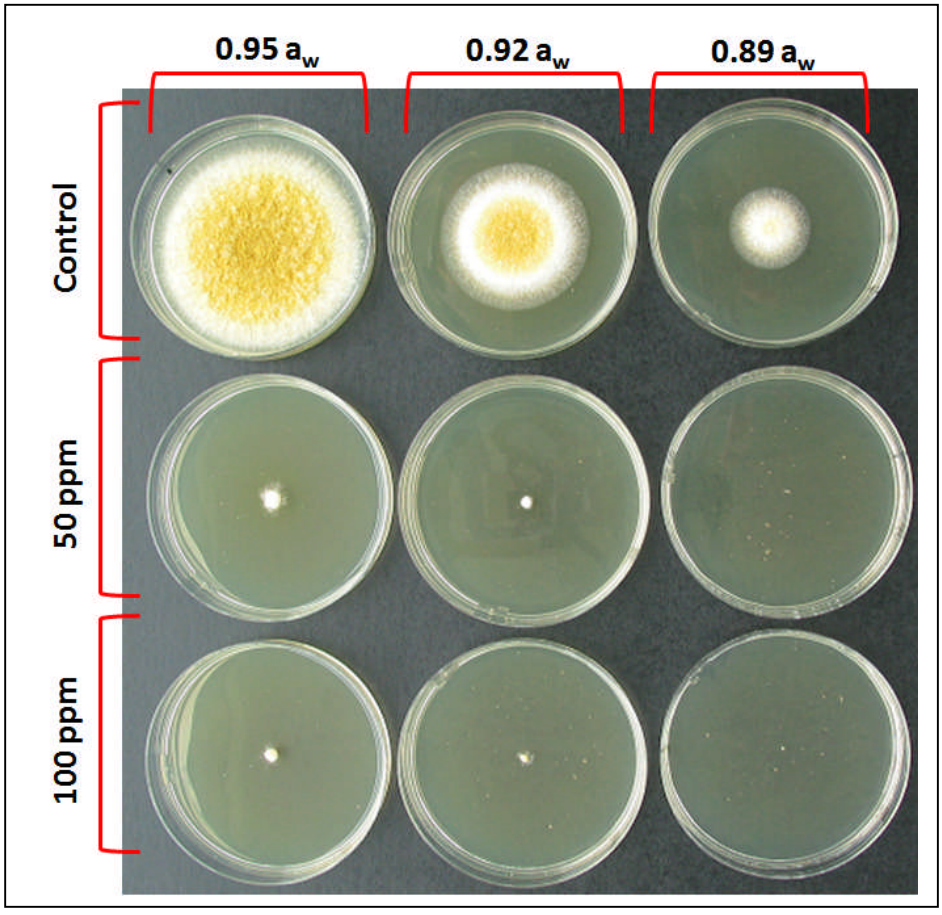


Figure 5.8. Influence of AS1 extract at different a_w levels on growth of *A. flavus* on YES medium at 25°C for 10 days.

Table 5.3. Analysis of variance of the effect of AS1, water activity (a_w) and their interaction on the growth rate of *A. flavus* on YES medium.

Effect	SS	DF	MS	F	P
Intercept	92.9580	1	92.95803	27225.64	0.00
AS1 (ppm)	101.4985	2	50.74927	14863.49	0.00
a_w	15.4714	2	7.73571	2265.64	0.00
AS1 x a_w	15.2026	4	3.80064	1113.14	0.00
Error	0.0615	18	0.00341		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

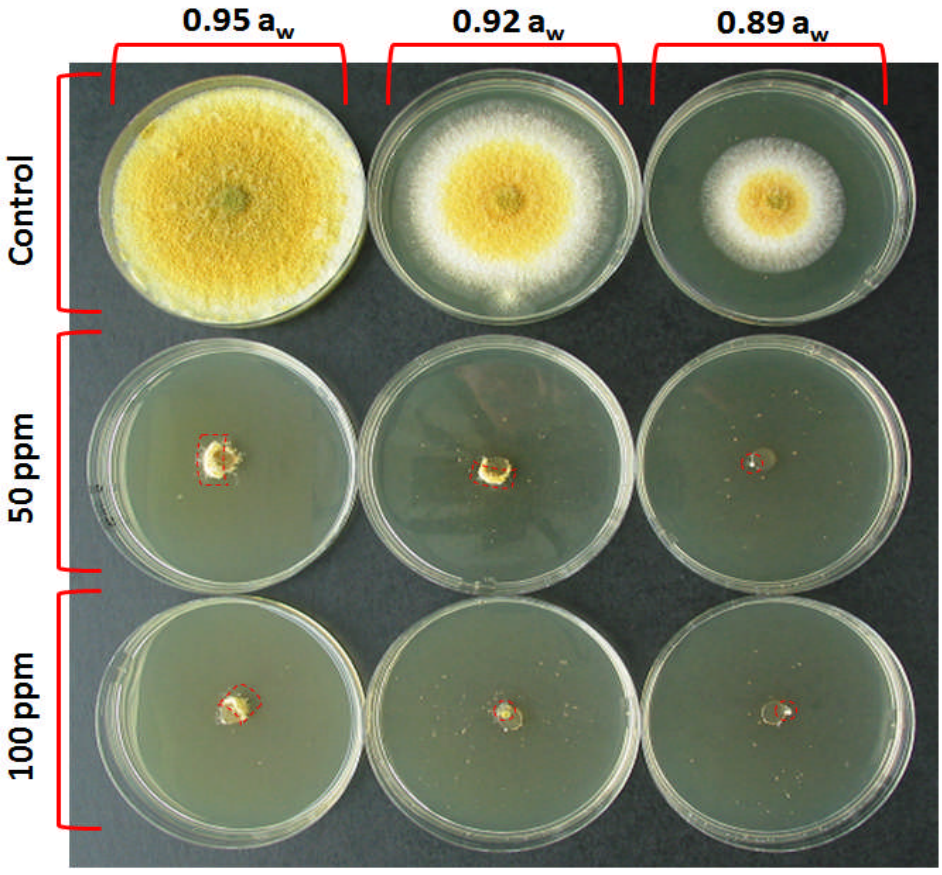


Figure 5.9. The inhibitory effect of AS1 at different a_w levels on the growth of *A. flavus* mycelium (one day old) after 10 days incubation.

The same pattern of the influence of AS1 on *A. flavus* growth on YES medium was observed on AFB1 production (Fig. 5.10). At 0.89 a_w the growth rate was very slow at 50 and 100 ppm and consequently no AFB_1 was detected in YES media. However, at 0.95 a_w growth at these doses was visible and AFB_1 production was also higher when compared with the lower a_w levels. Regardless AS1 concentration, AFB_1 concentrations were significantly decreased by decreasing the a_w levels. At 0.95 a_w, 50 and 100 ppm of AS1 significantly decreased the amount of AFB_1 by around 4 and 10 times respectively. This reduction increased at 0.92 a_w when compared with the control. Table 5.4 shows the statistical effects of AS1, a_w and their interaction on AFB_1 production by *A. flavus* ($P<0.05$). AS1 concentration was the major factor affecting AFB_1 production on media.

5.3.6 Efficacy of *Streptomyces* AS1 on *in situ* control of *A. flavus* on peanuts

Figure 5.11 illustrates the effect of *A. flavus* on AS1 populations over 14 days. The initial count of AS1 in the control samples and those treated with *A. flavus* was similar before storage. The colony forming units of AS1 in control samples (no *A. flavus*) statistically increased 1.5 log CFUs g^{-1} after 7 days. However, it significantly decreased again by the second incubation period when compared with that of 7 days. Occurrence of *A. flavus* on stored peanuts significantly inhibited AS1 population size over 7-14 days. The populations of *Streptomyces* AS1 were significantly affected by *A. flavus*, time and their interaction ($P<0.05$) on stored peanuts at 0.93 a_w (Table 5.5). The major impact was produced by incubation time.

Figure 5.12 shows that there was no antagonistic effect of the *Streptomyces* AS1 on *A. flavus* populations during the storage period of peanuts. Table 5.6 shows that ANOVA results for *Streptomyces spp*. (AS1), time and their interaction on *A. flavus* populations (log_{10} CFUs g^{-1} peanut) at 0.93 a_w. Time of storage had the main impact on the populations. However, no significant inhibitory effect was observed due to treatment with cells of *Streptomyces* AS1.

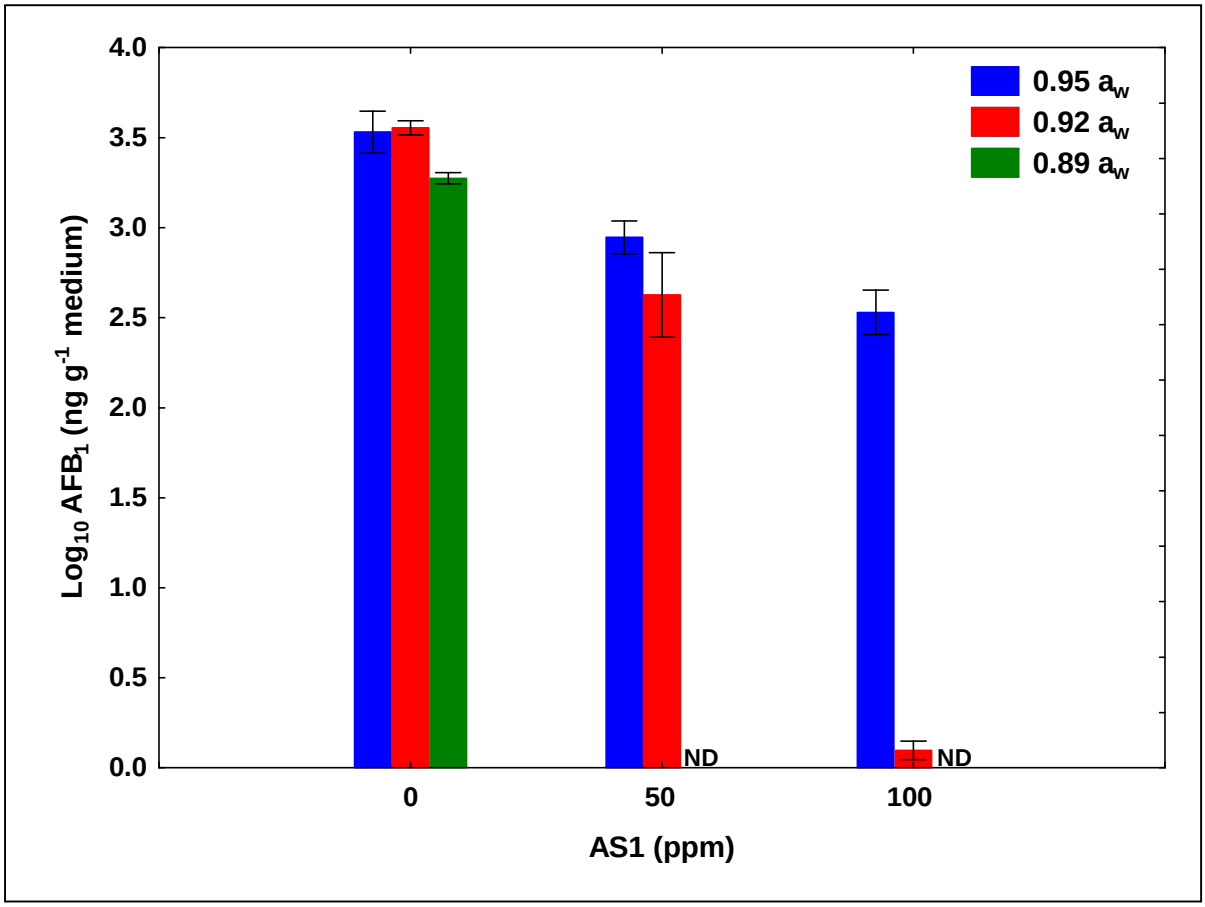


Figure 5.10. Effect of the ethyl acetate extract of AS1 culture on AFB_1 production by *A. flavus* on YES medium at 25°C for 10 days (Bars indicate standard error of the means). ND: None detected.

Table 5.4. Analysis of variance of the effect of AS1, water activity (a_w) and their interaction on the production of AFB_1 by *A. flavus* on YES.

Effect	SS	DF	MS	F	P
Intercept	114.8292	1	114.8292	3541.257	0.000000
AS1 (ppm)	30.4706	2	15.2353	469.847	0.000000
a_w	16.4508	2	8.2254	253.666	0.000000
AS1x a_w	11.7095	4	2.9274	90.278	0.000000
Error	0.5837	18	0.0324		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

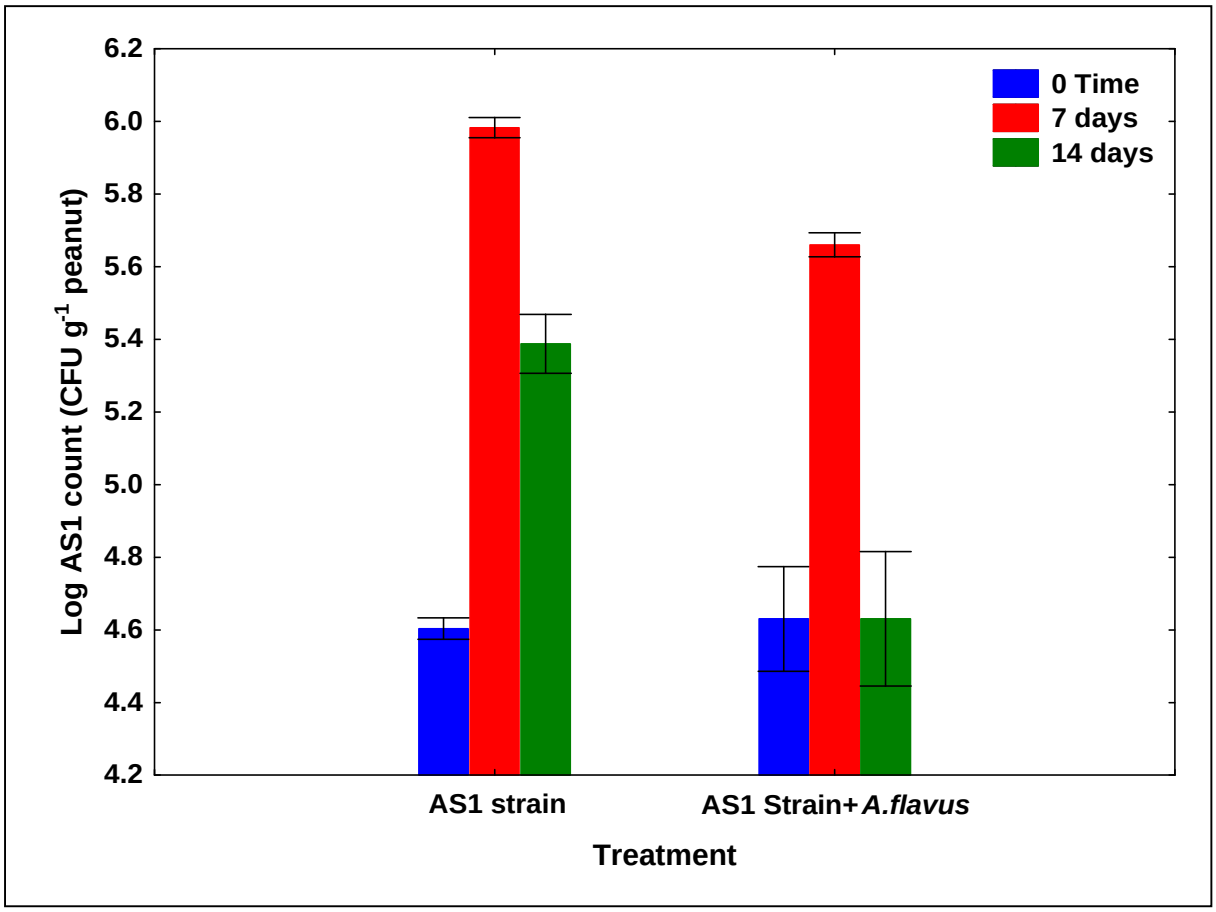


Figure 5.11. Populations of *Streptomyces* AS1 isolated from peanuts when treated with *A. flavus* at 0.93 a_w and 25°C. Bars indicate standard error of the means.

Table 5.5. Analysis of variance of the effect of *A. flavus* EGP-B07, time and their interaction on the populations of AS1 strain on peanuts at 0.93 a_w.

Effect	SS	DF	MS	F	P
Intercept	477.2593	1	477.2593	14776.79	0.000000
A. flavus	0.5543	1	0.5543	17.16	0.001364
Time	4.5288	2	2.2644	70.11	0.000000
A. flavus x time	0.4624	2	0.2312	7.16	0.008990
Error	0.3876	12	0.0323		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

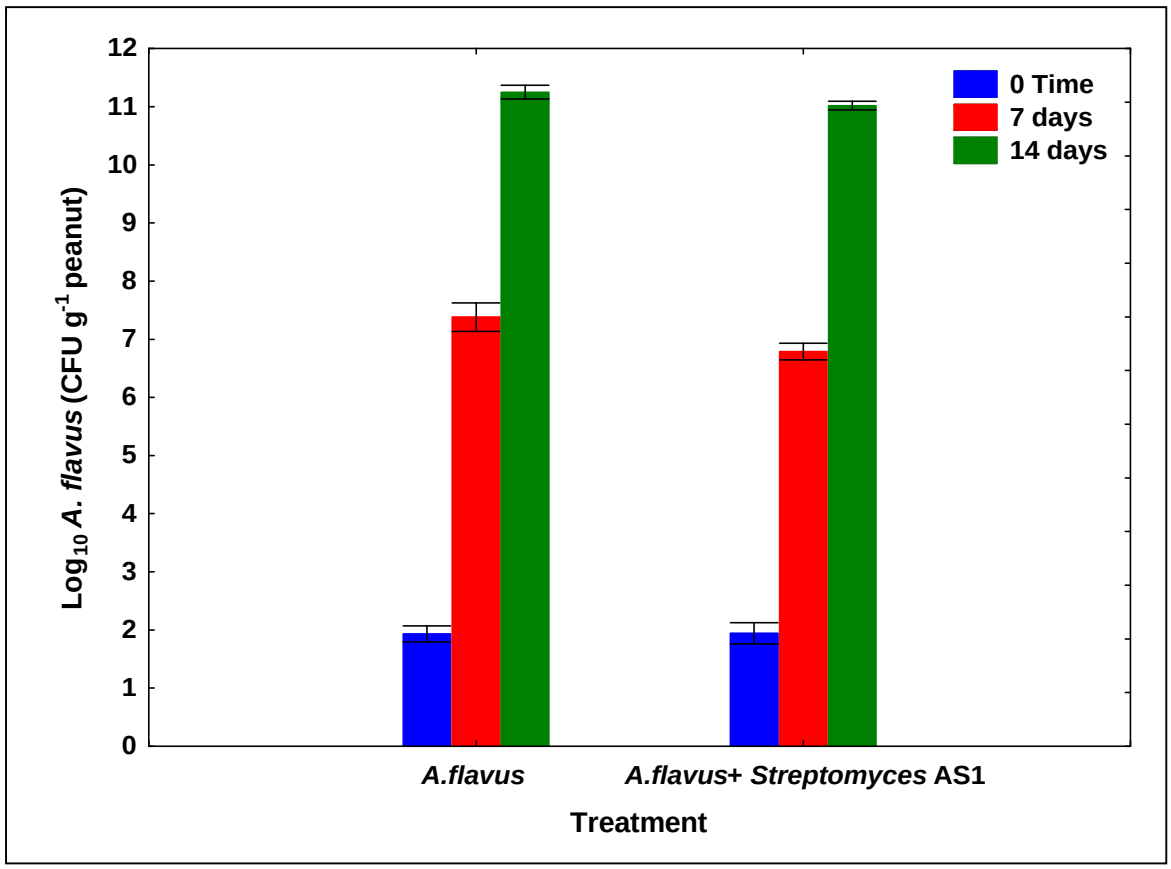


Figure 5.12. Populations of *A. flavus* EGP-B07 isolated from peanuts when treated with cells of the *Streptomyces* AS1 at 0.93 a_w and 25°C. Bars indicate standard error of the means.

Table 5.6. Analysis of variance of the effect of *Streptomeces* AS1, time and their interaction on the populations of *A. flavus* on peanuts at 0.93 a_w.

Effect	SS	DF	MS	F	P
Intercept	812.5643	1	812.5643	10637.93	0.000000
Streptomyces AS1	0.3307	1	0.3307	4.33	0.059558
Time	255.0141	2	127.5071	1669.30	0.000000
Streptomyces AS1xTime	0.2762	2	0.1381	1.81	0.205930
Error	0.9166	12	0.0764		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

5.3.7 Effect of AS1 metabolites on *A. flavus* and AFB_1 production on stored peanuts.

The influence of different concentrations of AS1 (1, 5 and 25) on the population of *A. flavus* (log_{10} CFUs g^{-1}) on peanuts at 0.93 a_w was examined over 14 days storage (Figure 5.13). At 7 and 14 days, the populations in control samples increased 5 and 9 log_{10} CFUs g^{-1} respectively, when compared with t=0. *A. flavus* populations were significantly decreased at 5 and 25 ppm of AS1 after 7 days storage when compared with the control (0 ppm). Table 5.7 shows significant effects of AS1 compounds, storage time and their interactions on the population of *A. flavus*. Storage time was the main significant effect.

The effect of same concentrations of AS1 (log_{10} ng g^{-1}) on AFB1 production on stored peanuts at 0.93 a_w for 14 days was studied (Figure 5.14). The best reduction of AFB_1 was obtained using 25 ppm AS1 through peanuts storage when compared with the amounts in control samples (0 ppm). AS1 extract concentrations, storage time (7, 14 days) and their interactions on the inoculated peanuts showed some influence on AFB_1 accumulation (Table 5.8).

Figure 5.15 shows the impact of higher concentrations of AS1 (50, 200 and 500 ppm) on populations of *A. flavus* (log_{10} CFUs g^{-1} peanuts) at 0.90 and 0.93 a_w after 7 and 14 days storage. The population was found to be significantly inhibited by AS1 at all doses, incubation periods and a_w levels. In general, the CFUs g^{-1} peanuts over all AS1 concentrations and incubation periods at 0.90 a_w were lower than those at 0.93 a_w. *A. flavus* in control samples (0 ppm) were affected by the grain humidity, decreasing by about 4 and 5 log_{10} CFUs g^{-1} in drier conditions (0.90 a_w) after 7 and 14 days storage, respectively. Overall, the best inhibitory effect was observed by treating samples with 200 and 500 ppm of AS1 at 0.90 and 0.93 a_w. Statistical analyses of the populations of *A. flavus* on peanuts, AS (50-500 ppm), storage (7, 14 days), water activity (a_w), two- and three- way interactions were statistically significant (Table 5.9). The major effects were produced by storage time followed by a_w and by AS1 metabolites.

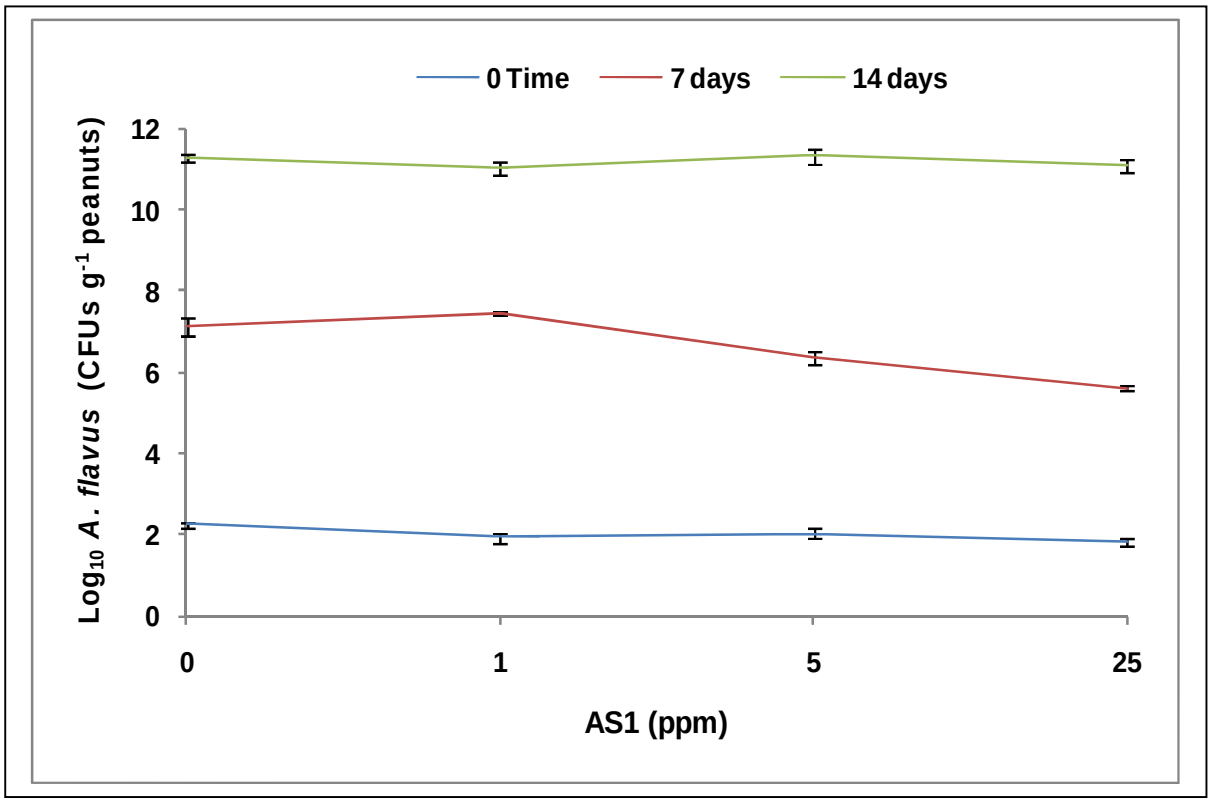


Figure 5.13. Efficacy of the metabolite AS1 (1-25 ppm) on *in situ* populations of *A. flavus* on peanuts stored at 0.93 a_w and 25°C. Bars indicate standard error of the means.

Table 5.7. Analysis of variance of the effect of AS1 (1-25 ppm), time and their interaction on the populations of *A. flavus* on Peanuts.

Effect	SS	DF	MS	F	P
Intercept	1577.204	1	1577.204	30323.10	0.000000
AS1 (ppm)	2.636	3	0.879	16.89	0.000004
Time	504.982	2	252.491	4854.36	0.000000
AS1xTime	3.755	6	0.626	12.03	0.000003
Error	1.248	24	0.052		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

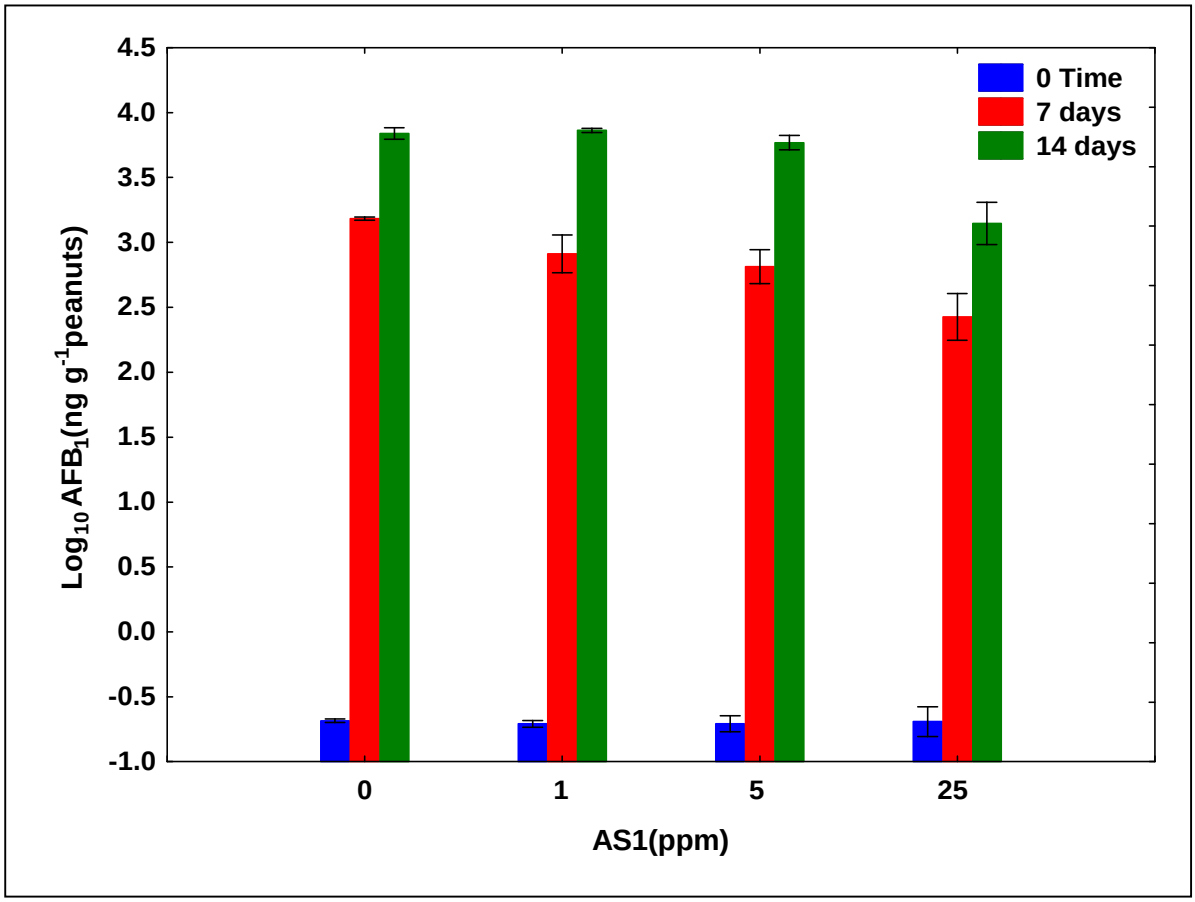


Figure 5.14. Efficacy of the metabolite AS1 (1-25 ppm) on *in situ* AFB_1 production by *A. flavus* on peanuts stored at 0.93 a_w and 25°C. Bars indicate standard error of the means.

Table 5.8. Analysis of variance of the effect of AS1 (1-25 ppm), time and their interaction on the production of AFB_1 by *A. flavus* on peanuts

Effect	SS	DF	MS	F	P
Intercept	134.0447	1	134.0447	4457.112	0.000000
AS1 (ppm)	1.2108	3	0.4036	13.421	0.000024
Time	128.3287	2	64.1643	2133.524	0.000000
AS1 x time	0.7234	6	0.1206	4.009	0.006396
Error	0.7218	24	0.0301		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

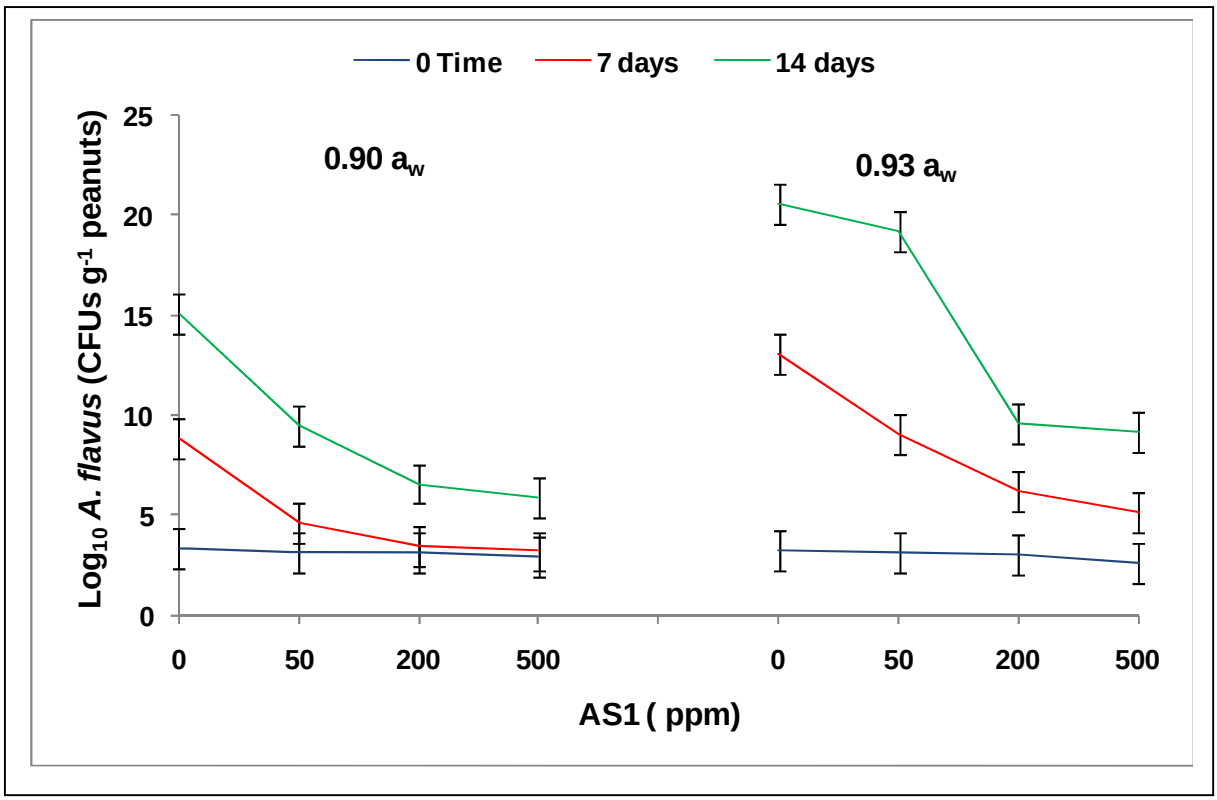


Figure 5.15. The effect of AS1 (50-500 ppm) on the control of *A. flavus* on peanuts at 0.90 and 0.93 a_w levels. Bars indicate standard error of the means.

Table 5.9. Analysis of variance of the effect of AS1 (50, 200, 500ppm), time, a_w and their interactions on the Log_{10} population of *A. flavus* on peanuts.

Effect	SS	DF	MS	F	P
Intercept	3108.942	1	3108.942	20737.00	0.000000
AS1 (ppm)	329.547	3	109.849	732.70	0.000000
Time	772.615	2	386.308	2576.72	0.000000
a_w	122.097	1	122.097	814.40	0.000000
AS1 x Time	172.684	6	28.781	191.97	0.000000
AS1 x a_w	22.460	3	7.487	49.94	0.000000
Time x a_w	69.468	2	34.734	231.68	0.000000
AS1 x time x a_w	17.900	6	2.983	19.90	0.000000
Error	5.697	38	0.150		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Figure 5.16 shows the effect of AS1 (50, 200 and 500 ppm) on the log_{10} of AFB_1 accumulation (ng g^{-1} peanuts) at two water availability condition (0.90 and 0.93 a_w) and over 7 and 14 days storage. There was a positive relationship between the populations of *A. flavus* and AFB_1 levels of T=0 and 7 days. However, it had found to be different over 14 days storage. At 0.90 a_w and after 7 days storage, AFB1 levels were significantly decreased over all AS1 concentrations when compared with that of control samples (0 ppm). However, the production was unaffected by treating samples with AS1 by the end of storage (14 days). The behaviour of AFB_1 production in peanuts by *A. flavus* at 0.93 a_w was different. Doses of 200 and 500 ppm of AS1 metabolites significantly controlled aflatoxin accumulation during the storage of peanuts (7-14 days) with reductions ranging between 1 and 2 log ng g-1 peanuts.

The effects of AS1 metabolites, storage time, a_w, time x a_w on AFB_1 production by *A. flavus* on peanuts were statistically significant (Table 5.10). However, those of AS1 x Time, AS1 x a_w (two way interaction) and AS1 x Time x a_w (three way interaction) were not significant. Storage periods of peanuts showed the major effect on AFB_1 accumulation.

5.3.8 The cytotoxicity activity of AS1 metabolite

The cytotoxicity activity of the ethyl acetate extract on brine shrimp (nauplii) is presented in Table 5.11. The 50% mortality (LC_{50}) of the extract was found to be 0.1 µg ml^{-1}. An approximate linear correlation was observed when the logarithm of concentration versus percentage mortality (Goldstein *et al.*, 1974) was plotted on the graph (Figure 5.17). Although there was no mortality in the control group, the test sample showed different mortality rates at different concentrations, which was found to increase with increasing concentration of AS1. It is difficult to compare the result with specific compounds because further tests are required to identify the *Streptomyces* species and to separate the crude extract into its pure compound components.

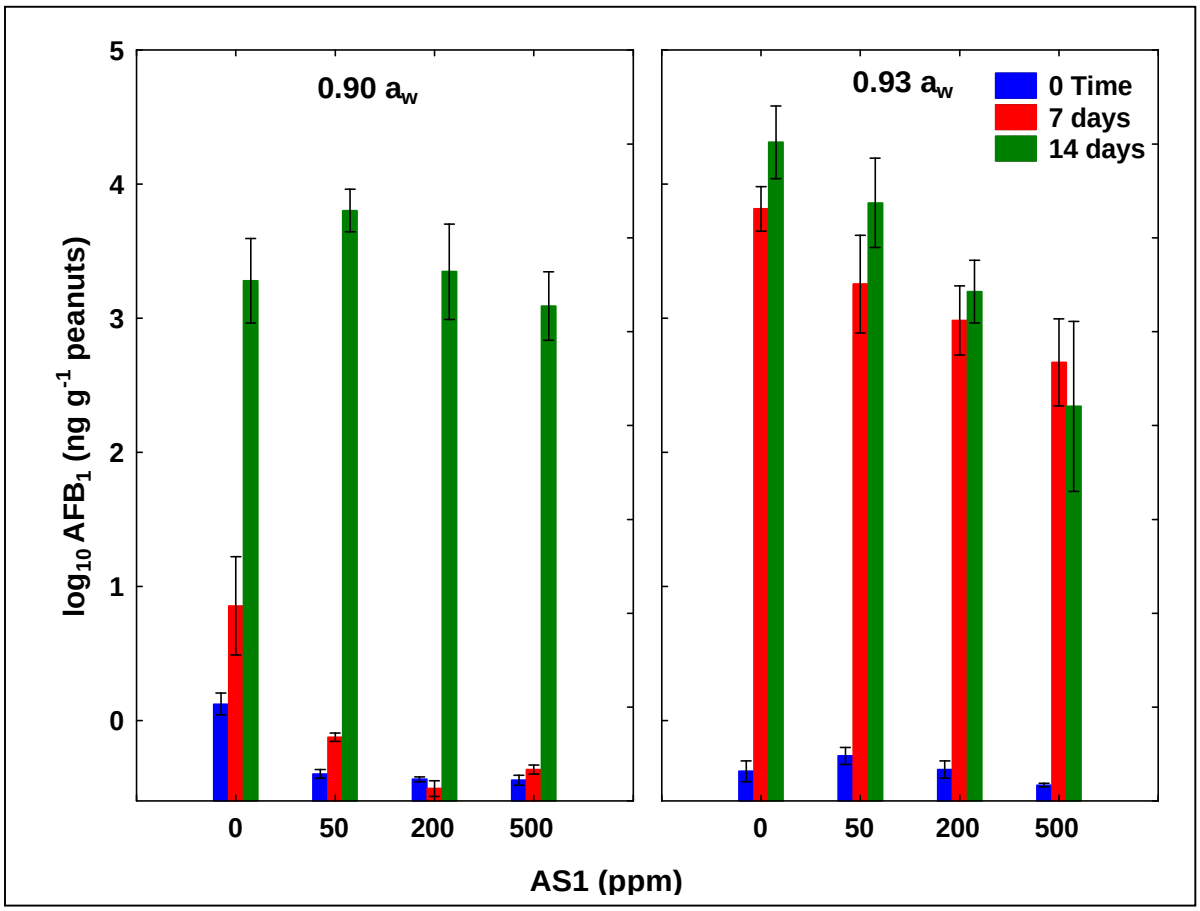


Figure 5.16. The ability of AS1 (50-500 ppm) to control AFB_1 accumulation by *A. flavus* on peanuts at 0.90 and 0.93 a_w levels. Bars indicate standard error of the means.

Table 5.10. Analysis of variance of the effect of AS1 (50,200,500ppm), time, water activity (a_w) and their interactions on the production of AFB_1 by *A. flavus* on peanuts

Effect	SS	DF	MS	F	P
Intercept	148.0592	1	148.0592	686.3601	0.000000
AS1 (ppm)	6.5914	3	2.1971	10.1852	0.000041
Time	135.7267	2	67.8633	314.5951	0.000000
a_w	17.3696	1	17.3696	80.5205	0.000000
AS1 x Time	2.4841	6	0.4140	1.9193	0.101286
AS1 x a_w	0.4996	3	0.1665	0.7720	0.516523
Time x a_w	38.9694	2	19.4847	90.3256	0.000000
AS1 x time x a_w	2.3222	6	0.3870	1.7942	0.124959
Error	8.6287	40	0.2157		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Table 5.11. The cytotoxic effect of ethyl acetate extract of AS1 metabolites (mg ml^{-1} DMSO).

Concentration (µg ml^{-1})	Log of conc.	No. of Napulii taken	No. of Napulii dead	No. of Napulii alive	% of mortality
0.1	-1	10	5	5	50
0.5	-0.3	11	8	3	73
1	0	12	10	2	83
2.5	0.4	9	8	1	89
5	0.7	10	9	1	90
10	1	12	11	1	92
25	1.4	10	10	0	100

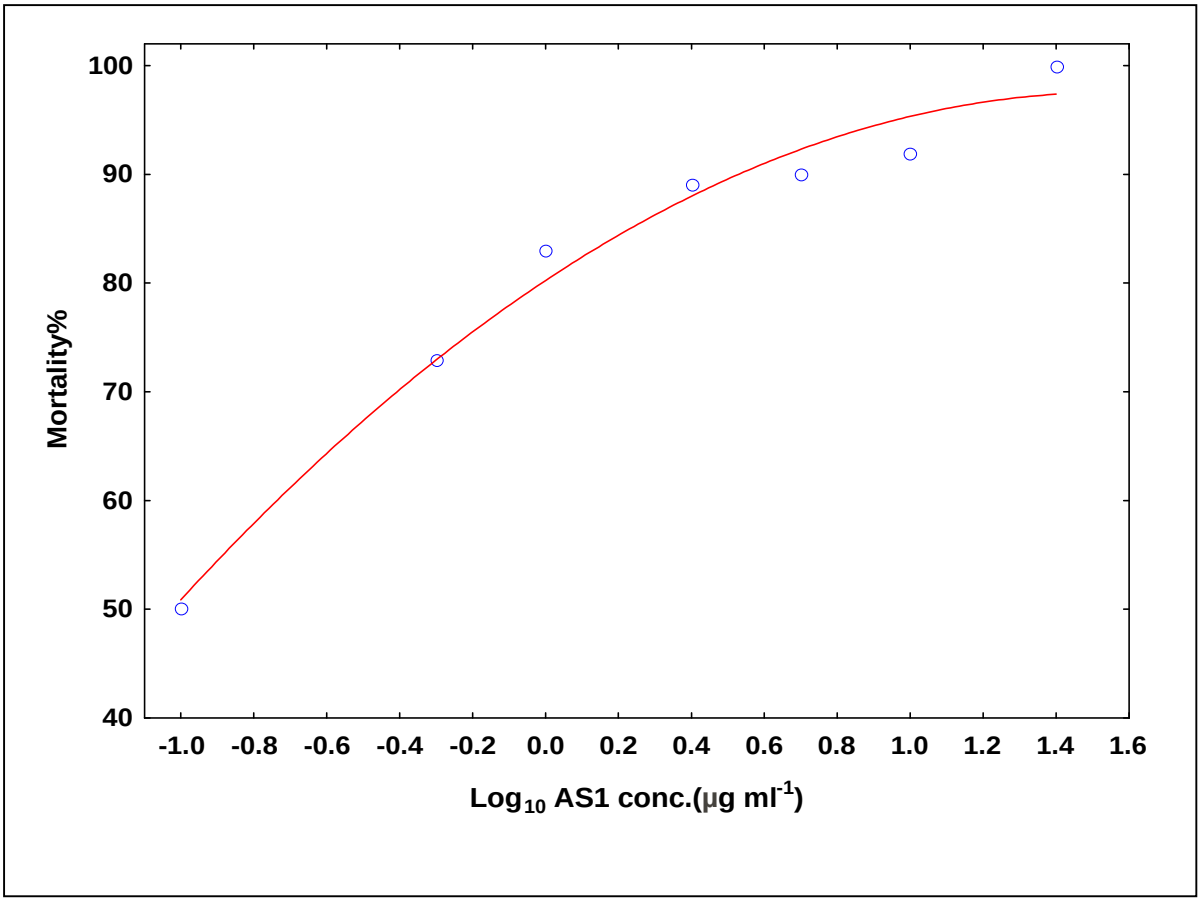


Figure 5.17. The polynomial correlation between logarithms of AS1 concentration versus percentage of nauplii mortality.

5.4 DISCUSSION

5.4.1 Isolation of actinomycetes from peanuts with potential for biocontrol of *A. flavus*

There are very few studies using actinomycetes for biocontrol of *Aspergillus* section *flavi* on peanuts, either pre- or post-harvest. Most of the successes to date have been achieved by applying certain non-toxigenic strains of *A. flavus* and *A. parasiticus* to soils around peanut plants. The applied strains occupy the same niche as the naturally occurring toxigenic strains and competitively exclude them when crops are susceptible to infection (Dorner, 2004). Based on the concept that biocontrol agents isolated from the crop will be better adapted, they will potentially be more effective for biocontrol than microorganisms isolated from other sources (Kerry, 2000). This study has screened actinomycete strains (D1-D5 and AS1) from in-shell peanuts from different regions of Egypt (Sultan and Magan, 2010). To select the best media for growth of each isolate, 5 media were tested (½ NA, YES, MEA, PMEA and MS). In general, ½ NA was the best medium for growth of actinomycetes and production of metabolites had antifungal activity. Growth of isolate AS1 completely inhibited *A. flavus* in that medium. This result was supported by measuring Indices of Dominance (I_D) which recorded 5/0 on ½ NA as well as on YES media. This isolate (AS1) was identified as a *Streptomyces* spp. The small percentage of actinomycetes that gave markedly antifungal activity (1/6) is similar to previous studies (e.g. Hacène *et al.*, 1994; Ouhdouch *et al.*, 2001; Boudemagh *et al.*, 2005; Dhanasekaran *et al.,* 2005).

Many studies have used bacteria and yeasts as biocontrol agents for controlling different pathogens in table grapes and apples (Castoria *et al.*, 2001), wheat (Druvefors *et al.*, 2002), peanut roots (Rojo *et al.*, 2007). Other studies used bacteria and yeasts to control mycotoxigenic fungi in food. Mokiou and Magan (2005) controlled *Penicillium verrucosum* growth and OTA production on wheat using formulated *Pichia anomala* cells. Kong *et al.* (2010) revealed that the marine bacterium *Bacillus megaterium* has potential biocontrol activity against *A. flavus* and aflatoxin biosynthesis on peanut kernels.

The diffusible metabolites of AS1 on ½ NA medium after 2, 5 and 10 days completely inhibited spore germination of *A. flavus* when compared with the control. Studies of the optimum incubation time and broth media for production of the antifungal compounds by AS1 showed that ½ NB was the best medium for production at 25°C and 5 days growth. Taechowisan *et al.* (2005) found that *Streptomyces aureofaciens* CMUAc130 produced antifungal compounds against *Colletotrichum musae* and *Fusraium oxysporum* after 5 days incubation in yeast extract-malt extract broth medium. However, Al-Bari *et al.* (2007) incubated *Streptomyces maritimus for* 14 days in YES medium at 37.5°C for antifungal metabolite production against fungi included *A. flavus.*

5.4.2 Antifungal activity of AS1 culture extracted in different solvents

In this study, four solvents (chloroform, petroleum ether, ethyl actate and butanol) were tested for their efficacy in extraction of antifungal compounds from AS1 cultures. Using the well diffusion method and just 50 µl of each extract (butanol, ethyl acetate and chloroform) on ½ NA medium achieved visible inhibition of *A. flavus* growth (28.1, 21.6 and 20.1% respectively). Previously, Boudjella *et al.* (2006) showed that n-butanol is most appropriate for antibiotic extraction from a new actinomycete strain isolated from Algerian soil. However, Ilić *et al.* (2005) recovered antibacterial metabolites from 9 isolates from the soils of south eastern Serbia using ethyl acetate. Al-Bari *et al.* (2007) reported that crude ethyl extracts from *Streptomyces maritimus* showed good antifungal activities against *A. flavus*. However, Dhanasekaran *et al.* (2005) reported chloroform extraction of 3 actinomycetes cultures isolated from the soil in India did not show any anti-microbial activity against the different pathogens tested.

5.4.3. *In vitro* effect of Ethyl acetate extract on *A. flavus* and AFB_1 production

Ethyl acetate extracts of AS1 appeared to reduce AFB_1 production the most. Although the butanol extract achieved the best inhibition of growth of *A. flavus* it had no effect on AFB_1 production. Both diffusible metabolites through cellophane and the crude extract (50, 100 ppm) completely inhibited conidial spore germination of *A. flavus* on YES at different a_w levels (0.95, 0.92 and 0.89 a_w). Complete inhibition was

also observed against fungal mycelia (one day old) using these concentrations of AS1. However, there was some recovery after 10 days incubation when the plates were inoculated directly with the fungal spore suspension (see Figure 5.10). This growth was decreased by increasing the concentration of AS1 and a_w level. Kavitha *et al.* (2010) recently demonstrated that 1H-indole-3-carboxylic acid extracted from *Streptomyces sp.* TK-VL_333 culture completely inhibited conidial germination of *Fusarium oxysporum* at 150 ppm. Cho *et al.* (2009) showed that the purified iturin of *B. pumilus* HY1 at 52 µg ml^{-1} methanol had a MIC_{50} against *A. flavus* on PDA medium. Palumbo *et al.* (2006) showed that some strains of *Bacillus* species isolated from almond reduced *A. flavus* growth to the extent that no visible mycelia growth occurred on YES and 2% broth almond media after 7 days.

In the current study, AFB_1 was completely inhibited in treated YES plates with AS1 metabolites at 0.89 a_w as a consequence of growth inhibition. Fifty and 100 ppm of AS1 significantly decreased AFB_1 levels at 0.95 and 0.92 a_w. Few studies have examined the metabolites from *Streptomyces* or bacteria to control aflatoxin production by *A. flavus* in media. Kondo *et al.* (2001) isolated aflastatin A (AsA, novel structure) from mycelial extracts of *Streptomyces sp*. MRI142. They reported that AsA completely inhibited aflatoxin production by *A. parasiticus* at 0.5 µg ml^{-1} in broth and agar media (potato dextrose) without affecting mycelia biomass of the fungus compared to the control. Kimura and Hirano (1988) suggested that *B. subtilis* NK-330 or NK-C-3 could synthesizes an inhibitory compound for growth and aflatoxins inhibition of *A. parasiticus* NRRL 2999 or *A. flavus* NRRL 3357.

5.4.4 The capability of *Streptomyces* AS1 to control of *A. flavus* in stored peanuts.

In the current study, *Streptomyces* AS1 and *A. flavus* were added to peanut samples (0.93 a_w) simultaneously to examine the capability of AS1 to control fungal growth. The growth of AS1 has affected by incubation time and the occurrence of *A. flavus* in peanuts. Although the initial count before storage (t=0) of *Streptomyces* AS1 (4.6 log CFUs g^{-1}) was much higher than that of *A. flavus* (1.9 log CFUs g^{-1}), the fungal populations (6.79 log CFUs g^{-1}) was higher than that by 7 days storage (5.7 log CFUs g^{-1}). The populations of *A. flavus* isolated increased whereas that of AS1 decreased after 14 days storage. *A. flavus* was dominant and it antagonised the AS1

strain on stored peanuts. A_w treatments as well as the substrate used in this study were the main factors affecting growth of both organisms. Peanuts are important substrates for the growth of different members of *Aspergillus* section *Flavi* (Pildain *et al.*, 2008). However, it is not very suitable for growth of strain AS1. At 0.93 a_w, growth of *A. flavus* was active while not for growth of strain AS1. Previously, Sanchis and Magan (2004) and Giorni *et al.* (2007) reported that *Asprgillus* section *flavi* were able to grow down to condition of 0.77 a_w and the optimal for the growth was 0.99 a_w. Also, Zenova *et al.* (2007) observed that some strains of *Streptomyces* grew most actively at 0.67 a_w with a limited development to the stage of germ tube extension but no mycelia growth occurred. However, these strains formed microcolonies with aerial mycelium at 0.92 a_w and completed spore formation only at 0.98 a_w.

Many studies have attempted to control *Aspergillus* section *Flavi* by using bacteria. Kimura and Hirano (1988) reported that treating moisten peanuts (37% MC) with *B. subtilis* NK-330 at higher concentration than that of *A. parasiticus* NRRL 2999 remarkably inhibited the growth. However, this is unrealistic as the peanuts will mould rapidly under these conditions. Also, Kong *et al.* (2010) demonstrated that the marine bacterium *B. megaterium* could be used as a biocontrol agent against post-harvest fungal diseases in peanuts caused by *A. flavus*. *Bacillus pumilus* HY1 isolated from Korean soybean sauce showed strong antifungal activity against *A. flavus* and *A. parasiticus* (Cho *et al.*, 2009).

5.4.5 *In situ* control of *A. flavus* and AFB_1 production using AS1 metabolites

Based on the previous results using AS1 extracts (50, 100 ppm) that gave >85% inhibition of *A. flavus* growth, lower concentration (1, 5, 25 ppm) was used *in situ* on stored peanuts. Moisten peanuts at 0.93 a_w was used in this experiment. Significant inhibition of the fungal population was just observed at 5 and 25 ppm in peanut samples after 7 days storage. Regarding AFB_1 inhibition by AS1, similar trends as for growth inhibition were observed in samples after 7 days storage. However, after 14 days, only 25 ppm AS1 significantly decreased AFB_1 production by *A. flavus*. This suggests that AS1 metabolites can affect AFB_1 production without any effect on growth.

For better efficacy of AS1 on *A. flavus*, higher concentrations were applied to stored peanuts (50, 200 and 500 ppm). Stored peanuts at 0.90 and 0.93 a_w were also examined. At 0.93 a_w, a significant effect on populations of *A. flavus* occurred. When compared to the controls (0 ppm), it decreased 8 and 11 log CFUs g^{-1} at 500 ppm after 7 and 14 days storage respectively. Generally, the populations of *A. flavus* at 0.90 a_w were lower than those of 0.93 a_w. However, the trend of inhibition was similar at both a_w levels. At 500 ppm AS1, significant inhibition by more than 6 log CFUs g^{-1} during the storage was observed when compared with the controls (0 ppm). Considering the impact of AS1 on AFB_1 production, it was limited in peanuts at 0.90 a_w when compared with that at 0.93 a_w. There was a positive relationship between the populations and AFB_1 accumulation on peanuts stored for 7 days at 0.90 a_w. However, after 14 days there was no significant effect on AFB_1 levels in samples at all concentration of AS1. This means AS1 inhibition of the *A. flavus* populations on stored peanuts produced the same amount of AFB_1 as the untreated samples (controls). This may be because the stress of a lower a_w. The inhibition by AS1 was different at the higher a_w treatment (0.93). At 200 and 500 ppm AS1 significantly inhibited AFB_1 production by *A. flavus* during storage. Sakuda *et al.* (1999) reported that 2 µg Aflastatin (AsA g^{-1} peanuts) reduced aflatoxin produced by *A. parasiticus* to only trace amounts without any effect on growth. Also, application of AsA on peanut plants was studied. Aflatoxin content was reduced by 50 and 98% at 2 and 10 mg $plant^{-1}$ respectively.

The possible mode of action of the metabolites produced by *Streptomyces* AS1 for inhibition of growth of *A. flavus* can be deduced from previous studies. For example, Kondo *et al.* (2001) reported that in an early step of the aflatoxin biosynthetic pathway, AsA inhibited norsolorinic acid (NA) production by *A. parasiticus* ATCC24690, a mutant blocked in aflatoxin biosynthesis. However, Castoria *et al.* (2001) found that both β-1,3-glucanase(s) and Nagase(s) acting against fungal walls, could actually play a role in the antagonism of *Aureobasidium pullulans* LS-30, since these activities were also detected in host wounds of grapes and apples.

5.4.6 Cytotoxicity of AS1 crude extract against brine shrimp

It was difficult to evaluate the toxicity of AS1 crude extract by doing bioassays against brine shrimp larvae because specific standards should used for comparison.

Thus although the ASI strain has been partially identified as being from the *Streptomyces* genus the species is still unknown. Further study is needed for identification. In the present investigation the LC_{50} of ethyl acetate extract was 0.1 µg ml^{-1}. A similar study by Al-Bari *et al.* (2007) found that the LC_{50} of ethyl acetate extract from *Streptomyces maritimus* and standard, vincristine sulphate, was found to be 14.125 and 0.758 µg ml^{-1}. They evaluate the extract as of being of moderate toxicity.

Chapter 6

CONCLUSION AND FUTURE WORK

6. CONCLUSION AND FUTURE WORK

6.1 Conclusion

The main objective of this research project was to show the importance of aflatoxigenic *Aspergilli* as well as ochratoxigenic *Aspergilli* strains in Egyptian peanuts. This led to consideration of different potential strategies to control aflatoxigenic *A. flavus* strains and consequently aflatoxin production. This section highlights the main findings from the various phases carried out in this research project.

A. Biodiversity of mycotoxigenic *Aspergillus* species in Egyptian peanuts

- Fungal populations in collected in-shell peanuts varied depending on the season, a_w and growing area.
- *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* were the most dominants fungi in in-shell peanuts of both seasons.
- Around 90% of *Aspergillus* section *Flavi* strains isolated from peanut seeds from different area in Egypt was aflatoxigenic.
- Based on analyses of aflatoxins on YES medium by HPLC followed by molecular identification and confirmation, more than 95% of the isolates were *A. flavus* (i.e., they produced only AFBs, not AFGs).
- None of the *Aspergillus* section *Nigri* strains isolated from Egyptian peanuts was able to produce OTA on media.
- 28% of *Aspergillus* section *Circumdati* from peanuts was ochratoxigenic isolates.

B. Control of *A. flavus in vitro* and *in situ* using preservatives

- A_w conditions played an important role in decreasing the growth rate at different concentrations of preservatives

- Significant inhibition was observed in the growth of *A. flavus* on YES media using all preservatives studied (phenolic antioxidants BHT, BHA, PP and OG; aliphatic salts SMB, SP and CP, and the antibiotic NM).

- In general, the inhibitory effect gradually increased with concentration of preservatives except that of SMB, which was inhibitory only at the highest concentration (2000 ppm) tested.

- OG was the best treatment affecting growth with the lowest LD_{50} (54-117 ppm) followed by BHA (113-154 ppm) at different a_w conditions (0.95, 0.92 and 0.89 a_w).

- Both inhibitory and stimulatory effects of preservatives on AFB_1 production were observed

- OG was the best antioxidant affecting AFB_1 production in media where it had the lowest ED_{50} (35-80 ppm) at all a_w levels.

- Within aliphatic salts, CP use gave the best reduction of AFB_1 production at all concentrations (500- 3000 ppm).

- The best inhibitory effect on AFB_1 synthesis by *A. flavus* using NM (1-25 ppm) was at 0.89 a_w which gave a gradual reduction ranging from 0.4 to 2.0 log units when compared with the controls.

- OG at 1000 and 2000 ppm gave best control of populations of *A. flavus* (4.3 to 5.6 log CFUs g^{-1} reduction) on stored peanuts after 2 weeks storage at 0.90 and 0.93 a_w.

- The pattern of OG impact on AFB_1 reduction in peanuts was different than that on colonisation. The doses 1000 and 2000 ppm significantly reduced the contamination over 2 weeks storage at 0.90 a_w and for one week at 0.93 a_w.

C. Control of *A. flavus* and AFB_1 production using gaseous ozone

- As a preliminary study, the impact of O_3 (30 min exposure) on the germination of two different strains of *A. flavus* on YES medium was evaluated under different a_w conditions.

- Germination was completely inhibited at > 100 ppm O_3 within 48 hrs except that at the lowest a_w level (0.89 a_w) which observed 3-12% germination at 100 ppm after 48 hrs.

- Sensitivity to ozonation varied with *A. flavus* strain.

- In general, the growth of both strains was unaffected by O_3 treatment (75 and 150 ppm). However, it significantly decreased the sporulation for 36 hrs. The same pattern of growth and sporulation was noticed by increasing the O_3 concentrations (100 and 300 ppm) and incubation time (72 hrs).

- AFB_1 production by both *A. flavus* strains was significantly inhibited at the lowest a_w (0.89 a_w) at 75, 150 ppm within 36 hrs; and at 100, 300 ppm within 72 hrs.

- Higher concentrations of O_3 (100, 200 and 400 ppm) were examined *in situ* on peanuts artificially inoculated with 10^3 and 10^5 spores g^{-1} of *A. flavus* EGP-B07 isolated from Egyptian peanuts.

- Viability of spores was significantly decreased by increasing O_3 concentration with no viable spores presented at 200 and 400 ppm in samples inoculated with 10^3 spores g^{-1}.

- A significant reduction of between 0.8 and 2.2 log CFUs g^{-1} was achieved in samples inoculated with 10^5 spores g^{-1} peanuts when compared with the populations before treatment.

- About 5 to 8 log CFUs reduction was accomplished on treated samples over 4 days storage when compared with control (using air).

- AFB_1 production in stored peanuts inoculated with both levels was only inhibited at 400 ppm of O_3 when compared with controls.

D. Biological control of *A. flavus* and aflatoxin production

- The ½ NA was the best media for growth and releasing antifungal metabolites by actinomycetes isolated from Egyptian peanuts.

- AS1 isolate was the best one showed antifungal activity against *A. flavus* which belonged to a *Streptomyces* spp.

- The optimal release of antifungal metabolites on ½ NB by AS1 *Streptomyces* was at 25°C after 5 days.

- Ethyl acetate was the best solvent for extracting the antifungal metabolites from AS1 broth culture.

- Compared with the controls on YES media at different a_w level (0.95, 0.92 and 0.89 a_w), >85% inhibition of *A. flavus* growth was observed using 50 and 100 ppm of AS1 ethyl acetate crude extract.

- AS1 metabolite at 50 and 100 ppm significantly decreased AFB_1 at 0.89 a_w and by around 4 and 10 times respectively at 0.95 a_w.

- No significant inhibitory effect of populations of *A. flavus* on stored peanuts was observed when treated with cells of *Streptomyces* AS1 at 25°C for 14 days.

- Generally, AS1 at low concentrations (1-25 ppm) had no effect on populations of *A. flavus* and AFB_1 production on stored peanuts at 0.93 a_w for 14 days when compared with the controls.

- Regardless of the AS1 concentrations the populations of *A. flavus* on peanuts at 0.93 a_w were significantly higher than those in drier conditions (0.90 a_w) over 14 days storage.

- The population was found to be significantly inhibited by AS1 at all doses with the best inhibitory effect at 200 and 500 ppm of AS1 at 0.90 and 0.93 a_w

- Doses of 200 and 500 ppm of AS1 metabolites significantly controlled aflatoxin accumulation during the storage of peanuts (7-14 days) with reductions ranging between 1 and 2 log ng g^{-1} peanuts. However, that effect remained just for 7 days storage with no effect by the end of storage (14 days) at 0.9 a_w.

6.2 Suggestions for future work

The following section lists some suggestions for continuing the present research which could eventually take it a step closer to having more applications to the food industry

A. Biodiversity of ochratoxigenic strains on Egyptian peanuts

- Further studies on ochratoxigenic strains from Egyptian peanuts
- Evaluation of the ochratoxigenic potential and identification of the isolated strains.
- Discrimination between these strains using molecular methods.

B. Control of *A.* section *flavi* and *A. circumdati* in stored peanuts using preservatives

- Applying OG and CP as well using higher concentrations to control the growth and toxin production in natural peanuts (0.6- 0.7 a_w) and intermediate moisture conditions for longer storage periods relative to EU legislative limits.
- Residue quantification on naturally stored peanuts of OG
- Toxicity tests for OG residues using brine shrimp larvae

C. Ozone treatment

- Further studies, concerning methodologies of O_3 applications on other microfungal species, like *Aspergillus section Circumdati* should be investigated in the future.
- Developing the way of sample exposure by changing the design of the reactor to give the best result of surface exposure of peanuts and other commodities
- Increasing exposure time to O_3 at low concentrations to control the growth of mycotoxigenic fungi on peanuts.

D. Biological control

- Identification of the species of *Streptomyces* AS1 morphologically and using molecular methods
- Applying *Streptomyces* AS1 against isolates of *Aspergillus* section *Circumdati*
- Efficiency of *Streptomyces* cells *in vitro* on the growth and ochratoxin production
- Effect of antifungal metabolites (AS1) on *Aspergillus* section C*ircumdati* growth and ochratoxin production
- Separate the crude extract of AS1 metabolites using HPLC-MS equipped with fraction collector and examination the collected fraction against *Aspergillus* section *Flavi* and *Aspergillus* section C*ircumdati*
- Choose the best fractions have antifungal activity to identify by NMR to recognize the compound in charge of the effect.
- Examine the toxicity of identified compound using bioassay (brine shrimp and mice).

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PUBLICATIONS

I. ORAL PRESENTATION

Sultan Y., Magan, N., 2009. Biocontrol of *Aspergillus flavus* and aflatoxin production in peanut using a *Streptomyces* species and its metabolites. *Cranfield Health Postgraduate Conference*, 16 September, 2009.

II. POSTER PRESENTATION

Sultan Y., Magan, N., 2007. Fungal community structure with special reference to *Aspergillus flavi* group and their control in groundnuts from Egypt. *Annual British Mycological Society Meeting*, 9-12 September 2007, Manchester, UK.

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Sultan Y., Magan, N., 2008. Control of *Aspergillus* section *flavi* and aflatxin production in peanut using biocontrol agent actinomycetes. *Cranfield Health Postgraduate Conference*, 18 September, 2008.

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III.PUBLICATIONS

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ORIGINAL PAPER

Mycotoxigenic fungi in peanuts from different geographic regions of Egypt

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Abstract To understand the importance of mycotoxigenic fungi in Egyptian peanuts, samples from five regions (Alexandria, El-Beheira, El-Sharqiya, El-Daqahelaya in northern Egypt and Asyut, southern Egypt) in two seasons (2007, 2008) were collected. *Aspergillus* was consistently the most frequent genus in seeds and in-shell peanuts and was the dominant mycotoxigenic component of the mycobiota. There was no direct correlation between the moisture content of the samples and the fungal populations on peanut seeds tested from different regions. The most common species were from *Aspergillus* section *Flavi* (4.7-78.3%), *Aspergillus* section *Nigri* (9.4–52.6%) and *Aspergillus* section *Circumdati* (5.1–30.9%). In the in-shell peanut samples, the lowest populations were recorded in El-Beheira and Asyut (3.7–4.0 $\log_{10}$ CFU g^{-1}) and the highest in Alexandria and Elsharqiya (4.1–6.0 $\log_{10}$ CFU g^{-1}). *Aspergillus* section *Flavi* and section *Nigri* were the most dominant, and *Aspergillus* section *Circumdati* were only found in samples in 2008. Both qualitative (coconut cream agar) and quantitative analyses (HPLC) were used to analyse the potential mycotoxin production by strains isolated from peanuts. Of a total of 88 *Aspergillus* section *Flavi* strains examined, 95% were *A. flavus* based on production of aflatoxin B_1 on yeast extract sucrose (YES) medium and confirmation using molecular analyses. Of 64 *Aspergillus* section *Circumdati* strains only 28% produced ochratoxin A (OTA), and were identified as *A. westerdijkiae*. No *Aspergillus* section *Nigri* strains produced OTA, and they were identified as *A. niger* (uniseriate). The presence of these toxigenic fungi indicates that there is a potential risk of mycotoxin contamination in Egyptian peanuts and suggests that problems can arise from contamination with both aflatoxins and perhaps also OTA.

Keywords Peanuts · Mycobiota · Mycotoxigenic fungi · *Aspergillus* section *Flavi* · *Aspergillus* section *Nigri* · *Aspergillus* section *Circumdati*

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Introduction

The mycotoxins produced by *Aspergillus* spp. of greatest significance in peanuts (*Arachis hypogaea* L.) and peanut products include aflatoxins (AFs) and ochratoxin A (OTA) (Pittet 1998). Peanuts are important substrates for the growth and subsequent AF production by different members of *Aspergillus* section *Flavi*: *A. flavus* Link, *A. parasiticus* Speare, *A. nomius*, *A. pseudotamarii* and *A. bombycis*. Recently, additional new AF-producing species have been isolated from peanuts in Argentina (*A.arachidicola* sp. nov. and *A.minisclerotigenes* sp. nov. (Pildain et al. 2008). The commonest species can infect peanuts and maize both pre- and post-harvest (Cotty 1997; Barros et al. 2003). Usually, AF contamination pre-harvest occurs under conditions of heat and drought stress during the latter stages of the growing season (Hill and Lacey 1983; Blankenship et al. 1984; Dorner and Cole 2002). Because peanuts are primarily used for food, strict regulatory limits for the amount of AF in peanut products have been established. For raw peanuts entering the EU, the level must be <4 μg kg^{-1} total AFs and have not more than 2 μg kg^{-1} AFB_1 (European Commission 2006).

Egypt is a major peanut exporting country and the European markets account for 68% of its peanut exports. Its production has increased steadily, as a result of increased

growing areas as well as significant increases in yields during the last two decades (1990 to 2005) from 2.1 to 3.1 t ha^{-1} (Diaz Rios and Jafee 2008). Peanuts are grown mostly in the north of the country. There are a large number of peanut producers, with many smallholders, resulting in an overall peanut cultivation area covering 61,000 ha (FAO 2003). Although some studies, especially in South America, have isolated ochratoxigenic fungi from peanuts, there are no legislative limits for this mycotoxin in peanuts or derived products, although these are in place for cereals (Van Egmond 2004).

In May 1999, the European Commission suspended the import of peanuts from Egypt due to the presence of AFs in concentrations in excess of the maximum levels specified in the EU regulations. Since then the Egyptian Government has been actively trying to reduce the AF content in peanuts. Since 2003, the EU only required random sampling in 20% of peanut consignments imported from Egypt for AFB1 and total AFs. Subsequently, Egypt has increased its market share in 2005 by more than U.S. $10 million (Diaz et al. 2008).

There have been previous studies on the mycobiota contamination of Egyptian peanuts (Moubasher et al. 1979, 1980; El-Hissy et al. 1981; El-Maghraby and El-Maraghy 1987, 1988; Youssef et al. 2008). However, none of these studies compared the toxigenic potential of the strains in peanut samples from different regions of Egypt. Furthermore, where mycotoxin analyses were carried out, this was qualitative only predominantly using thin-layer chromatography (TLC), with very limited quantification of AFs, or other mycotoxigenic fungi present. For example, recent studies in Argentina have found that *Aspergillus carbonarius* was commonly isolated from peanuts under South American cultivation conditions and that many of the strains isolated could produce OTA (Magnoli et al. 2007). No information is available on the isolation of *Aspergillus* section *Circumdati* species, or *Aspergillus* section *Nigri* and whether these may produce OTA.

The objectives of this study were: (1) to identify the dominant mycotoxigenic fungi in Egyptian peanuts (in shell, and seeds) in two seasons (2007, 2008) in five different regions of Egypt, (2) to examine and quantify the contributions of the mycotoxgenic fungi to the total populations, and (3) to examine the relative ratios of species from *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* which were able to produce AFs and OTA.

Material and methods

Collection of peanut samples

Triplicate raw peanut in shell samples (each 500 g) were collected after harvest in two seasons (2007, 2008) from five provinces in Egypt (Alexandria, El-Beheira, El-Daqahliya, El-Sharqiya, all from northern Egypt; and Asyut from southern Egypt). Half of each sample was shelled in a sterile flow bench to obtain the peanut seeds for mycobiota analyses. Samples were kept at 4°C, until fungal enumeration.

Determination of water activity and moisture content of peanuts

The water activity (a_w) of the peanut in-shell and peanut seed samples was determined using the AquaLab 3TE (Decagon Devices, Pullman, Wash., USA). A 10-g sub-sample of each replicate was also oven dried at 105°C for 24 h, and then cooled in a desiccator and re-weighed to a constant weight. The moisture content (MC) was calculated as the percentage of the wet weight (Deshpande et al. 1993).

Mycotoxigenic fungi isolation from samples

Direct plating A total of 45 peanut seeds (5 × 3 × 3 replicates) were directly plated onto Petri dishes of malt extract agar (MEA; Oxoid, Basingstoke, Hampshire, UK), Dichloran 18% glycerol agar (DG18, Oxoid) and MEA + 10% NaCl (0.95 a_w, MS; Oxoid) from each region. After incubation at 25°C for 7 days, the frequency of fungi was recorded. The isolates of *Aspergillus* section *Flavi, Aspergillus* section *Nigri and Aspergillus* section *Circumdati* were recovered from the peanut seeds and maintained on slants of MEA at 4°C, until examination for their toxigenic potential.

Serial dilutions The colonisation was assessed as colony-forming units (CFU) g^{-1} of dry weight peanut in-shell samples. Triplicate 10-g sub-samples from each region were weighed in stomacher bags containing 90 ml of distilled water and left for 10 min to soak. A total of four samples were homogenised for 15 min in the Colworth Stomacher 400. Serial dilutions (10^{-2}, 10^{-3}, 10^{-4}) were made and 200 µl from each dilution spread plated on MEA, DG18 and MS agar in triplicate. The plates were incubated at 25°C for 7–10 days and examined periodically. A colony counter was used for total counts and microscopic examinations were made for fungal genera classification. Individual colonies were sub-cultured for detailed examination. Data obtained on DG18 and MS showed no statistically significant differences, so data for only one of these media is presented.

For identification of species, comparisons were made with type strains from culture collections and reference to Pitt and Hocking (2009). Type strains in our laboratories included *Aspergillus flavus* (SRKC-G1907; USDA, USA);

A. parasiticus (SSWT 2999, USA), *Aspergillus carbonarius* (IMI 388653), *Aspergillus niger*. var *niger* (biseriate; IMI 388550); *A. niger* var. *niger* (uniseriate, IMI 387209), *Aspergillus westerdijkiae* (IBT 21991), *A. steynii* (IBT 22339), and *A. ochraceus* (IBT 11952).

Evaluation of toxigenic potential and identification

Two methods were used to assess this. We used a medium-based qualitative system and HPLC analyses for quantification of AFs and OTA production and molecular identification of *Aspergillus* section *Flavi* strains.

Species from the *Aspergillus* section *Flavi*, *Aspergillus* section *Nigiri* and *Aspergillus* section *Circumdati* isolated from peanut seeds were tested, respectively, for their ability to produce AFs (B1, B2, G1, G2) or OTA in coconut cream agar 50% (Dyer and McCammon 1994). The reverse side of the colony was observed daily for 6 days at 25°C under ultraviolet light at 365 nm in a dark cabinet to verify the presence of a blue fluorescent ring, an indication of the presence of AFs or OTA, thus characterising it as a toxigenic strain.

Quantitative analyses of mycotoxins

AF and OTA analyses were done using HPLC. Three replicates of the peanut samples from each region were analysed for AFs in the two seasons by extraction using aflaprep columns (Neogene Europe, wide bore). For the strains isolated from peanuts, three to five plugs were extracted from each colony after 10 days' incubation at 25°C on yeast extract sucrose (YES) agar (Fisher, Loughborough, Leicestershire, UK), transferred to a 2-ml Eppendorf tube and weighed (Filtenborg and Frisvad 1980). For AF extraction, 800 µl chloroform was added to each Eppendorf and shaken well for 1 h. The chloroform extract was transferred to a new vial and dried gently under air for derivatisation using the method of AOAC (2000) and then analysed quantitatively using HPLC. A 200-µl stock solution of AF mix standard in methanol (Supelco, Bellefonte, Pa., USA), containing 200 ng B1, 60 ng B2, 200 ng G1 and 60 ng G1, was dried under nitrogen gas and derivatised as for samples. Four concentrations were prepared for HPLC injection. For OTA extraction, 750 µl methanol was added, the sample shaken for 30 min and centrifuged for 10 min at 15,000 *g*. After filtration of the supernatant, the samples were analysed by HPLC. OTA 50 µg ml^{-1} benzene/acetic acid (99:1; Supelco, Bellefonte, Pa., USA) was used. A 50-µl sample of the stock was evaporated under nitrogen and dissolved with methanol to inject 10 ul from four different concentrations (125, 250, 375, 500 ng ml^{-1}). The detection limit of AFB_1 using HPLC was 0.012 and 0.8 ng g^{-1} peanut seeds and YES media, respectively, whereas the detection limit of OTA in YES media was 0.4 ng g^{-1}.

HPLC conditions The HPLC system used for AF and OTA analyses was an Agilent 1200 series system (Agilent, Berks., UK) with a fluorescence detector (FLD G1321A), an auto sampler ALS G1329A, FC/ALS therm G1330B, Degasser G1379B, Bin Bump G1312A and a C18 (Phenomonex, Luna 5 micron, 150 × 4.6 mm) column joined to a pre-column (security guard, 4 × 3-mm cartridge, Phenomenex Luna). The mobile phase was methanol/water/acetonitrile (30:60:10, v/v/v) using an isocratic flow rate of 1 ml min^{-1} at 360 nm excitation and 440 nm emission wavelengths and a 25-min run time for AF analyses. For OTA analyses, acetonitrile (57%)/water (41%)/acetic acid (2%) was isocratically used at the same flow rate at 333 nm excitation, 460 nm emission wavelengths. The run time for samples was 15 min, with OTA being detected at 5.75 min.

Molecular discrimination of *A. flavus* and *A. parasiticus*

DNA extraction and PCR amplification Genomic DNA of the strains was obtained using the DNAeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Differentiation between *A. flavus* and *A. parasiticus* strains isolated from Egyptian peanut samples was carried out according to González–Salgado et al. (2008). A pair of primers (Invitrogen), FLA1 (5′-GTAGGGTTCCTAGCGAGCC-3′) and FLA2 (5′-GG AAAAAGATTGATTTGCGTTC-3′), specific to *A. flavus* was used for that differentiation. PCR reactions were performed in 0.2-ml PCR Eppendorf tubes (Starlab, Milton Keynes, UK) using an Alpha Unit Block Assembly for PTC DNA Engine Systems (MJ Research, Waltham, USA). The PCR amplification protocol was as follows: one cycle of 5 min at 95°C, 26 cycles of 30s at 95°C (denaturation), 30 s at 58°C (annealing), 45 s at 72°C (extension) and, finally, one cycle of 5 min at 72°C. Amplification reactions were carried out in volumes of 25 ml containing 1 µl template DNA, 0.25 µl each primer (100 µM), 2.5 µl 10× PCR buffer, 2 µl $MgCl_2$ (25 mM), 0.625 µl dNTPs (40 mM) and 0.2 µl Taq DNA polymerase (5 U $µl^{-1}$) supplied by Qiagen (Hilden, Germany). PCR products were detected on 2% agarose ethidiumbromide gels in TAE 1× buffer (40 mM Tris–acetate, 1.0 mM EDTA). The 100-bp DNA ladder (Qiagen, Hilden, Germany) was used as the molecular size marker. Genomic DNA samples used in this work were tested using transcription of the housekeeping gene β-tubulin, Tub1-F (5′-GTCCGGTGCTGGTAACAACT-3′) and Tub1-R (5′-GGAGGTGGAGTTTCCAATGA-3′), obtained from IDT (Integrated DNA Technology, Iowa, USA) as a control. The PCR amplification protocol was as

Table 1 Water activity levels (a_w) and moisture content (MC%) of peanut in-shell and seeds collected from different Egyptian regions

		2007		2008	
Regions	Type of sample	a_w	MC%	a_w	MC%
Alexandria	Peanut in-shell	0.58	8.5	0.91	24.1
	Peanut seeds	0.56	7.0	0.91	26.1
El-Behiera	Peanut in-shell	0.50	8.3	0.54	7.9
	Peanut seeds	0.54	7.6	0.52	6.9
El-Sharqiya	Peanut in-shell	0.48	9.3	0.89	23.4
	Peanut seeds	0.51	8.4	0.92	24.4
El-Daqahliya	Peanut in-shell	0.54	8.8	0.56	8.9
	Peanut seeds	0.56	7.6	0.59	7.5
Asyut	Peanut in-shell	0.56	8.3	0.54	7.3
	Peanut seeds	0.53	6.1	0.50	5.8

previously detailed, except for changing the annealing temperature to 65°C and the number of denaturation cycles to 35.

The reference strains used in this work were *A. flavus* SRKC-G1907, *A. flavus* A 2092, *A. parasiticus* SSWT BN009-E, and *A. parasiticus* SSWT 2999. We are grateful to Dr. D. Bhatnagar and Dr. P. Cotty for supply of strains).

Statistical analysis

Data on populations of mycotoxigenic fungi and total counts (CFU g^{-1} of dry weight peanut in-shell) on DG18 and MS medium were logarithmically transformed before statistical analysis. This was required because of the wide range of variability (from single-digit numbers to numbers in hundreds/thousands) (Clewer and Scarisbrick 2001). Statistical significance were determined using Statistica version 9 (StatSoft, Tulsa, Okla., USA). Means of log CFU units was determined by analysis of variance (ANOVA, two and three ways) ($p < 0.05$). Fisher's LSD method ($\alpha = 0.05$) was applied to compare significant differences between regions and seasons.

Results and Discussion

Water activity and moisture content of peanut samples

Water activity levels (a_w) and moisture contents (MC%) were measured in an attempt to find any correlation with the fungal density of peanut samples from different regions of Egypt in 2007 and 2008 (Table 1). Values of either a_w or MC% of in-shell peanuts and seeds from each region were similar. Most of the samples had <0.59 a_w and 9.3 MC%. However, samples from Alexandria and El-Sharqiya in 2008 had the highest values (0.89–0.91 a_w, 23.4–26.1 MC %). Only in these two areas in 2008 were conditions conducive to colonisation by *A. flavus* and AF contamination, which can occur at >0.78–0.8 a_w (Lacey et al. 1991; Sanchis and Magan, 2004). The background AFB_1 concentrations found in the peanuts in 2007 and 2008 were very low, with the range being 0.8–0.41 and 0.09 to 0.23 μg kg^{-1}, respectively. Previous studies from markets, as opposed to directly from farmers, also suggest that *Aspergillus spp.* are predominant in Egypt (Moubasher et al. 1979; El-Maghraby and El-Maraghy 1987, 1988; Youssef et al. 2008).

Frequency of isolation and populations of mycotoxigenic fungi in peanuts from different geographic regions of Egypt in two seasons

Table 2 shows the populations (log_{10} CFU g^{-1} of in-shell peanut sample) of mycotoxigenic fungi and the total fungal populations from the five Egyptian regions in 2007 and 2008. Overall, the total fungal populations of samples collected in 2008, in particular from Alexandria and El-Sharqiya, was significantly higher when compared with 2007. High populations of *Cladosporium* and *Penicillium* species were present in samples from Alexandria and El-Sharqiya in 2008 (4–5 log_{10} units g^{-1}). However, *Wallemia sebi*, yeasts and *Acremonium* species were present in samples from the other regions in both seasons. *Aspergillus* section *Flavi* and *Aspergillus* section *Nigri* groups were the

Table 2 The toxigenic mycobiota populations which were isolated from in-shell peanuts (g^{-1} dry weight) in two seasons based on serial dilution. Means followed by different *superscript letters* are significantly different ($p = 0.05$)

Season	2007				2008			
Regions	*Aspergillus* section *Flavi*	*A.*section *Nigri*	*A. sec*tion *Circumdati*	Total fungal counts	*Aspergillus* section *Flavi*	*A.* section *Nigri*	*A.* section *Circumdati*	Total fungal counts
Alexandria	4.24^{a}±0.22	3.96^{a} ± 0.15	0.0	4.45^{a} ± 0.17	5.57^{a} ± 0.09	4.71^{a} ± 0.11	4.84^{a} ± 0.02	5.97^{a} ± 0.06
El-Behiera	3.00^{b}±0.21	3.84^{ab} ± 0.06	0.0	3.95^{b} ± 0.07	2.91^{b} ± 0.10	3.51^{b} ± 0.06	0.72^{b} ± 0.37	3.68^{b} ± 0.06
El-Sharqiya	3.68^{c}±0.30	3.54^{ab} ± 0.22	0.0	4.10^{ab} ± 0.12	5.56^{a} ± 0.01	4.79^{a} ± 0.17	4.27^{c} ± 0.14	5.93^{a} ± 0.03
El-Daqahilya	3.78^{c}±0.17	3.47^{bc} ± 0.16	0.0	4.06^{ab} ± 0.12	3.25^{b} ± 0.07	3.38^{b} ± 0.04	2.26^{d} ± 0.32	4.37^{c} ± 0.17
Asyut	3.21^{b}±0.18	3.74^{ac} ± 0.16	0.0	3.90^{b} ± 0.10	3.11^{b} ± 0.05	2.55^{c} ± 0.19	2.51^{d} ± 0.05	3.86^{b} ± 0.31

Table 3 Statistical analyses of significance of *Aspergillus* spp. (log CFU g^{-1} in-shell peanuts) in relation to regions, season and their interaction (*SS* sum of squares, *df* degree of freedom, *MS* mean square, *P* probability at $p = 0.05$)

Effect	SS	df	MS	F	P
Aspergillus section *Flavi*					
Intercept	440.3368	1	440.3368	5525.136	0.000000
Regions	18.5865	4	4.6466	58.304	0.000000
Season	1.8346	1	1.8346	23.019	0.000110
Regions × season	6.5233	4	1.6308	20.463	0.000001
Error	1.5939	20	0.0797		
Aspergillus section *Nigri*					
Intercept	419.3993	1	419.3993	6745.462	0.000000
Regions	5.6958	4	1.4239	22.902	0.000000
Season	0.0281	1	0.0281	0.451	0.509343
Regions × season	5.1241	4	1.2810	20.604	0.000001
Error	1.2435	20	0.0622		
Aspergillus section *Circumdati*					
Intercept	63.92400	1	63.92400	833.1498	0.000000
Regions	16.41431	4	4.10358	53.4837	0.000000
Season	63.92400	1	63.92400	833.1498	0.000000
Regions × season	16.41431	4	4.10358	53.4837	0.000000
Error	1.53451	20	0.07673		

most prevalent mycotoxigenic fungi in both seasons. Samples from Alexandria and El-Sharqiya collected in 2008 had the highest total populations among the regions examined, and subsequently the highest populations of mycotoxigenic fungi. The *Aspergillus* section *Circumdati* group was only found in samples from the 2008 season (0.7–4.8 $\log_{10}$ units g^{-1}).

Aspergillus section *Circumdati* was observed from all regions in 2008, but none in 2007. However, the other two mycotoxigenic groups were isolated in 2.9–5.6 and 2.6–4.7 $\log_{10}$ units g^{-1}, respectively, for the two seasons. A similar frequency was also found by Gonçalez et al. (2008) for *A. flavus* in full pod maturity (33.6%). In Brazil, Nakai et al. (2008) reported that in kernels, in addition to *A. flavus* (21.2%), *A. niger* was isolated but at a very low frequency (0.6%). However, they found that among representatives of the genus *Aspergillus*, only *A. flavus* (10.3%) was isolated from shells. Moubasher et al. (1979) found *A. flavus* to constitute 16.1, 8.4 and 27.2% of the total count in the shells, in covered and uncovered peanut seeds respectively. They also found that *A. niger* was recorded from 95% of peanut samples tested, constituting 20.7% of the total fungi and 35% of the total *Aspergillus*. Previously, El-Maghraby and El-Maraghy (1987) found *A. flavus* and *A. niger* comprised only 8.1 and 2.2% of the total fungi in Egyptian peanuts. With regard to *Aspergillus* section *Circumdati* group, Tripathi and Kumar (2007) found them to be present in only 12.7% of the total fungi in India. However, none of the studies in Egypt examined potential for mycotoxin production by these genera.

Table 3 shows the statistical analyses of the single and interaction between single factors of mycotoxigenic groups in relation to region and season. This shows that these were statistically significant effects of region and season on *Aspergillus* section *Flavi,* while for *Aspergillus* section *Nigri* season had no effect.

Figure 1 shows the frequency of isolation of the mycotoxigenic fungal groups from peanut seeds in the different regions in 2007 and 2008. The frequency of these fungal groups ranged from 0 to 78.3 and from 4.7 to 52.6% collected in the two years. Generally, the percentage of *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* groups in 2007 was higher than that isolated in 2008. In 2007, *Aspergillus* section *Flavi* group represented 78, 44, 27, and 25% of the total count in samples from El-Daqahelaya, Alexandria, El-Beheira, El-Sharqiya regions respectively. While, there was none found in samples from the southern region (Asyut). However, they were contaminated with *Aspergillus* section *Circumdati* (33%) in addition to *Eurotium* and

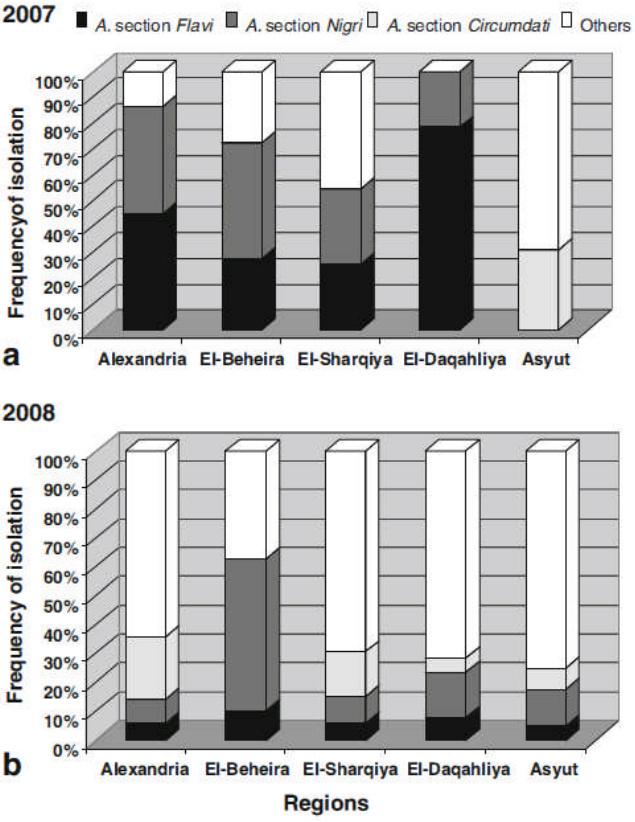

Fig. 1 Frequency of isolation of dominant groups from peanut seeds from different regions in (**a**) 2007 and (**b**) 2008 based on direct plating on MS medium for 7 days at 25°C

Table 4 AF- and OTA-producing ability of *Aspergillus* section *Flavi*, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* isolated from peanut seeds [*ND* not detected (below the detection limit using HPLC)]

Year	Group name	No. strains	Mycotoxin type	Positive producing strains	Concentration range (ng g^{-1} medium)
2007	*Aspergillus* section *Flavi*	56	AFB_1	51 (91%)[a]	10.9–248,460.5
	Aspergillus section *Circumdati*	12	OTA	5 (42%)[b]	96.6–2,871.8
	Aspergillus section *Nigri*	28	OTA	0 (0%)[c]	ND
2008	*Aspergillus* section *Flavi*	32	AFB_1	28 (87%)[a]	12.8–75,849.3
	Aspergillus section *Circumdati*	52	OTA	13 (25%)[b]	0.8–766.9
	Aspergillus section *Nigri*	50	OTA	0 (0%)[c]	ND

[a] *Aspergillus flavus* strains

[b] *Aspergillus westerdijkiae* strains

[c] *Aspergillus niger* strains

Alternaria as the dominant other genera (33 and 25% respectively). The second most important group was *Aspergillus* section *Nigri* which can produce OTA. Peanut seeds from El-Beheira contained the highest percentage frequency of *Aspergillus* section *Nigri* (45%) with the other regions having less in 2008. The frequency of isolation of *Eurotium* and *Acremonium* species from all regions was high when compared with that of the *Aspergillus* section *Flavi* group (4.7–10.2%), *Aspergillus* section *Circumdati* group (5.1–21.2%) and *Aspergillus* section *Nigri* groups (9.4–52.6%). Xerophilic *Eurotium* species represented 26%, 47% and 70% of the total fungal count in samples from El-Beheira, El-Daqahliya and Asyut region, respectively. However, *Acremonium* was dominant in Alexandria and El-Sharqiya samples.

El-Maghraby and El-Maraghy (1988) and Youssef et al. (2008) reported that *A. flavus* represented 4% and 4.9%, respectively, of the total fungi on untreated seeds. In the present study, *Aspergillus* section *Circumdati* group represented 5.1–21.2% of mycobiota populations in samples from all regions except from El-Beheira. In contrast, Youssef et al. (2008) did not find any *Aspergillus* section *Circumdati* group in peanut samples. In 2008, in the Alexandria and El-Sharqiya samples a higher frequency of *Penicillium* and *Acremonium* species were found in contrast to 2007 where *Eurotium* species were dominant. This may be linked to the higher a_w of these samples. This also suggests that samples from these regions were stored poorly after harvest (Pitt and Hocking 2009).

Toxigenic potential of isolated strains of mycotoxigenic genera

Screening of fungi isolated from peanut seeds for AF production by the fluorescence method on coconut cream agar medium revealed that among 56 and 32 *Aspergillus* section *Flavi* isolates in 2007 and 2008 almost all except for six and eight strains, respectively, produced a blue fluorescence (Table 4). The same medium was also used to examine *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* isolates for OTA production. No blue fluorescence was observed in any of the former group isolates tested (28 and 50 isolates in 2007 and 2008). Of the *Aspergillus* section *Circumdati* isolates in 2007 and 2008 (12 and 52, respectively), 50% and 31% were ochratoxigenic.

A total of 88 *Aspergillus* section *Flavi* isolates were tested by HPLC for quantification of the ability to produce AFs (B1, B2, G1, G2). A further 64 *Aspergillus* section *Circumdati and* 78 *Aspergillus* section *Nigri* were screened for production of OTA on a conducive YES medium. Table 4 also shows that there were differences between the qualitative and quantitative methods. For example, of 88 *Aspergillus* section *Flavi* isolates only five gave false-negative results. For *Aspergillus* section *Circumdati* of 64

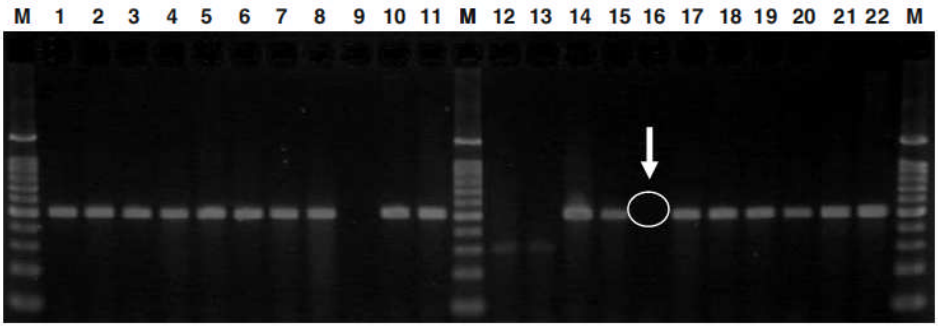

Fig. 2 PCR-based detection of *A. flavus* using FLA1/FLA2 primers. *Lanes 1–8:* tested strains. *Lane 9:* non-template control. *Lane 10: A. flavus* 2092. *Lane 11: A. flavus* SRKC-G1907. *Lane 12: A. parasiticus* SSWT BN009-E. *Lane 13: A. parasiticus* SSWT 2999. *Lanes 14–22:* tested strains. *M:* DNA molecular size marker

isolates, only four gave a false positive in the two seasons when compared with HPLC results. The mean aflatoxigenic *Aspergillus* section *Flavi* isolates for both seasons was 90% based on HPLC analysis. The concentrations of AFB_1 produced ranged from 10 to 100,000 ng g^{-1}. However, two (2.3%) isolates were able to produce AFB_1 >100,000 ng g^{-1}. The mean percentage ochratoxigenic *Aspergillus* section *Circumdati* isolates was 28% and the concentration range of OTA was from 1–3,000 ng g^{-1}. None of the 78 *Aspergillus* section *Nigri* isolates produced OTA. This suggests that most of the isolates from these groups were, respectively, *A. flavus*, *A. westerdijkiae* and *A. niger* (uniseriate).

Molecular analyses of *Aspergillus* section *Flavi* strains isolated from Egyptian peanuts were further tested using the primer pair FLA1 and FLA2. A single fragment of about 500 bp was only amplified when genomic DNA from *A. flavus* strains was used, but not from *A. parasiticus* strains where no product was observed. More than 95% of the tested *Aspergillus* section *Flavi* were *A. flavus* and this is shown in Fig. 2 as an example of the results. Isolate in lane 16 was *A. parasiticus* and non-aflatoxigenic as well. These result confirmed those obtained from analysis of isolates on YES medium for AFs. This showed that all the aflatoxigenic isolates produced AFB_1 and AFB_2 and none produced AFG_1 and AFG_2.

There was approximately 90% compatibility of the results between the coconut agar method and HPLC. Similar results were obtained by Giorni et al. (2007) who found that 73% of *Aspergillus* section *Flavi* strains isolated from maize showed fluorescence when inoculated on coconut extract agar and 70% of strains were positive when tested by HPLC. For both seasons, the mean *Aspergillus* section *Flavi* isolates producing AFs was 89.3%, and for OTA, 33.5% of *Aspergillus* section *Circumdati* isolates. However, no *Aspergillus* section *Nigri* group produced OTA. Nakai et al. (2008) found in Brazil that 93.8% of *Aspergillus* section *Flavi* isolated from Brazilian peanuts produced AFs. Also, Sánchez-Hervás et al. (2008) reported 64.1% of isolates from cocoa beans produced AFs. The results for OTA production by *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* groups in this study differ from those obtained by Sánchez-Hervás et al. (2008), who found that 49.2% of black *Aspergilli* strains were able to produce OTA in cocoa beans and suggested that *Aspergillus* section *Circumdati* is probably a relatively unimportant source of OTA in cocoa production. In contrast, *Aspergillus* section *Circumdati* species, especially *A. westerdijkiae*, were found to be commonly present on coffee beans (Noonim et al. 2008). Magnoli et al. (2007) reported that 32% of *Aspergillus* section *Nigri* isolated from Argentinean peanut seeds were OTA producers in culture. Additionally, Bayman et al. (2002) demonstrated that none of the isolates of *Aspergillus* section *Circumdati* or *Aspergillus melleus* from tree nuts and fig orchards produced OTA, although all isolates of *Aspergillus alliaceus* did produce the toxin. This is unusual as *Aspergillus* section *Nigri* isolates normally contaminate grapes, grape products and wine with OTA (Mateo et al. 2007).

In conclusion, the present study demonstrated the susceptibility of Egyptian peanut to colonisation with *Aspergillus* section *Flavi,* especially *A. flavus* isolates, *Aspergillus* section *Nigri* and *Aspergillus* section *Circumdati* groups as dominant fungi from different regions of Egypt. The a_w of the samples, season, and regions were important factors affecting the fungal populations. It may be important to not overlook the presence of ochratoxigenic fungi (28%) in peanuts in addition to aflatoxigenic species (90%) and to perhaps formulate guidelines for OTA as well. *Aspergillus* section *Nigri* group isolates from Egypt were unable to produce OTA in peanuts. This contrasts with results from South America. Poor post-harvest storage of peanuts in Egypt could increase the risk from contamination with AFs above the EU legislative limits.

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Fungal community structure with special reference to *Aspergillus flavi* group and their control in groundnuts from Egypt

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Introduction

- Peanuts are an important agricultural crop in Egypt, and is one of the major exporting countries into the EU.
- Peanut kernels are a good substrate for growth by *Aspergillus flavi* group, and subsequent aflatoxin contamination, especially under drought stress conditions.
- There is little knowledge of the diversity of this important fungal group in different regions of Egypt. There is also interest in non-toxic methods of control to reduce entry of aflatoxins into the peanut chain
- **The objective of this study were:** (a) a study of the diversity of contamination of peanuts in different regions of Egypt and (b) in vitro screening of anti-oxidants for potential control of growth and aflatoxin production.

Methods/Materials

- **Peanut samples:** These were cultivated in five regions of Egypt were collected (Alexandria, El-Beheira, Sharqiya, Daqahlaya and Assiut).
- **Media:** Malt Extract 10% Salt Agar (MS, 0.95 water activity) medium; Direct plating and serial dilutions for frequency of isolation and population diversity
- **In vitro efficacy of anti-oxidants:** Yeast Extract Sucrose agar (YES pH 6) at different aw levels: Effect of Octyl Gallate (OG), Butylated hydroxyanisole (BHA), Butyl hydroxytolunene (BHT), sodium metabisuplhite (SMB) and Potassium sorbate on the growth and aflatoxin B1 production. LD_{50} values were determined.
- **Toxin analyses:** Afaltoxin B1 analysis from YES discs; extraction by chloroform for 1 hour, derivatisation and HPLC analyses with fluorescence detection.

Results

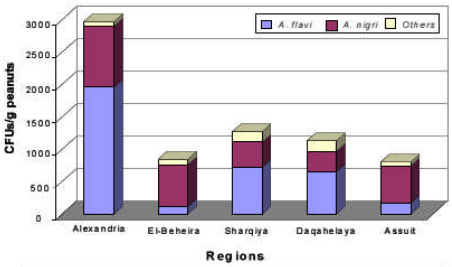

Figure 1. Total fungal count of in-shell peanut from different Egyptian regions on MS after 7 days at 25°C.

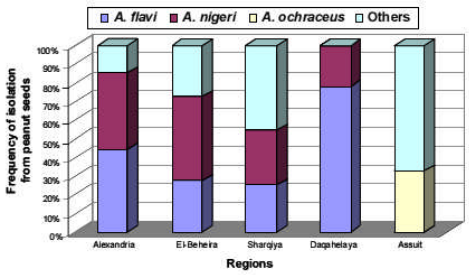

Figure 2. Frequency of isolation of dominant groups from peanut seeds from different Egyptian regions on MS after 7 days at 25°C.

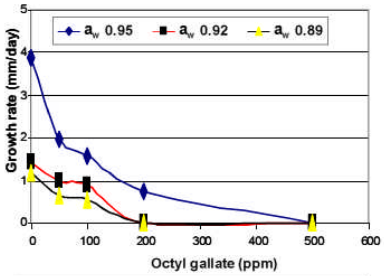

Figure 3. Effect of Octayl Gallate (OG) on in vitro growth of *A.flavus* at different water activity levels at 25°C.

Table 1. LD_{50} values for inhibition of growth of *A.flavus* when grown at different water availability conditions at 25°C. This shows that OG is by far the best compound of those screened.

Water activity:	0.982	0.955	0.928
Treatment			
PP	200	188	118
BHA	141	154	113
BHT	564	690	495
OG	85	117	54
SMB	2020	1750	1870

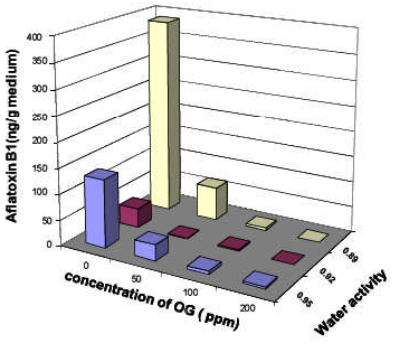

Figure 4. Effect of Octyl gallate (OG) on aflatoxin B1 production by *A.flavus* on YES at 25°C

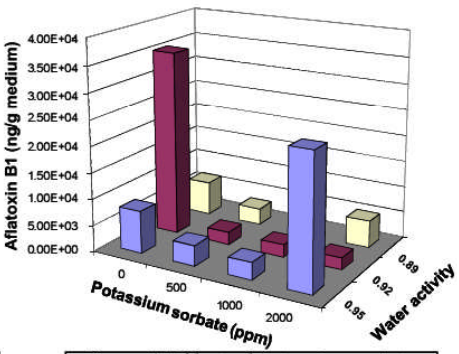

Figure 5. Effect of Potassium sorbate (PS) on aflatoxin B1 production by *A.flavus* on YES at 25°C

Conclusions

- *A.flavi* group are dominant colonisers of peanuts in most regions of Egypt, except Assiut.
- The second most important group were the *A.niger* section *nigri* group which can produce ochratoxins.
- Up to 90% of *A.flavi* group produce aflatoxins; <5% of *A.niger* section *nigri* produced ochratoxin
- Most anti-oxidants inhibited growth of the aflatoxigenic species. The best compound with the lowest LD_{50} value was octyl gallate over the water acativity range tested.
- However, aflatoxin B1 production was stimulated by some treatments. Octyl gallate gave the best toxin imnhibition at 100-200 ppm, which is within the allowable limits for food use.
- Work is in progress to examine binary and tertiary mixtures of these compounds for syngerstic effect and for testing in situ in storage.

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Acknowledgement: YYAS is grateful to NRC Institute and the Egyptian Higher Education Ministry for support

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Biocontrol of *Aspergillus* section *flavi* and aflatoxin production in peanut using a *Streptomyces* species and its metabolites

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Introduction

- Peanuts are an important agricultural crop in Egypt, and is one of the major exporting countries into the EU.
- Peanut kernels are a good substrate for growth by *Aspergillus flavi* group, and subsequent aflatoxin contamination, especially under drought stress conditions.
- Few studies have examined the potential of actinomycetes to control mycotoxigenic fungi in vitro and in situ and none for control of aflatoxins in peanuts.
- **The objective of this study were:** (a) to isolate and screen potential biocontrol strains (Actinomycetes) against *A.flavus* and minimise aflatoxin production in vitro, (b) to extract secondary metabolites, having antifungal activity from actinomycetes isolated from peanuts and (c) to examine the strain and its metabolites in situ for control of aflatoxins in peanuts

Materials & Methods

Isolation of actinomycetes from peanut samples: These were from 3 regions of Egypt (Alexandria, Daqahlaya and Assyut).
·**Media:** Half strength nutrient agar (1/2 NA), half strength nutrient broth (1/2 NB), Yeats extract sucrose agar (YES) and Malt Extract 10% Salt Agar (MS, 0.95 water activity); serial dilutions on 1/2 NA for actinomycetes isolation and total count and on MS for fungal count.
· **In vitro screening:** Six isolates of actinomycetes (5 from Daqahelaya and one from Asyut samples) were tested for in vitro antagonism against *A.flavus* on media using streak method (Fig.1).
· **Antifungal compound screening**: Screening for the efficacy of solvents in the extraction of antifungal compounds from isolate AS1 using well method (Fig 2).
In situ: Procedures of in situ antagonism against *A.flavus* on peanuts as in Fig. 3.
Toxin analyses: Afaltoxin B1 analysis from YES discs; extraction with chloroform for 1 hour, derivatisation and HPLC analyses with fluorescence detection.

Fig 1. Streak method of screening for antifungal efficacy

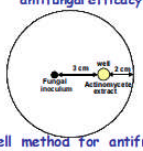

Fig 2. Well method for antifungal efficacy of solvent extracts

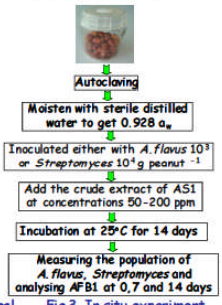

Fig 3. In situ experiment procedures

Results

Table 1. Relative competitiveness of actinomycetes against *A.flavus* in vitro at 25 °C for 7 days.

Inhibition indicators	Isolates / Medium	D1 Control	D1 Tested	D2 Control	D2 Tested	D3 Control	D3 Tested	D4 Control	D4 Tested	D5 Control	D5 Tested	AS1 Control	AS1 Tested
Fungal colony area (Cm^2)	YES	39.6	35.3	42	33.2	39.6	24.2	43	33.2	39.6	28.3	46.6	28.3
	½NA	25.6	14.6	27.4	12.9	25.6	13.2	29.3	16.3	27	15.3	25.6	0.0
Interaction score	YES	--	1/1	--	1/1	--	1/1	--	n.g.	--	2/2	--	5/0
	½NA	--	1/1	--	1/1	--	2/2	--	1/1	--	2/2	--	5/0

--, Not tested; n.g. not growth enough; 1/1, mutual intermingling; 2/2 mutual inhibition on contact; 3/3, mutual inhibition at a distance; 4/0. dominance on contact; 5/0. dominance at a distance (Magan and Lacey, 1984).

Table 2. AS1 solvent extract antagonism of *A.flavus* (colony area, cm2) on YES at 25°C.

Type of extract	Fungal colony area (cm ^{2}) Control	Treatment	% inhibition
Broth filtrate	19.6	16.8	14.3
Chloroform	20.4	16.3	20.1
Ethyl acetate	18.5	14.5	21.6

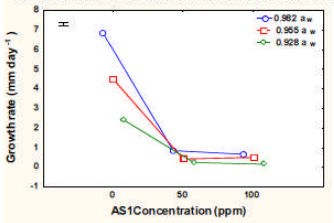

Fig. 4. Effect of the crude antifungal extracts of AS1 culture on *A.flavus* growth on YES medium.

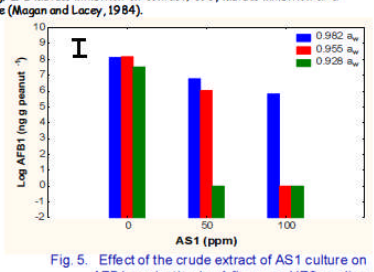

Fig. 5. Effect of the crude extract of AS1 culture on AFB1 production by *A.flavus* on YES medium.

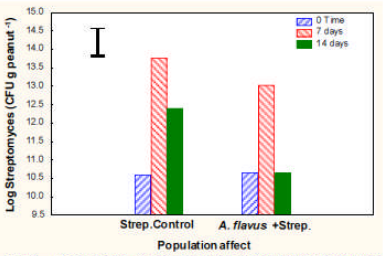

Fig 6. Populations of *Streptomyces* AS1 isolated from peanuts when treated with *A.flavus* at 0.928 a_w and 25°C

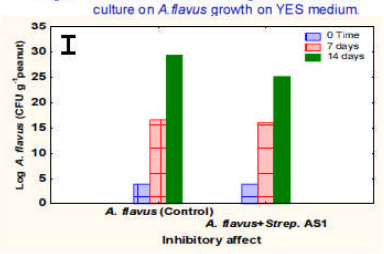

Fig.7. Populations of *A.flavus* isolated from peanuts when treated with cells of the *Streptomyces* AS1 at 0.928 a_w and 25°C.

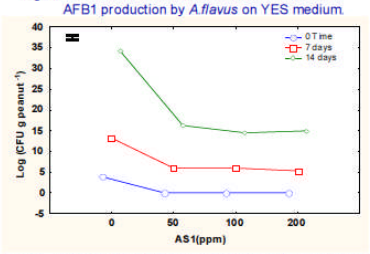

Fig 8. Efficacy of different concentrations of the metabolite AS1 on in situ populations of *A.flavus* on peanuts stored at 0.928 a_w at 25°C.

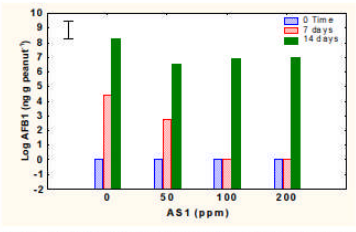

Fig 9. Effect of the metabolite AS1 on AFB1 production by *A. flavus* on peanuts stored at 0.928 a_w at 25°C.

* Vertical bars denote 0.95 confidence intervals

Conclusions

- **Isolate AS1 was found to have the highest antifungal activity of 6 isolates screened.**
- **Ethyl acetate extract achieved the best inhibition of *A.flavus*.**
- **Fifty and 100 ppm of AS1 significantly inhibited the growth of *A. flavus* and AFB1 production on YES at all a_w levels examined.**
- **Peanuts are not a good substrate for *Streptomyces* AS1 growth and production of antifungal metabolites and subsequently for controlling of *A.flavus* and AFB1 production.**
- **However, the metabolite AS1 at 50, 100 and 200 ppm (dissolved in ethanol) significantly inhibited growth of *A.flavus* on peanuts at 0.928 a_w during storage (14 days) and aflatoxin production for 7 days.**
- **Work in progress to examine the effect of AS1 on the growth of *A.flavus* and AFB1 production on peanuts at lower a_w levels.**

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Acknowledgement: YYAS is grateful to NRC Institute and the Egyptian Higher Education Ministry for support

APPENDIX

Table 1. Effect of ozone (ppm) on spore germination (%) of two different strains of *A. flavus* at 25°C for 48 hrs incubation. Mean followed by±SE

		Water activity level																			
		0.89					0.92					0.95					0.97				
		Ozone concentration (ppm)																			
strain	hrs	0	20	40	100	250	0	20	40	100	250	0	20	40	100	250	0	20	40	100	250
A. flavus	12	0.0	0.0	0.0	0.0	0.0	17.7±3.3	15.7±2.3	10.0±3.5	0.0	0.0	71±5.9	53.3±7.1	51.7±6.4	0.0	0.0	82.3±4.3	55.0±6.0	21.7±2.7	0.0	0.0
EGP-B07	24	5.8±1.4	14.3±3.2	11.8±3.0	0.0	0.0	86.0±5.0	70.3±2.9	74.3±2.2	0.0	0.0	100.0	61.3±5.2	52.3±4.3	0.0	0.0	100.0	74.0±4.6	66.0±7.2	0.0	0.0
	36	61.7±6.7	68.7±3.7	55.7±9.8	0.0	0.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0	100.0	0.0	0.0
	48	100.0	100.0	81.3±7.2	12.0±4.0	0.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0	100.0	0.0	0.0
A. flavus	12	0.0	0.0	0.0	0.0	0.0	54.0±2.1	26.3±3.5	64.0±6.7	0.0	0.0	67.0±7.8	3.7±1.2	0.0	0.0	0.0	44±3.7	0.0	0.0	0.0	0.0
SRRC-G	24	55±4.0	45±2.9	44±4.6	0.0	0.0	51.0±3.0	23.3±2.2	46.3±2.3	0.0	0.0	100.0	15.7±2.3	10.7±2.4	0.0	0.0	100.0	100.0	9.7±1.5	0.0	0.0
1907	36	65.7±7.4	58.7±5.4	57.7±11.3	0.0	0.0	88.3±2.9	34.7±3.2	56.0±1.2	0.0	0.0	100.0	79.0±3.2	70.7±2.3	0.0	0.0	100.0	100.0	73.0±5.5	0.0	0.0
	48	90.7±2.3	64.3±4.9	68.0±4.2	2.7±1.4	0.0	100.0	90.7±2.3	73.0±4.4	0.0	0.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0	100.0	0.0	0.0