CRANFIELD UNIVERSITY

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# ECOLOGY, CLIMATE CHANGE AND CONTROL STRATEGIES FOR *ASPERGILLUS FLAVUS* COLONISATION AND AFLATOXIN CONTAMINATION OF PISTACHIO NUTS

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ENVIRONMENT AND AGRIFOOD THEME
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PHD THESIS
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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD

# ABSTRACT

Pistachio nuts (*Pistacia vera* L.) have become one of the most important products in the economy of many countries including the USA, Iran, Syria, Greece, Turkey, China, EU and the Middle East. Pistachio nuts are very commonly colonized by spoilage mycobiota especially aflatoxigenic species because they are very hygroscopic and can adsorb water. *Aspergillus flavus can contaminate pistachio nuts with aflatoxins (AFs), especially aflatoxin B1 (AFB1) classified as a class 1a carcinogen. The objectives of this project were (a) to* examined the mycobiota and the aflatoxin producing strains of *Aspergillus* section *Flavi* species in pistachio nuts originating from different countries and sourced in the Kingdom of Saudi Arabia (KSA), (b) investigated the effect of the interactions between temperature and water activity ($a_w$) on the ecology and molecular ecology growth and $AFB_1$ production by *Aspergillus flavus* strains *in vitro* on pistachio nut-based media and in stored raw pistachio nuts (c) evaluate the effect of Climate Change (CC) interacting factors on growth and AFB1 production by strains of *A.flavus* and on relative genes expression of the *aflD* and *aflR* genes involved in the biosynthetic pathway for $AFB_1$ production, (d) examine whether acclimatisation to 1000 ppm $CO_2$ of *A.flavus* strains AB3 and AB10 for 5 generations affected growth and $AFB_1$ production; and (e) to examine the use of gaseous $O_3$ for the control of germination, *A.flavus* populations and $AFB_1$ contamination of stored pistachio nuts for up to 4 weeks. Pistachio samples were colonized by a range of *Aspergillus* and *Penicillium* species. In some samples, typically phyllosphere fungi such as yeasts, *Mucor*, *Rhizopus*, *Alternaria*, *Epicoccum* and *Phoma* species were isolated. 10 different species of *A.flavus* were isolated and molecularly identified. The relative toxigenic nature of strains was evaluated using selective media and HPLC and confirmed using molecular tools. Four strains were used in ecological studies and two (AB3, AB10) in other studies.

The ecological studies showed that optimum growth of AB3 and AB10 strains was at 0.98 or 0.95 $a_w$ and 30-35°C. The effect of the same factors on *aflR* gene expression of the two strains showed optimum condition at 30-35°C and 0.98 $a_w$; with optimum conditions for $AFB_1$ production at 35°C and 0.98 $a_w$ for strain AB3.

There was little difference between the effect of using a non-ionic (glycerol) or ionic (NaCl) to modify water stress in *in vitro* studies. The effect of interacting CC factors on growth of *A.flavus* colonisation was not significant. However, $AFB_1$ production was stimulated. With regards to *aflD* gene expression, at 35°C, the relative expression was higher in current $CO_2$ conditions (400 ppm) for both strains except that for strain AB3 the gene expression was higher at 1000 ppm $CO_2$ at 0.95 $a_w$. However, at 37°C, the expression was generally higher in the 1000 ppm $CO_2$ than with existing atmospheric $CO_2$ levels. The *aflR* gene expression was higher at 1000 ppm $CO_2$ at 37°C for both strains. $AFB_1$ production was higher at 35°C at the two $CO_2$ levels for both strains. At the same temperature, $AFB_1$ production was significantly increased at 1000 ppm $CO_2$ and 0.98 $a_w$. At 37°C, $AFB_1$ production was either decreased in strain AB3 or similar as in strain AB10 when exposed to 1000 ppm $CO_2$. This suggests that CC factors may have a differential effect depending on the interacting conditions of temperature (35 or 37°C) as in some cases for $AFB_1$. Acclimatisation influenced growth of one strain while there was no significant effect on another strain when colonising pistachio nuts. For $AFB_1$, the production was significantly stimulated after ten days colonisation after acclimatisation for one strain, while there was no significant increase for the other strain. This suggests that there may be intra-strain differences in effects of acclimatisation and this could influence mycotoxin contamination of such commodities as mixed population of contaminant fungi often occurs.

Exposure of conidia to gaseous $O_3$ initially had lower germination percentages when compared to the controls at different $a_w$ levels. Complete inhibition of germinations was observed after 12 h treatment of 200 ppm $O_3$ at 0.98 $a_w$. However, spore viability appeared to recover and the germination was increased after 24 h and reached 100% after 48 h. Growth rates of mycelial colonies were decreased with increasing of $O_3$ dose and colony extension was significantly inhibited by $O_3$ treatment at 0.98 $a_w$. Variable effects on $AFB_1$ production during exposure to $O_3$ treatment after *in vitro* exposure of colonies of *A.flavus* incubated for ten days at 30°C. The populations of *A.flavus* were significantly decreased by $O_3$ exposure; however, there was little difference between 50-200 ppm treatment

levels. A reduction in $AFB_1$ was only observed in the 50 ppm $O_3$ × 0.98 $a_w$ treatment in stored pistachio nuts. The relationship between exposure concentration × time of exposure and prevailing $a_w$ level to determine the efficacy in terms of toxin control needs to be better understand.

Keywords:

*A. flavus*, $AFB_1$, ecology, climate change, $O_3$, pistachio

# ACKNOWLEDGEMENTS

I am grateful to Allah (God) for giving me this opportunity to learn and expand knowledge.

This work would not be a success without the people, in one way or another, helped during the period of the project. I am especially grateful to my parents, grandparents and siblings for their encouragement, prayer, assistance and moral support.

A heartfelt gratitude goes to my wife, Doaa, and my children, Warif and Abdulaziz, for their patience and moral support throughout my research. I have to admit that it was not easy for both of us, my wife and I, to study abroad away from extended families with responsibility for two children. I am glad now she has just graduated and this humble piece of work is wholeheartedly dedicated. My family served as my inspiration to achieve my goal.

I would like to express my gratitude to my supervisor, Professor Naresh Magan, for his leadership, guidance, assistance and patience throughout the period of this work.

I am very grateful to Dr. Angel Medina for his advice and help during my research. I am thankful for everybody at Cranfield Applied Mycology group for their support and friendships.

A special thank goes to my entire friends in the UK and in the KSA for their communications and encouragements.

I also acknowledge Taif University for sponsoring this project and the Saudi culture bureau in London for their help and supervision.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACN | Acetonitrile |
| AFB1 | Aflatoxin B1 |
| ANOVA | Analysis of Variance |
| AOAC | Association of Official Analytical Chemists |
| $a_w$ | Water activity |
| BLAST | Basic Local Alignment Search Tool |
| CCA | Coconut cream agar medium |
| cDNA | Complementary deoxy ribonucleic acid |
| $CO_2$ | Carbon dioxide |
| Ct | Threshold cycle |
| CFU | Colony Forming Unit |
| DG18 | Dichloran (18%) glycerol agar |
| DNA | Deoxy ribonucleic acid |
| d | Day |
| ERH | Equilibrium relative humidity |
| EU | European Union |
| et al. | *et alii* |
| FAO | Food and Agricultural Organisation |
| FDA | Food and Drug Administration |
| G | Gram |
| h | Hour |
| HPLC-FLD | High Pressure Liquid Chromatography-Fluorescence Detector |
| IARC | International Agency for Research on Cancer |
| ITS | Internal transcribed spacer |

| | |
|---|---|
| L | Litre |
| ʎ | Wavelength |
| log | Logarithm |
| µ | Micro ($10^{-6}$) |
| m | Milli ($10^{-3}$) |
| M | Molar (mol/L) |
| MEA | Malt Extract Agar medium |
| MeOH | Methanol |
| mRNA | Messenger Ribonucleic Acid |
| min | Minutes |
| NaCl | Sodium chloride |
| n | Nano ($10^{-9}$) |
| $O_3$ | Triatomic oxygen (ozone) |
| OTA | Ochratoxin |
| PCR | Polymerase Chain Reaction |
| PMA | Pistachio Melt Media |
| ppb | Parts per billion |
| PPM | Part per Million |
| PBS | phosphate buffered saline |
| qPCR | Real-time Polymerase Chain Reaction |
| RH | Relative Humidity |
| RNA | Ribonucleic Acid |

| | |
|---|---|
| RNAse | Ribonuclease |
| TFA | Trifluoroacetic Acid |
| UV | Ultraviolet |
| WHO | World Health Organization |
| YES | Yeast Extract Sucrose medium |

# CHAPTER 1
# General Introduction, Literature Review and Research Objectives

## 1.1 LITERATURE REVIEW

### 1.1.1 GENERAL INTRODUCTION

Pistachio nuts (*Pistacia vera* L.) have become one of the most important products in the economy of many countries in some parts of the world including the USA, Iran, Syria, Greece, Turkey, China, EU and the Middle East. In 2016/2017, the world pistachio production was about 777,000 tonnes (USDA, 2017). The USA is the biggest producer with 407,000 tonnes followed by Iran with 153,000, and then by Turkey producing almost 153,000 tonnes. Other minor producers include Syria, China and Turkey are minor producers of this commodity.

The primary target customers of pistachio nuts in general are Europe as well as East Asia, Central Asia and the Middle East. There is competition between producers because it is a high value product and the quality of the product, including possible contamination with mycotoxins, becomes an important consideration. This is demonstrated by the competition between Iran and USA in a global trade war of pistachio production and export when Iran was placed under pressure to join the World Trade Organisation (WTO) to have access to world trade and meet the safety requirements in relation to quality and the accepted legislative limits on aflatoxins (AFs) in these nuts. Because of AFs contamination

of pistachio nuts in Iran, caused by colonization with *Aspergillus flavus*, their access to the EU market was compromised. As a result, the USA pistachio trade increased in the EU market from 4 to 23%, while, in contrast, the Iranian trade decreased from 95 to 70% for the period from 1992 to 2002. This was predominantly due to the implementation of the new European Community regulation on AFs. Pistachios are a high value perishable nut which can be colonized by *A.flavus* pre-harvest in the orchard or during the processing stages including harvesting, storage, packaging and transportation. This can lead to mycotoxin contamination which can be harmful to humans. In 2005, Iran submitted an application to be a member of WTO which was accepted after a period of time. This membership has influenced the Iranian export percentage of pistachios positively.

With regard to the Kingdom of Saudi Arabia (KSA), the quantity of imported pistachios has increased throughout the years from 80 tonnes in 1966 to 4,113 tonnes in 2008, and it reached a peak in 2006 with 6,951 tonnes (Mongabay, 2008). There is also information that the KSA has exported 372 tonnes pistachio nuts in 2007 and 2008. KSA also stores and processes the nuts for export to other Middle Eastern countries.

### 1.1.2 Fungal diversity of pistachio nuts in different countries

A wide variety of fungi are present in pistachio nuts, especially when there is damaged hulls and nuts (Denizel et al., 1976, a) at harvest time, during post-harvest operations, transport and storage. This depends on the temperature,

relative humidity, and whether Good Agriculture Practice (GAP) has been introduced, and soil and storage hygiene conditions are effectively managed. The fungal diversity will vary from region to region and some of the fungi are able to produce mycotoxins. Different fungal genera including *Alternaria, Aspergillus, Penicillium, Cladosporium, Eurotium, Fusarium, Rhizopus, Epicoccum, Trichoderma,* and *Ulocladium* can infect and decay pistachio nuts. *Aspergillus, Fusarium* and *Penicillium* genera contain the key mycotoxigenic species (Fernane et al., 2010a). Previous studies have shown that the most significant mycotoxins associated with pistachio nuts are AFs, which are produced by *A. flavus, A parasiticus and A. nomius,* and ochratoxin A (OTA), which is produced by *A. ochraceus*, *A. carbonarius* and *P. verrucosum* (Scott, 1993; Peterson, 2000). Pistachio nutmeat infections by *A.flavus* can occur due to early splits in the pistachio shells at the time of harvest (California Pistachio Research Board, 2009). They can thus be infected by *Aspergillus* spp. during both pre- and post-harvest operations. These are the main sources of mycotoxins in this food product (Ciegler, 1972; Mojatahedi et al., 1979). In Spain, *A.flavus* was found in 30% of 50 samples of pistachio nuts, whereas, *Aspergillus* section *Nigri, A. ochraceus* and *P. verrucosum* were found in 40%, 2% and 26% of the samples, respectively (Fernane et al., 2010a). A study of 31 samples of pistachio nuts collected from different regions in Algeria showed that *Penicillium* spp., *Aspergillus* section *Nigri* and *A.flavus* were isolated from the 38%, 30% and 22% samples, respectively (Fernane et al. 2010b). Soil-borne fungi such as *Fusarium, Phytophthora* and *Verticillium* in addition to *A.flavus* were found to contaminate pistachio nuts and contributed to the reduction in export from Iran (Luttfullah and

Hussain, 2011). Fourteen and thirteen different species of *Aspergillus* were isolated from pistachio nuts pre-harvest including *A.flavus, A.parasiticus,* and *A.niger* in the USA and Iran, respectively (Mojtahedi et al., 1979; Doster and Michailides, 1994). A survey done in the KSA to evaluate the risk derived from improper storage conditions for salted and non-salted pistachio samples collected from three different regions showed that *A.flavus* and *A.niger* were present in all the samples (Nawar, 2008). However, no detailed studies of AFs contamination or ecology of the species associated with mycotoxin contamination have been carried out.

### 1.1.3 *Aspergillus flavus* and aflatoxin contamination of pistachio nuts pre- and post-harvest

Figure 1.1 shows the life cycle of *A.flavus* in a pistachio orchard. In autumn/winter seasons, mummies fall down with infected pistachio nuts. *A.flavus* survives at this stage in a form of sclerotia in soil. In spring/summer seasons, climate conditions became more appropriate for *A.flavus* to start growing and sporulation occurs. The conidia got transferred by air resulting in pistachio nuts infection by *A.flavus*.

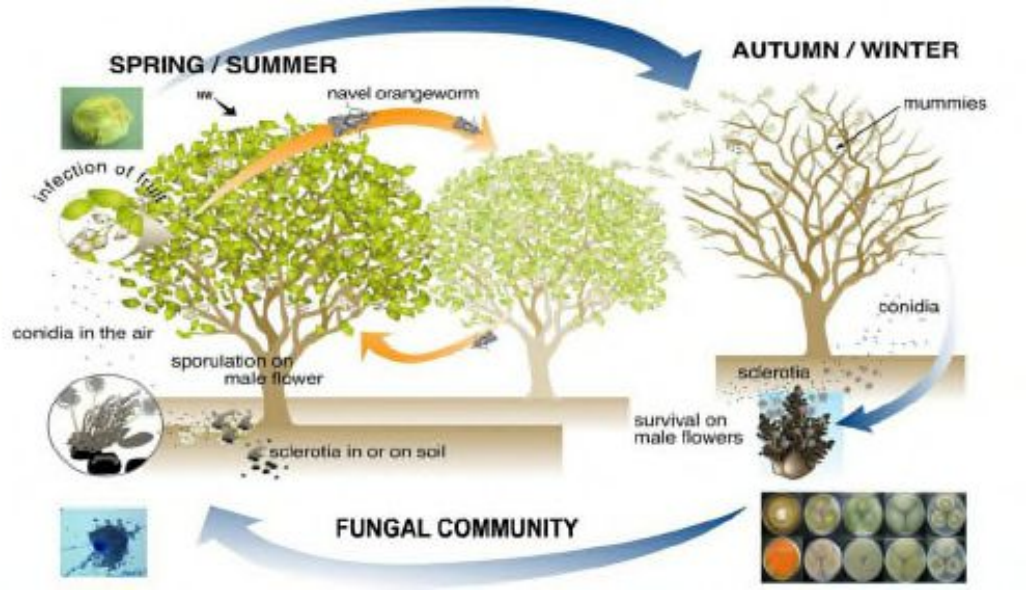


**Figure 1.1 Life cycle of *Aspergillus flavus* in a pistachio orchard**

Aflatoxins are important because they are classified as Class 1A carcinogens by the WHO and International Agency of Research on Cancer (IARC) as shown in Table 1.1. It is thus the most toxic natural toxin produced by a fungal species. Aflatoxins are a group of secondary metabolites produced by the aflatoxigenic fungi *A.flavus, A.parasiticus, and A.nomius* (Varga et al., 2011; Roehuck and Maxuitenko, 1994). Aflatoxins have been shown to cause serious clinical problems to humans and animals, because of their toxicity and carcinogenicity (Eaton and Groopman, 1994). Aflatoxicosis is a condition as a result of the ingestion of moderate to high levels of AFs in contaminated food or feedstuffs.

Over the years, it has been demonstrated by toxicological and clinical studies that several types of animals such as hamsters, rats, trout, ducklings, rabbits and a number of other vertebrates, are susceptible to AFs poisoning. Additionally, acute aflatoxicosis can lead to death, liver failure, rapid progressive jaundice, oedema of the limbs, pain, vomiting, necrosis and cirrhosis (CDC,

2004; Lewis *et al*., 2005). Several outbreaks caused by ingesting high levels of AFs from contaminated food in humans have been reported in several countries such as India, Kenya, Thailand and Malaysia (CAST, 2003). In 1974, in western India, more than 150 villagers were affected by aflatoxicosis, causing the most widespread outbreak of aflatoxicosis in humans. Of the 397 cases, 108 deaths were reported (Krishnamachari *et al.,* 1975).

**Table 1.1 Mycotoxins classifications according to carcinogenicity to humans (adapted from IARC, 2002).**

| Group | Description | Mycotoxins |
|---|---|---|
| **Group 1** | Carcinogenic to humans | Aflatoxin |
| **Group 2A** | Probably carcinogenic to humans | - |
| **Group 2B** | Possibly carcinogenic to humans | Fumonisins, Ochratoxin A and Sterigmatocystin |
| **Group 3** | Not classified as carcinogenic to humans | Citrinin, Patulin, Zearalenone and Tricothecenes |
| **Group 4** | Probably not carcinogenic to humans | - |

In 2004, an incident of AFs poisoning in Kenya was documented involving 317 cases, of which 125 deaths were reported, due to the consumption of maize contaminated with AFs, resulting in the largest and most severe outbreak of acute aflatoxicosis documented worldwide (CDC, 2004; Lewis et al., 2005). Recently, the IARC reported that AFs are responsible for stunting in infants and young children in Lower Middle Income Countries (LMICs), especially in Sub-Saharan Africa (IARC, 2016). Chronic aflatoxicosis, as opposed to acute aflatoxicosis, can lead to “slow” pathological conditions, immune suppression

and cancer. Therefore, a lot of countries worldwide have enforced strict legislative limits on the total amount of AFs and aflatoxin $B_1$ ($AFB_1$) acceptable in food and feedstuffs for human and animal consumption. Overall, the limits for $AFB_1$ in food products range from 0 to 30 µg/kg, while those for total AFs range from 0 to 50 µg/kg.

Table 1.2 shows the maximum tolerable level of AFs in nut products in different countries, while Table 1.3 shows the maximum tolerable level of AFs in nut products in the European Union (FAO, 2003). KSA, as a member of the Gulf Cooperation Council (GCC) with some other countries established maximum limits of total AFs in nuts and nut products as indicated in Table 1.4.

Reduction of $AFB_1$ is also necessary in order to control $AFM_1$ in food and feeds especially milk. When $AFB_1$ got metabolised $AFM_1$ is excreted (Prandini et al., 2009). The presence of this mycotoxin in milk and milk products is important issue, especially for children and infants, who are more susceptible than adults. Thus prevention fungal growth and minimisation of $AFB_1$ contamination could help to control this matter (Iqbal et al., 2015).

**Table 1.2 Maximum tolerable level of aflatoxin in nut products in different countries.**

| Country | Aflatoxin | Level (µg/kg) |
|---|---|---|
| **Canada** | $B_1 + B_2 + G_1 + G_2$ | 15 |
| **Israel** | $B_1$ | 5 |
| **New Zealand** | $B_1 + B_2 + G_1 + G_2$ | 15 |
| **Philippines** | $B_1 + B_2 + G_1 + G_2$ | 20 |

**Table 1.3 Maximum tolerable levels of aflatoxins (µg/kg) in nut products in the European Union (adapted from FAO, 1997).**

| Foodstuff | $B_1$ | Sum of $B_1$, $B_2$, $G_1$ and $G_2$ |
|---|---|---|
| **Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs** | 8.0 | 15.0 |
| **Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs** | 5.0 | 10.0 |
| **Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs** | 2.0 | 4.0 |

**Table 1.4 Total aflatoxins limits in Australia, Canada, Codex, GCC, Nigeria, India, USA and South Africa in foodstuffs (adapted from European Mycotoxins Awareness Network, 2012).**

| Country | Nut and nut products | Total aflatoxins (µg/kg) |
|---|---|---|
| **Australia** | Peanuts and Tree nuts | 15 |
| **Canada** | Nut and nut products | 15 |
| **Codex** | Peanuts, almonds, shelled Brazil nuts, hazelnuts pistachios intended for further processing | 15 |
| **GCC*** | | |
| **Nigeria** | Almonds, hazelnuts, pistachios, shelled Brazil nuts, "ready-to-eat" | 10 |
| **India** | Groundnut (shelled) | 30 |
| **USA** | Brazil nuts, peanuts and peanut products, pistachio products | 20 |
| **South Africa** | Peanuts | 15 |

**Members of GCC are Saudi Arabia, United Arab Emirates, Kuwait, Bahrain, Oman, Yemen and Qatar*

In Iran, an analysis of 10,068 samples of pistachio nuts showed that $AFB_1$ was detected in 36% of the samples with a mean of 5.9 µg/kg. $AFB_1$ exceeded the maximum level in Iran (5 µg/kg) in 11.8% of the samples. With regard to total AFs, only 7.5% of the same samples exceeded the maximum limit (15 µg/kg) and the majority were below the limit with a mean of 7.3 µg/kg (Cheraghali et al., 2007). Pistachio samples were also analysed in Turkey during the period of 1998 to 2002 according to the Turkish Ministry of Agriculture and Rural Affairs. The highest detected amount of $AFB_1$ in 523 samples was 113 µg/kg with a mean range of 1 to 3.8 µg/kg (Fernane et al. 2010b). Analysis of pistachio nut samples in Mexico showed that samples contained AFs >20 µg/kg (Joint FAO/WHO Expert Committee on Food Additives, 1998). Another study in Tunisia found that AFs contaminated 76.1% of the pistachio nut samples (Ghali et al., 2009). However, detailed studies and data on pistachio nuts consumed in KSA in terms of fungal biodiversity and ecology and control of AFs in this commodity are scarce. Neamatallah and Serdar (2013) found that nut samples, including pistachios, collected from the main outlet in the holy city of Mekkah were contaminated with $AF_s$ exceeding the maximum tolerable limit for AFs in Saudi Arabia (SASO, 1998).

### 1.1.4 Aflatoxin biosynthesis gene cluster

The genes involved in the production of AFs are clustered together and include about 25 genes (see Figure 1.2). They include two regulatory genes in the pathway which are *aflR* and *aflS.* These genes are responsible for the transcriptional activation of the specific sequence in the DNA-binding protein. The absence of *aflR* or *aflS* gene means no aflatoxin production (Yu et al., 2004).

An early structural gene, *aflD*, in the biosynthetic pathway is important in the intial stages of the biosynthetic pathway for secondary metabolite production (Yu et al., 2011). A specific toxin-producing type strain of *A.flavus* (NRRL3357) was used in the present study for comparison with those isolated from pistachio nuts. This strain was used as its full genome sequence has been reported Nierman *et al*. (2015).

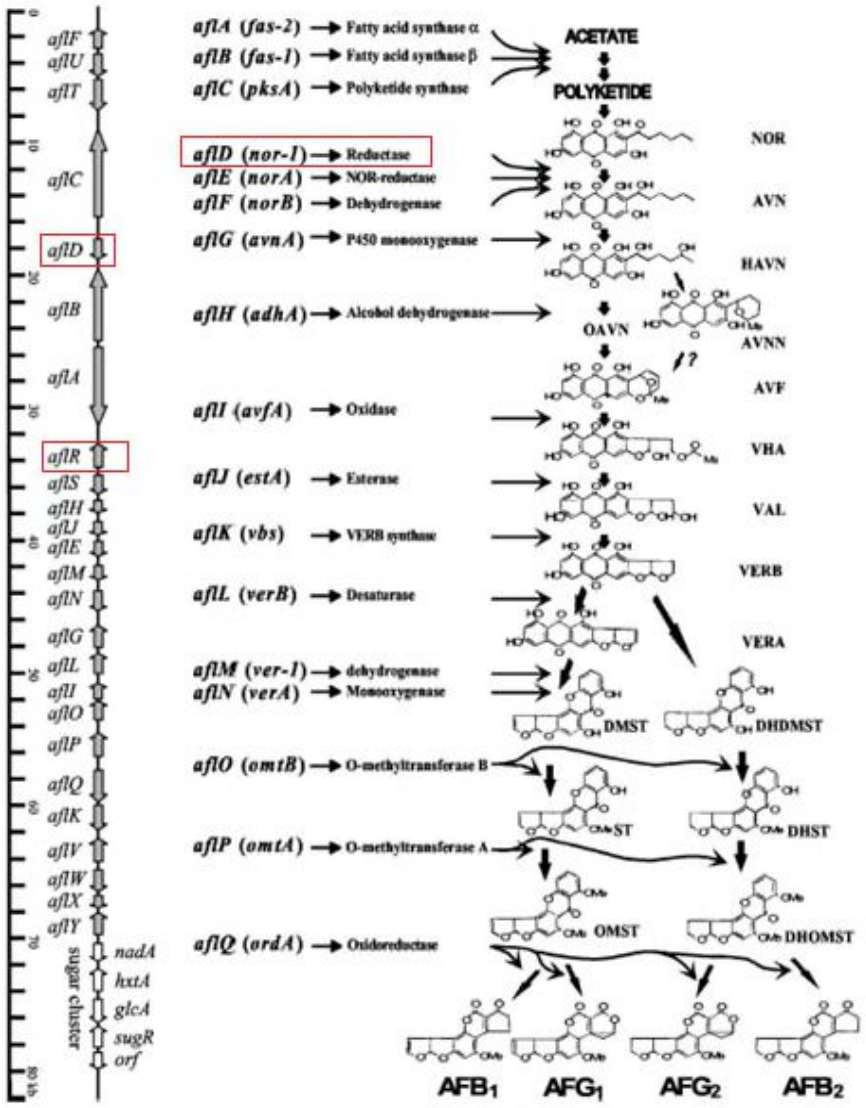


Figure 1.2 The gene cluster responsible for aflatoxin biosynthesis in *A.flavus* and *A.parasiticus*. The genes highlighted are *aflR*, regulatory gene and *aflD*, structural gene of the biosynthetic pathway (adapted from Yu *et al.,* 2004).

Abdel-Hadi et al. (2012) showed that environmental factors and time influenced the relative expression of the *aflD* gene. They were also able to show that the activity of the *aflD* gene was related to the prevailing $a_w$ and temperature conditions. They were also able to correlate the activity of this gene with growth and $AFB_1$ production by *A.flavus*. Interestingly, they also found that growth of *A,flavus* occurred over a wider range of $a_w$ and T, than that for $AFB_1$ production.

### 1.1.5 Role of ecophysiological factors in growth and aflatoxins production by *Aspergillus flavus*

**(a) Temperature (°C)**

Temperature is one of the most important ecophysiological factors which significantly affect fungal proliferation and subsequently the mycotoxin production. According to fungal tolerance to temperature, fungi can be classified into five groups; psychrotolerant, psychrophilic, mesophilic, thermotolerant and thermophilic. Some spoilage fungi*, e.g. A.fumigatus* and *Humicola lanuginose*, are able to grow at temperatures >35°C (Magan, 2007). Fungi, in general, are able to grow over the temperature range of 10-35°C. The temperature range for mycotoxin production can often be narrower than that for spore germination or fungal growth, and the optimum temperature for growth and mycotoxin production can also be different. For example, for *A.carbonarius,* the growth optimum is between 30-35°C, while that for OTA production is around 15-25°C (Michell et al., 2004). Similarly, for *P.verrucosum* it was shown that OTA production was optimum under lower temperature conditions than that for growth (Sanchis and Magan, 2004; Cairns et al., 2005). Recently, for *A.flavus*, it was shown that the optimum growth temperature was between 28-30°C *in vitro* and

in maize. However, the optimum conditions for AFs production are slightly different (Giorni et al., 2009; Abdel-hadi et al., 2012). Abdel-Hadi et al. (2012) found that the optimum growth conditions for *A.flavus* are 30°C with freely available water while 15°C was very marginal for growth. Optimum conditions for $AFB_1$ production were between 25-30°C with freely available water (≈99.5% ERH) and this changed to 30-35°C to 0.95 $a_w$. Thus, temperature is an important parameter which must be monitored regularly to minimise mycotoxin contamination. According to the California Pistachio Research Board (2009), Good Agriculture Practice (GAP) and Good Processing Practices (GPP) for pistachio must be applied very carefully with clear control points to minimise colonisation by spoilage and mycotoxigenic fungi and insect pests to ensure quality conservation. Key important points recommended for monitoring in the pistachio production and processing chain are:

a) Documentation should be available in order to trace the product back to identify and to ensure that the specifications have been met in terms of quality criteria including harvesting, drying and AFs contamination
b) Water sources are critical in pistachio production as water is used in field operations including irrigation and pesticide application, as well as the use of water in pistachio processing operations such as washing and subsequent drying. Thus, water can be a direct source of microbial contamination
c) Documentation of any materials applied to pistachio orchards including supplier details and method of application and actual dates of application prior to harvest must be available

d) The application of pesticides must be in accordance with suppliers instructions and applied and appropriate rates to ensure that no residues remain on the nuts during post-harvest processing

e) AFs and Navel orangeworm (*Amyelois transitella*) must be controlled effectively by growers pre-harvest to ensure subsequent yield and quality of pistachios for downstream processing

f) Timely harvesting, drying and storage of pistachios are essential to conserve and maintain pistachio nut quality and prevent microbiological safety issues

g) Field workers must be fully trained in Good Hygienic Practice (GHP) to minimise potential problems with food-borne pathogens

**(b) Water availability ($a_w$)**

Water is required by all organisms for effective functioning of the cells. Particular groups of yeasts and filamentous fungi have evolved to have the ability to adapt and grow in environments where water stress is imposed. The importance of water availability for microbial activity was first identified and related to the total water content of substrates by Scott (1957). He recognised that all the water in a substrate was not available to a microorganism because a percentage was tightly bound to the substrate. The available fraction which would allow microbial growth was defined as the water activity ($a_w$). He defined $a_w$ as a measure of the amount of water available in a substrate for microbial growth relative to that of pure water when at equilibrium at a standard temperature and pressure (Magan, 2007).

Various terms have been used to describe the microorganisms which have the ability to survive water stress conditions. Most terms have included xerophilic (*dry-loving*), osmophilic (*osmotic-pressure-loving*), xerotolerant (*dry-tolerating*), osmotolerant (*osmotic-pressure-tolerating*) or halophilic (*salt-loving*). The two most appropriate terms for fungi are osmophilic and xerophilic. Osmophilic describes the organisms which can thrive in high-osmotic pressure environments (Magan, 2007) while xerophilic is defined as those which are able to grow at ≤0.85 $a_w$ during any stage of their lifecycle (Pitt, 1997). Sanchis and Magan (2004) showed that the profile for growth and AFs production by *A.flavus* strains from maize and peanuts in relation to $a_w$ and temperature interactions were 35ºC - 0.95 $a_w$ and 33ºC - 0.99 $a_w$ for growth and toxin production respectively. However, few studies have examined the ecology of *A.flavus* strains isolated from pistachio nuts. To date, no detailed studies have been done with *A.flavus* strains isolated from pistachios from KSA in relation to growth and $AFB_1$ production.

**(c) Storage conditions**

According to Hazard Analysis Critical Control Point framework (HACCP), harvesting, drying, storage conditions are considered to be critical control points (CCPs) in the supply chain for pistachio nut production. Effective ventilation also plays a vital role in preventing fungal growth. Good Manufacturing Practice (GMP) is aimed at keeping the product at safe moisture content during storage, thus protecting the product from any possible damage by insects or pests and preventing any possible fungal contamination. Key elements, including effective drying prior to storage, stock organisation, physical facilities and environmental

conditions, must be taken into account in order to reduce and prevent biological hazards.

The required storage conditions for pistachios differ from stage to stage. At the post-harvest handling stage, fresh pistachios are sometimes stored temporarily. They should be cooled and held before hulling at 0°C and the relative humidity should be <70%. At the drying stage, firstly, pistachios are dried for about three hours at a temperature around 82°C to reduce the moisture content to around 12%. Secondly, they are dried further for one to two days at temperatures of <49°C to reach a moisture content of 5 to 6%. At post-drying storage, the pistachios are usually stored in 10°C or below and at 65 to 70% relative humidity to minimise deterioration including growth of *A.flavus.* Eventually, at the post-processing storage, the $a_w$ of pistachios should be kept at <0.70 at 25°C (moisture content <7%) and RH kept at <70% in order to avoid any mould growth. Temperature should be kept around 0-10°C depending on the storage time. The lower the temperature, the longer the storage life. However, pistachio nuts have high lipid content and are thus very hygroscopic. They can thus absorb moisture from the atmosphere easily during transportation between climatic zones. Thus care is needed to minimise this occurring to prevent spoilage and mycotoxin contamination.

Decreased $O_2$ <5% helps to control insects and maintain the quality of pistachio nuts. This can be done using packaging in nitrogen or by vacuum packaging. However, because they are very hygroscopic due to the fatty acid content they

can easily absorb moisture during transport and storage resulting in deterioration in quality and sometimes result in an increase in toxin contamination.

### 1.1.6 Impact of climate change on *Aspergillus flavus* growth and toxin production

The effects of climate change (CC) on food security is receiving significant attention world-wide in the last five years (Magan et al., 2011; Medina et al., 2014; Medina et al., 2015; Medina et al., 2017). It has been suggested that the current environmental conditions for crops to grow may change significantly in the next 25-50 years. Temperature is expected to rise due to the expected increase of atmospheric $CO_2$ due to levels of man-made industrial activity This increase might be in the range +2 to +4°C with $CO_2$ levels doubling or tripling from 350-400 to 700-800 or 1000-1200 ppm (Magan et al. 2011; Medina et al., 2017). This needs to be considered in relation to possible fluctuations in water availability especially very wet and very dry extreme conditions. The effect of temperature × $a_w$ on growth and $AFB_1$ production by *A.flavus* on colonisation of maize and groundnuts has previously been determined (Sanchis and Magan, 2004; Magan and Aldred, 2007). However, these studies did not examine the effect of increasing temperature or drought stress in the presence of elevated $CO_2$ conditions (Magan et al., 2011). Studies on maize have shown that CC interacting factors may not affect growth of *A.flavus* but that $AFB_1$ production was significantly stimulated (Medina et al., 2015a,b). In addition, this was supported by increased expression of one of the regulatory genes (*aflR*) involved in the biosynthetic pathway for AFs production. Studies with *A.carbonarius* and

*A.westedijkiae* in coffee under CC conditions have suggested differential effects on OTA production with the former species having no changes in growth or OTA production, with no effect on the former species, while for the latter species, OTA production was stimulated (Akbar et al., 2016). However, there have been no studies on whether CC factors will influence growth or $AFB_1$ production by strains of *A.flavus* on pistachio nuts. This may be important to understand whether under such CC conditions the risk of AFs in pistachio nuts is higher or lower.

Climate Change factors can impact on crops leading to mycotoxin contamination during both pre- and post-harvest phases. For example, pistachio nuts can become infected by fungi due to early split caused by heat/drought stress (Cotty & Jaime-Garcia, 2007). Additionally, heavy rain and late harvesting may result in toxin contamination if the nuts are not dried properly. Chauhan et al. (2008) described an increase in $AFB_1$ contamination in peanuts due to increases in ambient temperature and decreases in rainfall in Australia. Management practices for post-harvest operations applied using a Hazard Analysis Critical Control Point (HACCP) framework may require significant modifications under CC scenarios (Magan et al., 2011). More studies are needed to understand the impact of CC on stored food ecosystems, including pistachio nuts.

Recently, some interesting studies were carried out with regard to CC scenarios and acclimatisation of fungal pathogens (Vary et al., 2015). They grew *Fusarium graminearum* for 20 generations in CC conditions of 650 ppm $CO_2$. These acclimatised strains were found to be more virulent and cause increased

symptoms in ripening wheat. While the effect on type B trichothecenes was not examined, this does suggest that acclimatisation needs to be examined in relation to xerophilic fungi such as *A.flavus* and AFs production.

### 1.1.7 Control strategies

Strategies to reduce the activity of toxigenic fungi and production of mycotoxins, including AFs are critical in key food commodities to minimise consumers' exposure to these toxins. Biological, chemical and physical approaches have been used in many stored crops, including maize and peanuts, but few have been applied in pistachio nuts. One approach has been the use of microbial antagonists alone or in combination with other methods to try and reduce pistachio nut contamination with AFs pre- and post-harvest. Atoxigenic strains of *A.flavus* as well as bacteria, yeasts and other filamentous fungi have been found to reduce colonisation and toxin contamination in pistachio nuts (Tsitsigiannis et al., 2012). In other commodities, e.g. maize and peanuts, atoxigenic *A.flavus* strains have been successfully used pre- and post-harvest for effective control of AFs (Mohale et al., 2013; Bandyopadhyay et al., 2016). Studies by Mohale et al. (2013) and Sixtos-Rodriguez (2017) showed that atoxigenic strains of *A.flavus* were able to control $AFB_1$ production *in vitro* and *in situ* in stored maize. Efficacy was influenced by the ratio between toxigenic/atoxigenic spores of the strains and by temperature and $a_w$ level.

Physical techniques using gaseous treatment of stored commodities with sulphur dioxide ($SO_2$) or ozone ($O_3$) including pistachio nuts has been previously

examined to try and control toxin contamination post-harvest. $SO_2$ is commonly used to control microbial spoilage in foods and beverages. It is also used as an antioxidant to prevent browning in both fresh and dried fruits and vegetables. However, it has been found to be more effective against spoilage moulds than against bacteria (Magan, 1993). Its mode of action is via mutagenic effects on microbial activity. Applegate and Durrant (1969) studied the synergistic action of $SO_2$-$O_3$ on preservation of peanuts. They found that both gasses, when applied separately, were found to be phytotoxic. However, when applied together, potential effects were better in terms of control of spoilage fungi and less impact on the plants. No studies have been previously reported with such gaseous treatment for controlling growth and $AFB_1$ production by *A.flavus* in pistachio nuts. The present work however, only focussed on the application of $O_3$ as a means of controlling the proliferation of the pathogen and its subsequent toxin production.

Ozone is commonly generated in the summer because of the reactions between photochemicals in the presence of sunlight. Elevated levels of $O_3$ have been found to be effective at inhibiting water contaminating microorganisms. However, it is a very corrosive gas and dissociates/degrades very quickly. It has been previously shown that small increases in $O_3$ concentrations (40-60 ppb) can influence the mycobiota on plant surface and perhaps the ability to produce toxins (Manning, 1995). Slight increases in $O_3$ have been previously shown to change the mycobiota on leaf surfaces (Magan et al., 2006). Previous studies have examined the efficiency of electrochemically generated $O_3$ on activity of aflatoxigenic fungi and AFs in pistachio nuts inoculated with *A.parasiticus.* This

showed that population density of *A.parasiticus* after $O_3$ treatment of pistachios was decreased by 90-92% and total AFs in treated pistachio nuts decreased by 45-74% (Mir Abu Al-Fotuhi et al., 2008). More recently, Mylona et al. (2014) showed that gaseous $O_3$ (100-400 ppm) was effective against *Fusarium verticillioides in vitro* and *in situ* in maize grain and inhibited fumonisin production. However, the gaseous $O_3$ was less effective against spores and mycelial growth than against toxin production. For spores, initial inhibition was followed by recovery, growth and fumonisin production. Sultan (2010) however, found that gaseous $O_3$ was very effective in inhibiting germination of conidia of *A.flavus* but it had little efficacy in controlling mycelial growth of *A.flavus* strains isolated from peanuts. Studies have been carried out on $O_3$ treatment of Brazil nuts and showed that exposure oto $O_3$ affected growth of the mycobiota and decreased $AFB_1$ contamination. They used three concentrations of $O_3$ (10, 14 and 31.5 mg/L) for five hours and examined the effect after 180 days. They found that exposure for this period was effective for inhibiting growth of *A.flavus* and *A.parasiticus,* although they were still able to grow during the initial days immediately after exposure; especially those exposed to the lower $O_3$ levels. They suggested that AFs were degraded by this treatment although more evidence is needed to support this (Giordano et al., 2012).

## 1.2 RESEARCH AIMS AND OBJECTIVES

### 1.2.1 Research Aims

The overall aims of the research project was to study the mycobiota of pistachio nuts, to understand the impacts of CC factors on *A.flavus* growth and $AFB_1$

production, and to examine the possible control strategies to inhibit *A.flavus* growth and $AFB_1$ contamination. This also required a good understanding of the ecophysiological behaviour of molecular ecology of *A.flavus* on pistachio nuts.

### 1.2.2 Research Objectives

**1.** To examine the mycobiota biodiversity and $AFB_1$ contamination of raw and salted pistachio nuts obtained from the KSA

**2.** To understand the ecology of *A.flavus* strains isolated in (1) in relation to interacting $a_w$ × temperature conditions on growth and $AFB_1$ production *in vitro* on pistachio-based media and *in situ* on stored pistachio nuts

**3.** To determine the ecological ranges for growth and $AFB_1$ production and relate this to biosynthetic gene expression of key genes such as *AflR* and *AflD* in the biosynthetic pathway for $AFB_1$ production using q-PCR

**4.** To evaluate the impact of interacting CC factors (+4°C, drought stress and 2-3× existing $CO_2$ levels) on growth and $AFB_1$ by *A.flavus* strains *in vitro* and *in situ* on raw pistachio nuts. The adaptation and acclimatisation of the *A.flavus* strains were also examined to evaluate whether this affected growth and $AFB_1$ production.

**5.** To examine the use of gaseous $O_3$ to control conidial germination, mycelial growth and $AFB_1$ production by strains of *A.flavus in vitro* and *in situ* under different $a_w$ storage regimes

Figure 1.3 summarises the programme of work carried out in the different phases of the project and the layout of the thesis

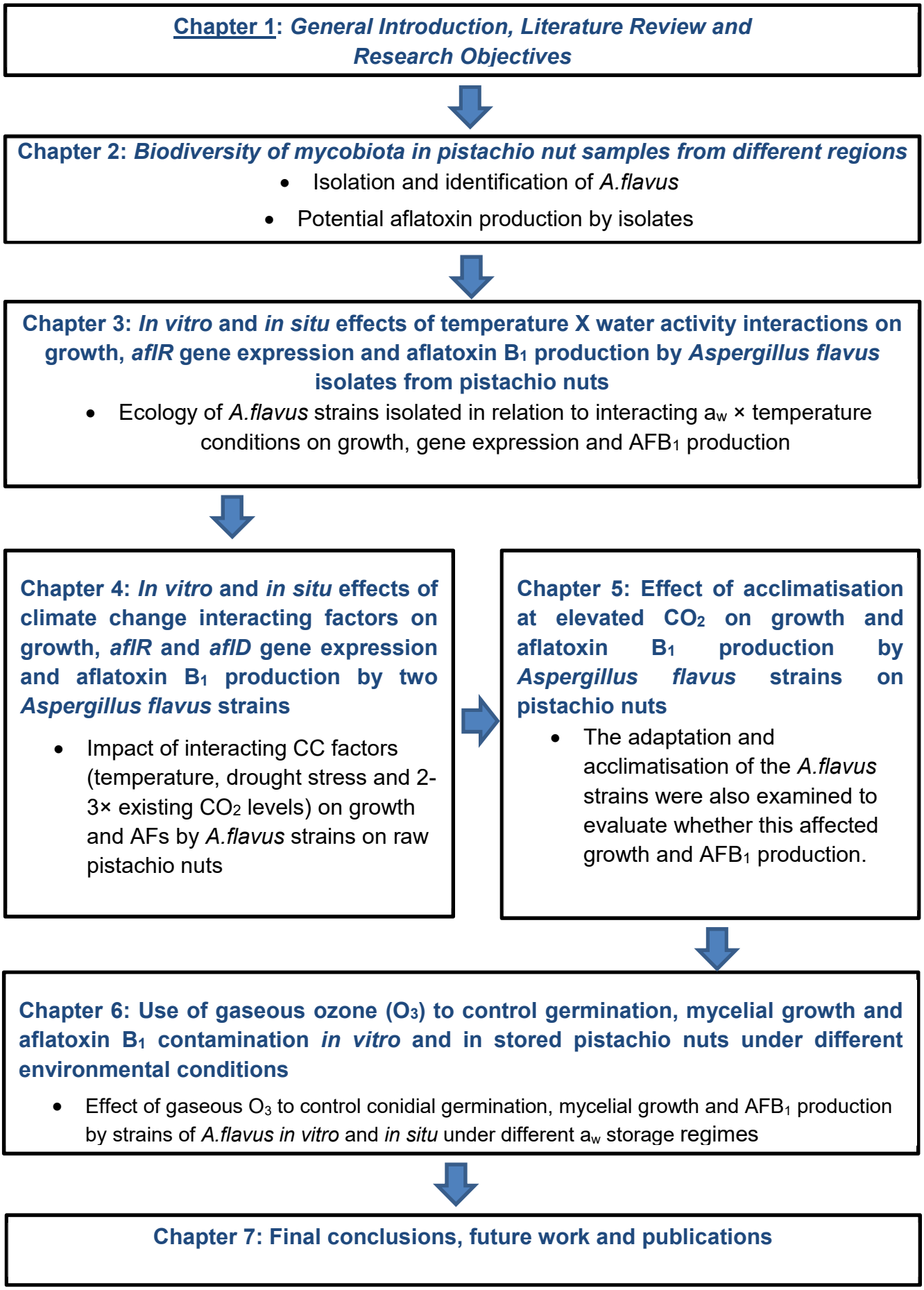


**Figure 1.3** **Diagrammatic representation of the layout of the Chapters of the thesis.**

# CHAPTER 2
# Biodiversity of mycobiota in pistachio nut samples from different regions obtained in the KSA

## 2.1 INTRODUCTION

Mycobiota is defined as the naturally present fungal flora community in a specific habitat or niche. A wide variety of fungi are present in and on pistachio nuts, especially when there are damaged hulls and nuts (Denizel et al., 1976, b). According to Kallsen et al. (2009) nutmeat infections can occur due to early splits in the pistachio shells at harvest time, during post-harvest operations, transport and storage. This also depends on factors including temperature, relative humidity, and whether GAP has been applied, and the drying and the hygiene of the storage facilities are effectively managed. Pistachio nuts can be contaminated with different fungal genera including *Alternaria, Aspergillus, Penicillium, Cladosporium, Eurotium, Fusarium, Rhizopus, Epicoccum, Trichoderma,* and *Ulocladium.* The key genera responsible for potential mycotoxin contamination are the *Aspergillus, Fusarium* and *Penicillium* (Fernane et al., 2010a). Previous studies showed that the key mycotoxins associated with pistachio nuts are aflatoxins (AFs), which are produced by *A.flavus* and *A. parasiticus,* and ochratoxin A (OTA), produced by *A. westerdijkiae, A. carbonarius and P. verrucosum* (Peterson, 2000; Scott, 1993). Pistachio nut infections by *A.flavus* can occur due to early splits in the pistachio shells at the time of harvest (Kallsen et al., 2009). In Spain, *A.flavus* was found in 30% of 50 samples of pistachio nuts (Fernane et al., 2010a). A study for 31 samples of pistachio nuts collected from

different regions in Algeria showed 22% of the samples were contaminated by *A.flavus* (Fernane et al. 2010b). *A.flavus* was also found to contaminate pistachio nuts and contributed to the reduction in exports from Iran (Luttfullah and Hussain, 2011). Fourteen and thirteen different species of *Aspergillus* were isolated from pistachio nuts pre-harvest including *A.flavus, A.parasiticus,* and *A.niger* in the USA and Iran, respectively (Doster and Michailides, 1994; Mojtahedi et al., 1979). A survey done in the KSA to evaluate the risk derived from improper storage conditions for salted and non-salted pistachio samples collected from three different regions showed that *A.flavus* was predominant (Nawar, 2008). Neamatallah and Serdar. (2013) found that nut samples, including pistachios, collected from the main outlet in the holy city of Mekkah were contaminated *A.flavus* with AFs also being present. However, a detailed study of the mycobiota and the contamination with *A.flavus* strains which can produce AFs has not been carried out in detail.

The objectives of this Chapter were:

1. To examine the mycobiota biodiversity and mycotoxin production in raw and salted pistachio nut samples (8 x 2) originating from different countries but sourced from different markets in the Kingdom of Saudi Arabia (KSA).
2. To isolate and identify aflatoxigenic strains of *A.flavus* from these samples using morphological (conventional) and molecular methods
3. To quantify the $AFB_1$ contamination of the pistachio nut samples obtained from the KSA.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Sampling of pistachio nuts

The raw pistachio nut samples used in the present study were sourced from the USA and packed in the UK. The salted pistachio nut samples (8 × 2 replicates; 500 g of each sample) were collected from different markets in the KSA. These samples were imported into the KSA from the USA (three samples), Iran (three samples) and Syria (two samples). These samples were sealed in plastic bags and transported to the UK. All samples, raw and salted, were refrigerated at 4°C until further analyses. In addition, shelled raw pistachio nut samples were also obtained from Sussex Wholefoods Company, UK.

### 2.2.2 Measurement of moisture content and water activity of pistachio nut samples

Moisture content (MC) is a quantitative analysis of the total amount of water in a given sample. The oven-drying method was used in the present study to measure the MC of pistachio nut samples. Five gram (5 g) shelled pistachio nut samples were weighed on an analytical balance. The weight was recorded before and after the drying process. The samples were placed in a 50 mL beaker after the initial weighing and placed in a pre-heated oven (Genlab, UK) at 100 ± 0.5°C for 24 h. Dried samples were then cooled and re-weighed to obtain the final dry weights.

However, MC alone does not give a clear indication of whether the water amount present in the samples would affect fungal colonisation. Thus, the $a_w$ of the samples was also measured using a $a_w$ analyser (AquaLab TE4, Labcell, USA). First, the temperature in the measuring chamber was equilibrated by leaving the analyser on for 15 min. Three to four pistachio nuts were placed in a single layer in the measuring container of the analyser which was then inserted into the measuring chamber. The $a_w$ of the samples were measured in triplicates at the prevailing stable temperature (25°C).

### 2.2.3 Preparation of fungal growth media

Malt Extract Agar (MEA) and Dichloran-Glycerol 18 Agar (DG-18), which were both obtained from Oxoid (UK), were used for fungal cultivation. MEA is a general medium that supports the growth of a wide range of fungi. It is a non-selective medium. DG-18 is a selective medium which supports growth of selected yeasts and moulds from dried and semi-dried foods. To inhibit bacterial growth, chloramphenicol (Fisher, UK) was added to the fungal growth media before autoclaving. Growth media were prepared separately according to manufacturer's instruction (MEA 50 g and DG-18 31.5 g; in 1 L distilled water) and autoclaved (Meadowrose, England) at 121°C for 15 min at 15 psi. Autoclaved media were then poured into 9 cm diameter sterile Petri dishes and left to solidify. Solidified media were stored at 4°C until further use.

Yeast Extract Sucrose (YES; yeast extract 20 g, sucrose 150 g, $MgSO_4 \cdot 7H_2$ 1 g, agar 20 g, distilled water 1 L) was used for sub-culturing and purifying the strains for later use in ecological studies.

The capability of AFs production by isolates was examined on Coconut Cream Agar (CCA) medium under U-V light. A 50% coconut cream (Blue Dragon, Indonesia) was dissolved in water while being gently heated. A 2% agar technical No. 3 (Oxoid, UK) was added to the mixture. The mixture was autoclaved for 15 min at 121°C, left to cool, mixed well, and then poured into 9 cm diameter sterile Petri dishes and left to solidify. Solidified media were stored at 4°C until further use (Sultan and Magan, 2010).

### 2.2.4 Fungal isolation and enumeration

The mycobiota of the pistachio nut samples were investigated and the fungal populations determined using MEA and DG-18 media using two different methods. The first was by direct-plating of nuts separately on MEA or DG-18; triplicates of five nuts and shells per Petri dish. The frequency of isolation (%) of fungi was recorded after incubation at 25°C for 7 d. Serial dilution was the second technique used. Populations of fungi (CFUs/g) were assessed by comminuting 10 g sub-samples in 90 mL of sterile distilled water containing a small amount of Tween-20. Four samples were homogenised in a Colworth Stomacher 400 and serial dilutions were made. 200 µL of each dilution was spread-plated on MEA and DG18 plates in triplicate. A colony counter was used for total colony counts

after incubation at 25°C for 7–10 d, and microscopic examinations were made to identify the fungal genera. Individual colonies were sub-cultured onto YES media for isolation and detailed examination and identification.

### 2.2.5 Molecular identification of *Aspergillus flavus* strains isolated from the pistachio nut samples

**(a)** DNA extraction

Five strains of *A.flavus* were isolated from pistachio nut samples. The isolated strains were grown on MEA and inoculated onto the conducive YES medium. After 5 d, the mycelium was harvested from the Petri dish with the aid of a spatula. This was frozen with liquid nitrogen and ground to a fine powder in a mortar with a pestle. A total of 100 mg of the powder was transferred to a 2 mL Eppendorf tube and then mixed with 500 µL CTAB lysis buffer (5 g D-sorbitol, 2 g N-lauroylsarcosine, 1.6 g/L CTAB, 1.4 M NaCl, 20 mM $Na_2$EDTA, 2 g PVPP, 0.1 M Tris-HCL, pH 8.0) containing 5 µL β-mercaptoethanol. The tube was vigorously shaken for 15 s and then vortexed for 30 s after adding 10 µL proteinase K solution (10 mg/mL). The samples were then incubated for 1 h at 65°C. The sample was centrifuged at 13,000 rpm for 5 min at 4°C to obtain two different layers. The supernatant was then transferred into a new 2 mL Eppendorf tube by pipetting, and 500 µL chloroform (HPLC grade) was added and mixed briefly by vortexing for 30 s. Samples were then centrifuged at 13,000 rpm for 20 min at 4°C. Samples were then was incubated at 37°C for 1 h and 10 µL RNase solution (10mg/mL) was added. After adding 500 µL chloroform, the tube was vortexed for 30 s and centrifuged at 13,000 rpm for 5 min at 4°C. The upper layer was again transferred to a new Eppendorf and mixed with 500 µL cold isopropanol.

Later, sample was centrifuged at 10,000 rpm for 2 min at 4°C and supernatant was removed. After adding 1 mL 70% ethanol (HPLC grade), samples were vortexed briefly and centrifuged at 13,000 rpm for 2 min at 4°C, the supernatant was discarded and DNA was re-suspended in 100 µL Tris-EDTA (TE) buffer (pH 8.0) and stored at -20°C until used as template for PCR amplification.

**(b)** PCR amplification

Two primer pairs, ITS1-ITS2 and ITS3-ITS4, were used to perform PCR reactions for the ITS1-5.8S-ITS2 region (Bellemain, et al. 2010, Table 2.1). The mixture of PCR reaction consisted of Buffer 10X (5 µL), $Cl_2Mg$ (3 µL), DNTPs (1 µL), *Taq* polymerase (0.5 µL) and 2 µL of each primer (10 µM) in a total reaction volume of 50 µL. The amplification program used was: 1 cycle of 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, and finally 1 cycle of 5 min at 72°C. Agarose gel (2%) stained with 10 µL Safeview nucleic acid stain was used to separate the PCR products (NBS Biologicals, Cambridgeshire, UK). The size of the PCR product was determined by using a DNA molecular size marker of 2.1–0.15 kbp (Promega BioSciences, CA, USA) with the aid of Genesnap v.7.09.02 image acquisition software (Syngene, Cambridge, UK).

**Table 2.1 Oligonucleotide sequences of the primers.**

| Primers | Primer sequence | Position |
|---|---|---|
| ITS1 | TCCGTAGGTGAACCTGCGG | 1761-1779 |
| ITS2 | GCTGCGTTCTTCATCGATGC | 2024-2043 |
| ITS3 | GCATCGATGAAGAACGCAGC | 2024-2043 |
| ITS4 | TCCTCCGCTTATTGATATGC | 2390-2409 |

**(c)** Sequence analysis

Amplification products were purified and sequenced at Macrogen (the Netherlands). Applied Biosystems Sequence Scanner (v. 1.0) and CLC Sequence Viewer (v. 6.6.1) software were used to analyse the sequences. The NCBI database (http://www.ncbi.nlm.nih.gov) was used to align and compare sequences obtained with the ones previously deposited. The identification of fungal species was based on the BLAST tool of the NCBI database. When assigning an isolate for a species name, only results over 99 % similarity with an isolate's ITS sequence was considered on BLAST search.

### 2.2.6 Preliminary screening of aflatoxigenic potential of *Aspergillus flavus* strains isolated from pistachio nut samples using Coconut Cream Agar

$AFB_1$ production was screened using the fluorescence technique, based on growth on a 50% Coconut Cream Agar (CCA) medium. A total of four strains and a type strain (NRRL strain) were grown on CCA for 7-10 d (Sultan and Magan, 2010). Ultra-violet light was used in the dark at 360 nm to check whether or not any blue fluorescence occurred on the reverse side of the colony. Blue fluorescence was an indication that AFs have been produced.

### 2.2.7 Quantification of aflatoxin $B_1$ from pistachio nut samples

The separation and detection of $AFB_1$ were done using HPLC. Two replicates of each sample were analysed by extraction using Aflaprep columns (Neogene Europe, wide bore) and derivatised using the method of the AOAC (2005). For

the four isolated strains, eight plugs were extracted from each colony from each strain following 10 d incubation at 25°C on YES and transferred into a 2-mL Eppendorf tubes and weighed. For $AFB_1$ extraction, 1 mL chloroform was added to the plugs in each Eppendorf tube and shaken well for 1 h. The chloroform extract was transferred to a new Eppendorf and dried gently under air for derivatisation using the method of the AOAC (2005) and then analysed quantitatively using HPLC. A 200-µL stock solution of AF mixed standard in methanol containing 250 ng $B_1$, 250 ng $B_2$, 250 ng $G_1$ and 250 ng $G_2$ was dried under nitrogen gas and derivatised as for samples for the construction of linear calibration curve which in turn was used in the quantification of actual $AFB_1$ concentration obtained from chromatograms.

### 2.2.8 Statistical analysis

Three replicates for each treatment were used in all experimental studies. Means were obtained by taking the average of each three measurements with the standard error of the means (± SE; standard error). Analysis of Variance (ANOVA) was applied to analyse the variation of means with 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov using Minitab statistical software. Fisher's Least Significant Difference (LSD) was checked to identify differences between the means with $p < 0.05$ as significant difference using the same statistical software.

## 2.3 RESULTS

### 2.3.1 Fungal isolation frequency from pistachio nut samples

The relative isolation frequency of the dominant genera and species from salted pistachio nut samples was examined. Figures 2.1 and 2.2 show that *A.flavus* was isolated from the pistachio nuts and shells, respectively. *A.flavus* was isolated from all pistachio nut samples except the Syrian ones, and only from the shells of the USA samples. In addition to *A.flavus*, other fungi including *Eurotium*, *Cladosporium*, *Penicillium*, *Rhizopus, Mucor and A.niger* were also isolated.

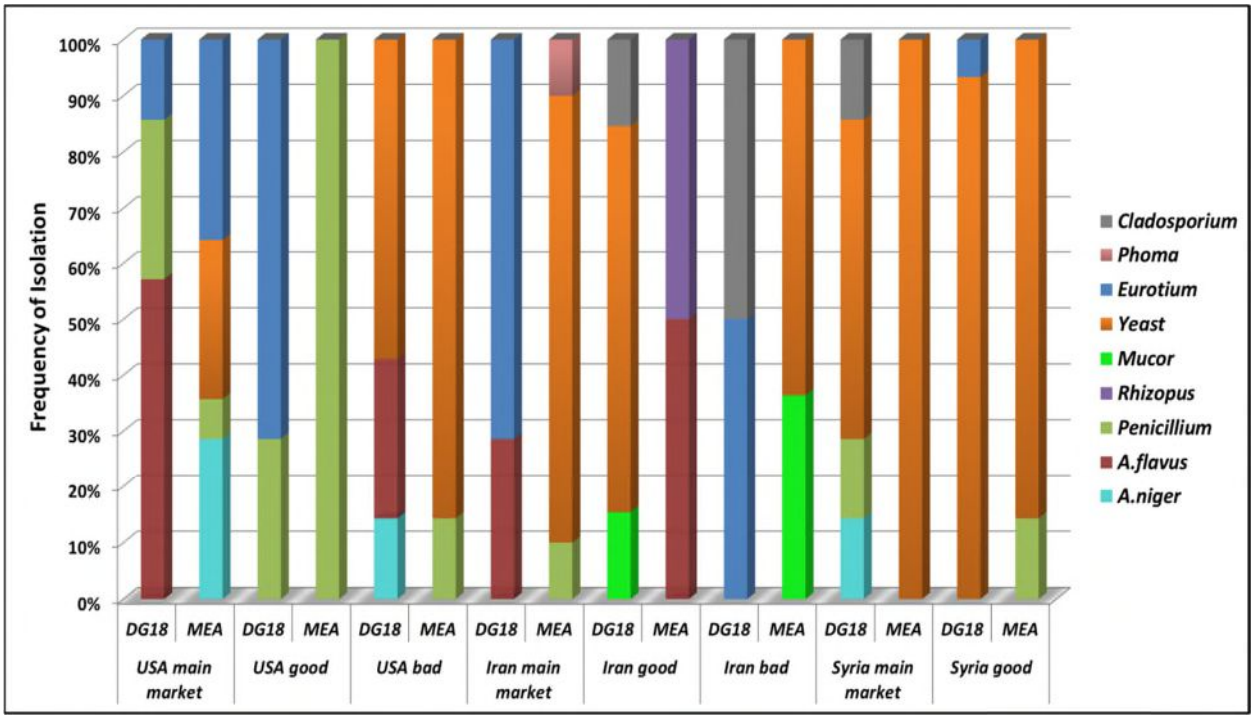


**Figure 2.1 Frequency of isolation of dominant fungi from different pistachio nuts based on direct plating on two different media, DG18 and MEA, incubated at 25°C for seven days.**

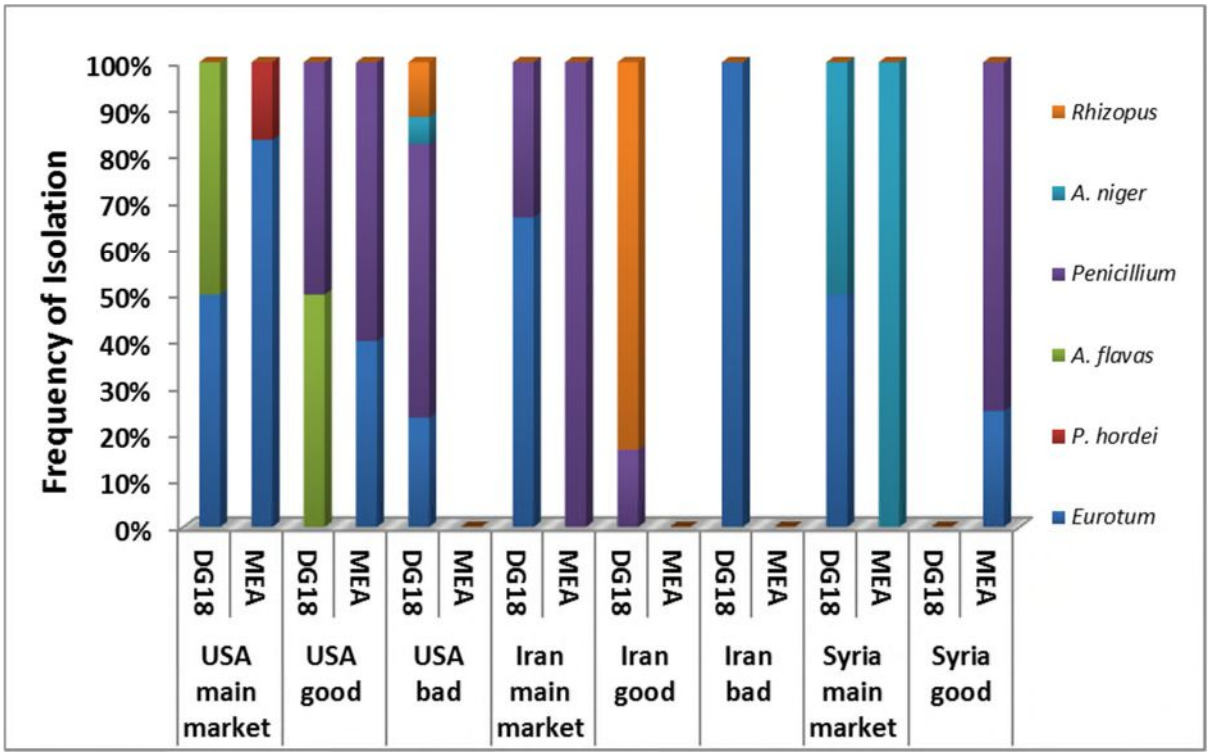


**Figure 2.2 Frequency of isolation of dominant fungi from different pistachio shells based on direct plating on two different media, DG18 and MEA, incubated at 25°C for seven days.**

### 2.3.2 Fungal load of raw and salted pistachio nut samples

Figure 2.3 shows the initial total fungal populations ($log_{10}$ CFUs/g dry weight) from four salted pistachio samples. This shows that the total population was higher in all samples on MEA than DG18. All samples had almost similar total CFU populations on MEA. The sample from Syria, obtained from the main market, had the highest total fungal populations whereas Iranian samples had the lowest total fungal load on DG18 (grey and red columns in Figure 2.3 respectively). There was a significant difference in the total CFUs in the Iranian samples examined on DG18 and MEA. For the Syrian samples there was no significant difference.

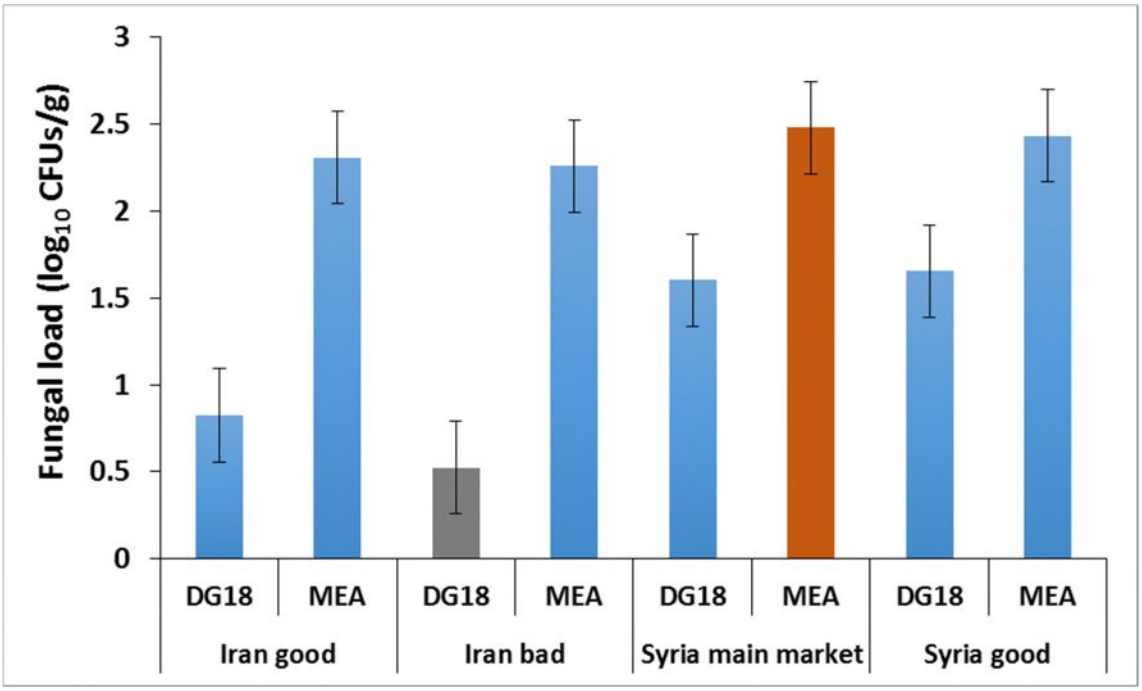


**Figure 2.3 Total fungal populations ($log_{10}$ CFUs/g dry weight) isolated from different pistachio samples based on serial dilutions on two different media, DG18 and MEA, incubated at 25°C for 5-7 days. Bars indicate standard error (SE) of the means.**

### 2.3.3 Molecular identification of *Aspergillus flavus* strains isolated from pistachio nut samples

Following the isolation of *A.flavus,* confirmation of their identity was required. This was done based on molecular techniques using the ITS region. Table 2.2 shows the sequencing results of isolated strains and type strain using ITS1 & 2 and ITS 3& 4 primer pairs. All strains were confirmed to be *A.flavus* with a similarity percentage ≥99%. For strain AB3, results based on the similarity percentage showed that it is more likely to be *A.oryzae* (100%) then *A.flavus* (98%). However, further studies show the ability of strain AB3 to produce $AFB_1$ which confirmed that this was indeed *A.flavus* (*A.oryzae* does not produce AFs).

**Table 2.2 Sequencing results of isolated strains and type strain using ITS1&2 and ITS 3&4 primer pairs for molecular identification.**

| Sample ID | GenBank ID | | Genus | Species | Similarity % | |
|---|---|---|---|---|---|---|
| | ITS1&2 | ITS3&4 | | | | |
| Type strain | M1204.653 | BP4 | *Aspergillus* | *flavus* | 100 | 99 |
| AB3 | A4S3_13 | SCAU-F-142 | *Aspergillus* | *flavus oryzae* | 98 | 100 |
| AB4 | SV/09-05 | UOMS28 | *Aspergillus* | *flavus* | 96 | 99 |
| AB5 | M1204.653 | M1204.653 | *Aspergillus* | *flavus* | 100 | |
| AB10 | M1204.653 | LPSC 1183 | *Aspergillus* | *flavus* | 100 | 99 |

### 2.3.4 Aflatoxin $B_1$ contamination of pistachio nut samples collected from the KSA

In the present study, eight pistachio nut samples were obtained from the KSA and replicates of each sample were analysed. All samples were imported into KSA from different countries including the USA, Iran and Syria. The data in Figure 2.4 shows that the poor quality samples from the three countries contained the highest levels of contamination. Overall however, the $AFB_1$ levels were below the EU regulation limits (5 ng/g).

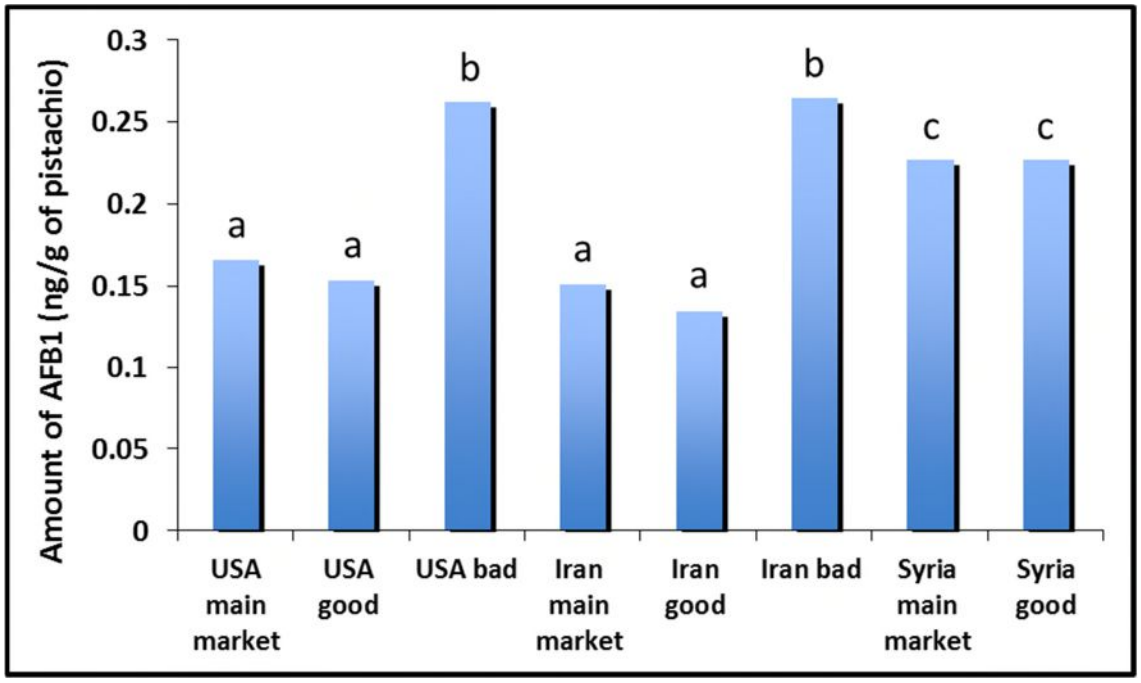


**Figure 2.4 The aflatoxin $B_1$ contamination of the pistachio nut samples obtained from different markets in the Kingdom of Saudi Arabia. Different letters indicate significant difference ($p < 0.05$).**

## 2.4 DISCUSSION

### 2.4.1 Mycobiota of pistachio nuts

The mycobiota level in the present study was relatively low when salted pistachio nuts were examined, perhaps because they had already been roasted and salted. This may have decreased the fungal load in the samples. Despite this, *A.flavus* was present and isolated from most of the samples.

Previously, *A.flavus* was found to be present in samples in a survey done in the KSA to evaluate the risk derived from improper storage conditions for salted and non-salted pistachio samples collected from three different regions in the country (Nawar, 2008). In the present study, all isolates were confirmed molecularly using

ITS regions as *A.flavus* strains except for strain AB3 when the sequencing percentage was closer to *A.oryzae* than *A.flavus.* $AFB_1$ studies showed the ability of strain AB3 to produce $AFB_1$ which confirmed that this was indeed *A.flavus* as *A.oryzae* is not capable of AFs production since it lacks the biosynthetic genes involved in secondary metabolite production (personal communication; Dr D. Bhatnagar).

In the present study a range of up to 10 different *Aspergillus* species in samples from the USA and Iran were found. A similar range of species were found in other surveys of pistachio nuts from these countries including *A.flavus, A.parasiticus,* and *A.niger* (Mojtahedi et al., 1979; Doster and Michailides, 1994). In Spain, *A.flavus* was found in 30% of 50 samples of pistachio nuts (Fernane et al., 2010a); while in Algeria, the mycobiota was dominated by *Penicillium* spp. (38%), *Aspergillus* section *Nigri* (30%) and *A.flavus* (22%) (Fernane et al. 2010b). However, these studies did not use molecular identification methods or examine the ecology of the *A.flavus* strains in terms of either growth or $AFB_1$ production.

### 2.4.2 Aflatoxin $B_1$ contamination of pistachio nut samples

Although the detected $AFB_1$ levels in the different samples were below the legislative limits, this does suggest that there is an inherent problem with regard to contamination of these nuts and that harvesting, drying and storage systems need to be carefully managed to ensure that the levels do not exceed the limits. This study has also provided strains of *A.flavus* which produced $AFB_1$ which can

be subsequently used in ecological studies and for examining the impact of climate change conditions and minimisation approaches using $O_3$ during storage.

Previously, $AFB_1$ was detected in 36% of the total of 10,068 pistachio nuts samples with a mean of 5.9 µg/kg in Iran with 11.8% of the samples exceeding the maximum Iranian level of 5 µg/kg. With regard to total AFs, only 7.5% of the same samples exceeded the maximum limit (15 µg/kg) and the majority were below the limit with a mean of 7.3 µg/kg. Similar results were obtained in another study by Cheraghali et al. (2007). Pistachio samples from Turkey in the period 1998 to 2002 showed a mean range of detected $AFB_1$ from 1 to 3.8 µg/kg (Fernane et al. 2010b). Analysis of pistachio nut samples in Mexico showed that samples contained high contamination levels with AFs >20 µg/kg (Joint FAO/WHO Expert Committee on Food Additives (JECFA) 1998). In Tunisia, A study concluded that AFs contaminated pistachio nut samples represented 76.1% (Ghali et al. 2008).

This study was predominantly aimed at obtaining information on the relative contamination of pistachio nuts from different sources consumed in the KSA. IT also provided a source of *A.flavus* strains for ecological studies to better understand the relationship between environmental factors and the capacity for strains of *A.flavus* to grow and produce AFB1 which is presented in Chapter 3.

# CHAPTER 3
# *In vitro* and *in situ* effects of temperature × water activity interactions on growth, *aflR* gene expression and aflatoxin $B_1$ production by *Aspergillus flavus* isolates

## 3.1 INTRODUCTION

It is known that warm and humid conditions are best for fungal colonisation of raw commodities, especially by *A.flavus,* which may lead to aflatoxins (AFs) contamination. However, as *A.flavus* is a xerophilic fungus, it is often able to grow under conditions where other non-xerophilic fungi are not as active or competitive. Drought stress can lead to cracking of the pods in groundnuts or shell split in pistachio nuts and allow ingress by *A.flavus* and *A.parasiticus* resulting in significant AFs accumulation. For *A.flavus,* the germination occurs over wider ranges of temperature and water activity ($a_w$) than those for growth, while the range for AFs production is often narrower than those for growth (Sanchis and Magan, 2004). While a number of studies have been carried out on *A.flavus* ecology in terms of growth and aflatoxin $B_1$ ($AFB_1$) production in maize and groundnuts, data on the effect of interacting environmental conditions on colonisation of pistachio nuts and optimum and marginal conditions for toxin production are scarce (Giorni et al., 2009; Abdel-Hadi et al, 2011).

Molecular ecology studies have also suggested that relative expression of the structural *aflD* gene in relation to $a_w$ × temperature conditions both *in vitro* and in stored peanuts was related to the imposed environmental stress. (Abdel-Hadi et

al., 2011). However, this previous study did not examine the effect on the regulatory gene *aflR* which is an important downstream gene, and together with the *aflS* gene, critical for the biosynthesis of AFs (Schmidt-Hyedt et al., 2011). No molecular ecology studies have been conducted to examine the effect of interacting environmental conditions of temperature × $a_w$ on colonisation and toxin production on raw pistachio nuts.

The objective of this Chapter was therefore to understand the ecology of isolated and identified strains of *A.flavus* in Chapter 2 in relation to the effect of interacting environmental factors on (a) growth, (b) *aflR* gene expression and (c) $AFB_1$ *in vitro* and *in situ* on raw pistachio nuts.

## 3.2 MATERIALS AND METHODS

### 3.2.1 *Aspergillus flavus* strains

Four strains of *A.flavus* isolated from pistachio nut samples and molecularly identified (in Chapter 2) were used in the present Chapter. They were coded as AB3, AB4, AB5 and AB10. In addition, one type strain (NRRL3357) was kindly provided by Prof. Deepak Bhatnagar from the Southern Regional Research Centre, New Orleans, LA, USA, and used for comparison.

### 3.2.2 Preparation of *in vitro* growth media

The growth medium used was a 3% milled pistachio nut agar (PMA). To prepare this medium, raw unsalted pistachio nuts were milled to a powder form in a

homogeniser. The milled pistachio powder was then sieved to obtain a uniform size. Thirty gram (30 g) of the fine pistachio powder and 20 g bacteriological agar were added to 1 L distilled water. The $a_w$ was modified using the ionic solute NaCl and the non-ionic solute glycerol to 0.90, 0.93, 0.95, 0.98 and 0.995 $a_w$ according to Dallyn and Fox (1980). The treatments were then autoclaved at 121°C for 15 min (Meadowrose, England). After autoclaving, the PMA was cooled, mixed thoroughly, and then poured onto 9 cm sterile Petri plates and allowed to completely cool and solidify. The media were refrigerated in separate closed polyethylene bags at 4°C until use.

### 3.2.3 Fungal inoculation and growth rate measurement of *Aspergillus flavus* isolates on pistachio agar

The PMA plates were equilibrated at 25°C and then centrally inoculated with the strains of *A.flavus* (4 isolates + type strain) inoculum consisted of a conidial spore suspension of each strain made from fresh 5-7 day old growing cultures on PMA at 25°C. The cultures surface was gently scraped with a sterile loop and conidia transferred into sterile 25 ml Universal vials containing 10 ml sterile water + 0.1% Tween 80 solution (Acros Organics, UK). The concentration of the spore suspension was determined using a haemocytometer (Marienfeld, Germany) and a light microscope (Olympus, Japan), and adjusted by dilution with sterile water to $10^6$ spores/mL. The inoculated treatments and replicates were incubated at 20, 25 and 30°C. Growth was assessed by measuring colony diameters of *A.flavus* every day for up to 10 d. Measurements of three replicates of each treatment was

recorded on an excel sheet. The radial growth rates (mm/d) was obtained by computing the radial data into the $y = mx + c$ equation, where $y$ was radius (mm), $x$ was day (d) and $m$ was the growth rate (mm/d). $c$ was set to intercept at 0 by assuming at day 0, the radius was 0.

### 3.2.4 Colonisation of pistachio nuts under different temperature × water activity conditions by *Aspergillus flavus* isolates

#### (a) Moisture adsorption curve for pistachio nuts

To accurately modify the $a_w$ of the raw pistachio nuts, a water adsorption curve was developed. The relationship between added water and aw was obtained by adding known amounts of water to 5 g subsamples of raw pistachio nuts in 25 mL Universal glass bottles. These were shaken, sealed and left at 4oC overnight to equilibrate. After equilibration at 25°C the $a_w$ was determined for each subsample using the Aqualab TE4 as detailed in Section 2.2.2. This water adsorption curve was subsequently used to accurately determine the amounts of water necessary to obtain the target level of $a_w$ in experimental studies. The moisture adsorption curve of pistachio nuts is illustrated in Figure 3.1.

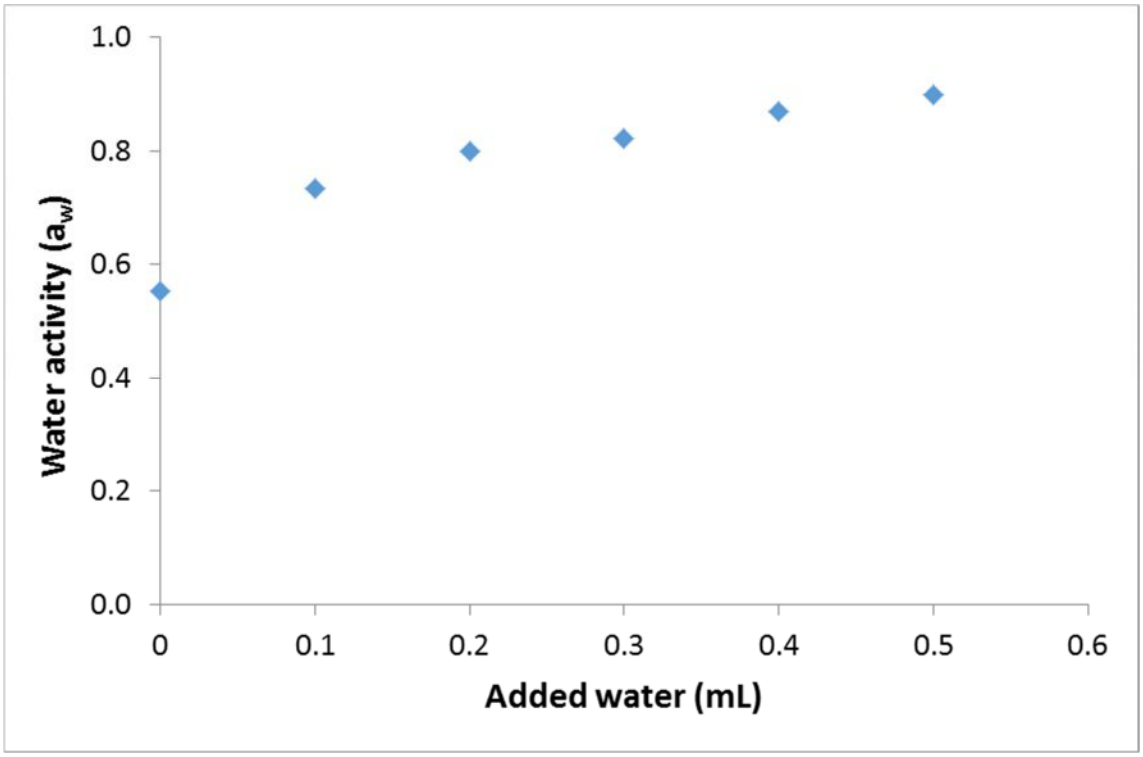


**Figure 3.1 Moisture adsorption curve depicting water activities of raw pistachio nuts following addition of different amounts of water (mL) at 25°C.**

### (b) Fungal inoculation and growth rate measurement of *Aspergillus flavus* isolates on pistachio nuts

Single layers of pistachio nuts were spread in 9 cm Petri plates. These were centrally inoculated with an individual strain of *A.flavus* (AB3, AB10) using a hyphal plug of germinating spores of each strain. The experiment was performed twice with three replicates per treatment at 25, 30 and 35°C. The Petri plates were placed in large surface-sterilised plastic chambers where the ERH was maintained with glycerol/water solutions (500-750 mL) to the target $a_w$ levels of the nut treatments. The colonisation rates were measured on a daily bases for up to 10 d.

### 3.2.5 Gene expression studies

Sampling was performed after five and ten days incubation in triplicate for gene expression studies. After each incubation time, the biomass was aseptically harvested, immediately frozen in liquid nitrogen, and stored at -80°C for later RNA extraction.

**(a) RNA isolation from pistachio nuts**

Using the bead-beating method by Leite et al. (2012), RNA was extracted with some modifications. One hundred fifty mg of frozen biomass was placed into an autoclaved 2 mL extraction tube containing 0.5 mm sized glass beads. 1 mL of RLT buffer (RNeasy® Plant Mini Kit; Qiagen, Germany) supplemented with 10 µL of β-mercaptoethanol was added. The tubes were immediately frozen in liquid nitrogen. After a quick vortex to help disrupt the mycelium, samples were then placed on ice. The mycelium disruption was performed in a bead beater Precellys 24 (Bertin Technologies, Montigny le Bretonneux, France) with 3D motion at 6,500 rpm. The tubes were agitated for 25 s followed by a 5 s interval and another 25 s of agitation. This mixture was centrifuged at 10,000 rpm for 5 min at 4°C in a temperature controlled centrifuge system. A pre-frozen 2 mL Safe-Lock tube (Eppendorf, Germany) was used to place the collected supernatant. According to instructions of the RNeasy® Plant Mini Kit (Qiagen, Germany), the RNA purification was carried out. RNA obtained was eluted in 50 µL of RNase free water and kept at -80°C until used for reverse transcription. The RNA concentration and purity (A260/A280 ratio) were determined

spectrophotometrically using a 2.5 µL aliquot on the PicodropTM (Spectra Services Inc., USA).

**(b) Primers and probes**

Nucleotide sequences of primers and probes used in the present study are listed in Table 3.1 (Medina et al., 2014). The design of the primer pairs of aflRtaq1/aflRTaq2 and the hydrolysis probe AflRprobe was based on the *aflR* gene involved in the aflatoxin biosynthetic pathway. The AflRprobe was labelled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher Black Hole Quencher 2 (BHQ2).

**Table 3.1 Nucleotide sequences of primers for RT-qPCR assay designed on the basis of *aflR* and *β-tubulin* genes.**

| Gene | Primers and probes | Primer sequence | Position |
|---|---|---|---|
| *aflR* | aflRTaq1 | TCGTCCTTATCGTTCTCAAGG | 1.646[a] |
| | aflRTaq2 | ACTGTTGCTACAGCTGCCACT | 1.735[a] |
| | aflRprobe | [FAM]AGCAGGCACCCAGTGTACCTCAAC[BHQ2] | 1.6889[a] |
| β-tubulin | Bentaq1 | CTTGTTGACCAGGTTGTCCAT | 65[b] |
| | Bentaq2 | GTCGCAGCCCTCAGCCT | 99[b] |
| | benprobe | [CY5]CGATGTTGTCCGTCGCGAGGCT[BHQ2] | 82[b] |

[a] Positions are in accordance with the published sequence of *aflR* gene of *Aspergillus flavus* (GeneBank accession no. AF441435.2).

[b] Positions are in accordance with the published sequence of β-tubulin gene of *Aspergillus flavus* (GeneBank accession no. AF036803.1).

**(c) Reverse transcription to convert cDNA from mRNA**

To amplify the regulatory gene *aflR* of the aflatoxin biosynthetic pathway as the target genes, a RT-qPCR assay (Bio-Rad CFX96 Real Time PCR Detection

System; Bio-Rad, UK) was performed as described by Medina et al. (2014). The β-tubulin gene was used as a control gene.

**(d) Amplification of *aflR* gene through real-time quantitative PCR (RT-qPCR)**

The RT-qPCR reaction mixture (12.5 µL) was prepared in triplicates in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Bio-Rad, UK). Three replicates of a RNA control sample together with a template-free negative control were also included in the runs. The TaqMan system with primers and probes were used in all cases. The reaction mixtures consisted of 6.25 µL Premix Ex TaqTM (Takara Bio Inc., Otsu, Shiga, Japan), 830 nM of each primer, 330 nM of each probe, and 1.5 µL of cDNA template in a final volume of 12.5 µL. The optimal thermal cycling conditions included an initial step of 10 min at 95°C and all 45 cycles at 95°C for 15 s, 55°C for 20 s and 72°C for 30 s. $C_t$ determinations were automatically performed by the instrument using default parameters.

Data analysis was performed using the software CFX Manager™ Software (Bio-Rad, UK). Relative quantification of the expression of *aflR* gene was carried out using the housekeeping gene β-tubulin as an endogenous control to normalise the quantification of the mRNA target for differences in the amount of total cDNA added to the reaction in the relative quantification assays and used for all treatments. The expression ratio was calculated as previously described by Livak and Schmittgen (2001). Before using the above method, it was tested to show that experimental treatments did not influence expression of the internal control

gene, and the amplification efficiencies of the target and reference genes were practically equal (93.1% for *aflR* and 95.2 % for β-tubulin genes). This method allows calculation of the expression ratio of a target gene between a tested sample and its relative calibrator ("control" sample). In this work, the calibrator corresponded to *A.flavus* grown at 30°C and 0.90 $a_w$.

The $C_t$ raw data were obtained from the BIO-RAD detection system and the calculations were performed by Microsoft Excel®. The statistical design was factorial CRD, 2 factors and the statistical analysis obtained using SPSS® software.

### 3.2.6 Aflatoxin $B_1$ quantification

**(a) Preparation of aflatoxin standards**

A 200 µL stock solution of AFs mix standard in methanol (Romer, UK) containing 250 ng $AFB_1$ was prepared and pipetted into 2 mL Eppendorf tubes for overnight evaporation until drying in a fume cupboard as for samples.

**(b) Extraction of aflatoxin $B_1$**

Agar plugs were taken across the colonies using a 3 mm diameter cork-borer. The agar plugs were placed in pre-weighed 2 mL Eppendorf tube and weighed again. Five-hundred µL of HPLC-grade chloroform was added to the tubes and shaken for 30 min using KS 501 digital orbital shaker (IKA®, Germany). The chloroform extract was transferred to new Eppendorf and gently air-dried for derivatisation.

**(c) Derivatisation of aflatoxin $B_1$ extract**

Derivatisation of $AFB_1$ extract was performed according to Association of Official Analytical Chemists (AOAC). First, 200 µL hexane was added to the tube followed by 50 µL of triflouroacetic acid. The mixture was vortexed for 30 s and left for 5 min. A mixture of water:acetonitrile (9:1) was then added to the tube, and vortexed for 30 s and left for 10 min to allow for separation of the layers. Next, the aqueous layer was filtered using syringe nylon filters (13 mm × 0.22 µm; Jaytee Biosciences Ltd., UK) into amber salinized 2 mL HPLC vials (Agilent, USA) before HPLC analysis. All analytical reagents were of HPLC-grade.

**(d) Quantification of aflatoxin $B_1$ with High Performance Liquid Chromatography HPLC**

A reversed-phase high performance liquid chromatography with fluorescence detector (HPLC-FLD) was used to quantify the $AFB_1$. An Agilent 1200 series HPLC system was used. It consisted of an in-line degasser, auto sampler, binary pump and a fluorescence detector (excitation and emission wavelengths of 360 and 440 nm, respectively). Separation was achieved through the use of a $C_{18}$ column (Phenomenex Gemini; 150 × 4.6, 3 µm particle size; Phenomenex, USA) preceded by a Phenomenex Gemini $C_{18}$ 3 mm, 3 µm guard cartridge. Isocratic elution with methanol:water:acetonitrile (30:60:10, v/v/v) as mobile phase was performed at a flow rate of 1.0 mL/min. The injection volume was 20 µL. A set of standards was injected (1 to 5 ng $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ per injection) and standard curves generated by plotting the area underneath the peaks against the amounts of $AFB_1$ standard injected.

### 3.2.7 Quantification of aflatoxin $B_1$ in pistachio nuts

The same procedure as in Section 3.2.6 was applied for $AFB_1$ quantitative analyses with a modification as colonised pistachio nuts were initially cleaned up using Immunoaffinity Columns (IAC) and to subsequently elute the bound $AFB_1$. The IAC are based on a specific antibody-analyte binding technology (AflaStar™; Romer Labs, Austria). They contained a gel bed with toxin-specific antibodies coupled to the gel particles. These antibodies capture the analyte in a sample and release them again after the washing step.

The dried pistachio nut samples (25 g) were ground and weighed. Acetonitrile/water 60/40 (100 mL) was used as an extraction solvent. The mixture was blended for 3 min and the extract was filtered into smaller sample container. PBS buffer was used for sample dilution, then the diluted extract was allowed to pass through the IAC column with a flow rate between 1-3 mL/min. The column was rinsed with 2×10 mL sterilised distilled water. HPLC-grade methanol (1.5 - 3 mL) was applied to the column and the eluent was collected in a new vial and left to dry overnight before the derivatisation step as detailed in Section 3.2.6.

### 3.2.8 Statistical analysis

Three replicates per treatment were used in all experiments. Some experiments were repeated twice. Means were obtained by taking the average of each three measurements with the standard error of the means (± SE). Analysis of Variance (ANOVA) was applied to analyse the variation of means with 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov using Minitab statistical software. Fisher's Least Significant Difference (LSD) was checked to identify differences between the means with $p < 0.05$ as significant difference using the same statistical software.

## 3.3 RESULTS

### 3.3.1 Effect of temperature × water activity on growth rates of *Aspergillus flavus* strains on a pistachio nut-based medium

The effect of temperature and $a_w$ was examined for all four *A.flavus* strains as well as the type strain (Figures 3.2 to 3.6). The highest growth rate for all strains was at 0.98 $a_w$ on this medium. Relative growth rates were slightly faster at 30°C than 20-25°C. However, for strains AB3 and AB4 the growth rate was higher at 25°C than 30°C at 0.98 $a_w$ (see Figures 3.3 and 3.4). Growth at 0.85 $a_w$ was tested when the strain showed the ability to grow at 0.90 $a_w$. Strains AB3 and AB10 had a similar growth patterns to the type strain; and they were thus selected for more detailed *in vivo* studies (Figure 3.7). This shows a growth comparison of the growth of the 5 strains on PMA media modified with glycerol at 0.98, 0.95 and 0.93 $a_w$. Strains show different growth behaviours. AB3 strain has similar growth behaviour when compared to the type strain, while the other strains had a higher growth rates at 0.95 $a_w$ + 30°C.

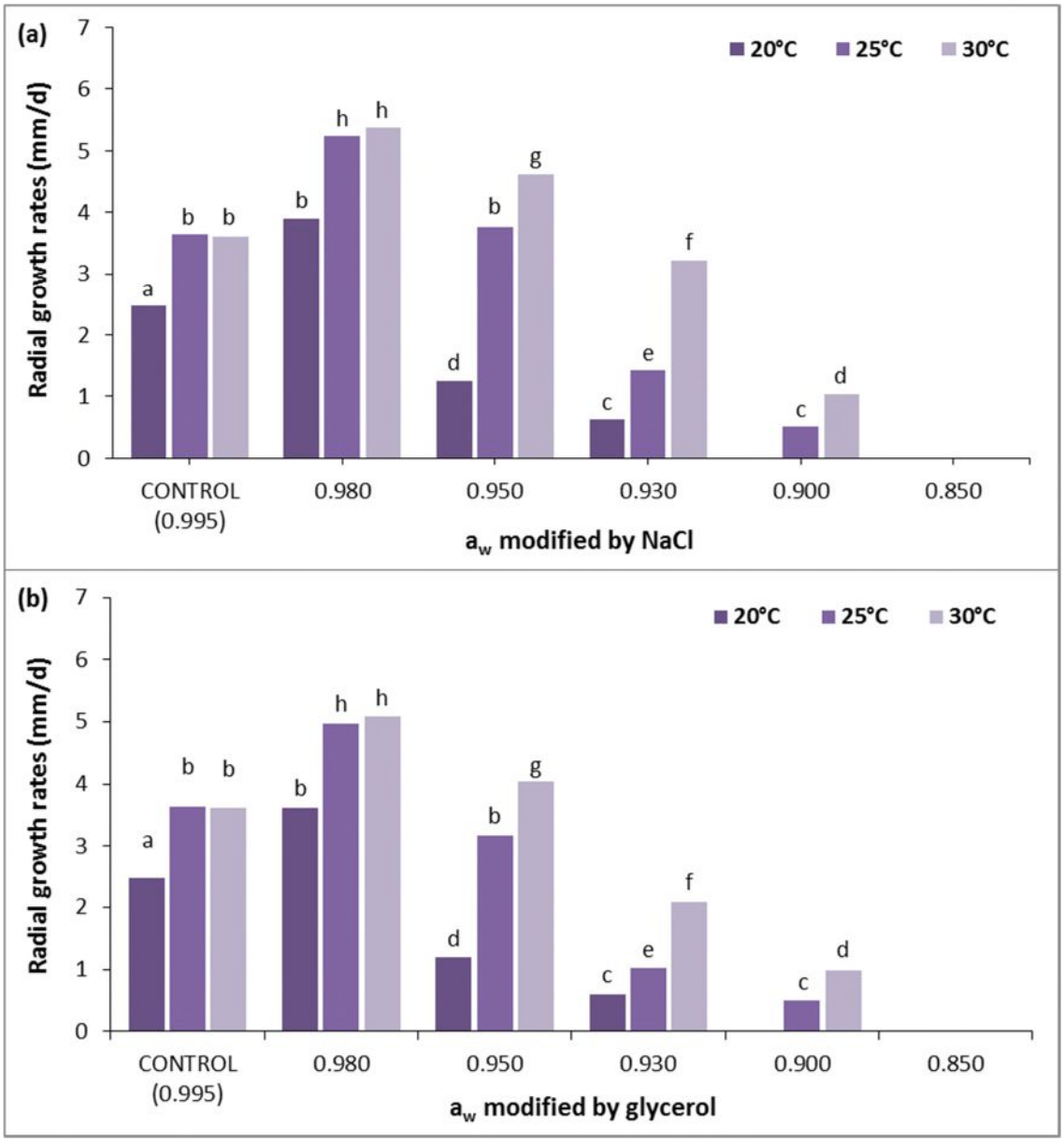


**Figure 3.2 Growth rates (mm/day) of *Aspergillus flavus* type strain (NRRL3357) grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

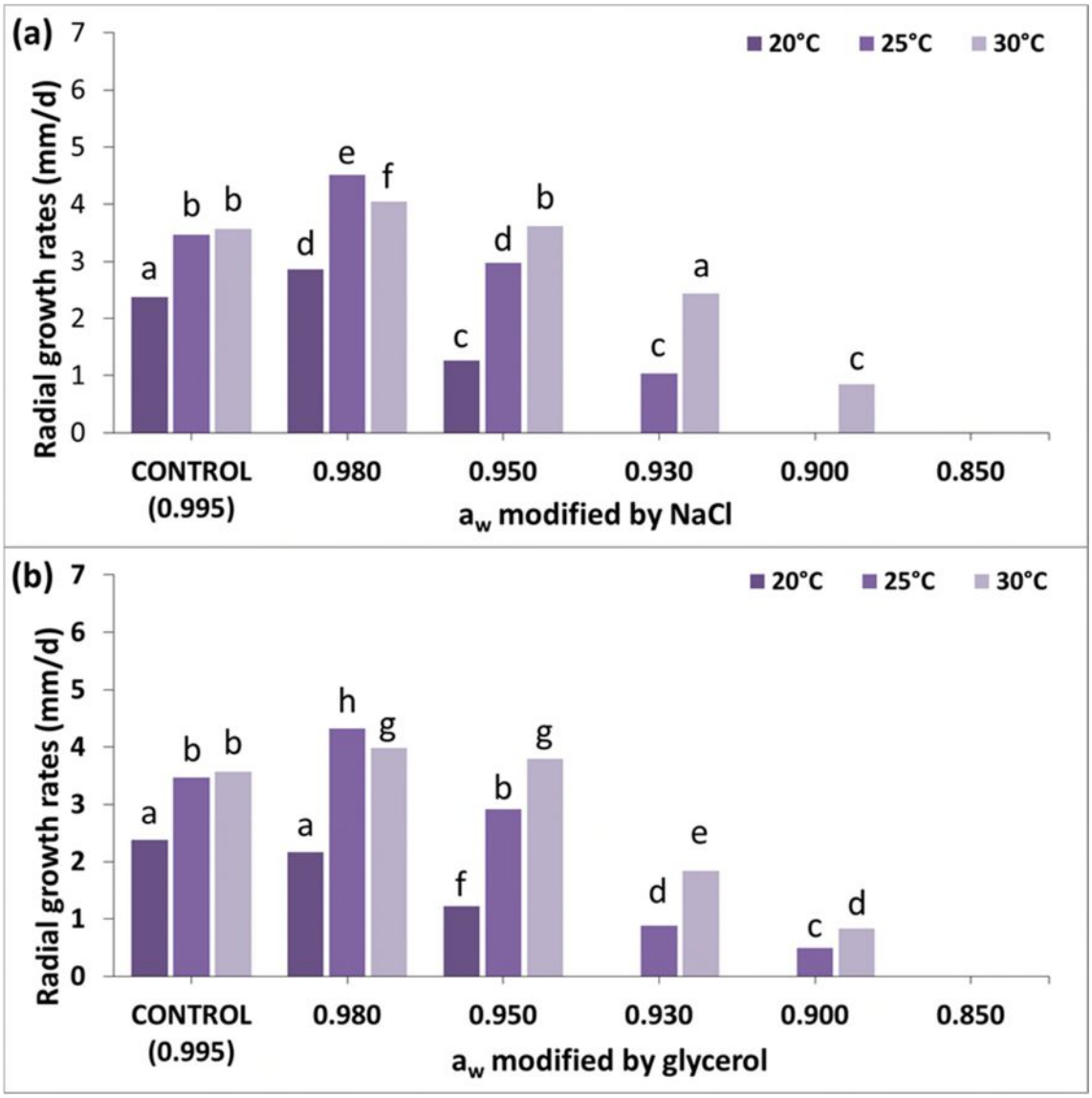


**Figure 3.3 Growth rates (mm/day) of *Aspergillus flavus* AB3 grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

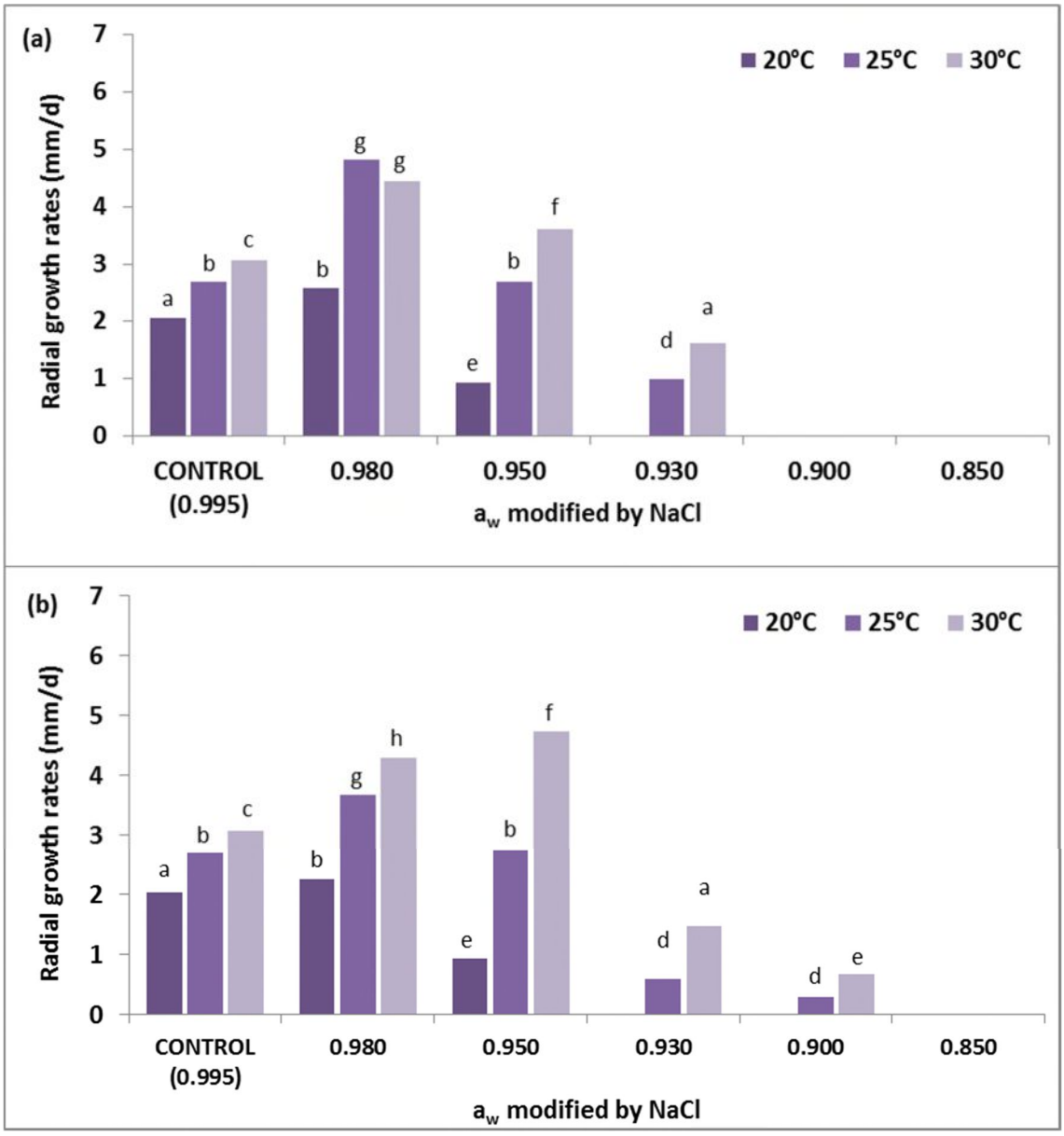


**Figure 3.4 Growth rates (mm/day) of *Aspergillus flavus* AB4 grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

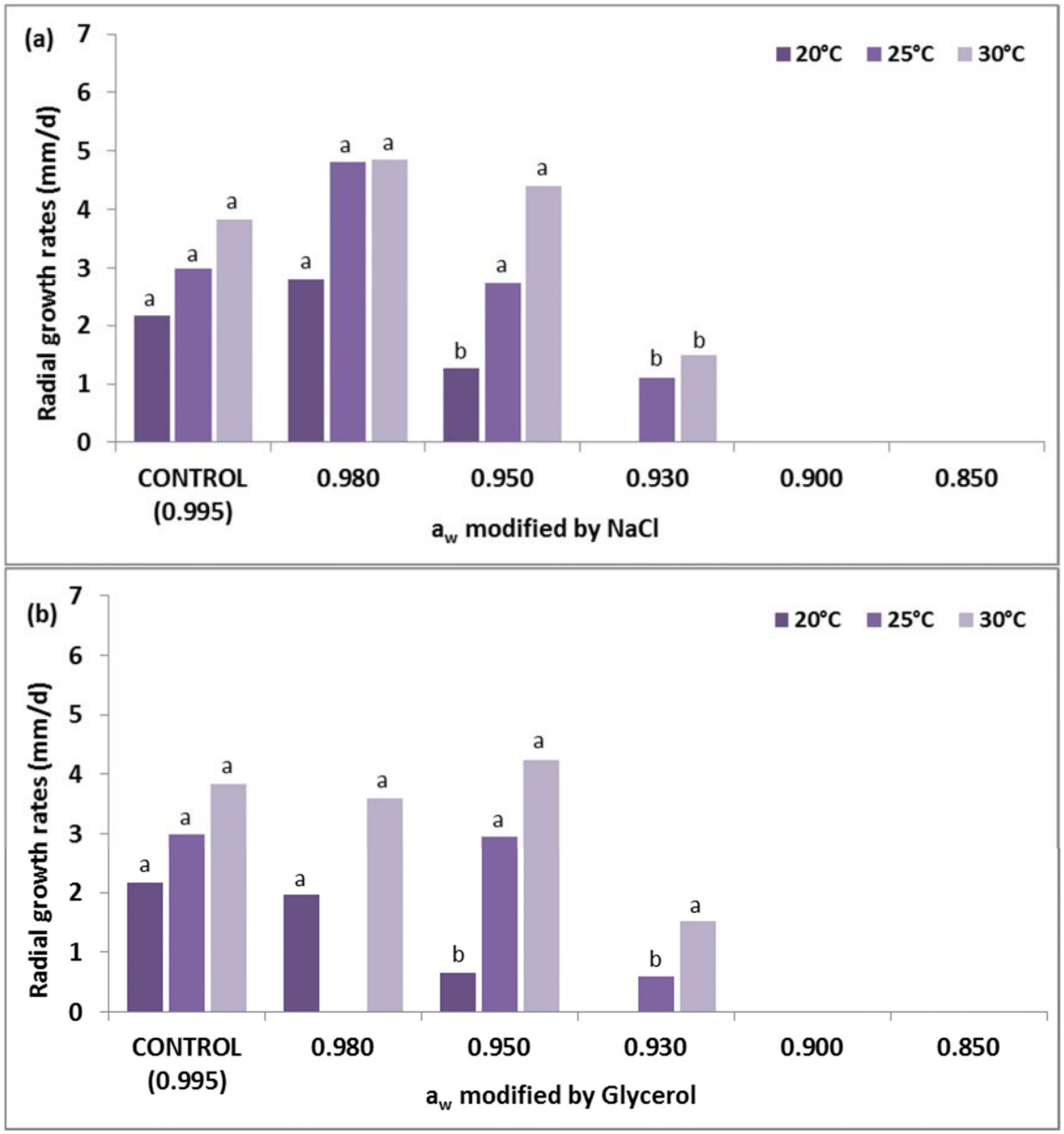


**Figure 3.5 Growth rates (mm/day) of *Aspergillus flavus* AB5 grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

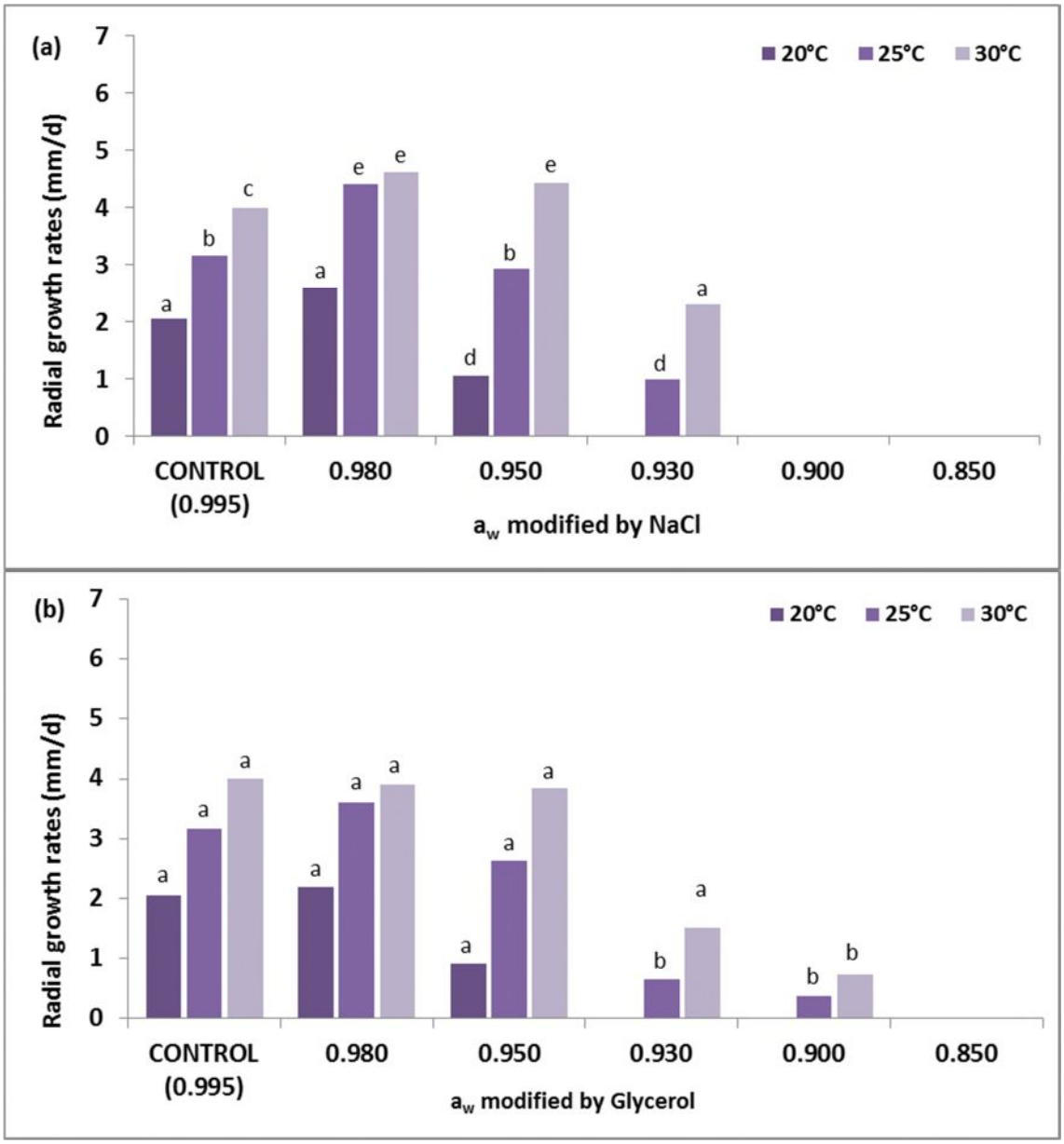


**Figure 3.6 Growth rates (mm/day) of *Aspergillus flavus* AB10 grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

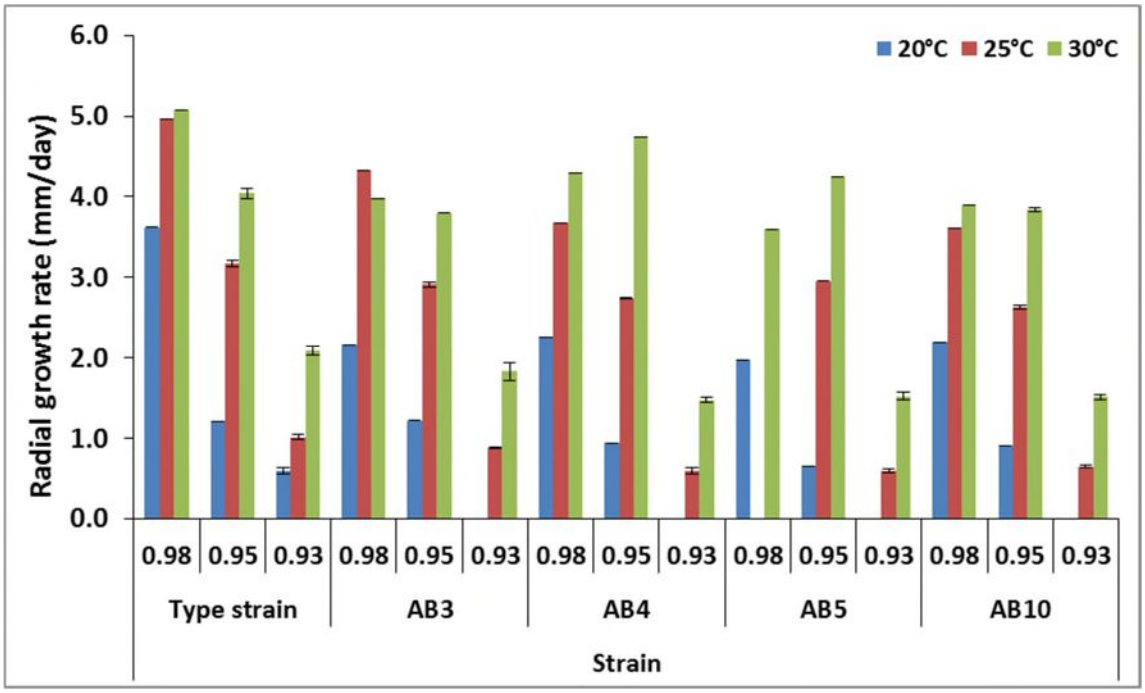


**Figure 3.7 Growth rates (mm/day) of *Aspergillus flavus* strains grown on pistachio nut-based medium with $a_w$ modified with glycerol to 0.98, 0.95 0.93, and incubated at 20, 25, 30°C. Data are means of triplicates. Bars indicate standard error of the mean.**

### 3.3.2 Effect of temperature × water activity on aflatoxin $B_1$ (ng/g) production of *Aspergillus flavus* strains on pistachio nut-based medium

Figures 3.8 to 3.10 show the $AFB_1$ production of *A.flavus* type strain, strain AB3 and AB10 respectively. Based on this data, the amount of $AFB_1$ production were always higher at 30°C for most strains except the type strain (Figure 3.8) where toxin production was higher at 25 than 30°C when the $a_w$ was modified to 0.95 with glycerol. In all other strains, production of $AFB_1$ occurred, but predominantly at 30˚C in the control treatment (0.995 $a_w$) as shown in Figure 3.10 for strain AB10. Strains AB4 and AB5 showed very similar result to AB10 (hence results

are not shown here). With the exception of the type strain, no $AFB_1$ was detected at 20°C except for AB3 at 0.95 and 0.98 $a_w$. This strain was the most similar in behaviour to the type strain (see Figure 3.9). Consequently, AB3 strain was chosen to be tested for all further studies and AB10 was also chosen as a representative of other strains isolated from pistachio nuts.

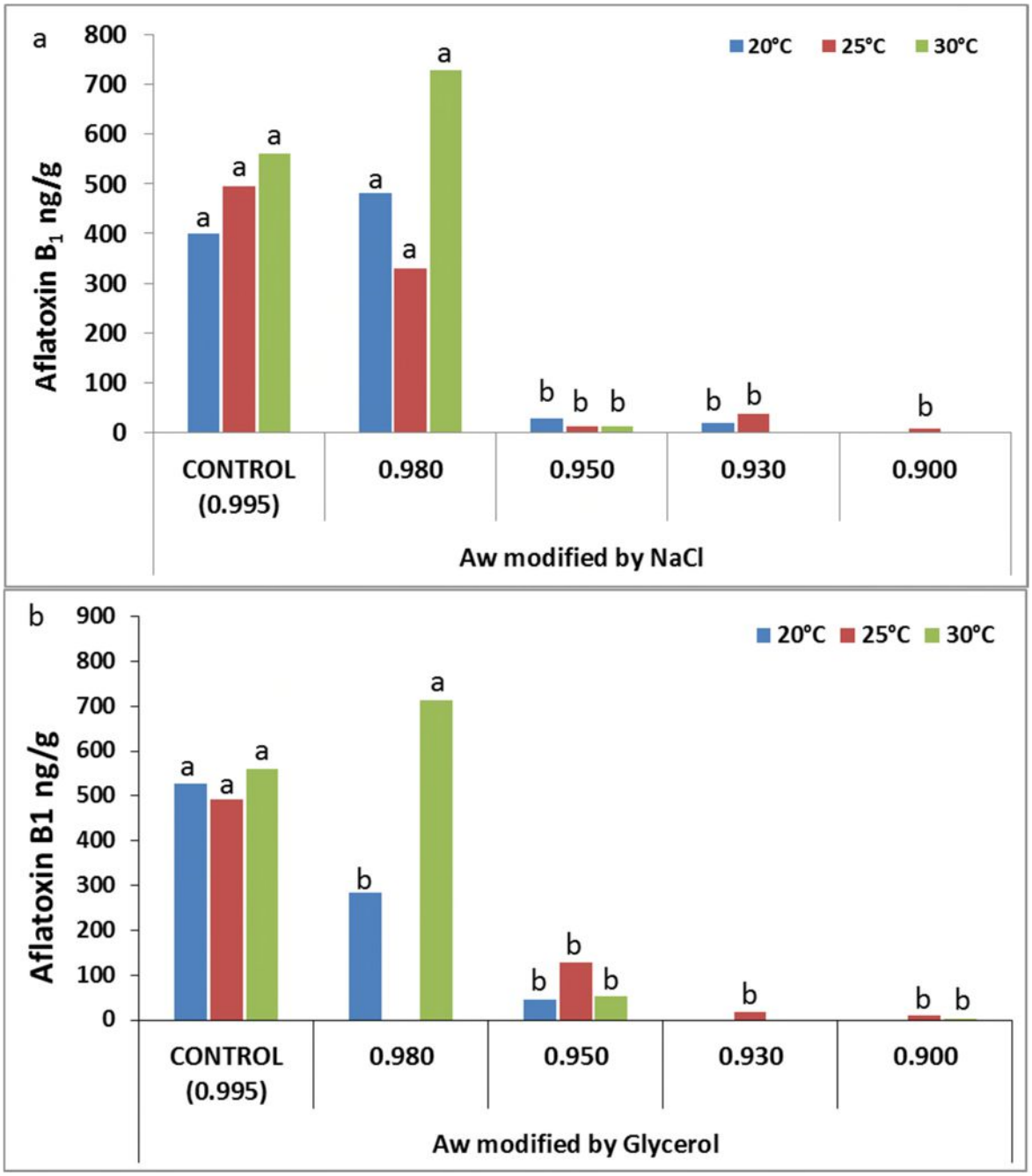


**Figure 3.8** The aflatoxin $B_1$ production (ng/g) by the type strain of *Aspergillus flavus* (NRRL3357) grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).

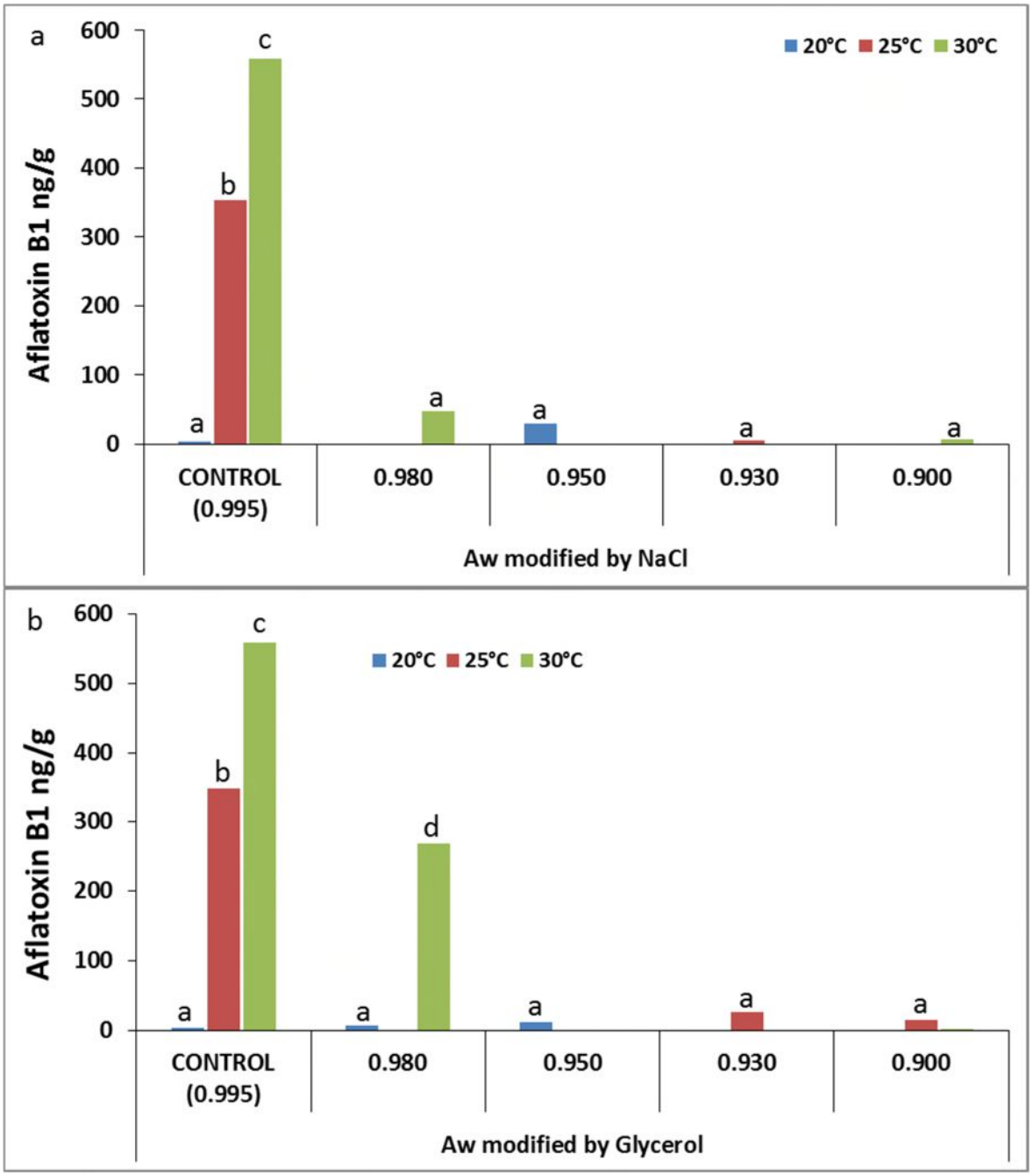


**Figure 3.9 The aflatoxin $B_1$ production (ng/g) by *Aspergillus flavus* AB3 strain grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

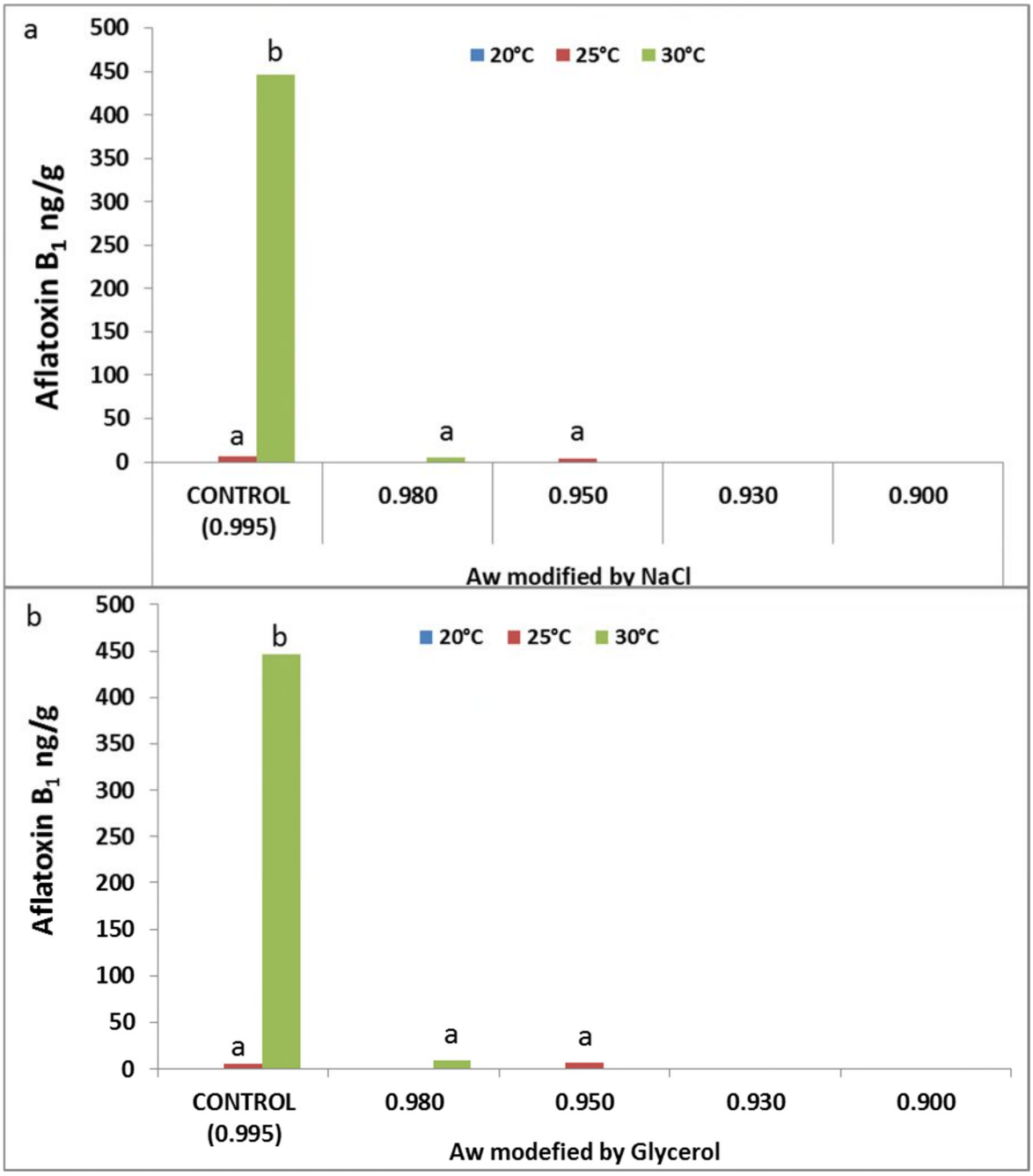


**Figure 3.10 The aflatoxin $B_1$ production (ng/g) by *Aspergillus flavus* AB10 strain grown on pistachio nut-based medium with $a_w$ modified with (a) NaCl and (b) glycerol, and incubated at 20, 25, 30°C. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

### 3.3.3 Effect of temperature × water activity on growth rates (mm/d) of *Aspergillus flavus* strains on stored pistachio nuts

Figures 3.11 and 3.12 show the colonisation rates of layers of raw pistachio nuts for two selected strains, AB3 and AB10. Both strains showed similar behaviour as the growth rate increasing when temperature or $a_w$ were increased. There was no growth observed at 0.90 $a_w$ when incubated at 25°C. Strain AB10 showed slightly faster colonisation rates than strain AB3. There was no significant difference between the growth rate of the two strains at 25 and 30°C and 0.95 and 0.98 $a_w$. However, at 35°C growth was almost double the rate at 30°C at all $a_w$ levels tested for both strains.

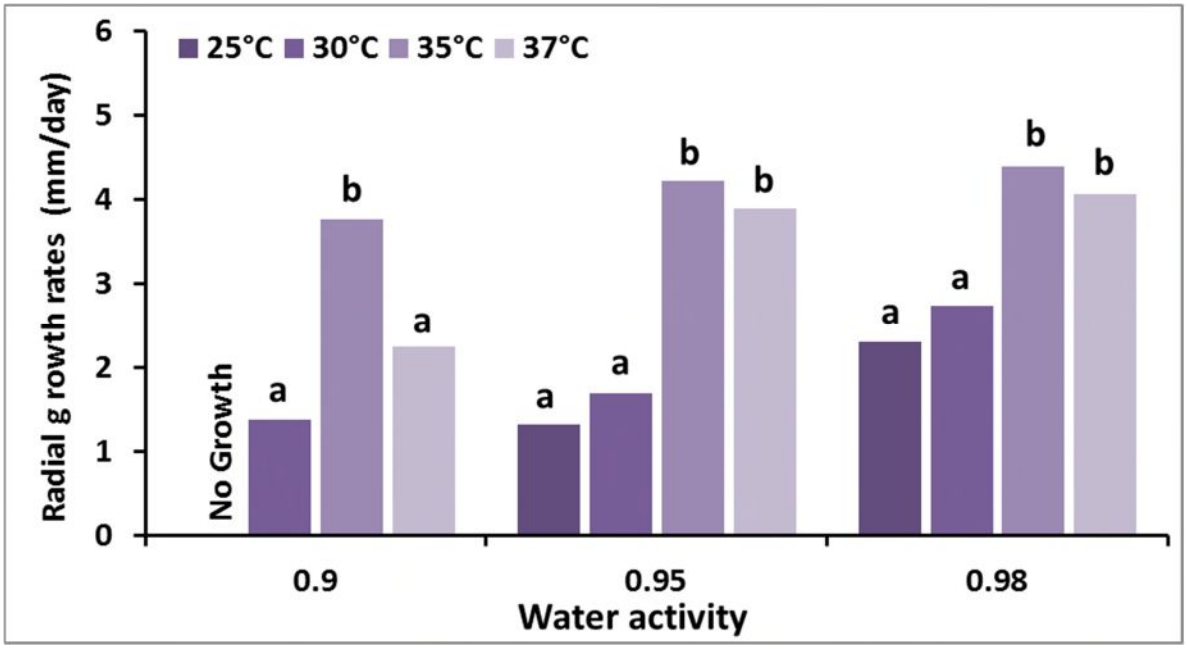


**Figure 3.11 Growth rates (mm/day) of *Aspergillus flavus* AB3 grown on raw pistachio nuts at different $a_w$ and incubation temperatures. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

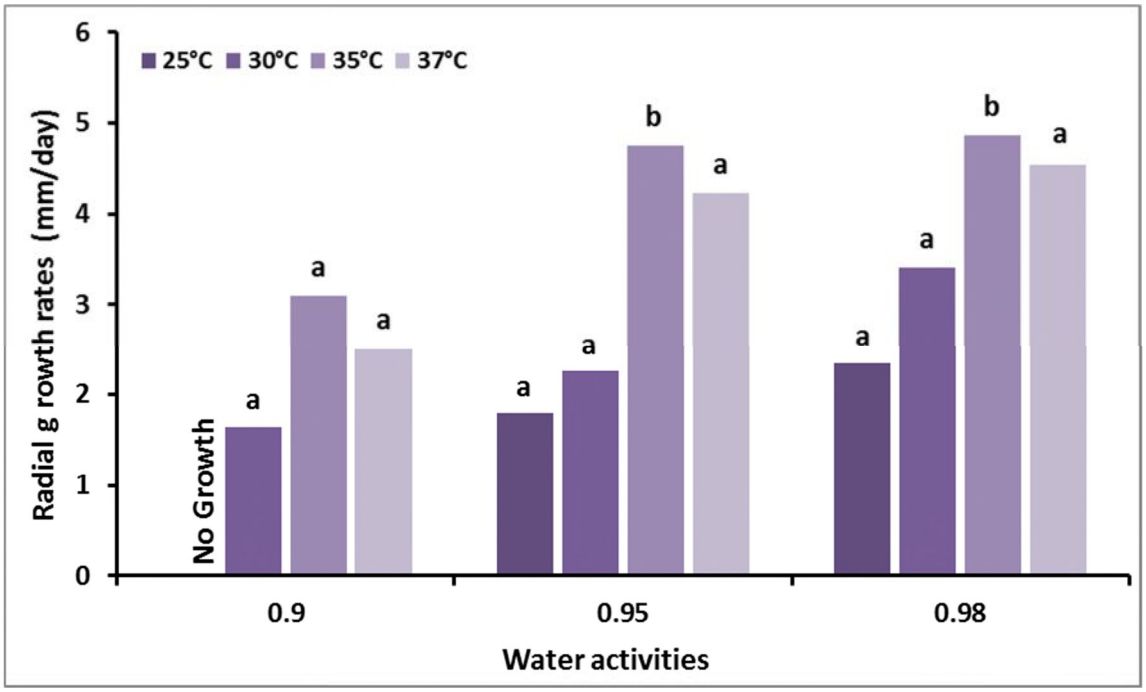


**Figure 3.12 Growth rates (mm/day) of *Aspergillus flavus* AB10 grown on raw pistachio nuts at different $a_w$ and incubation temperatures. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

### 3.3.4 Effect of temperature × water activity on relative expression of the *aflR* regulatory gene in the biosynthetic pathway for aflatoxin $B_1$ production

Figures 3.13 and 3.14 show the effect of temperature and $a_w$ on the expression of the *aflR* regulatory gene of strains AB3 and AB10. For AB3, the expression was higher at 35°C and 0.98 $a_w$. For AB10 however, the expression reached its highest point at the same $a_w$ at 30°C.

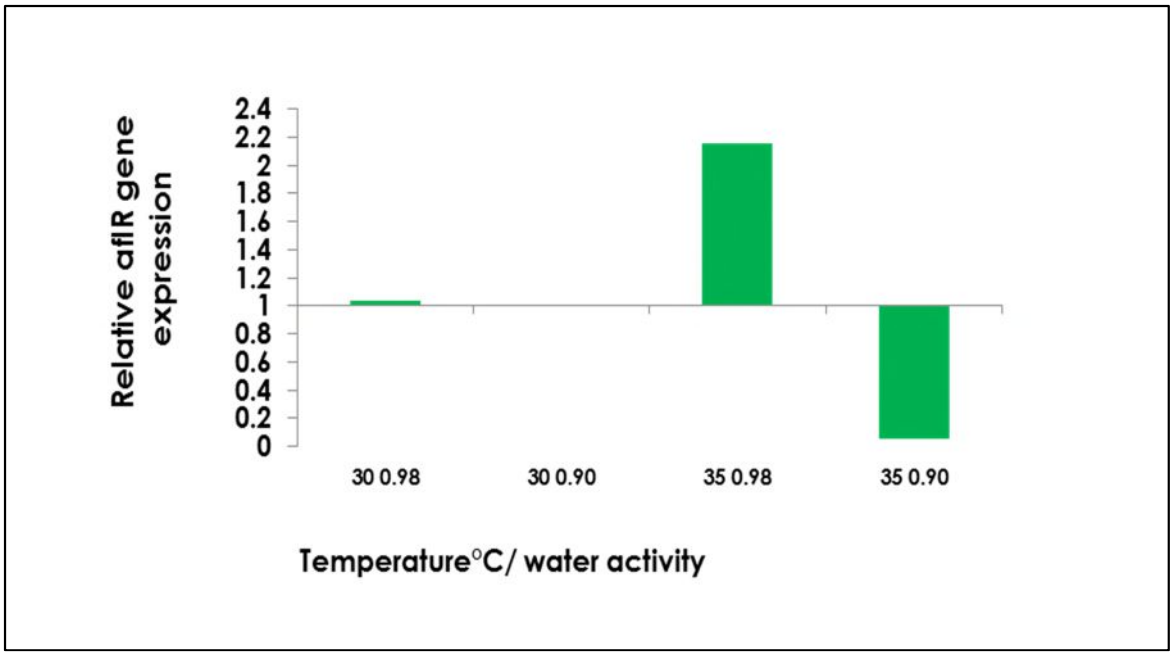


**Figure 3.13 Effect of temperatures (30 and 35°C) and water activities (0.90 and 0.98 $a_w$) on the relative expression of the *aflR* regulatory gene of *Aspergillus flavus* AB3 following five days incubation on a single layer of pistachio nuts.**

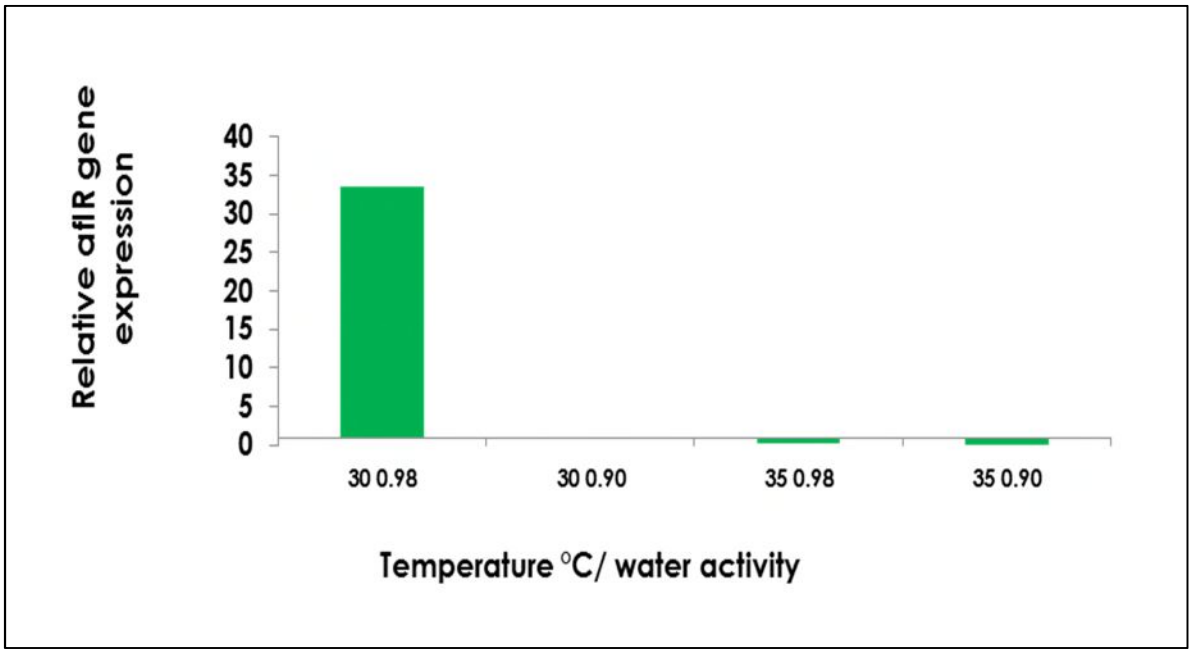


**Figure 3.14 Effect of temperatures (30 and 35°C) and water activities (0.90 and 0.98 $a_w$) on the relative expression of the *aflR* regulatory gene of *Aspergillus flavus* AB10 following five days incubation on a single layer of pistachio nuts.**

### 3.3.5 Effect of temperature × water activity on aflatoxin $B_1$ (ng/g) production of *Aspergillus flavus* strains on pistachio nuts

Figures 3.15 and 3.16 show the effect of $a_w$ × temperature on $AFB_1$ production by strains AB3 and AB10 on pistachio nuts, respectively. The amount of $AFB_1$ was significantly higher at 35°C and 0.98 $a_w$ for strain AB3 strain (Figure 3.15). For AB10 strain, the production of $AFB_1$ was significant at 0.98 $a_w$ at 30 and 35°C. Higher $AFB_1$ was produced at 0.95 $a_w$ at 25°C (Figure 3.16). No $AFB_1$ was detected at 35°C except at 0.98 $a_w$ for both strains.

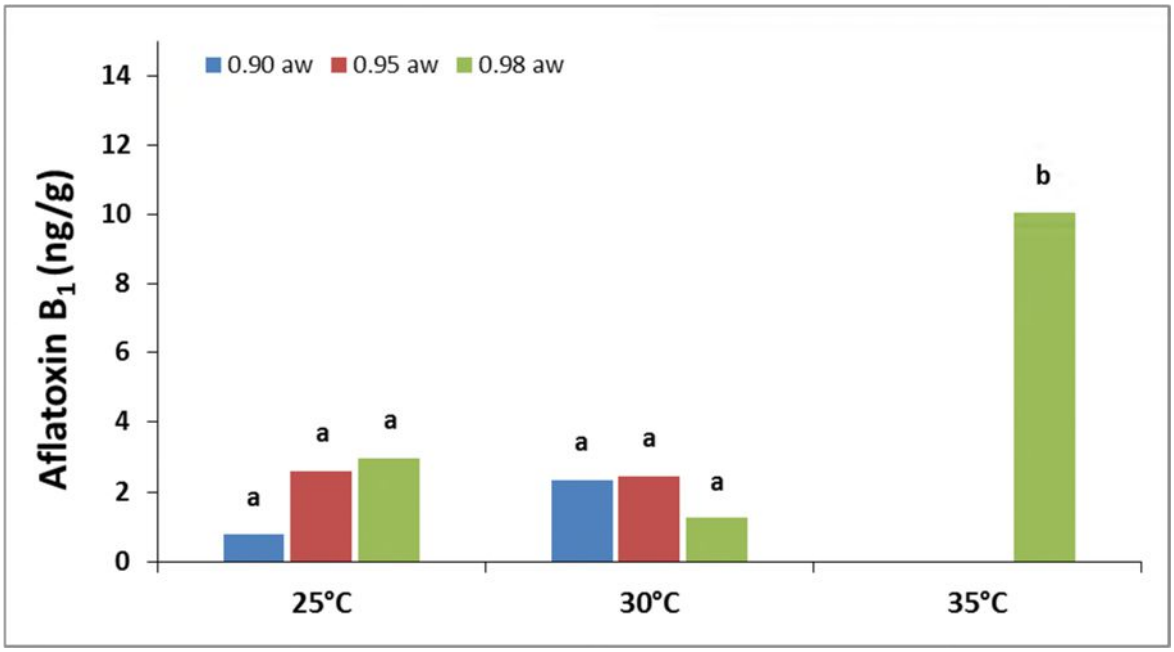


**Figure 3.15 The aflatoxin $B_1$ production (ng/g) by *Aspergillus flavus* AB3 strain grown on pistachio nuts at different water activities and temperatures. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

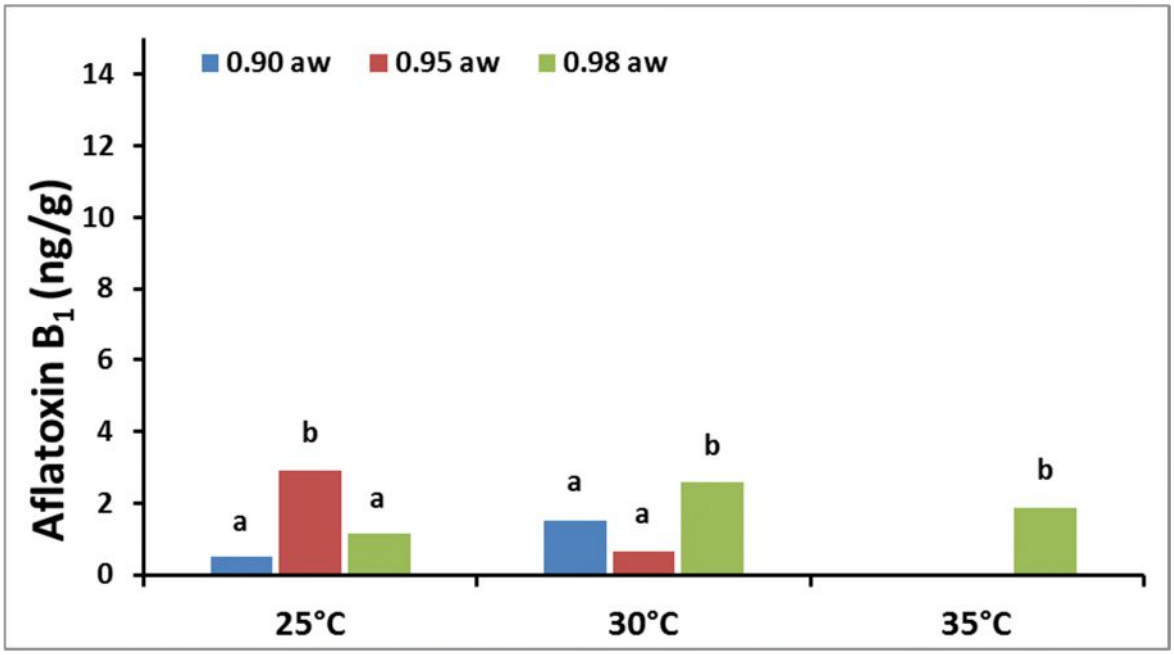


**Figure 3.16 The aflatoxin $B_1$ production (ng/g) by *Aspergillus flavus* AB10 strain grown on pistachio nuts at different water activities and temperatures. Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$).**

## 3.4 DISCUSSION

### 3.4.1 Ecology of *Aspergillus flavus in vitro* and *in situ*

*A.flavus* was able to grow effectively when incubated at 25, 30, 35°C over a range of $a_w$ levels, both *in vitro* and *in situ*. However, the range of conditions for $AFB_1$ production was narrower with best production at 30°C in modified media, at 0.98 and 0.955 $a_w$; and at 35°C + 0.98$a_w$ by strain AB3 on pistachio nuts.

Colonisation rate for *in vitro* was generally greater than rates observed *in situ*. Moreover, $AFB_1$ production was considerably higher *in vitro* when compared with that produced *in situ*. This could be because of the better access to the nutrients in the media allowing the fungi to grow faster and produce more toxins. The

temperature of 35°C was included for the *in situ* to examine growth rate and $AFB_1$ at higher temperature as the *A.flavus* strains showed the ability to grow and produce $AFB_1$ at 30°C, the highest tested temperature for the *in vitro* study. This suggests that strains of *A.flavus* from pistachios are able to grow at higher temperatures than found in other commodities such as maize and peanuts (Giorni et al., 2009).

Previously, for isolates of *A.flavus* from maize, Giorni et al. (2009) and Abdel-Hadi et al. (2012) found optimum growth of *A.flavus* was between 28-30°C *in vitro*, whereas, the optimum conditions for AFs production were slightly different. Thus, temperature must be monitored in agricultural products at all times, in order to control mycotoxin contamination. About 70% of *A.flavus* isolates from a total of 204 fungal isolates were able to produce aflatoxin $AFB_1$ and $AFB_2$ on pistachio nuts (Fermane et al., 2010a). Aldars-García et al. (2015) used a predictive mycology strategy to predict potential AFB1 contamination of pistachio nuts based on environmental conditions. They found that using this approach it was possible to predict 70-81 % of samples correctly with regard to growth and in 67-81% of the cases contamination with $AFB_1$ based on the models developed from their *in vitro* studies.

The results obtained in the present study show that the interaction between temperature × $a_w$ had a significant impact on *A.flavus* growth and $AFB_1$ production. Overall, some strains isolated from pistachio nuts were able to grow

at much higher temperatures than those from maize. This could have implications for the impacts of climate change on growth and perhaps $AFB_1$ production.

The relative increase in expressions of the regulatory *aflR* gene, affected by temperatures and $a_w$, suggests that there is an impact on the biosynthetic pathway involved in secondary metabolite production by *A.flavus.* It also suggests that the stimulation of the gene by these two interacting environmental factors is similar to what was found in isolates from maize. Abdel-Hadi et al. (2010) also showed that there was a correlation between $AFB_1$ production and the expression of the structured *aflD* gene. It has also been previously shown that temperature × $a_w$ interactions were related to the ratio of the two regulatory genes (*aflR* and *aflS*) (Abdel-Hadi et al., 2012). Medina at el. (2014) found a relationship between the different environment conditions and the relative expression of genes (regulatory and structural) which correlated directly with the production of $AFB_1$. The same review illustrated that the effect of interacting conditions of temperature × $a_w$ and $CO_2$ may not affect *A.flavus* growth significantly but, on the other hand, $AFB_1$ can be significantly stimulated. This was confirmed by the stimulation of expression of both the *aflD* and *aflR* genes. This leads to interest in examining the impact of temperature × $a_w$ × $CO_2$ on isolates from pistachio nuts and whether $AFB_1$ stimulation may occur on stored pistachio nuts under climate change conditions.

# CHAPTER 4
# *In vitro* and *in situ* effects of climate change interacting factors on growth, *aflD* and *aflR* gene expression and aflatoxin $B_1$ production by two *Aspergillus flavus* strains

## 4.1 INTRODUCTION

The prevailing climate is expected to change significantly in the next 25-50 years in many regions of the world where the temperature and atmospheric $CO_2$ is increasing due to human industrial activity. It has been estimated that the $CO_2$ concentration which is approx. 403 ppm (NASA, 2017) will increase to 700-800 ppm (2×) or to 1000-1200 ppm (3×) in the future. At the same time this will lead to an increase in temperature by 2-4°C. Furthermore, the rainfall patterns are also expected to change with more extreme wet and dry periods occurring (Wu et al., 2011). It has been suggested that interactions between these three factors may have a significant impact on fungal diseases of staple food crops and perhaps also on contamination of food and feed with mycotoxins (Magan et al. 2011; Medina et al. 2014; Medina et al. 2015a,b; Medina et al., 2017).

The effect of temperature × $a_w$ on growth and aflatoxin $B_1$ ($AFB_1$) production by *A.flavus* has been determined in commodities such as maize and groundnuts (Sanchis & Magan, 2004; Magan & Aldred, 2007). However, these studies did not include the effect of increasing temperature or drought stress in the presence of elevated $CO_2$ conditions (Magan et al., 2011). A recent study by Medina et al. (2015a) examined the effect of the three-way interaction between the CC factors

on *A.flavus* growth and $AFB_1$ production. They found that the interaction of these three factors had no significant effect on growth. However, they resulted in a significant stimulation of the expression of biosynthetic genes involved in aflatoxin production *(aflD, aflR*) and on $AFB_1$ production. This was shown to occur when comparisons were made between 34 and 37°C in combination with elevated $CO_2$ and drought stress. This increase was especially profound at 37°C and 0.92 $a_w$. There is thus interest in determining whether similar effects may occur in *A.flavus* infection of pistachio nuts and whether this would represent an additional risk in terms of increased contamination with $AFB_1$.

The objectives of the present Chapter were therefore to examine the effect of three way interactions of the CC factors of temperature (35 vs 37°C) x $a_w$ (0.98 vs 0.95 and 0.93 aw) x $CO_2$ (400 vs 1000 ppm) on (a) growth, (b) $AFB_1$ production and (c) expression of structural and regulatory genes (*aflD*, *aflR* respectively) *in vitro* on a pistachio nut-based medium and and (d) *in situ* on stored pistachio nuts, for two strains of *A.flavus* isolated from pistachio nuts.

## 4.2 MATERIALS AND METHODS

### 4.2.1 *Aspergillus flavus* strains

Two strains of *A.flavus* isolated from pistachio nuts were used in this study. They had a similar ecophysiological behaviour as the type strain (NRRL3357) used in previous experiments. These strains were AB3 and AB10.

### 4.2.2 Preparation of *in vitro* growth media and inoculation

The medium used was a 3% milled pistachio nut agar (PMA). For PMA preparation, unsalted pistachio nuts were ground to a powder in a homogeniser. Thirty grams (30 g) of the fine pistachio powder, and 20 g bacteriological agar were added to 1 L distilled water. The $a_w$ was modified using the non-ionic solute glycerol to 0.93, 0.95 and 0.98. The amounts of glycerol required were based on those provided by Dallyn and Fox, (1980). The treatments were then autoclaved at 121°C for 15 min (Meadowrose, England). After the autoclaving the PMA, the molten cooled media were mixed thoroughly and poured onto 9 cm sterile Petri plates and allowed to completely cool and solidify. Plates were then centrally inoculated with the *A.flavus* strains (AB3 and AB10). Three replicates of each strain were placed in the CC environmental chambers and the ERH was maintained with glycerol/water solutions (2 x 500 mL) in beakers to maintain the target $a_w$ levels.

### 4.2.3 Climate change experimental system

The environmental chambers used included an inlet and outlet valve on either side and could be sealed during exposure and storage (Medina et al., 2015a). The *in vitro* and *in situ* treatments and replicates were placed into individual separate chambers. They were flushed with either air (400 ppm) or 1,000 ppm $CO_2$ (speciality gases; British Oxygen Company, Guildford, Surrey, UK). The chambers were flashed with the required $CO_2$ concentrations at a rate of 2 L/min to replace 3× chambers volume. This process was repeated every two days after

growth measurements were recorded and the chambers sealed again. The treatments were incubated at 35 and 37°C (Medina et al., 2015a).

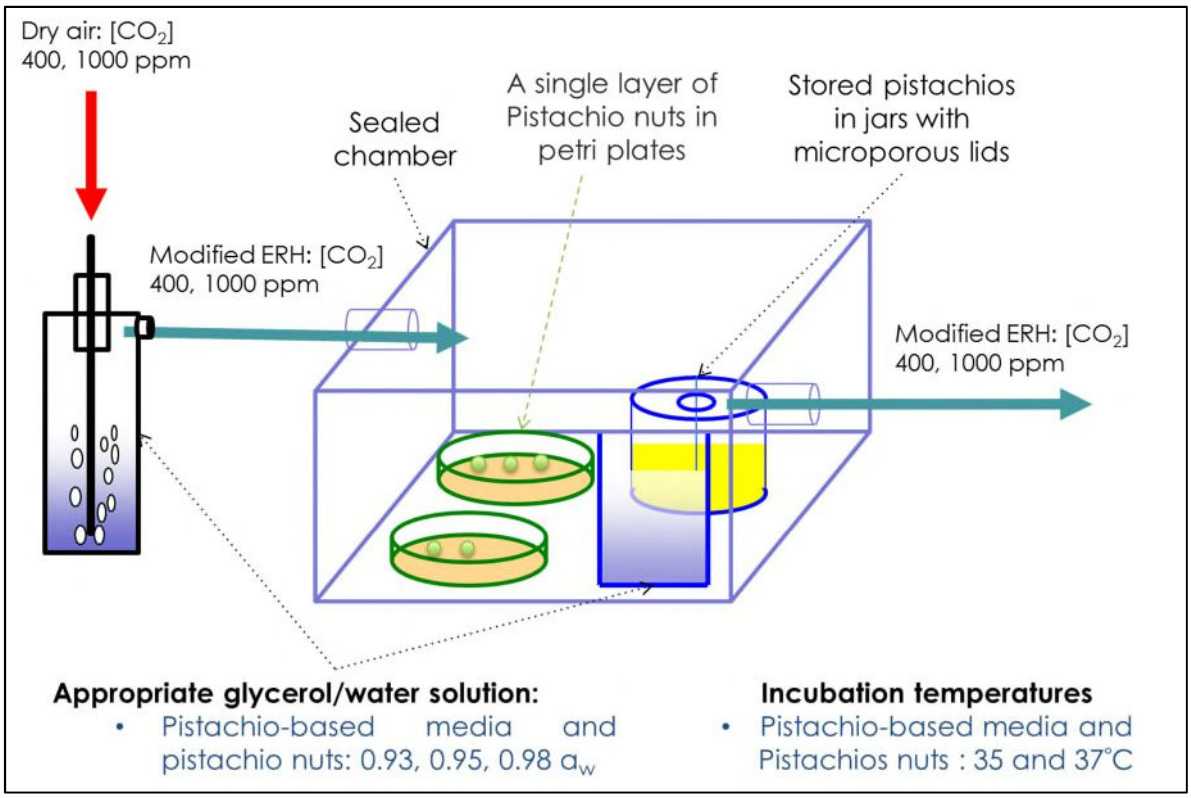


**Figure 4.1 Climate change experimental system used in these studies.**

### 4.2.4 Colonisation of pistachio nuts by *Aspergillus flavus* isolates and incubation conditions

#### (a) Moisture adsorption curve

The $a_w$ of the pistachio nuts was modified based on the moisture sorption curve that was constructed previously in Chapter 3 for ecological studies. The $a_w$ of pistachio nuts was modified to 0.93, 0.95 and 0.98 with the addition of known amounts of water and equilibrated at 4°C overnight.

**(b) Inoculation and colony measurement**

Single layers of treatment pistachio nuts were spread into 9 cm Petri plates. These were centrally inoculated with an individual *A.flavus* strain (AB3 and AB10). The experiment was carried out with three replicates per treatment at 35 and 37°C. Inoculated plates were placed into the environmental chambers and flushed with $CO_2$ as described previously. The colonisation rates were measured in two directions at right angles to each other, every two days for ten days.

### 4.2.5 Gene expression studies

Sampling was carried out following ten days incubation in triplicate for gene expression studies. The biomass was aseptically harvested, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

**(a) RNA isolation from pistachio nuts**

Using the bead-beating method published by Leite et al. (2012), RNA was extracted with some modifications. One hundred fifty mg of frozen biomass was transferred into an autoclaved 2 mL extraction tube containing 0.5 mm sized glass beads. 1 mL of RLT buffer (provided by the RNeasy® Plant Mini Kit) (Qiagen, Germany) supplemented with 10 µL of β-mercaptoethanol was added. The tubes were immediately frozen in liquid nitrogen. After a quick vortex to help disrupt the mycelium, samples then were placed on ice to be thawed. The tubes were agitated for 25 s followed by a 5 s interval and another 25 s of agitation. This mixture was centrifuged at 10,000 rpm for 5 min at 4°C in a temperature

controlled centrifuge system. A pre-frozen 2 mL Safe-Lock tube (Eppendorf, Germany) was used to place the collected supernatant. According to instructions of the RNeasy® Plant Mini Kit (Qiagen, Germany), the RNA purification was carried out. RNA obtained was eluted in 50 µL of RNase free water and kept at -80 °C until used for reverse transcription. The RNA concentration and purity (A260/A280 ratio) were determined spectrophotometrically using a 2.5 µL aliquot on the PicodropTM (Spectra Services Inc., USA).

**(b) Primers and probes**

Nucleotide sequences of primers and probes used in this study are included in Table 4.1 (Medina et al., 2014). The design of the primer pairs of nortaq-1/nortaq2 and aflRtaq1/aflRTaq2 and the hydrolysis probe norprobe and *AflR*probe were respectively designed based on the *aflD* and *aflR* genes involved in the aflatoxin biosynthetic pathway. The primer pair bentaq1/bentaq2 and the hydrolysis probe benprobe were designed and relied on the β-tubulin gene. The norprobe and *AflR*probe were labelled at the 5′ end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher Black Hole Quencher 2 (BHQ2). However, the reporter cyanine-5 (CY5) was used for the benprobe and labelled at the 5′ end with the quencher BHQ2.at the 3′ end.

**Table 4.1 Nucleotide sequences of primers for RT-qPCR assay designed on the basis of *AflD, AflR* and β-tubulin genes.**

| Gene | Primers and probes | Primer sequence | Position |
|---|---|---|---|
| *AflD* | nortaq1 | GTCCAAGCAACAGGCCAAGT | 516[a] |
| | nortaq2 | TCGTGCATGTTGGTGATGGT | 562[a] |
| | norprobe | [FAM]TGTCTTGATCGCGCCCG[BHQ2] | 537[a] |
| *AflR* | aflRTaq1 | TCGTCCTTATCGTTCTCAAGG | 1.646[b] |
| | aflRTaq2 | ACTGTTGCTACAGCTGCCACT | 1.735[b] |
| | aflRprobe | [FAM]AGCAGGCACCCAGTGTACCTCAAC[BHQ2] | 1.6889[b] |
| β-tubulin | Bentaq1 | CTTGTTGACCAGGTTGTCCAT | 65[c] |
| | Bentaq2 | GTCGCAGCCCTCAGCCT | 99[c] |
| | benprobe | [CY5]CGATGTTGTCCGTCGCGAGGCT[BHQ2] | 82[c] |

*[a] Positions are in accordance with the published sequence of the aflD gene of Aspergillus flavus (GeneBank accession no. XM_002379908.1).*
*[b] Positions are in accordance with the published sequence of aflR gene of Aspergillus flavus (GeneBank accession no. AF441435.2).*
*[c] Positions are in accordance with the published sequence of ß-tubulin gene of Aspergillus flavus (GeneBank accession no. AF036803.1).*

**(c) Reverse transcription to convert cDNA from mRNA**

To amplify the structural gene nor-1 (*aflD*) and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway as the target genes, a quantitative RT-qPCR assay was used. The β-tubulin gene was used as a control gene (Abdel-Hadi et al., 2010; Medina et al., 2014)

**(d) Amplification of *aflD* and *aflR* genes through real-time quantitative PCR (RT-qPCR)**

The Bio-Rad CFX96 Real Time PCR Detection System (Bio-Rad, U.K) was used to perform two RT-qPCR assays to amplify the *aflD* gene and the housekeeping

β-tubulin gene in the first one, and the other one to quantify the *aflR* gene expression using the β-tubulin gene as control (Medina et al., 2014). They were prepared in triplicates of 12.5µL reaction mixture in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Bio-Rad). Three replicates of a RNA control sample together with a template-free negative control were also included in the runs. The TaqMan system with primers and probes were used in all cases. The reaction mixtures consisted of 6.25 µL Premix Ex TaqTM (Takara Bio Inc., Otsu, Shiga, Japan), 830 nM of each primer, 330 nM of each probe, and 1.5 µL of cDNA template in a final volume of 12.5 µL. The optimal thermal cycling conditions included an initial step of 10 min at 95°C and all 45 cycles at 95°C for 15 s, 55°C for 20 s and 72°C for 30 s. $C_t$ determinations were automatically performed by the instrument using default parameters.

Data analysis was carried out using the software CFX Manager™ Software (Bio-Rad). Relative quantification of the expression of *aflD* and *aflR* genes were carried out using the housekeeping gene β-tubulin as an endogenous control to normalise the quantification of the mRNA target for differences in the amount of total cDNA added to the reaction in the relative quantification assays and used for all treatments. The expression ratio was calculated as previously described by Livak and Schmittgen (2001). Prior analysis, it was tested to show that the experimental treatments did not influence expression of the internal control gene, and the amplification efficiencies of the target and reference genes were practically equal (93.1% for *aflR* and 95.2% for β-tubulin genes). This method allows calculation of the expression ratio of a target gene between a tested

sample and its relative calibrator ("control" sample). In this work, the calibrator corresponded to *A.flavus* strains grown at 35°C and 0.98 $a_w$ at atmospheric air (400 ppm $CO_2$).

The $C_t$ raw data were obtained from the BIO-RAD detection system and the calculations were performed by Microsoft Excel®. The statistical design was factorial CRD, 2 factors and the statistical analysis obtained using SPSS® software.

### 4.2.6 Quantification of aflatoxin $B_1$ production

As described previously in Sections 3.2.6 and 3.2.7 for *in vitro* and *in situ* studies respectively.

### 4.2.7 Statistical analysis

Three replicates per treatment were used in all experimental studies. Means were obtained by taking the average of each three measurements with the standard error of the means (± SE). Analysis of Variance (ANOVA) was applied to analyse the variation of means with 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov using Minitab statistical software. Fisher's Least Significant Difference (LSD) was used to identify differences between the means with $p < 0.05$ as significant difference using the same statistical software.

## 4.3 RESULTS

### 4.3.1 *In vitro* effect of climate change factors on growth of *Aspergillus flavus* strains on pistachio-based media

The effect of changing $CO_2$ exposure levels (400 and 1,000 ppm) at two different temperatures (35 and 37°C) and three levels of water stress (0.93, 0.95 and 0.98 $a_w$) on the growth of two strains of *A.flavus* (AB3 and AB10) on pistachio-based media are shown in Figures 4.2 and 4.3 respectively. For both strains, growth was slightly increased, but not significantly, when exposed to 1,000 ppm $CO_2$ at both temperatures and all the $a_w$ levels tested when compared to the control $CO_2$ treatment.

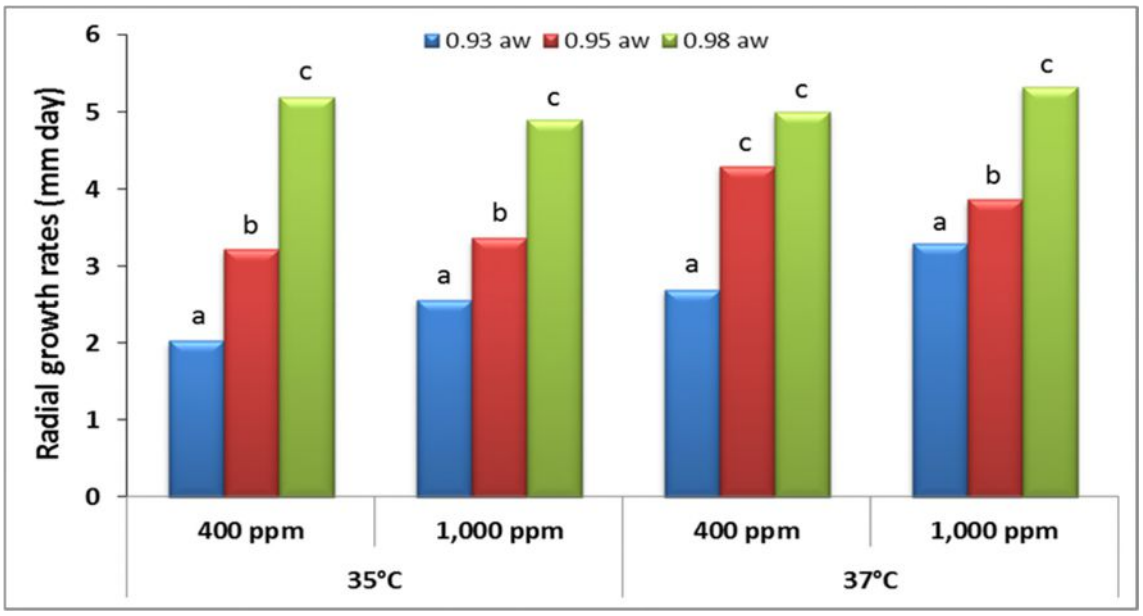


**Figure 4.2 Growth rates (mm/day) of *Aspergillus flavus* AB3 grown on pistachio-based media and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

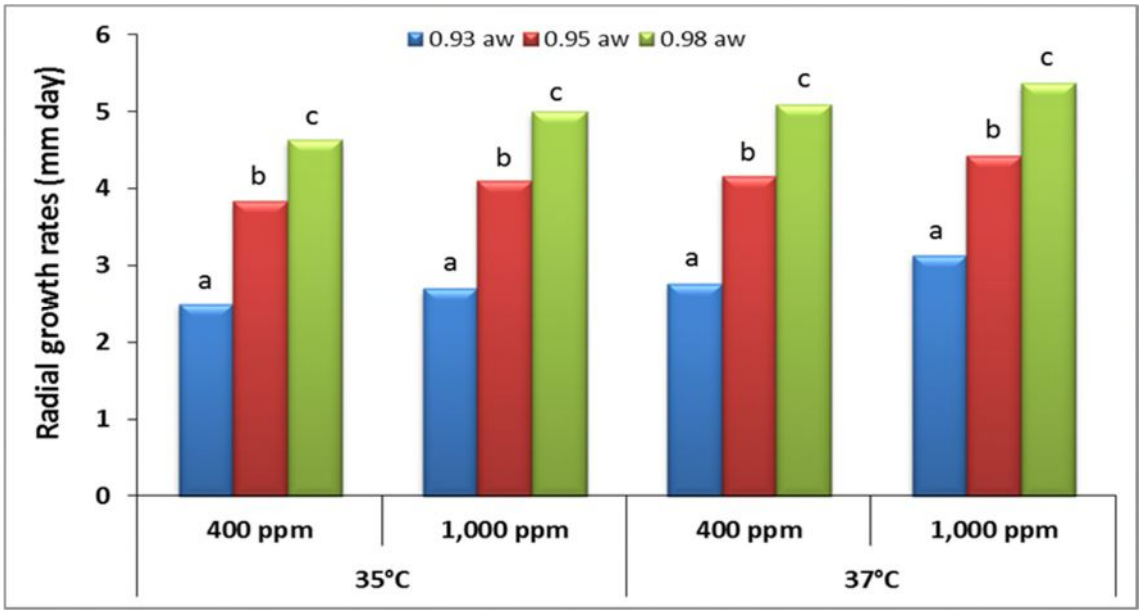


**Figure 4.3 Growth rates (mm/day) of *Aspergillus flavus* AB10 grown on pistachio-based media and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

### 4.3.2 *In situ* effect of climate change factors on growth of *Aspergillus flavus* strains on pistachio nuts

The effect of changing $CO_2$ exposure levels (400 and 1,000 ppm) at two different temperatures (35 and 37°C) and three levels of water stress (0.93, 0.95 and 0.98 $a_w$) on the growth of two strains of *A.flavus* (AB3 and AB10) on pistachio nuts are shown in Figures 4.4 and 4.5. Results were similar to that of *in vitro* study. For both strains, growth was increased, but not significantly, when exposed to 1,000 ppm $CO_2$ at both temperatures and all the $a_w$ levels tested when compared to the control $CO_2$. However, growth of the AB3 strain was slightly decreased at 1,000 ppm $CO_2$ at the lowest $a_w$ tested (0.93 $a_w$) in the two incubation temperatures.

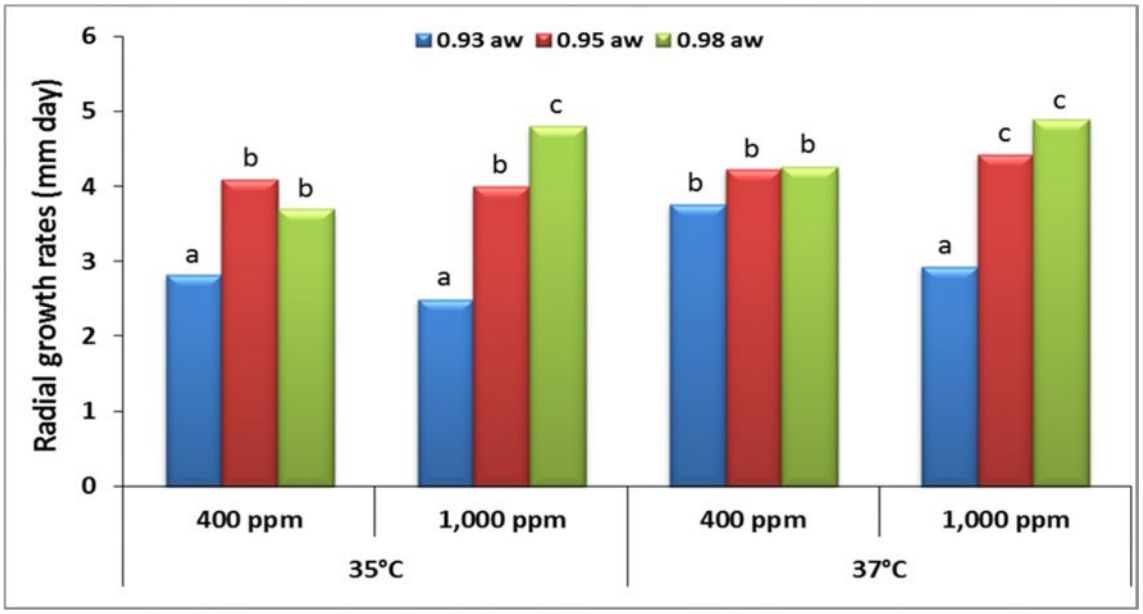


**Figure 4.4** **Growth rates (mm/day) of *Aspergillus flavus* AB3 grown on single layers of raw pistachio nuts and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

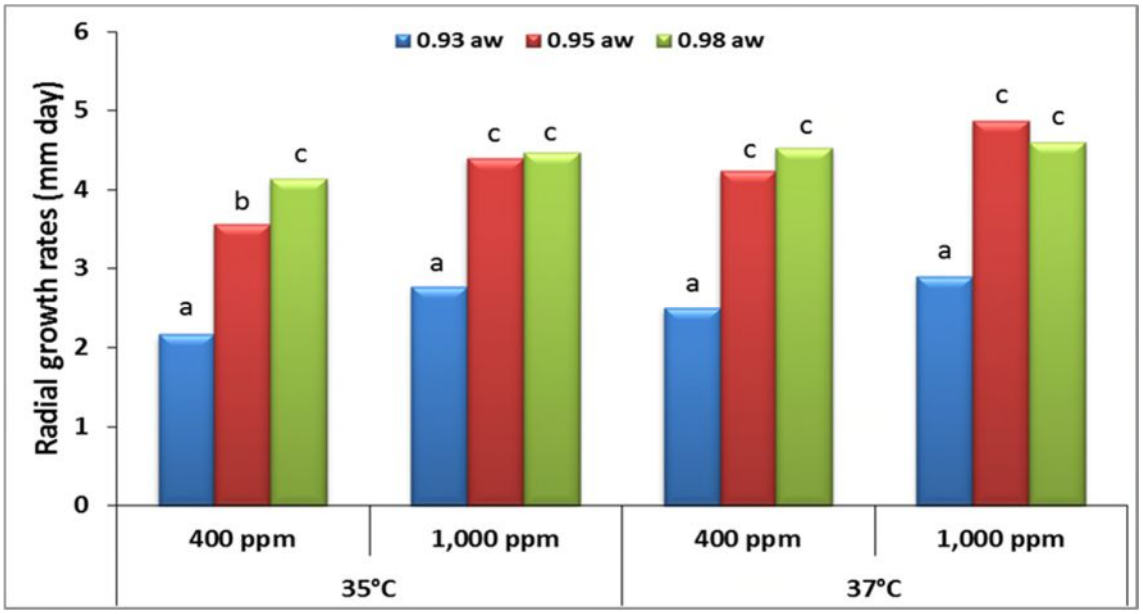


**Figure 4.5 Growth rates (mm/day) of *Aspergillus flavus* AB10 grown on single layers of raw pistachio nuts and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

### 4.3.3 *In vitro* effect of climate change interacting factors on aflatoxin $B_1$ production from *Aspergillus flavus* strains on pistachio-based media

Figures 4.6 and 4.7 show the effect of the interaction between CC factors on $AFB_1$ production by two strains of *A.flavus* (AB3 and AB10) respectively on pistachio based media. Generally, $AFB_1$ production was higher at 35°C at the two $CO_2$ levels (400 and 1,000 ppm $CO_2$) for both stains. At the same temperature, the production was increased significantly at 1000 ppm $CO_2$ and 0.98 $a_w$ when compared with atmospheric air at 400 ppm $CO_2$. At 37°C, $AFB_1$ production by the *A.flavus* strains when exposed to 1,000 ppm $CO_2$ was either decreased as in strain AB3 (Figure 4.6), or similar as in strain AB10 (Figure 4.7). This indicates

that the three-way interaction affected $AFB_1$ production especially when $CO_2$ was increased (from 400 to 1,000 ppm) under optimum conditions (35°C, 0.98 $a_w$, and 400 ppm $CO_2$).

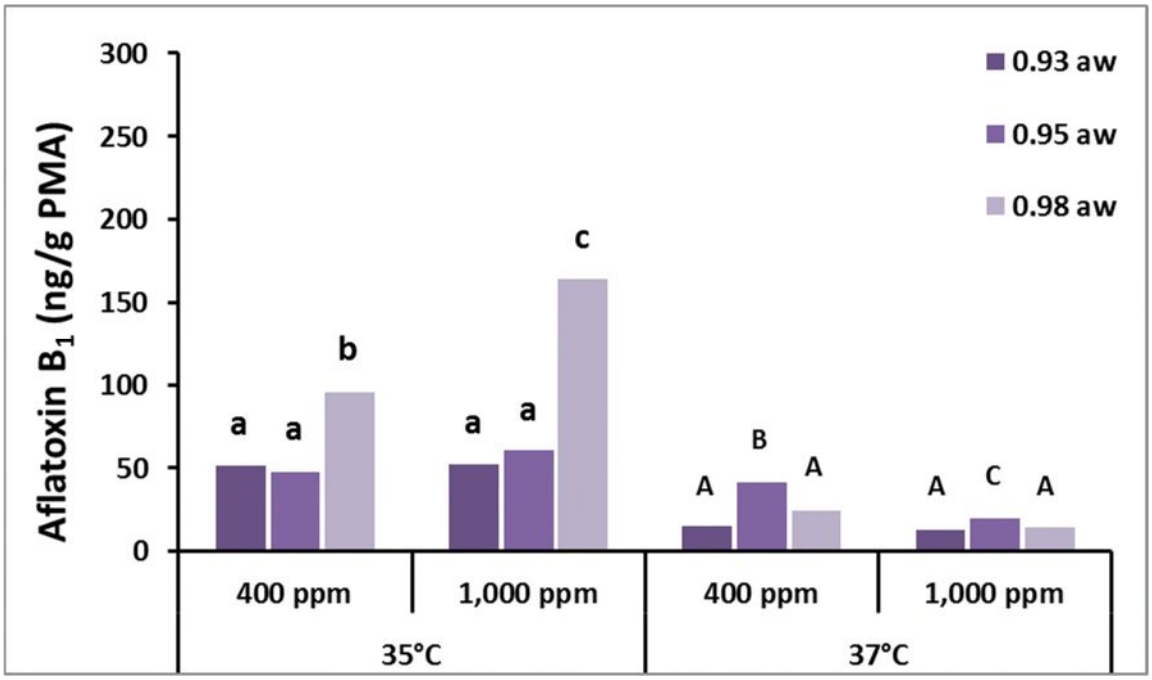


**Figure 4.6 Aflatoxin $B_1$ production by *Aspergillus flavus* AB3 grown on pistachio-based media and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

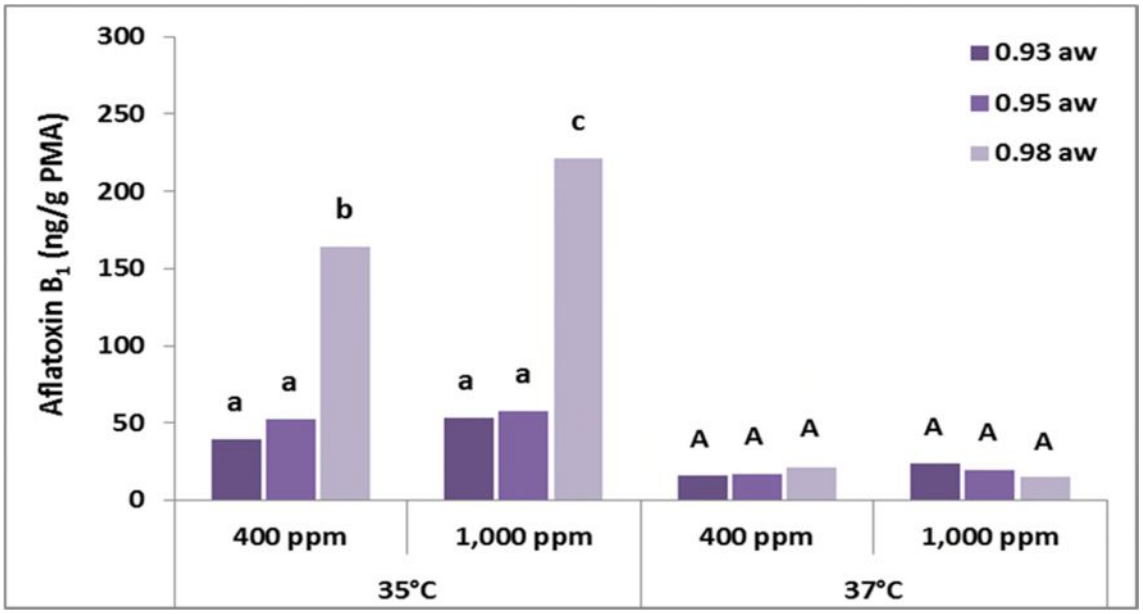


**Figure 4.7 Aflatoxin $B_1$ production by *Aspergillus flavus* AB10 grown on pistachio-based media and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

### 4.3.4 *In situ* effect of climate change interacting factors on aflatoxin $B_1$ production from *Aspergillus flavus* strains on pistachio nuts

Figures 4.8 and 4.9 show the effect of the three-way interaction between temperatures × $a_w$ × $CO_2$ on $AFB_1$ production by the two strain of *A.flavus* (AB3 and AB10) on layers of stored pistachio nuts. There was an increase in $AFB_1$ production when exposed to 1,000 ppm $CO_2$ for both strains at both temperatures and all aw levels except for 0.98 at 37°C for AB10 (Figure 4.9) where the $AFB_1$ production decreased after exposure to higher concentration of $CO_2$. For strain AB3, $AFB_1$ production was significantly higher at 1,000 ppm $CO_2$ when compared with 400 ppm $CO_2$ at 35°C and 0.98 $a_w$ (Figure 4.8). A significant increase in $AFB_1$

production was also found for strain AB10 at 35°C and 0.95 $a_w$ when exposed to 1,000 ppm $CO_2$.

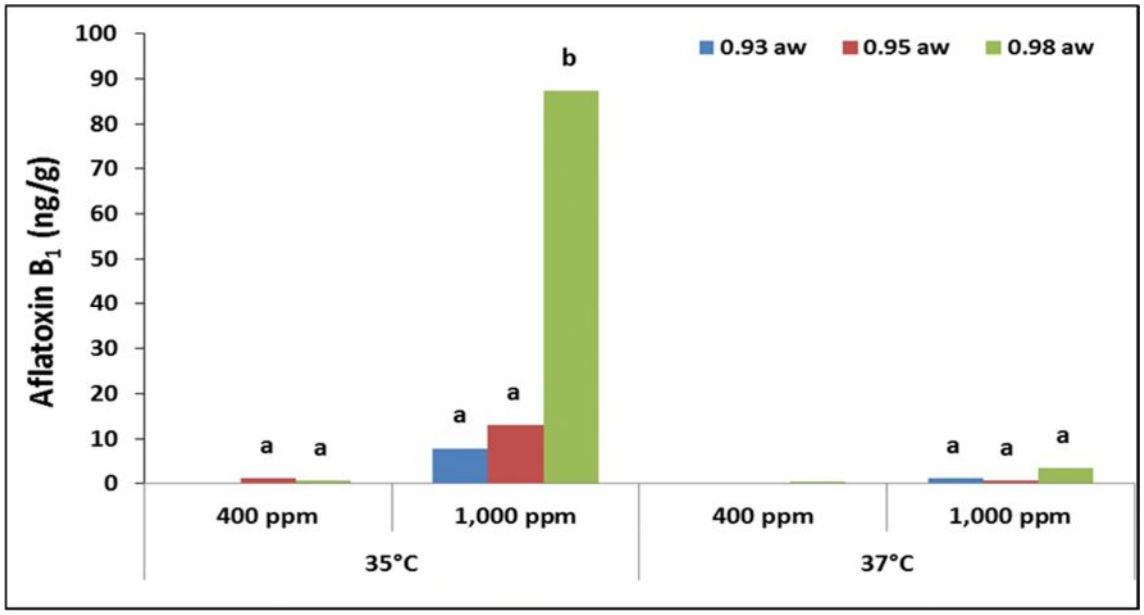


**Figure 4.8 Aflatoxin $B_1$ production by *Aspergillus flavus* AB3 grown on single layers of raw pistachio nuts and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

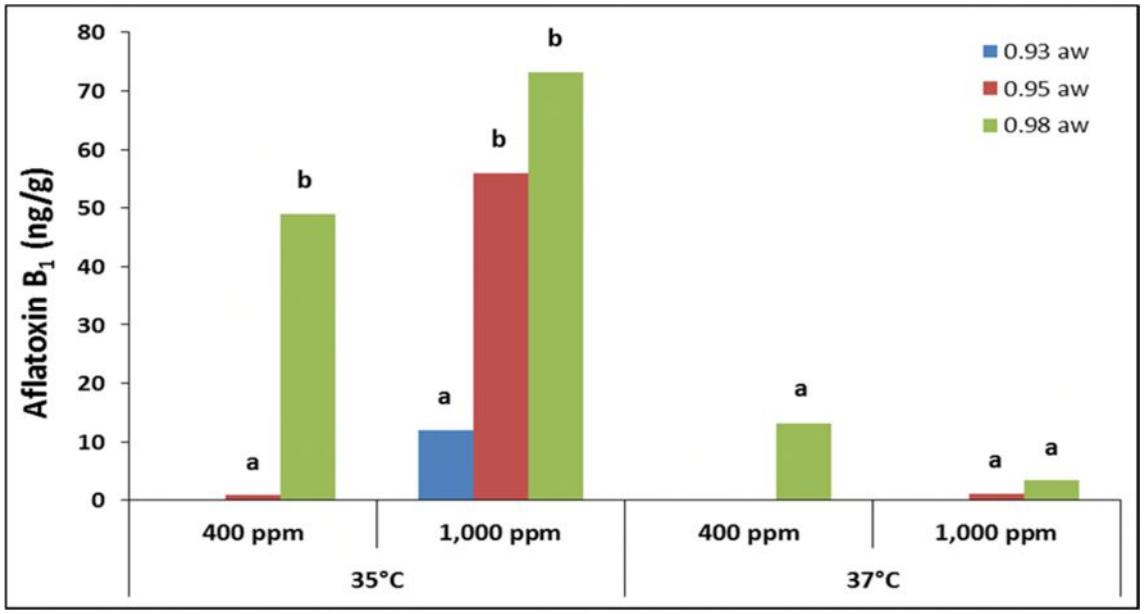


**Figure 4.9 Aflatoxin $B_1$ production by *Aspergillus flavus* AB10 grown on single layers of raw pistachio nuts and incubated at 35 and 37°C under different concentrations of $CO_2$ (a) 400 ppm and (b) 1,000 ppm and different water activity levels (0.93, 0.95, 0.98 $a_w$). Data are means of triplicates. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

### 4.3.5 *In vitro* effect of climate change interacting factors on relative genes expression of the *aflD* and *aflR* genes in the biosynthetic pathway for aflatoxin $B_1$ production

Figures 4.10 and 4.11 show the effect of the three-way interacting treatments on the expression of the *aflD* structural and *aflR* regulatory genes of the two strains of *A.flavus* (AB3 and AB10) respectively. The control conditions (calibrator) were 35°C, 400 ppm $CO_2$, 0.98 $a_w$. Regarding *aflD,* at 35°C, the relative expression was higher at 400 ppm $CO_2$ for both strains. However, for strain AB3, the expression was higher at 1,000 ppm $CO_2$ and 0.95 $a_w$. However, at 37°C, the expression was generally increased at 1,000 ppm $CO_2$ when compared with existing atmospheric $CO_2$ levels. With regard to the regulatory *aflR* gene, the

expression was higher in the 1000 ppm $CO_2$ treatment at 37°C for both strains. This suggests that the interaction between the three CC factors stimulated the expression of this gene which parallels the effects on production of $AFB_1$. Unfortunately, because of time issues it was not possible to carry out similar quantification of biosynthetic genes in the *in situ* experiments.

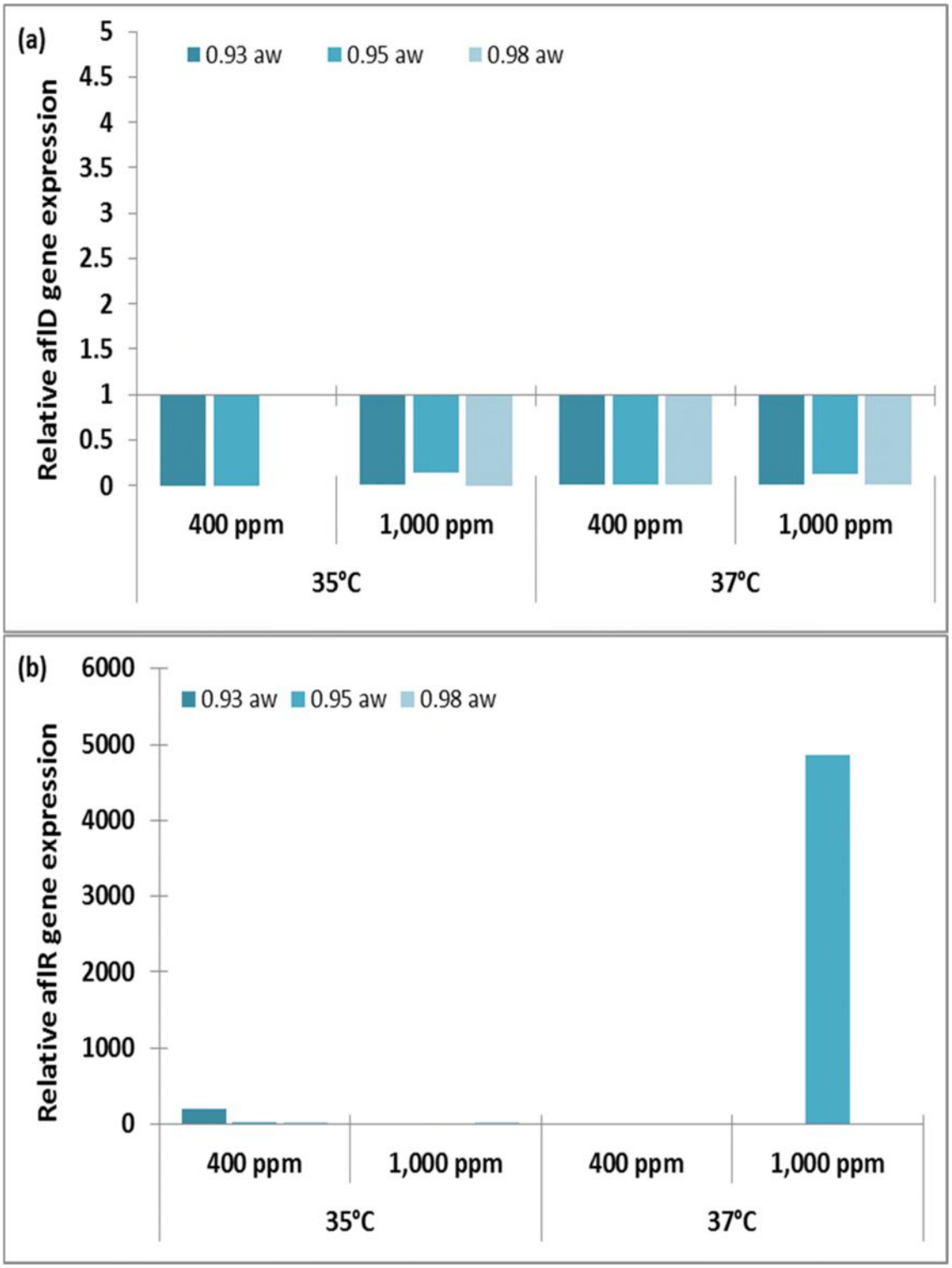


**Figure 4.10 Effect of water activities (0.93, 0.95, 0.98 $a_w$) × $CO_2$ levels (400, 1,000 ppm) × incubation temperatures (35, 37°C) on relative expression of (a) *aflD* structural gene and (b) *aflR* regulatory gene of *Aspergillus flavus* strain AB3 *in vitro*.**

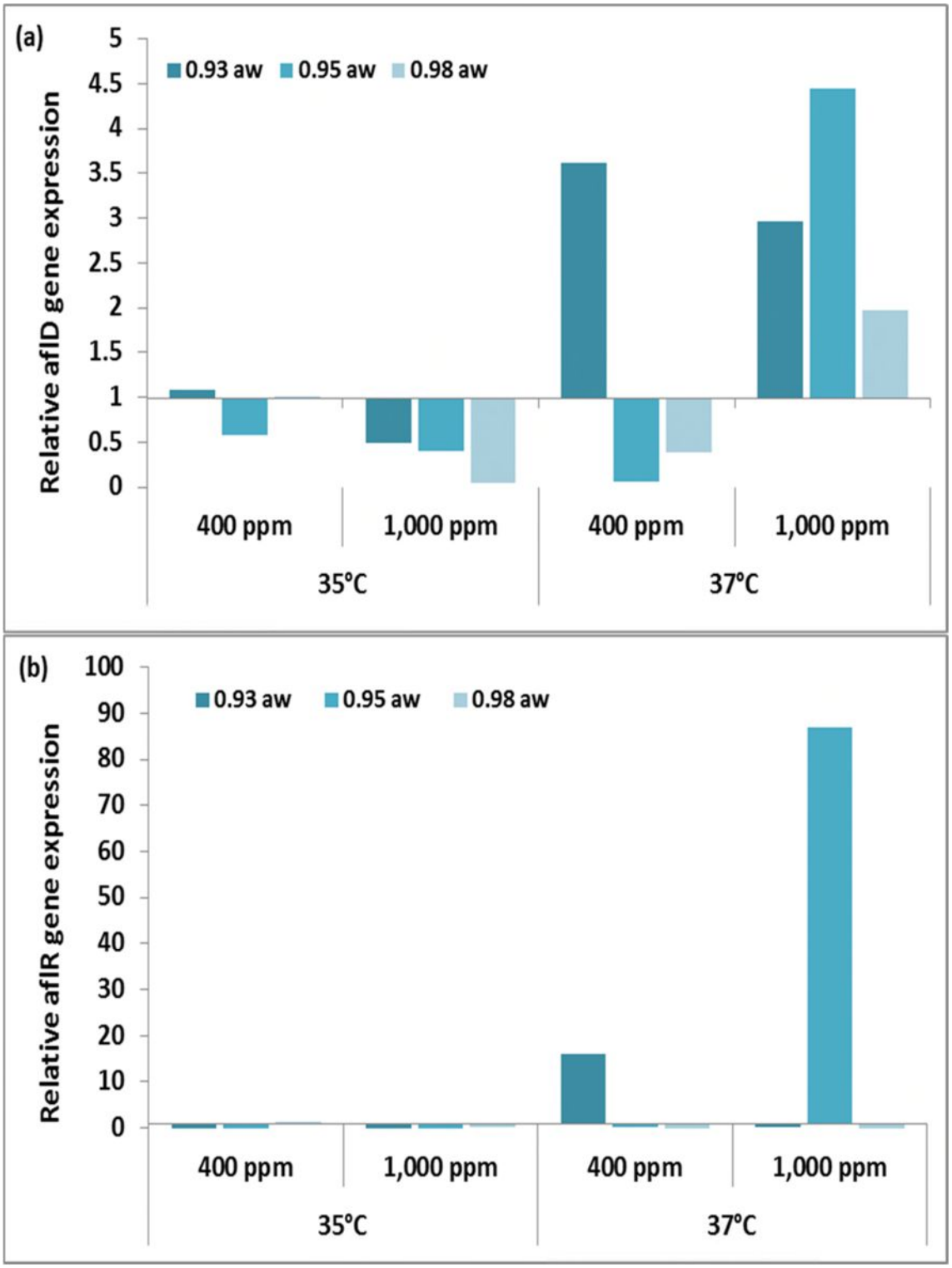


**Figure 4.11 Effect of water activities (0.93, 0.95, 0.98 $a_w$) × $CO_2$ levels (400, 1,000 ppm) × incubation temperatures (35, 37°C) on relative expression of (a) *aflD* structural gene and (b) *aflR* regulatory gene of *Aspergillus flavus* strain AB10 *in vitro*.**

## 4.4 DISCUSSION

The study was the first to try and examine the effect of CC interacting factors of temperature, water stress and elevated $CO_2$ on *A.flavus* colonisation rate, biosynthetic genes expression and $AFB_1$ production in pistachio nuts. Overall, this showed that growth of *A.flavus* was not significantly affected by the interactions. However, the relative expression of the biosynthetic genes (*aflD* and *aflR),* and $AFB_1$ production were affected, often with increased levels in the CC conditions examined.

The only other similar study which has been carried out on the effect of the three interacting CC factors on *A.flavus* and $AFB_1$ production was the recent work by Medina at el. (2015b). They found that the interaction had no significant effect on growth of *A.flavus.* In contrast, the three-way interacting factors significantly stimulated both *aflD* and *aflR* gene expression and $AFB_1$ production. These studies were carried out on a condusive YES medium for secondary metabolite production. Subsequent studies (Medina et al., 2016; 2017) showed that stimulation also occurred in stored maize grain although the stimulation was not as high as that found in the *in vitro* studies.

In the present study, in most cases, for strain AB3, *AflD* and *AflR* gene expression paralleled the production of $AFB_1$. For the structural gene *aflD,* expression was generally related to $AFB_1$ quantities except when strain AB3 was grown at 35ºC + 1,000 ppm $CO_2$ and 0.98 $a_w$. $AFB_1$ produced was higher than the control conditions (35°C, 0.98 $a_w$, and 400 ppm $CO_2$) whilst the *aflD* gene expression

was lower when compared with the calibrator. This may be because *aflD* is a structural gene early in the pathway and since the treatments were sampled after ten days, the optimum expression of this gene may have already occurred much earlier. However, the regulatory gene *aflR* expression for the same strain was found to parallel the production of $AFB_1$. Overall, this suggests that $CO_2$ affects $AFB_1$ production because there was an increase in amounts at 35°C + 1,000 ppm $CO_2$ at 0.98 $a_w$ when compared to the control. The $AFB_1$ production profiles of the two *A.flavus* strains were similar and correlated with *aflR* gene expression. Medina at el. (2015b) found that the maximum relative expression of *aflD* gene was at 34°C and with 400 ppm $CO_2$ with a decrease in expression with elevated $CO_2$ and water stress while the *aflR* gene expression was found to be significant only at 0.92 $a_w$ and 650 ppm $CO_2$. In contrast, the expression of both *aflD* and *aflR* genes increased significantly at 37°C under treatment conditions of 0.95 and 0.92 $a_w$ and 650 and 1000 ppm $CO_2$ and these increases were associated with the increase of $AFB_1$ production. Recent studies of the effect of two way $a_w$ × temperature interactions and three way CC interacting factors have shown that there are significant changes in the transcriptome of *A.flavus* both *in vitro* and in stored maize. This secondary metabolite pathways, sugar transporters and other related gene clusters were found to significantly change using RNAseq studies (Medina et al., 2015b; Gilbert et al., 2017).

The impact of interacting CC factors on different mycotoxigenic fungi may however vary with differential effects. Recent studies of CC factors on colonisation and ochratoxin A (OTA) production by strains of *A.westerdijkiae* and

*A.carbonarius* on coffee-based media and in stored coffee showed some differences (Akbar et al., 2016; Medina et al., 2017a). Akbar et al. (2016) showed that for *A.westerdijkiae*, while growth was relative unaffected, OTA production was stimulated by CC interacting factors, both *in vitro* and *in situ*. However, for *A.carbonarius* there was no effect on growth or OTA production. Thus differential effects of CC factors on mycotoxin production could occur and for each mycotoxigenic species and food chain, studies are needed to identify in which species stimulation of mycotoxin production might occur. In addition, it is known that pest reproduction might increase under CC scenarios. More damage to ripening crops may also influence toxin production and perhaps also the ratio of related toxins (Medina et al., 2017a). In additional, the impact of acclimatisation needs to be addressed. Studies by Vary et al. (2015) showed that growing *F.graminearum* for 20 generation in elevated $CO_2$ resulted in higher infection of ripening wheat, with increased head blight symptoms. Perhaps mycotoxin production could also be influenced. More studies are needed on the acclimatisation of fungal pathogens and their effect on crops under CC scenarios to obtain more accurate data on implications for mycotoxins contamination of economically important commodities (Medina *et al*, 2017b). Questions include what would mycotoxins patterns look like in the future under CC condition? Will mycotoxins classifications and its carcinogenicity change? These questions need to be addressed. The next Chapter (5) addresses the issue of acclimatisation with regard to *A.flavus* colonisation and $AFB_1$ production in pistachio nuts.

# CHAPTER 5
# Effect of acclimatisation in elevated $CO_2$ on growth and aflatoxin $B_1$ production by *Aspergillus flavus* strains on pistachio nuts

## 5.1 INTRODUCTION

Global warming has been receiving significant interest because of the recent concerns on its potential impacts on food security and food quality. The challenge of increasing crop productivity to meet the demand of an ever growing world population may be further exacerbated by climate change (CC) scenarios (Wheeler, 2013). Under CC condition, plant growth and physiology will be affected and also result in exposure to more fungal pathogens and pests. This may lead to more suitable environmental conditions for fungal infection and mycotoxins contamination (Battilani, 2016; Medina et al., 2017). Vary *et al*. (2015) examined the effect of wheat exposure to 650 ppm *vs* 350 ppm $CO_2$ in controlled plant growth chambers. They found that there were effects on both plant physiology and severity of wheat diseases. Exposure of wheat plants during anthesis onwards to CC resulted in modifications in stomatal number and position and physiology of the plant. They also grew *Septoria* and *Fusarium graminearum* for 10 and 20 generations respectively and examined the infection and disease symptoms in elevated $CO_2$ conditions. They found that the acclimatised strains of the two fungal pathogens were able to increase their infection of wheat under CC conditions. This was based on both disease symptoms and on fungal biomass measurements using molecular biology approaches. Unfortunately, while there

was an increase in *Fusarium* Head Blight symptoms they did not analyse whether type B trichothecenes were also increased.

Practically no acclimatisation studies have been previously carried out with other mycotoxigenic fungi (Medina et al., 2017). Pistachio nuts are known to be more prone to contamination with $AFB_1$ when there is increased insect infection and poor post-harvest practices, which could, under CC scenarios, predispose the nuts to *A.flavus* infection and perhaps increased $AFB_1$ contamination. The potential for acclimatisation allowing better tolerance of CC environmental conditions and thus exacerbating toxin contamination has not been examined in any staple commodities. There have been no studies on the impact that acclimatisation of *A.flavus* strains to elevated $CO_2$ may have on colonisation and $AFB_1$ contamination.

The objectives of the present study were to examine whether acclimatisation to 1,000 ppm elevated $CO_2$ of *A.flavus* strains AB3 and AB10 (for five generations) would affect (a) mycelial colonisation and (b) $AFB_1$ production under interacting CC conditions (+2$^oC$ × 0.98 or 0.93 $a_w$ × 1,000 ppm $CO_2$) and compare this with non-acclimatised strains under existing and future environmental conditions for the first time.

## 5.2 MATERIALS AND METHODS

### 5.2.1 *Aspergillus flavus* strains

Two strains of *A.flavus were* selected for this study due to their ecophysiological similarities to the type strain based on preliminary experiments. These strains were AB3 and AB10.

### 5.2.2 Acclimatisation experimental system

Both *A.flavus* strains were acclimatised to 1,000 ppm elevated $CO_2$ incubated at 37°C for five generation. Each generation was incubated for 7-10 days on pistachio-based media in the CC growth chambers. This was repeated five times.

### 5.2.3 Colonisation of pistachio nuts by *Aspergillus flavus* isolates and incubation conditions

For experiments with non-acclimatised and acclimatised strains, the $a_w$ of the unsalted pistachio nuts was modified to 0.98 and 0.93 $a_w$ by adding known amounts of water based on the previously developed moisture sorption curves and left overnight at 4°C for equilibration as described in Chapter 3. These were inoculated with either the acclimatised or non-acclimatised strains by scratching the surface of the fungi gently using a sterilised inoculation loop as described in section 4.2.4. They were incubated under existing conditions: 35°C + 400 ppm $CO_2$ + 0.98 or 0.93 $a_w$, and CC conditions of 37°C + 1,000 ppm $CO_2$ + 0.98 or 0.93 $a_w$. All experiments were carried out in environmental growth chambers as previously described (Medina et al., 2015). At the end of the experiments, the

pistachio nuts were dried, milled and extracted for $AFB_1$ analyses using the IAC columns and quantification using HPLC-FLD after five and ten days incubation.

### 5.2.4 Quantification of aflatoxin $B_1$ production

As described previously in Section 3.2.6.

### 5.2.5 Statistical analysis

Three replicates per treatment were used in all experimental studies. Means were obtained by taking the average of each three measurements with the standard error of the means (± SE). Analysis of Variance (ANOVA) was applied to analyse the variation of means with 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov using Minitab statistical software. Fisher's Least Significant Difference (LSD) was used to identify differences between the means with $p < 0.05$ as significant difference using the same statistical software.

## 5.3 RESULTS

### 5.3.1 *In situ* effects of acclimatisation on growth of *A.flavus* strains and $AFB_1$ contamination of pistachio nuts

Figure 5.1 shows the effects of acclimatisation on growth of two strains of *A.flavus* (AB3 and AB10). This shows that for strain AB3 acclimatisation influenced growth at 0.98 $a_w$ while for strain AB10 there was no significant effect on colonisation of layers of raw pistachio nuts. With regards to $AFB_1$ production, Figure 5.2 shows

the effect of acclimatisation on the production by strain AB3. This shows that $AFB_1$ was significantly stimulated, especially after 10 days colonisation. However, for the other strain there was no significant increase when compared to the control (Figure 5.3). $AFB_1$ was not detected at 0.93 $a_w$ in the control conditions (400 ppm $CO_2$ + 35°C) for both strains.

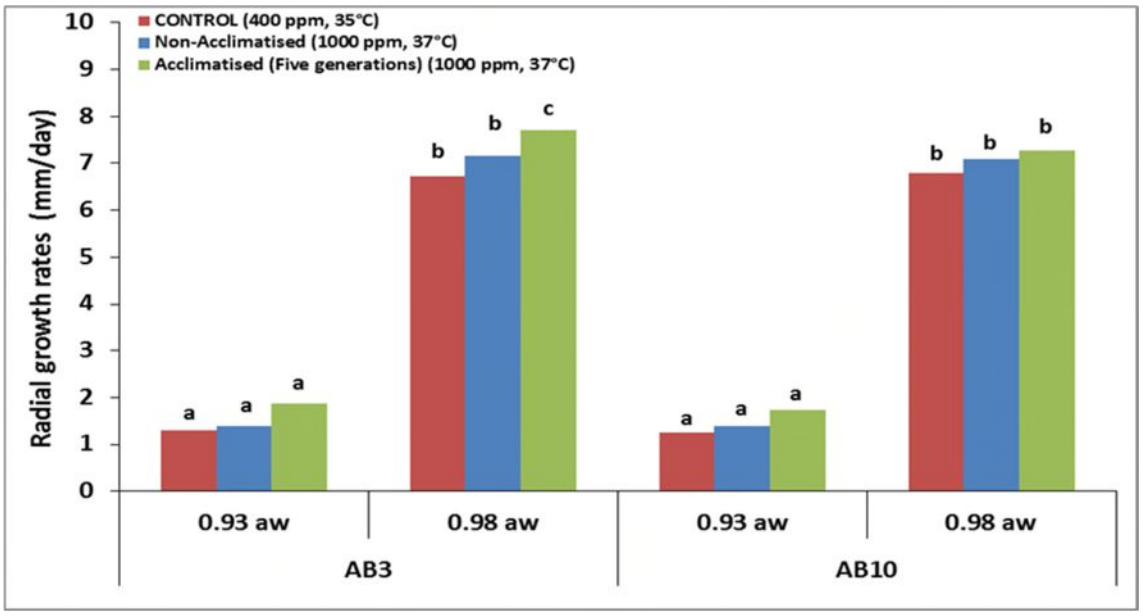


**Figure 5.1 Radial growth rates (mm/day) of non-acclimatised and acclimatised (five generations) strains of *Aspergillus flavus* (AB3 and AB10) on layers of raw pistachio nuts incubated at 35°C + 400 ppm $CO_2$ and 37°C + 1,000 ppm $CO_2$. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

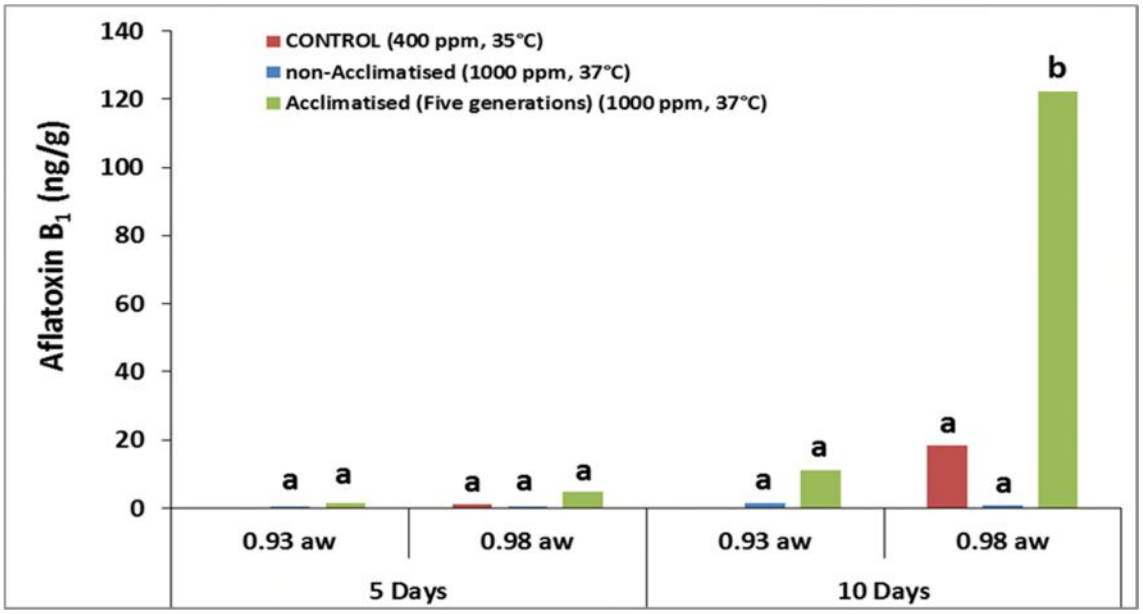


**Figure 5.2 Aflatoxin $B_1$ production of non-acclimatised and acclimatised (five generations) of *Aspergillus flavus* AB3 on layers of raw pistachio nuts incubated at 35°C + 400 ppm $CO_2$ and 37°C + 1,000 ppm $CO_2$. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

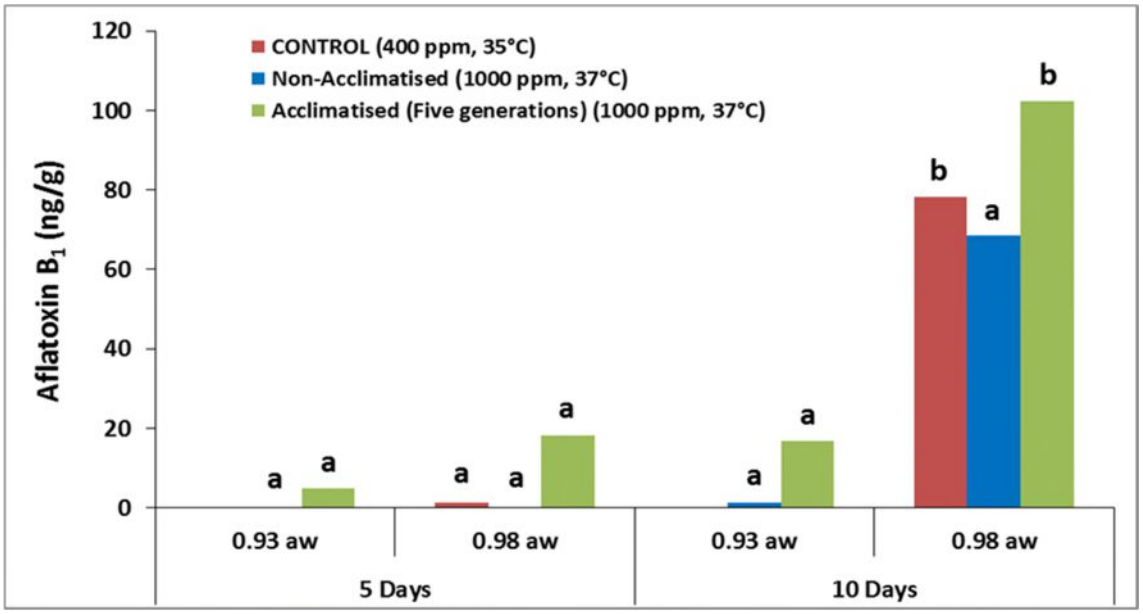


**Figure 5.3 Aflatoxin $B_1$ production of non-acclimatised and acclimatised (five generations) of *Aspergillus flavus* AB10 on layers of raw pistachio nuts incubated at 35°C + 400 ppm $CO_2$ and 37°C + 1,000 ppm $CO_2$. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

## 5.4 DISCUSSION

This study suggests that the effect of acclimatisation needs more investigation. The results differed for two strains, both isolated from pistachio nuts. Results were differential with one strain showing faster growth and clear stimulation of $AFB_1$ production while the other no difference from the control. It may be that comparisons need to be made between 5, 10 or 20 generations in CC scenarios to determine the real impact of adaptation and resilience to these factors and what influence this may have on toxin biosynthesis. Recently, Vary et al. (2015) used 10 and 20 generations of pathogens of wheat. They did not examine inter-strain differences. This certainly needs to be investigated in more detail.

a) Previous studies of CC effects on growth and ochratoxin A (OTA) production also suggested that CC environmental factors has differential effects on growth and OTA production by strains of *A.westerdijkiae* (stimulated) and *A.carbonarius* (no effect). Also perhaps molecular ecology approaches and RNAseq may help to better understand the possible role of acclimatisation in relation to any changes in the transcriptome and in relation to key biosynthetic pathways. Studies also need to examine whether other mycotoxins produced by *A.flavus* such as cyclopiazonic acid may be changed under acclimatisation scenarios (Medina et al., 2017a). Acclimatisation for 10-20 generations in elevated $CO_2$ is required to better understand the development of tolerance and effects on secondary metabolite production in CC scenarios.

# CHAPTER 6
# Use of gaseous ozone ($O_3$) to control germination, mycelial growth and aflatoxin $B_1$ contamination *in vitro* and in stored pistachio nuts under different environmental conditions

## 6.1 INTRODUCTION

Ozone ($O_3$) is commonly generated in the summer because of the reactions between photochemicals in the atmosphere in the presence of sunlight. However, it is a very corrosive gas and dissociates/degrades very quickly. It has been previously shown that small increases in $O_3$ concentrations (40-60 ppb) can influence the mycobiota on plant surfaces and perhaps the ability to produce toxins (Manning, 1995; Magan et al., 2006). Previous studies have examined the efficiency of electrochemically generated $O_3$ on the activity of aflatoxigenic fungi and AFs in pistachio nuts inoculated with *A.parasiticus.* This showed that the population density of *A.parasiticus* after $O_3$ treatment was decreased by 90-92% and decreased total AFs by between 45-74% in pistachio nuts (Mir Abu Al-Fotuhi et al., 2008). More recently, Mylona et al. (2014) demonstrated that gaseous $O_3$ (100-400 ppm) was effective against *Fusarium verticillioides in vitro* and *in situ* in maize grain and inhibited fumonisin $B_1$ production. For spores, initial inhibition was followed by recovery, growth and fumonisin $B_1$ production. Sultan (2010) however, found that gaseous $O_3$ was very effective in inhibiting germination of conidia of *A.flavus* but it had little efficacy in controlling mycelial growth of *A.flavus* strains on peanut-based media, regardless of $a_w$. Studies have been carried out on $O_3$ treatment of Brazil nuts and showed that exposure to $O_3$ affected growth

of the mycobiota and decreased AFs (Giordano et al., 2012). They used three concentrations of $O_3$ (10, 14 and 31.5 mg/L) for five hours and examined the effect after 180 days storage. They found that exposure for this period was effective with growth of both *A.flavus* and *A.parasiticus* inhibited. However, at low concentrations of $O_3$ the fungi were still able to grow. However, the quality of the nuts can be affected with some studies suggesting that tainting can occur due to the effect of $O_3$ on the lipids in the nuts. Sensitivity of fungal species to $O_3$ exposure may vary and depends on exposure period and concentration applied. It can also be influenced by water content and conidial morphology (Hibben and Stotzky, 1969; Antont-Babu and Singleton, 2009).

No previous studies have examined the effect of gaseous $O_3$ as a control measure for germination, colonisation and $AFB_1$ contamination in stored pistachio nuts. Thus the aim of the present chapter was to examine the use of gaseous $O_3$ to control (a) *in vitro* conidial germination, (b) *in vitro* mycelial growth and $AFB_1$ production, and (c) mycelial growth and $AFB_1$ production on pistachio nuts inoculated with *A.flavus* and stored under different $a_w$ regimes.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Apparatus for ozone generation and experimental system

$O_3$ was generated in the laboratory using a C-Lasky series $O_3$ generator (model CL010DS; AirTree Ozone Technology Co., Taiwan) as shown in Figure 6.1. This equipment generates $O_3$ by corona discharge between two tubs with no metals involved for efficiency improvement, generation stability and less energy

consumption. Generated $O_3$ was directed into the exposure chamber using a Teflon tube which was properly connected to the generator. For safety reason, the experiment was carried out in a fume cupboard to prevent $O_3$ from spreading into the laboratory atmosphere. Two different systems were used for $O_3$ exposure for *in vitro* and *in situ* assays. $O_3$ concentration was measured using an $O_3$ analyser (Model UV-100, Eco Sensor, Santa Fe, New Mexico 87505, USA) as shown in Figure 6.1 which was connected to the chamber to measure the exit gas accurately. Experimental set-ups were performed as follows:

**(a) Exposure system of $O_3$ for in vitro germination and mycelial growth assays**

A five Litre (5 L) airtight glass jar was used for these *in vitro* studies. The $O_3$ inlet of the system was connected from the generator to the lid of the jar using a Teflon tube which was inserted into the bottom of the jar. The outlet of the system was also in the lid of the jar and connected to the analyser using a Teflon tube. The flow rate of generated $O_3$ used was 6 L/min. Figure 6.1 shows the idea of the experimental setup used. The only modification was that petri plates with pistachio-based media were placed inside the jar with no lids as the treated sample. Plates were placed on top of each other with at least 2 cm spacing allowed between them to insure exposure to ozone.

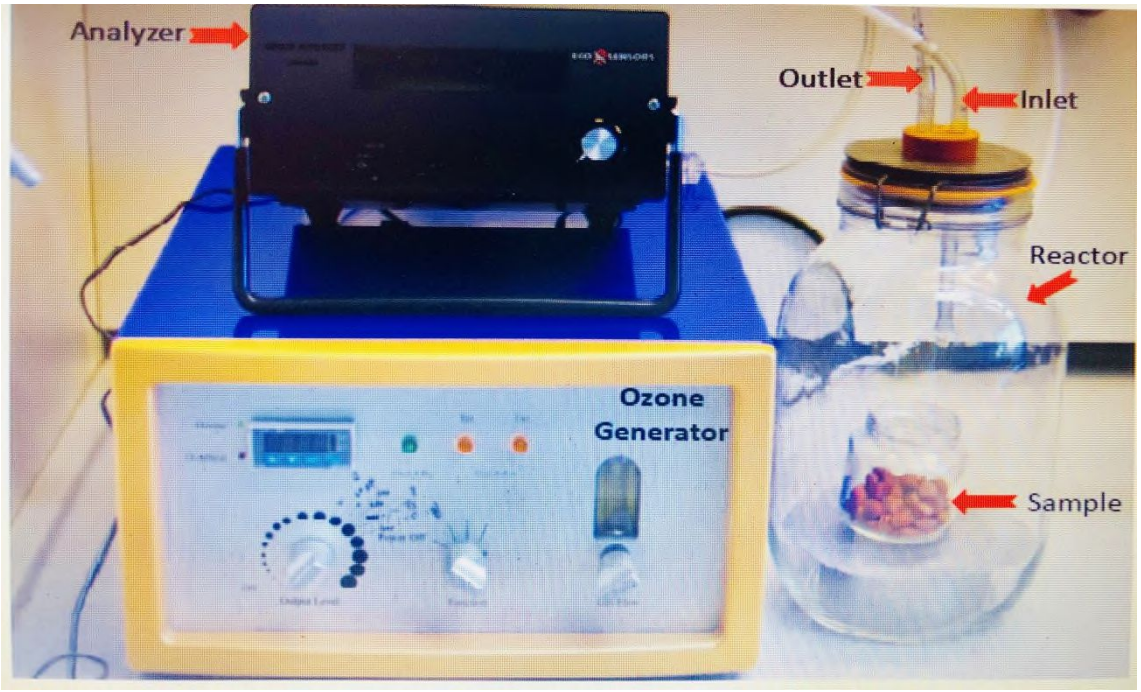


**Figure 6.1 Experimental setup using (5 L) airtight glass jar including the ozone generator and the analyser.**

### (b) Exposure system of $O_3$ for the *in situ* study

The exposure chamber for *in situ* experiments was a 100 mL volume glass tube. The tube was capable of containing about 45-50 g of pistachio nuts. These were placed inside the column and the $O_3$ was forced upwards *via* an inlet at the bottom of the tube coming from the generator and the outlet at the top which was connected to the $O_3$ analyser. The flow rate of generated $O_3$ was 6 L/min (Figure 6.2).

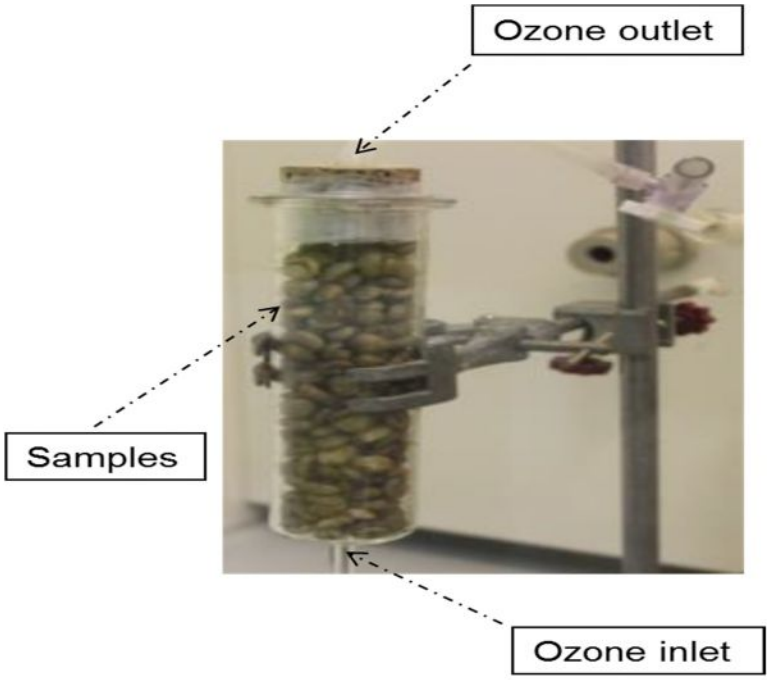


**Figure 6.2 Ozone experimental system**

### 6.2.2 Fungal strains, media, spore suspension and water activity

One strain of *A.flavus* (AB3) was used in this trial. This was chosen as it was representative of those studied previously. Pistachio-based media was used for spore germination and mycelial growth studies. This was prepared as described previously in Section 3.2.2. For spore suspension, fresh cultures of AB3 strain were prepared on PMA and incubated at 25°C for 5-7 days. AB3 cultures surface was gently scraped and transferred into sterile Universal vials containing sterile water + 0.1% Tween 80 solution (Acros Organics, UK). The concentration of the spore suspension was determined using a haemocytometer (Marienfeld, Germany) and a light microscope (Olympus, Japan), and adjusted by dilution to $10^6$ spores/mL. Targets $a_w$ values (0.98 and 0.93) for PMA and stored pistachio nut experiments were obtained as described previously in Sections 3.2.2 and

3.2.4. For total population enumeration, Malt Extract Agar was used and prepared as described previously in section 2.2.3.

### 6.2.3 Effect of $O_3$ on conidial spore germination of *A.flavus*

Four different treatments were examined including three concentrations of $O_3$: 0 (control), 50, 100, 200 ppm $O_3$ at two different $a_w$ levels detailed previously and incubated at 30°C. Samples exposed to air were used as controls for each $a_w$. The experiment was carried out in triplicate. 100 µL of $10^6$ spore suspension were spread onto PMA media and allowed to dry. Lids were taken off the plates and the media placed inside an airtight glass jar for $O_3$ exposure for 30 min at a flow rate 6 L/min as explained in Section 6.2.1 (a). The Petri plates were separated by 2-3 cm to ensure exposure of each plate. After exposure, plates were placed into plastic boxes which were maintained at the same $a_w$ levels with glycerol/water solutions (500 mL × 2) and stored at 30°C. Three agar plugs were taken every 12 h from each plate using cork-borer and placed on a glass microscope slide (Fisher, UK). The agar plugs were then stained with lactophenol cotton blue (ProLab Diagnostics, UK) and covered with a glass slip. Each plug was then examined under the microscope and germination was recorded. Spores were considered to have germinated when the length of the germ tube was longer than the diameter of the spore. A total of 150 single spores per replicate were examined (50 single spores per agar plug; 450 per treatment) (Bosch *et al.*, 1995; Marín *et al.*, 1998). The average germination was used to calculate the percentage germination in each $O_3$ treatment.

### 6.2.4 *In vitro* effects of $O_3$ on mycelial growth and $AFB_1$ contamination

PMA media were inoculated centrally with 10 µL of spore suspension made from AB3 strain and incubated at 30°C in replicates and allowed to grow for 2 and 5-6 days in the 0.98 and 0.93 $a_w$ treatments respectively. Measurements of colonies were recorded and plates were exposed to $O_3$ with no lids for 30 min using the system described in Section 6.2.1 (a). $O_3$ concentrations were 50, 100, 200 and air as a control. $A_w$ of media and ERH during incubation after $O_3$ exposure was adjusted to 0.93 and 0.98 $a_w$. After exposure, plates were covered and incubated at 30°C. Colony diameters were recorded on a daily basis for each treatment and compared with the control. Agar plugs were taken after day ten from each plate and stored at -20°C for $AFB_1$ analysis as described previously in Section 3.2.6.

### 6.2.5 *In situ* effect of $O_3$ on fungal population and $AFB_1$ production on irradiated pistachio nuts

120 g of gamma-irradiated raw pistachio nuts (12-15 KGys, Steris, Swindon,UK) were weighed and place in sterilised bottles (eight bottles) for each treatment (40 g per replicate) as summarised in Table 6.1. Pistachio nuts were rehydrated using the moisture adsorption curve and mix well and left overnight at 4°C to equilibrate to the target $a_w$ levels of 0.93 and 0.98 as described in Section 3.2.4. A conidial suspension of $10^6$ spores was prepared. After equilibration, 1 mL of the spore suspension was added to the pistachio nuts and mixed very well. One pistachio nut was taken and placed in a 10 mL sterile water containing tween 80 in a 25 mL Universal bottle for serial dilution to assess the populations of *A.flavus*

present. Three replicate of each treatment (40 g each) were exposed to $O_3$ (50, 100 and 200ppm, and air) for 30 min at a flow rate of 6 L/min as described in Section 6.2.1 (b).

**Table 6.1 Summary of *in situ* experimental design for the effect of $O_3$ on fungal population and $AFB_1$ production on irradiated stored pistachio nuts**

| | **0.93 $a_w$** | **0.98 $a_w$** |
|---|---|---|
| **Amount per treatment** | 120 g | 120 g |
| **Amount per replicate** | 40 g | 40 g |
| **Air (control; 0 ppm $O_3$)** | 40 g × 3 replicates = 120 g | 40 g × 3 replicates = 120 g |
| **50 ppm $O_3$** | 40 g × 3 replicates = 120 g | 40 g × 3 replicates = 120 g |
| **100 ppm $O_3$** | 40 g × 3 replicates = 120 g | 40 g × 3 replicates = 120 g |
| **200 ppm $O_3$** | 40 g × 3 replicates = 120 g | 40 g × 3 replicates = 120 g |

Immediately following exposure, a weighed pistachio nut was taken and placed in a Universal bottle for serial dilution of populations. The rest of the nuts were placed in solid culture vessels with microporous lids (Magenta, Sigma Ltd, UK; Figure 6.3). These vessels were previously autoclaved at 121°C for 15 min with an aluminium foil cover (Kimura and Hirano, 1988). Vessels containing samples were then placed in plastic chambers with glycerol/water solutions to maintain the ERH and treatments were stored for four weeks at 30°C before $AFB_1$ analysis. Total populations after this storage period was also examined as described

previously. The remaining pistachio nuts were stored at -20°C for later $AFB_1$ analysis.

Serial dilution samples were soaked for 20 mins and then vigorously shaken using a vortex mixer. From each treatment/replicate serial dilutions were made (-1, -2 and -3). For each concentration, three replicates were made and 100 µL was spread on MEA media using a sterile spreader and these were incubated at 30°C for seven days before colonies were counted for *A.flavus* populations (Figure 6.4). Table 6.2 summarises the conditions used in this study.


**Figure 6.3 Pistachio nuts placed in glass culture vessels with microporous lids.**


**Figure 6.4 Colonies of *A.flavus* being counted using a colony counter.**

**Table 6.2 Summary of *in situ* experimental conditions used for the effect of $O_3$ on fungal population and $AFB_1$ production on irradiated pistachio nuts.**

| Factor | Conditions |
|---|---|
| **$O_3$** | 50 , 100 and 200 + air (*control; 0 ppm $O_3$*) |
| **$A_w$** | 0.93 and 0.98 |
| **Time** | 30 min (*60 min exposure had similar result to 30 min in previous studies*) |
| **Temperature** | 30°C (*optimum temperature*) |
| **Strain** | AB3 |

Pistachio nuts were also direct-plated on MEA medium and incubated for seven days at 25°C to confirm the absence of contamination following irradiation (Figure 6.5).

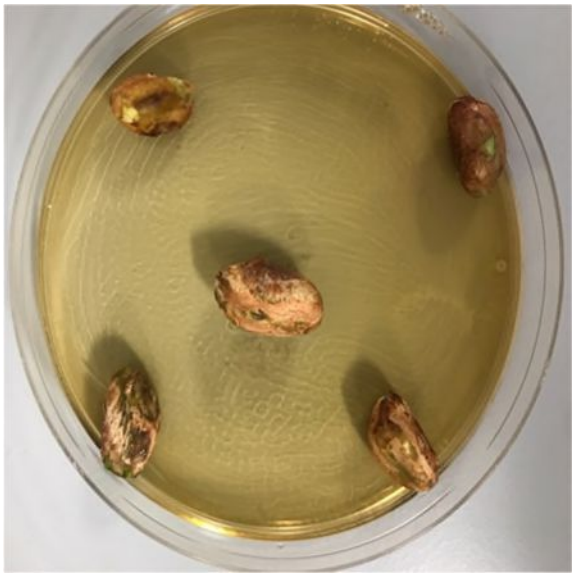

**Figure 6.5 Direct-plating of irradiated pistachio nuts on Malt Extract Agar. Picture was taken seven day after incubation at 25°C.**

### 6.2.6 Statistical analysis

Three replicates per treatment were used in all experimental studies. Means were obtained by taking the average of three measurements with the standard error of the means (± SE). Analysis of Variance (ANOVA) was applied to analyse the variation of means with 95% confidence interval. Normal distribution of data was checked by the normality test Kolmogorov-Smirnov using Minitab statistical software. Fisher's Least Significant Difference (LSD) was used to identify differences between the means with $p < 0.05$ as significant difference using the same statistical software.

## 6.3 RESULTS

### 6.3.1 Effect of $O_3$ on spores germination of *A.flavus in vitro*

Figure 6.6 shows the effect of $O_3$ treatment at 50, 100, 200 ppm and control (air) on the mean conidial germination of *A.flavus* AB3 on PMA media adjusted to 0.93

and 0.98 $a_w$ and incubated at 30°C for up to 48 h. $O_3$ treated samples showed lower germination percentages compared to the control at both $a_w$ levels. However, all treatments including the control showed a recovery in germination after 48 h for all treatments. No germination was recorded from samples treated with 200 ppm $O_3$ at 0.98 $a_w$ and all $O_3$ doses at 0.93 $a_w$ after 12 h. However, over time, there appeared to be a recovery with germination being delayed but not lethally affected.

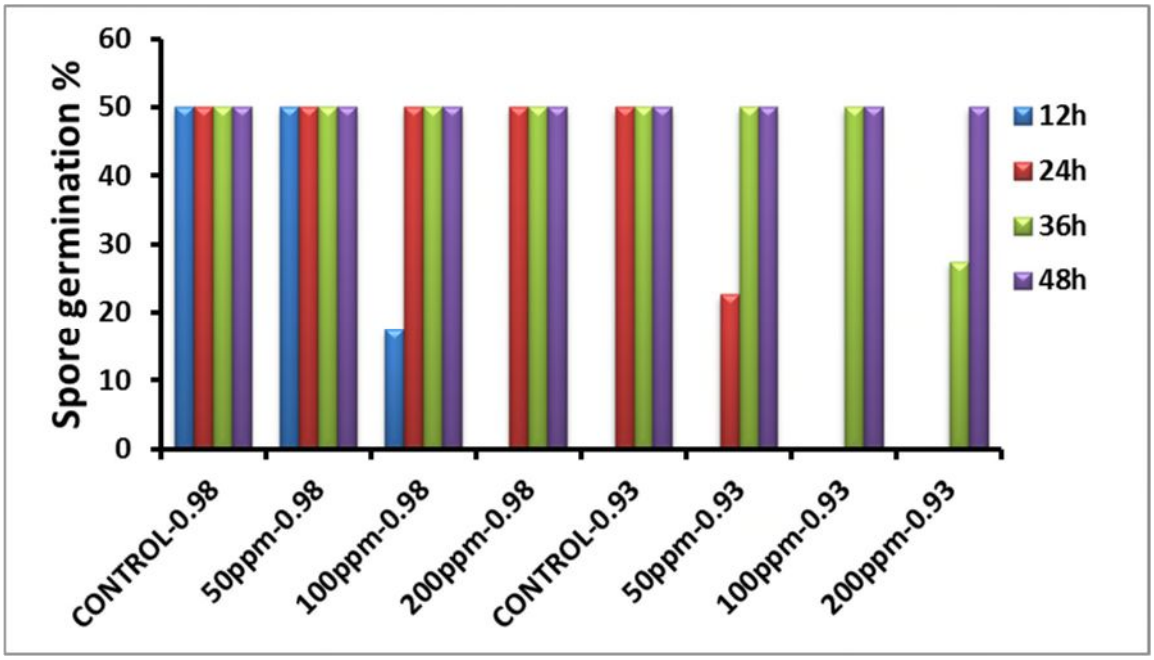


**Figure 6.6 Effect of ozone on spore germination of *Aspergillus flavus* AB3 at 0.93 and 0.98 $a_w$ following incubation at 30°C. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation.**

### 6.3.2 *In vitro* effect of $O_3$ on mycelial growth of *Aspergillus flavus* AB3

Figure 6.7 shows the effect of $O_3$ treatment on mycelial growth of *A.flavus* AB3 on PMA media at 30°C for 4-6 days at two $a_w$ levels (0.98; 0.93). Colonies were exposed to 50, 100, 200 ppm $O_3$ and compared with the control after 30 min exposure at 6 L/min prior to incubation. Mycelial extension was significantly inhibited after $O_3$ treatment when compared with the control at 0.98 $a_w$. However, while growth rates were markedly decreased with increasing $O_3$ concentration the effects were not significant. At 0.93 $a_w$, growth rates were slightly higher after $O_3$ treatment.

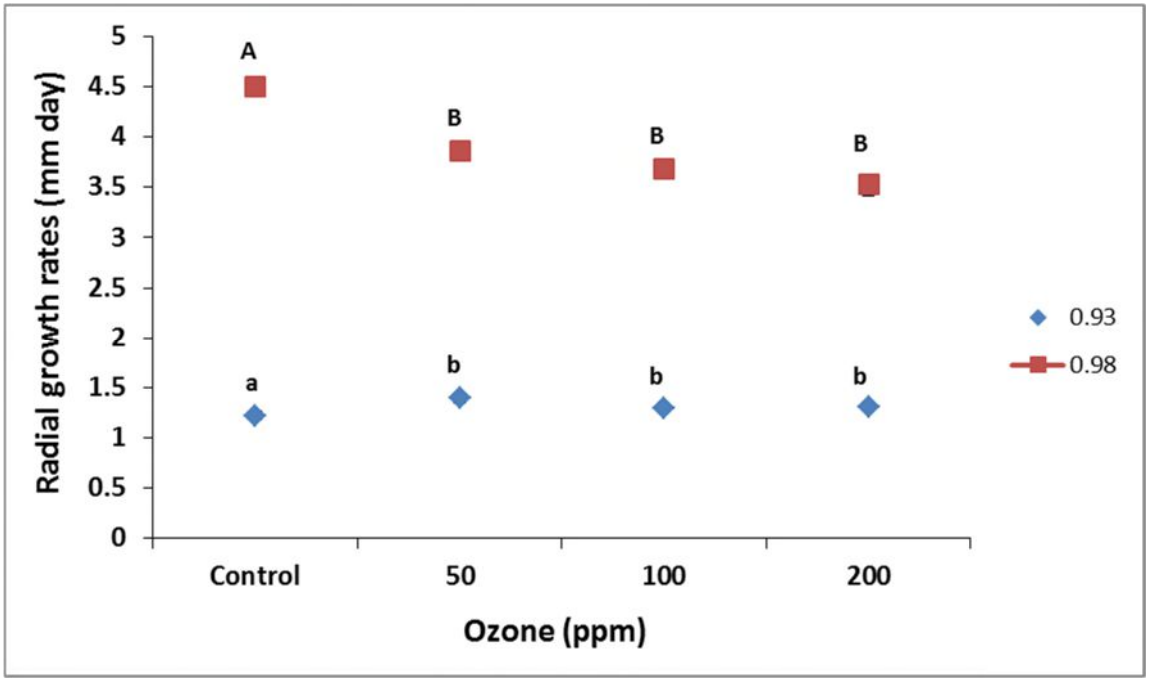


**Figure 6.7 Effect of ozone treatment on growth rate of *Aspergillus flavus* AB3 on pistachio-based media at 30°C for 4-6 days at two $a_w$ levels. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

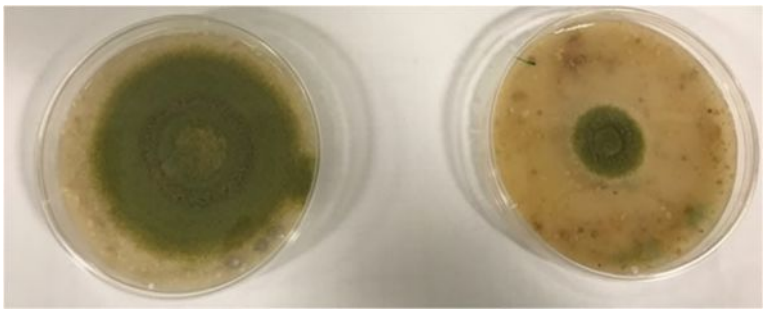

**Figure 6.8 Colony size of *Aspergillus flavus* on 90 mm Ø plates containing PMA adjusted to 0.98 $a_w$ (left) and 0.93 $a_w$ (right) following two days incubation at 30°C.**

### 6.3.3 *In vitro* effect of $O_3$ on $AFB_1$ contamination

The effect of $O_3$ treatment at 50, 100, 200 and control (air) on the production of $AFB_1$ by *A.flavus* AB3 is shown in Figure 6.9. $AFB_1$ amounts were significantly greater after $O_3$ treatments at all exposure levels when compared to the untreated control in the 0.98 $a_w$ treatment. However, at 0.93 $a_w$ there was no significant effect on toxin production.

### 6.3.4 *In situ* effect of $O_3$ on fungal population

Figure 6.10 shows the effect of $O_3$ exposure on the total populations of *A.flavus* AB3 (CFUs) isolated form the stored pistachio nut treatments before and after treatment. Total populations of *A.flavus* were slightly increased in control samples after exposure to air for 30 min compared with the control before exposure. Immediately after inoculation, a significant reduction of CFUs was observed from $\log_{10}$ 5.3 CFUs (control after exposure to air) to $\log_{10}$ 1.4 CFUs, after exposed to

50 ppm, and less than $log_{10}$ 1.0 CFUs after 100 and 200 ppm exposure at 0.98 $a_w$. For 0.93 $a_w$, populations did not change for the control before and after 30 min air exposure. However, total population was decreased significantly when treated with $O_3$. For both $a_w$ levels, it seemed that higher $O_3$ doses of 100 and 200 ppm did not have any increased efficacy when compared to 50 ppm $O_3$ exposure. Generally, $O_3$ treatment affected *A.flavus* populations, reducing the overall contamination levels, especially at 50 ppm $O_3$. There was also no significant difference between $O_3$ concentrations of exposure on the populations.

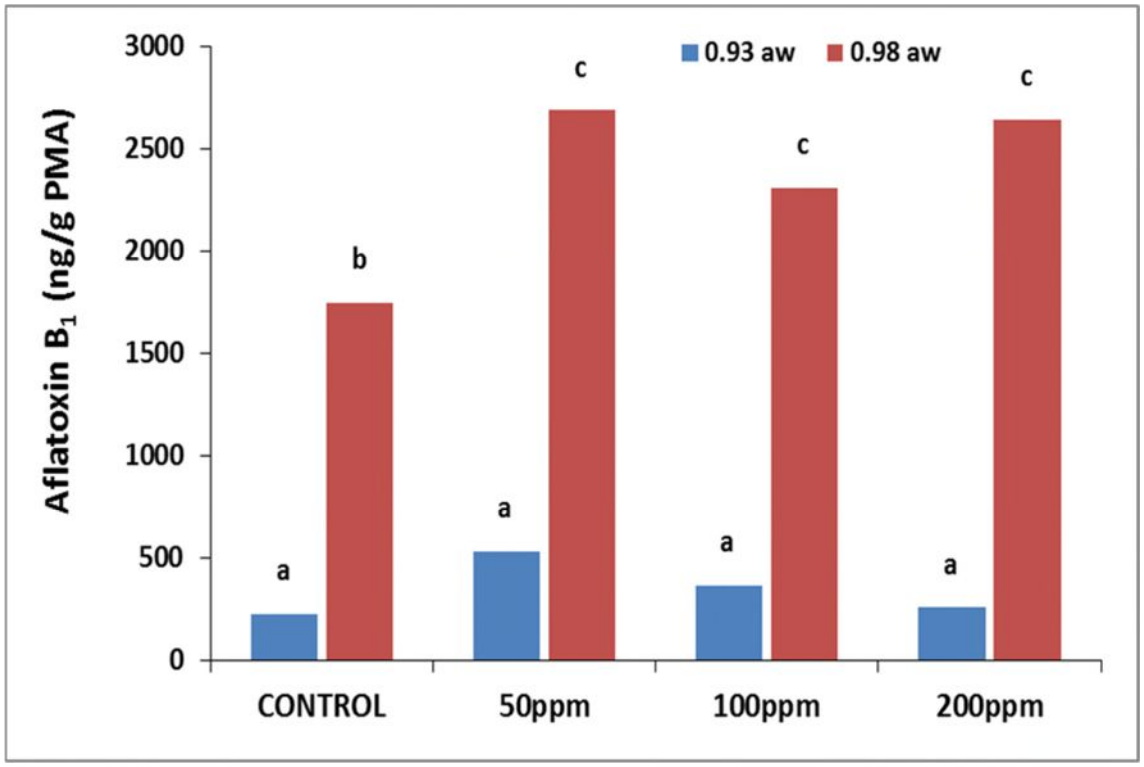


**Figure 6.9 Effect of ozone treatment on $AFB_1$ production by *Aspergillus flavus* AB3 at 0.93 and 0.98 $a_w$ on pistachio-based media following ten days of incubation at 30°C. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

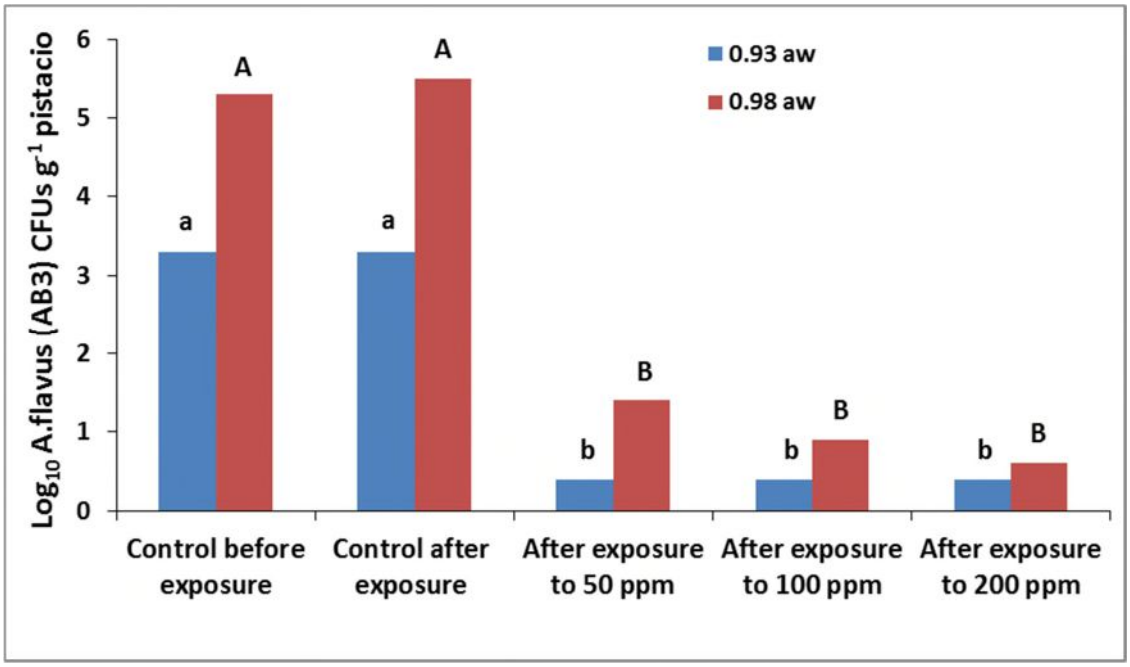


**Figure 6.10 Effect of ozone treatment on population of *Aspergillus flavus* AB3 at 0.93 and 0.98 $a_w$ on pistachio-based media following ten days of incubation at 30°C. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

### 6.3.5 *In situ* effect of $O_3$ on $AFB_1$ production on irradiated pistachio nuts

Figure 6.11 shows the effect of $O_3$ treatments on $AFB_1$ production at 0.93 and 0.98 $a_w$ after storage for four weeks at 30°C. $AFB_1$ contamination was always higher in the treated samples compared to the controls at both $a_w$ and all $O_3$ levels except for 50 ppm $O_3$. At this concentration and 0.98 $a_w$ the $AFB_1$ was significantly reduced.

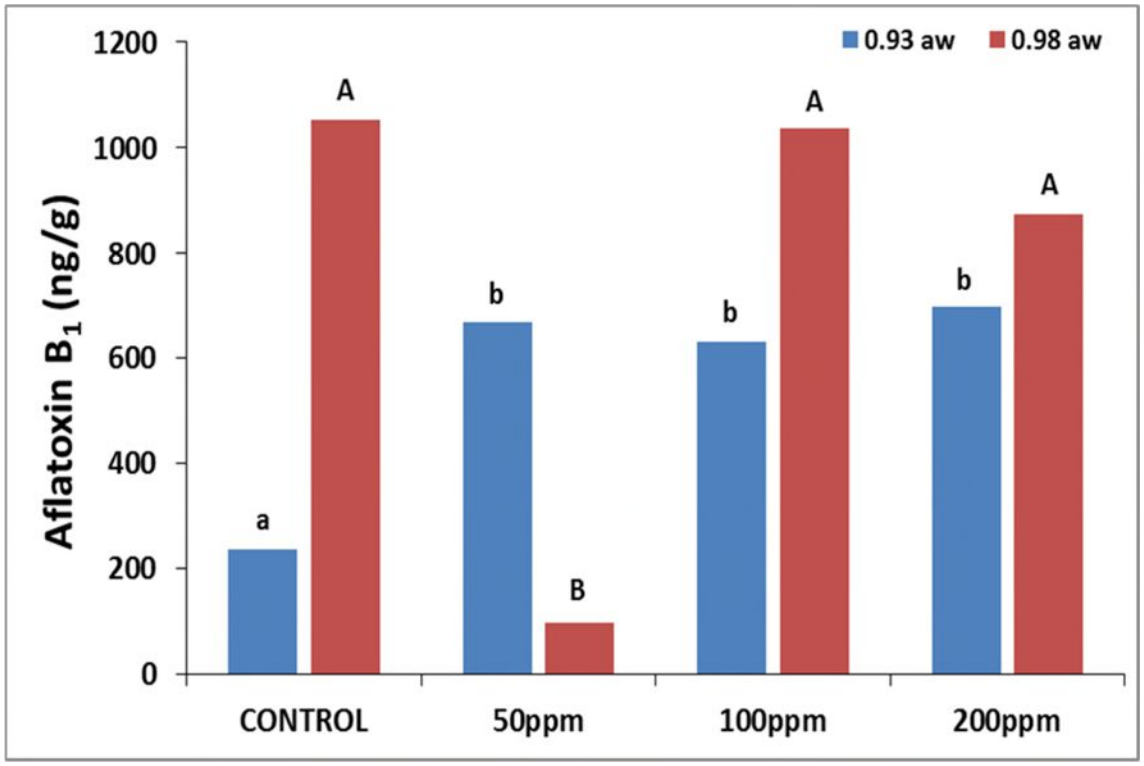


**Figure 6.11 Effect of ozone treatment on $AFB_1$ production by *Aspergillus flavus* AB3 at 0.93 and 0.98 aw on irradiated pistachio nuts following four weeks of incubation at 30°C. Colonies were exposed to ozone for 30 min at 6 L/min prior to incubation. Data are means of triplicates. Different letters indicate significant differences between treatments ($p < 0.05$) by Fisher's Least Significant Difference (LSD).**

## 6.4 DISCUSSION

### 6.4.1 *In vitro* effects on germination and mycelial extension

The efficacy of gaseous $O_3$ for the control of conidial germination and mycelial growth of *A.flavus* AB3 was evaluated in the present study. Treatment of 200 ppm $O_3$ at 0.98 $a_w$ showed complete inhibition of germinations after 12 h. However, spore viability appeared to recover and the germination was increased after 24 h and reached 100% after 48 h. Overall, exposure of conidia to $O_3$ initially had lower germination percentages when compared to the controls at both $a_w$ levels.

For mycelial growth, the study showed that the mycelial extension was significantly inhibited by $O_3$ treatment at 0.98 $a_w$. However, growth rates decreased with increasing $O_3$ dose.

Very few studies have studied the *in vitro* effect of $O_3$ on spore germination and growth of *A.flavus* and none on pistachio nut media. A previous study by Zotti et al. (2008) found that $O_3$ treatment of 3-day old *A.flavus* colonies for 3 h inhibited growth and spores completely. However, when the same colony was 6- and 9-days old, the efficacy decreased. Additionally, they found that there are different sensitivity levels among species with *A.flavus* being less sensitive than *A.niger*. However, in their study $a_w$ modification was not considered and the $O_3$ concentrations used was only 1 ppm. Mylona et al. (2014) examined the *in vitro* effect of $O_3$ treatment at 100 and 200 ppm for 30 min on spore germination of *F.verticillioides*. They reported that spore germination recovered over 8-10 days following incubation at 0.98 and 0.94 $a_w$ after initially observing complete inhibition after 24 h. They also found that doubling the exposure time did not improve the efficacy of $O_3$. Sultan (2010) found that the conidial germination by *A.flavus* strains from peanuts was significantly inhibited by 100-200 ppm $O_3$. However, they did not examine temporal effects on subsequent potential recovery of spore germination. Akbar (2016) exposed strains of *A.carbonarius* and *A.westerdijkiae*, responsible for ochratoxin contamination in coffee to up to 500 ppm $O_3$ and found that it was difficult to control the ochratoxigenic germination and growth of these species, regardless of $a_w$ level used or exposure period.

Sensitivity of fungal species to $O_3$ exposure may vary and depend on exposure period and concentration applied. It can also be influenced by water content and conidial morphology (Hibben and Stotzky, 1969; Antont-Babu and Singleton, 2009). Reduction of fungal growth can be obtained in high moisture after treatment with up to 1,000-15,000 ppm $O_3$ for 1 h (White et al., 2013). Giordano et al. (2012) found that $O_3$ treatment of Brazil nuts affected growth of the mycobiota and decreased aflatoxin amounts. They also found that exposure for a period of 5 h was effective and inhibited growth of *A.flavus* and *A.parasiticus*, although they were still able to grow during the first days after exposure. Thus $O_3$ levels, exposure time together with the other influencing factors, including temperature and ERH, need to be investigated deeper on fungal growth and toxin production. Aldars-García et al (2017) used a predictive mycology strategy to investigate the probability of detection between germination, growth and $AFB_1$ production by *A.flavus* on pistachio extract media. They found that $AFB_1$ production is a stage far from germination. Their model showed that when growth has started, $AFB_1$ was started to be produced after six days. In percentage, 40-57% $AFB_1$ detection probability was predicted when growth probability is 100%.

### 6.4.2 *In vitro* and *in situ* effects of $O_3$ on $AFB_1$ contamination

In the present study, $AFB_1$ was analysed after *in vitro* exposure of colonies of *A.flavus* to $O_3$ for ten days at 30°C. There appeared to be variable effects on $AFB_1$ production during exposure to $O_3$ treatment. The increase in toxin may be due to $O_3$ exposure acting as an environmental stress resulting in the biosynthesis of more toxin as a defence reaction. Also it may be that the $O_3$ has

an effect on the pistachio-based medium changing the nutritional matrix. Previously, Sultan at el. (2010) examined *A.flavus* under exposure to $O_3$ (100-300 ppm). However, in these studies a condusive YES medium was used. In this case, use of a defined medium + exposure to $O_3$ resulted in a significant decrease of $AFB_1$ production in mycelial colonies. However, Sultan (2010) analysed the toxin after just three days while in the present study this was extended to ten days on a pistachio-nut based medium to simulate nutritional conditions. This could partially explain the differences in the outcome between the two studies. A previous study by Mason et al. (1997) showed that exposure of *A.flavus* colonies for five days inhibited asexual conidial sporulation. This suggests that perhaps the effect of $O_3$ on the whole life cycle of *A.flavus* would provide useful information on which phase might be more sensitive to such treatment. The present study is the first to examine in detail the *in situ* effect of gaseous $O_3$ on colonisation and toxin production by *A.flavus* in stored pistachio nuts. Overall, while the populations of *A.flavus* were significantly decreased by $O_3$ exposure, there was little difference between 50-200 ppm treatment levels. Indeed, a reduction in $AFB_1$ was only observed in the 50 ppm $O_3$ × 0.98 $a_w$ treatment. It may be that a reduction in overall populations of *A.flavus* allows more rapid subsequent colonisation by the surviving inoculum on the rich nutrient source. In naturally contaminated pistachio nuts (see Chapter 2) the mycobiota highly varied. Thus the niche will be occupied by a fungal community including *A.flavus* and it would have to compete with these other fungi, some of which may survive $O_3$ treatment. Thus, further studies may be required to better understand the relationship

between exposure concentration × time of exposure and prevailing $a_w$ level to determine the efficacy in terms of toxin control.

The studies by Mylona et al. (2014) certainly suggest that natural contamination of maize grain can be reduced by exposure to $O_3$ concentrations. However, it may be more difficult to reduce mycotoxin production by specific fungi without longer term exposure to $O_3$. Akbar (2016) found that even with 500 ppm $O_3$ it was very difficult to significantly reduce ochratoxin A contamination of stored coffee by colonisation by *A.westerdijkiae* and *A.carbonarius*. The capability for perhaps relatively rapid DNA repair after exposure may be responsible for viability of some conidia to be conserved after exposure. This could be related to pigmentation or repair systems which help the cells to recover and subsequently grow and colonise the substrate.

# CHAPTER 7
# Final conclusions, future work and publications

## 7.1 FINAL CONCLUSIONS

The overall objectives of this work were achieved in the programme of work carried out. The biodiversity of mycobiota on pistachio nut samples collected from different sources in the KSA was examined in detail for the first time and demonstrated the presence of a range of aflatoxigenic strains of *A.flavus* which were confirmed using molecular techniques. A detailed study of the ecology of growth and $AFB_1$ production by these strains were determined and showed that growth and toxin production occurred in pistachio-based matrices in the presence of both non-ionic and ionic solutes. This suggests that even salted pistachios may be at risk from toxin contamination under conducive storage conditions if *A.flavus* is present. The studies on pistachio nuts largely confirmed *in vitro* studies and the boundary conditions for growth and $AFB_1$ was established. This suggests that growth and toxin production occurs at higher temperatures than that seen previously in strain from maize and groundnuts. This formed the foundation for subsequent studies to examine the impact of interacting climate change (CC) environmental factors on growth and toxin production for the first time. This showed that CC effects varied with strain of *A.flavus* and in some cases growth only or growth and $AFB_1$ production was modified by CC environmental factors. The stimulation of $AFB_1$ production by some strains of *A.flavus* is similar to that seen previously for *A.flavus* strains on maize where growth was not affected but toxin production was significantly stimulated. Also this study showed that strains

of *A.flavus* from pistachio nuts grew effectively at much higher temperatures than expected from previous studies on strains from maize. The acclimatisation study is the only one to have been carried out with *A.flavus*. Although only 5 generations were used in these studies there were some indications that growth and perhaps $AFB_1$ production may be stimulated when the strains have been adapted or more tolerant to CC by repeated cultivation in these environmental conditions. For control strategies gaseous $O_3$ was examined as a potential method for controlling the different phases of growth. These studies suggested that while it was possible to temporarily inhibit germination of conidia of *A.flavus* they remained viable and could recover and germinate subsequently. While populations of *A.flavus* were significantly decreased by 50-200 ppm $O_3$ when exposed for 30 mins there was enough inoculum remaining to result in significant $AFB_1$ contamination. This suggests that perhaps longer time frames may be needed for exposure to kill conidia and significantly reduce conidial viability or much higher concentrations are required which would have human exposure consequences and thus dangerous. Also, since pistachio nut flavour is very important because it is a high value nut product, any tainting due to $O_3$ reactions with lipids in the nuts would reduce the quality. Alternative gases, e.g. $SO_2$, could be employed to examine whether efficacy would be effective for germination and toxin control in stored pistachio nuts.

Summary of detailed Conclusions from each Chapter are given below:

**CHAPTER 2: Biodiversity of mycobiota on pistachio nut samples from different regions obtained in the KSA**

a) The mycobiota levels in the present study were relatively low when salted pistachio nuts were examined. Despite this, 10 different *A.flavus* species were present and isolated from most of the samples.

b) All isolates were confirmed molecularly using ITS regions as *A.flavus* strains.

c) *A.flavus* strains isolates showed their capability to produce $AFB_1$ under UV light on Coconut Cream Agar**.**

d) Quantitative analysis using HPLC proved that $AFB_1$ was detected from those samples, although the concentrations were below the EU legislative limits.

**CHAPTER 3: *In vitro* and *in situ* effects of temperature × water activity interactions on growth, *aflR* gene expression and aflatoxin $B_1$ production by *Aspergillus flavus* isolates**

a) The interaction between temperature × $a_w$ had a significant impact on *A.flavus* growth and $AFB_1$ production.

b) *A.flavus* was able to grow effectively when incubated at 25-35°C over a range of $a_w$ levels, both *in vitro* and *in situ*.

c) Growth and toxin production occurred on pistachio-based media when ionic or non-ionic solutes were used suggesting good tolerance to NaCl.

d) Colonisation rate for *in vitro* was generally greater than rates *in situ.*

e) The range of conditions for $AFB_1$ production was narrower with optimum production at 30°C in pistachio-based media, at 0.98 and 0.955 $a_w$; and at 35°C + 0.98 $a_w$ by strain AB3 on pistachio nuts.

f) $AFB_1$ production was considerably higher *in vitro* when compared with the amounts produced *in situ*.

g) The study suggested that there is an impact of temperatures and $a_w$ on the biosynthetic pathway involved in secondary metabolite production by *A.flavus* based on the results obtained for expressions of the regulatory *aflR* gene in the *in vitro* studies

**CHAPTER 4: *In vitro* and *in situ* effects of climate change interacting factors on growth, *aflD* and *aflR* gene expression and aflatoxin $B_1$ production by two *Aspergillus flavus* strains**

a) The relative expression of the biosynthetic genes (*aflD* and *aflR),* and $AFB_1$ production were affected by interacting CC interaction factors.

b) Growth of *A.flavus* was not significantly affected by the interactions.

c) The expression of the structural gene *aflD* was generally related to $AFB_1$ quantities.

d) $CO_2$ affects $AFB_1$ production because there was an increase in toxin amounts at 35°C + 1000 ppm $CO_2$ at 0.98 $a_w$ when compared to the control (400 ppm $CO_2$).

**CHAPTER 5: Effect of acclimatisation in elevated $CO_2$ on growth and aflatoxin $B_1$ production by *Aspergillus flavus* strains on pistachio nuts**

a) The results of the study differed for two strains, both isolated from pistachio nuts.

b) One strain was showed faster growth and clear stimulation of $AFB_1$ production after 5 generations of acclimatisation while the other strain showed no difference from the control.

c) This study suggests that the effect of acclimatisation needs more investigation.

**CHAPTER 6: Use of gaseous ozone ($O_3$) to control germination, mycelial growth and aflatoxin $B_1$ contamination *in vitro* and in stored pistachio nuts under different environmental conditions**

a) Exposure of conidia to $O_3$ (50-200 ppm) initially inhibited germination when compared to the controls at both $a_w$ levels.

b) Complete inhibition of germinations was observed after 12 h treatment of 200 ppm $O_3$ at 0.98 $a_w$.

c) Spore viability appeared to recover and the germination subsequently increased after 24 h and reached 100% after 48 h.

d) Growth rates decreased with increasing $O_3$ exposure dose and mycelial extension was significantly inhibited by $O_3$ treatment at 0.98 $a_w$.

e) Variable effects on $AFB_1$ production after exposure to $O_3$ treatment occurred in the *in vitro* experiments with colonies of *A.flavus* incubated for ten days at 30°C.

f) $O_3$ exposure may act as an environmental stress resulting in the biosynthesis of more toxin as a defence reaction. Also it may be that the $O_3$ has an effect on the pistachio-based medium changing the nutritional matrix.

g) The populations of *A.flavus,* when inoculated onto pistachio nuts, were significantly decreased by $O_3$ exposure; however, there was little difference between 50-200 ppm treatment levels.

h) A reduction in $AFB_1$ was only observed in the 50 ppm $O_3$ × 0.98 $a_w$ treatment in the *in situ* study.

i) The relationship between exposure concentration × time of exposure and prevailing $a_w$ level to determine the efficacy in terms of toxin control needs better understanding.

j) Alternative treatments are needed to try and minimise $AFB_1$ contamination of pistachios during the drying and storage phase

## 7.2 Future recommendations

b) A more detailed survey of the relative contamination of pistachio nuts from different sources is required.

c) Examination of the impact of temperature × $a_w$ × $CO_2$ on isolates from pistachio nuts and on $AFB_1$ stimulation on stored pistachio nuts under climate change conditions.

d) Acclimatisation for 10-20 generations in elevated $CO_2$ is required to better understand the development of tolerance and effects on secondary metabolite production in CC scenarios.

e) The data obtained in this study could be effectively utilised to develop prediction model of the relative risk of growth and $AFB_1$ contamination during storage as part of a Decision Support System

f) In terms of impacts of CC scenarios, more studies are necessary to examine whether other toxins such as cyclopiazonic acid which is also produced by *A.flavus* trains could become more important or whether the ratios of different toxins changes. More detailed molecular ecology approaches with the use of RNAseq approaches to better understand the possible role of acclimatisation in relation to key biosynthetic pathways.

g) Alternative more effective gaseous treatments are necessary to try and control growth and minimise $AFB_1$ contamination of pistachios, especially during the critical drying and storage phases.

## 7.3 PUBLICATION AND TRAINING

1. Medina, A., Akbar, A., Baazeem, A., Rodriguez, A. & Magan, N. (2017). Climate change, food security and mycotoxins: do we know enough? ***Fungal Biology Reviews 31, 143-154.***

2. Abstract submitted, and poster presented at the *British Mycological Society (BMS) Annual scientific Meeting, 10th -13th Septemper 2013, Cardiff,* ***WALES***

Cranfield Health

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# Mycobiota and aflatoxigenic strains of *A.flavas* from pistachio nuts from different origins collected from Saudi Arabia

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## Introduction

Pistachio nuts (*Pistacia vera L.*), is perishable if infected by fungi whether in the orchard or during other stages including improper conditions of storage and transportation. Nowadays, Pistachio nuts play significant rule in the income of the production countries such as USA, Iran and Syria. Nuts are very commonly colonized by spoilage mycobiota especially aflatoxigenic species because of the very hygroscopic and can adsorb water. This can influence people health and world trade between producers and importers. This study has examined the mycobiota and the aflatoxin producing strains of *Aspergillus* section *Flavi* species isolated from different production regions including Iran, Syria and USA collected from different markets in Saudi Arabia.

## Methods/Materials

A number of 8 samples of pistachio collected from different markets in Saudi Arabia were used in this study. Samples were imported to Saudi from USA, Iran and Syria (500g of each sample).

***Direct plating:*** Samples were directly plated onto Petri plates on malt extract agar (MEA) and dichloran 18% glycerol agar (DG18). Shells were also separately plated directly on the same media. The frequency of isolated fungi was recorded after incubation at 25° C for 7 days.

***Serial dilutions:*** The colonisation was assessed as colony- forming units (CFU) g-1 of dry weight pistachio out of shells. Each sample was homogenised using Colworth Stomacher 400 (10g in 90ml) and the dilutions were made. An amount of 200 µl of each dilution was spread plated on MEA and DG18 in triplicate. A colony counter was used for total counts after incubation at 25° C for 7–10 days and microscopic examinations were made for fungal genera classification. Individual colonies were sub-cultured for detailed examination.

***Toxigenic potential of isolated A. flavas strains:*** AF production was screened using the fluorescence technique, based on growth on a coconut cream agar medium. A total of 4 isolates and a type strain were grown on coconut cream agar (CCA) for 7-10 days. Ultra violet light was used in the dark at 360 nm to check whether or not any blue fluorescence occurred on the reverse side of the colony. This was an indication that aflatoxin was being produced.

***Quantitative analyses:*** AF analyses were done using HPLC. Two replicates of each sample were analysed by AF extraction using aflaprep columns (Neogene Europe, wide bore) and derivatised using the method of AOAC (2000). For the four isolated strains, Eight plugs were extracted from each colony from each strain after 10 days' incubation at 25° C on yeast extract sucrose (YES) and transferred to a 2-ml Eppendorf tubes and weighed. For AF extraction, 1ml of chloroform was added to the plugs in each Eppendorf and shaken well for 1 h. The chloroform extract was transferred to new Eppendorfs 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 containing 200 ng B1, 60 ng B2, 200 ng G1 and 60 ng G1 was dried under nitrogen gas and derivatised as for samples. 200µl/ml was the concentration injected for HPLC.

## Results

**Figure 1.** Total fungal populations (Log$_{10}$ CFUs g$^{-1}$ dry weighted) isolated from different pistachio samples based on serial dilutions on two different media, DG18 and MEA, incubated at 25ºC for 5-7 days.

**Figure 3.** Frequency of isolation of dominant fungi from different pistachio shells based on direct plating on Dichloran glycerol DG18 agar base (DG18) and Malt extract agar (MEA) medium incubated at 25° C for 7 days.

**Figure 2.** Frequency of isolation of dominant fungi from different pistachio samples based on direct plating on Dichloran glycerol DG18 agar base (DG18) and Malt extract agar (MEA) medium incubated at 25° C for 7 days.

**Figure 4.** HPLC result for pistachio samples showing the detected amount of AFB1 (ng/g) for each sample.

## Conclusions

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**Acknowledgements** A. Baazeem wishs to acknowledge the support and supervision received from Prof. N. Magan and Dr. A. Medina.

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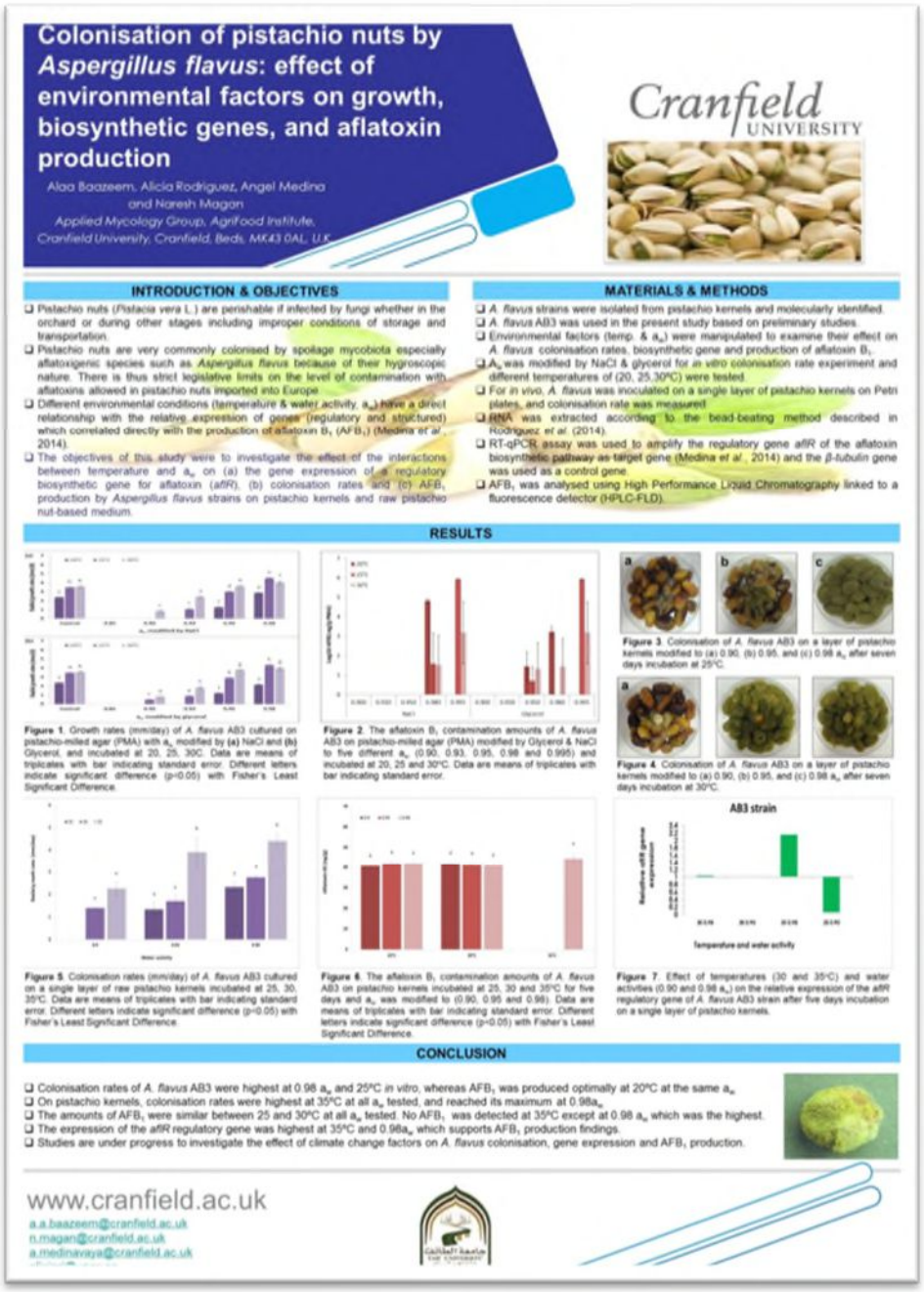
3. Abstract submitted, and poster presented at the *British Mycological Society (BMS) Annual scientific Meeting, International Meeting on the Invasive Fungus, 7th – 9th September 2015, Manchester,* ***ENGLAND****.*

4. The same poster was presented on the *Science for the Green Economy Conference – A National Debate in Technology and Governance for the Green Economy, 13th and 14th January 2016, Cranfield,* ***ENGLAND***



**Colonisation of pistachio nuts by *Aspergillus flavus*: effect of environmental factors on growth, biosynthetic genes, and aflatoxin production**

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**INTRODUCTION & OBJECTIVES**

- Pistachio nuts (*Pistacia vera* L.) are perishable if infected by fungi whether in the orchard or during other stages including improper conditions of storage and transportation.
- Pistachio nuts are very commonly colonised by spoilage mycobiota especially aflatoxigenic species such as *Aspergillus flavus* because of their hygroscopic nature. There is thus strict legislative limits on the level of contamination with aflatoxins allowed in pistachio nuts imported into Europe.
- Different environmental conditions (temperature & water activity, $a_w$) have a direct relationship with the relative expression of genes (regulatory and structured) which correlated directly with the production of aflatoxin $B_1$ ($AFB_1$) (Medina *et al.*, 2014).
- The objectives of this study were to investigate the effect of the interactions between temperature and $a_w$ on (a) the gene expression of a regulatory biosynthetic gene for aflatoxin (*aflR*), (b) colonisation rates and (c) $AFB_1$ production by *Aspergillus flavus* strains on pistachio kernels and raw pistachio nut-based medium.

**MATERIALS & METHODS**

- *A. flavus* strains were isolated from pistachio kernels and molecularly identified.
- *A. flavus* AB3 was used in the present study based on preliminary studies.
- Environmental factors (temp. & $a_w$) were manipulated to examine their effect on *A. flavus* colonisation rates, biosynthetic gene and production of aflatoxin $B_1$.
- $A_w$ was modified by NaCl & glycerol for *in vitro* colonisation rate experiment and different temperatures of (20, 25,30ºC) were tested.
- For *in vivo*, *A. flavus* was inoculated on a single layer of pistachio kernels on Petri plates, and colonisation rate was measured.
- RNA was extracted according to the bead-beating method described in Rodriguez *et al.* (2014).
- RT-qPCR assay was used to amplify the regulatory gene *aflR* of the aflatoxin biosynthetic pathway as target gene (Medina *et al.*, 2014) and the *β-tubulin* gene was used as a control gene.
- $AFB_1$ was analysed using High Performance Liquid Chromatography linked to a fluorescence detector (HPLC-FLD).

**RESULTS**



**Figure 1.** Growth rates (mm/day) of *A. flavus* AB3 cultured on pistachio-milled agar (PMA) with $a_w$ modified by **(a)** NaCl and **(b)** Glycerol, and incubated at 20, 25, 30C. Data are means of triplicates with bar indicating standard error. Different letters indicate significant difference ($p<0.05$) with Fisher's Least Significant Difference.



**Figure 2.** The aflatoxin $B_1$ contamination amounts of *A. flavus* AB3 on pistachio-milled agar (PMA) modified by Glycerol & NaCl to five different $a_w$ (0.90, 0.93, 0.95, 0.98 and 0.995) and incubated at 20, 25 and 30ºC. Data are means of triplicates with bar indicating standard error.



**Figure 3.** Colonisation of *A. flavus* AB3 on a layer of pistachio kernels modified to (a) 0.90, (b) 0.95, and (c) 0.98 $a_w$ after seven days incubation at 25ºC.



**Figure 4.** Colonisation of *A. flavus* AB3 on a layer of pistachio kernels modified to (a) 0.90, (b) 0.95, and (c) 0.98 $a_w$ after seven days incubation at 30ºC.



**Figure 5.** Colonisation rates (mm/day) of *A. flavus* AB3 cultured on a single layer of raw pistachio kernels incubated at 25, 30, 35ºC. Data are means of triplicates with bar indicating standard error. Different letters indicate significant difference ($p<0.05$) with Fisher's Least Significant Difference.



**Figure 6.** The aflatoxin $B_1$ contamination amounts of *A. flavus* AB3 on pistachio kernels incubated at 25, 30 and 35ºC for five days and $a_w$ was modified to (0.90, 0.95 and 0.98). Data are means of triplicates with bar indicating standard error. Different letters indicate significant difference ($p<0.05$) with Fisher's Least Significant Difference.




**Figure 7.** Effect of temperatures (30 and 35ºC) and water activities (0.90 and 0.98 $a_w$) on the relative expression of the *aflR* regulatory gene of *A. flavus* AB3 strain after five days incubation on a single layer of pistachio kernels.

**CONCLUSION**

- Colonisation rates of *A. flavus* AB3 were highest at 0.98 $a_w$ and 25ºC *in vitro*, whereas $AFB_1$ was produced optimally at 20ºC at the same $a_w$.
- On pistachio kernels, colonisation rates were highest at 35ºC at all $a_w$ tested, and reached its maximum at 0.98$a_w$.
- The amounts of $AFB_1$ were similar between 25 and 30ºC at all $a_w$ tested. No $AFB_1$ was detected at 35ºC except at 0.98 $a_w$ which was the highest.
- The expression of the *aflR* regulatory gene was highest at 35ºC and 0.98$a_w$ which supports $AFB_1$ production findings.
- Studies are under progress to investigate the effect of climate change factors on *A. flavus* colonisation, gene expression and $AFB_1$ production.



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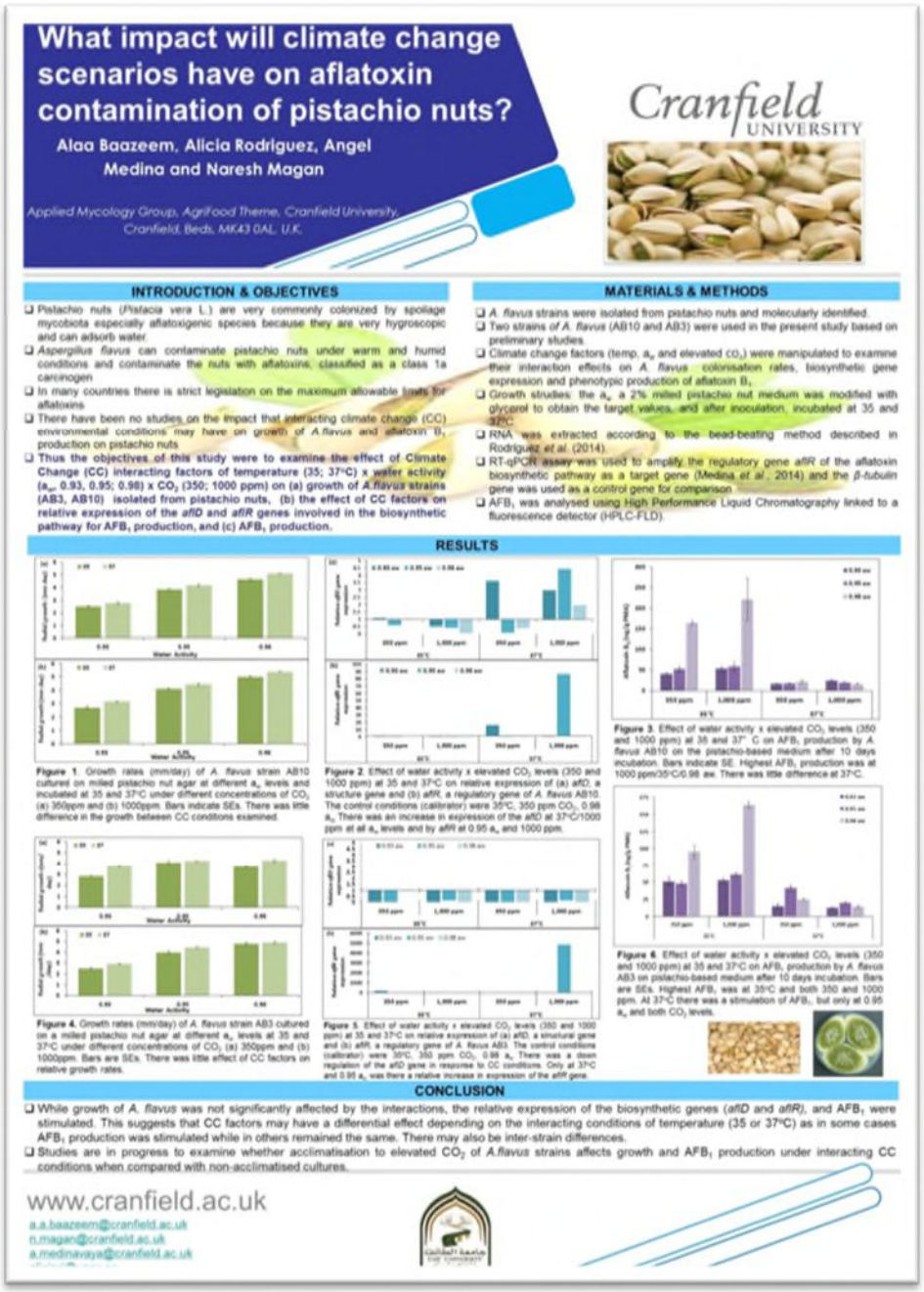
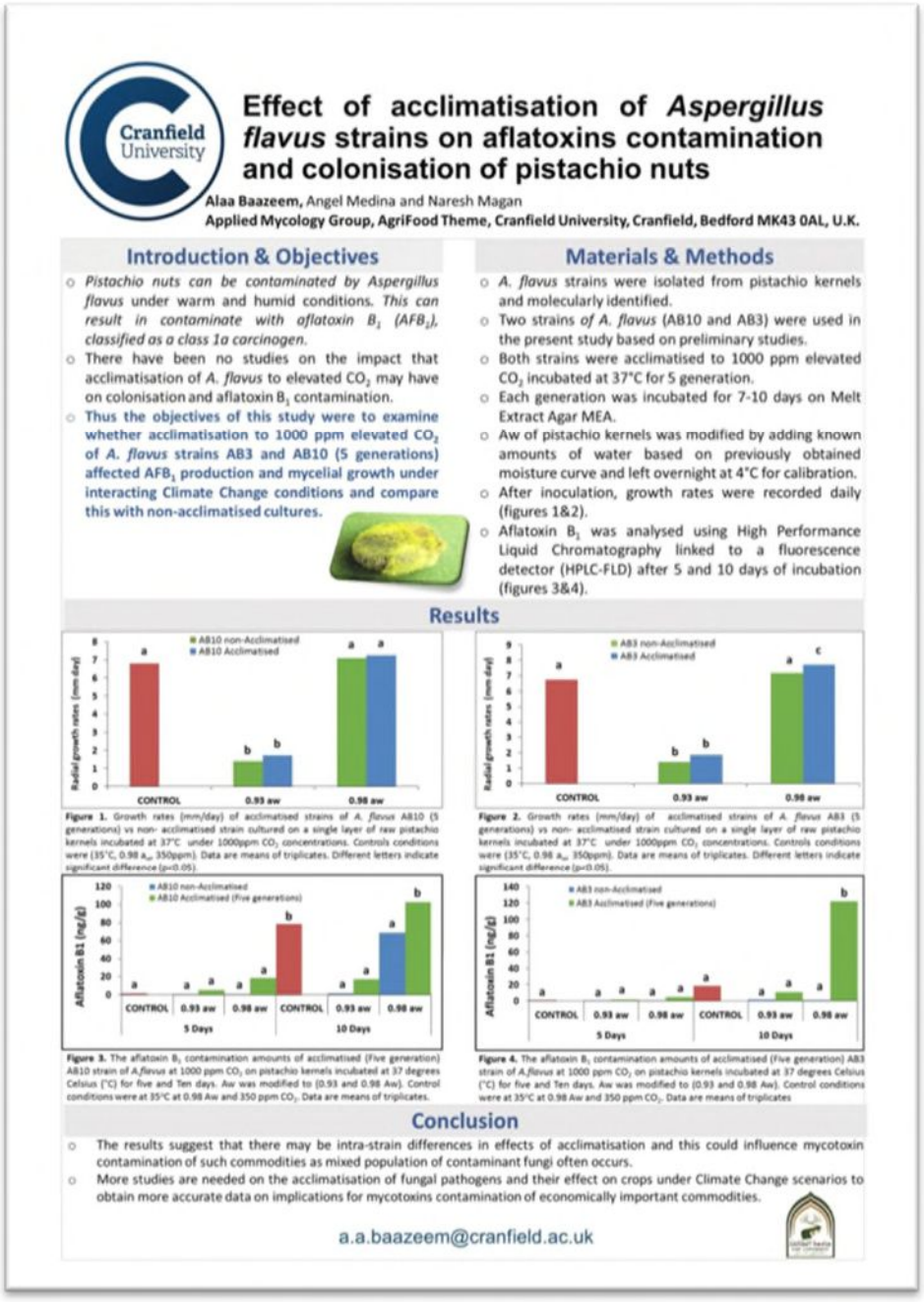
5. Abstract submitted, and poster presented at the *World Mycotoxins Forum (WMF), 6th – 10th June 2016, Winnipeg,* ***CANADA***.

6. The same poster was presented on the *Microbiology Society event, Focused Meeting 2016: The Dynamic Fungus, 5th – 7th September 2016. Exeter,* ***ENGLAND***



7. Abstract submitted, and poster presented at *1ST MYCOKEY Conference, 11th – 15th September 2017, Ghent,* ***BELGIUM****.*



# Effect of acclimatisation of *Aspergillus flavus* strains on aflatoxins contamination and colonisation of pistachio nuts

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## Introduction & Objectives

- *Pistachio nuts can be contaminated by Aspergillus flavus* under warm and humid conditions. *This can result in contaminate with aflatoxin* $B_1$ *(*$AFB_1$*), classified as a class 1a carcinogen.*
- There have been no studies on the impact that acclimatisation of *A. flavus* to elevated $CO_2$ may have on colonisation and aflatoxin $B_1$ contamination.
- **Thus the objectives of this study were to examine whether acclimatisation to 1000 ppm elevated $CO_2$ of *A. flavus* strains AB3 and AB10 (5 generations) affected $AFB_1$ production and mycelial growth under interacting Climate Change conditions and compare this with non-acclimatised cultures.**

## Materials & Methods

- *A. flavus* strains were isolated from pistachio kernels and molecularly identified.
- Two strains *of A. flavus* (AB10 and AB3) were used in the present study based on preliminary studies.
- Both strains were acclimatised to 1000 ppm elevated $CO_2$ incubated at 37°C for 5 generation.
- Each generation was incubated for 7-10 days on Melt Extract Agar MEA.
- Aw of pistachio kernels was modified by adding known amounts of water based on previously obtained moisture curve and left overnight at 4°C for calibration.
- After inoculation, growth rates were recorded daily (figures 1&2).
- Aflatoxin $B_1$ was analysed using High Performance Liquid Chromatography linked to a fluorescence detector (HPLC-FLD) after 5 and 10 days of incubation (figures 3&4).

## Results




**Figure 1.** Growth rates (mm/day) of acclimatised strains of *A. flavus* AB10 (5 generations) vs non- acclimatised strain cultured on a single layer of raw pistachio kernels incubated at 37°C under 1000ppm $CO_2$ concentrations. Controls conditions were (35°C, 0.98 $a_w$, 350ppm). Data are means of triplicates. Different letters indicate significant difference ($p<0.05$).




**Figure 2.** Growth rates (mm/day) of acclimatised strains of *A. flavus* AB3 (5 generations) vs non- acclimatised strain cultured on a single layer of raw pistachio kernels incubated at 37°C under 1000ppm $CO_2$ concentrations. Controls conditions were (35°C, 0.98 $a_w$, 350ppm). Data are means of triplicates. Different letters indicate significant difference ($p<0.05$).




**Figure 3.** The aflatoxin $B_1$ contamination amounts of acclimatised (Five generation) AB10 strain of *A.flavus* at 1000 ppm $CO_2$ on pistachio kernels incubated at 37 degrees Celsius (°C) for five and Ten days. Aw was modified to (0.93 and 0.98 Aw). Control conditions were at 35°C at 0.98 Aw and 350 ppm $CO_2$. Data are means of triplicates.




**Figure 4.** The aflatoxin $B_1$ contamination amounts of acclimatised (Five generation) AB3 strain of *A.flavus* at 1000 ppm $CO_2$ on pistachio kernels incubated at 37 degrees Celsius (°C) for five and Ten days. Aw was modified to (0.93 and 0.98 Aw). Control conditions were at 35°C at 0.98 Aw and 350 ppm $CO_2$. Data are means of triplicates

## Conclusion

- The results suggest that there may be intra-strain differences in effects of acclimatisation and this could influence mycotoxin contamination of such commodities as mixed population of contaminant fungi often occurs.
- More studies are needed on the acclimatisation of fungal pathogens and their effect on crops under Climate Change scenarios to obtain more accurate data on implications for mycotoxins contamination of economically important commodities.

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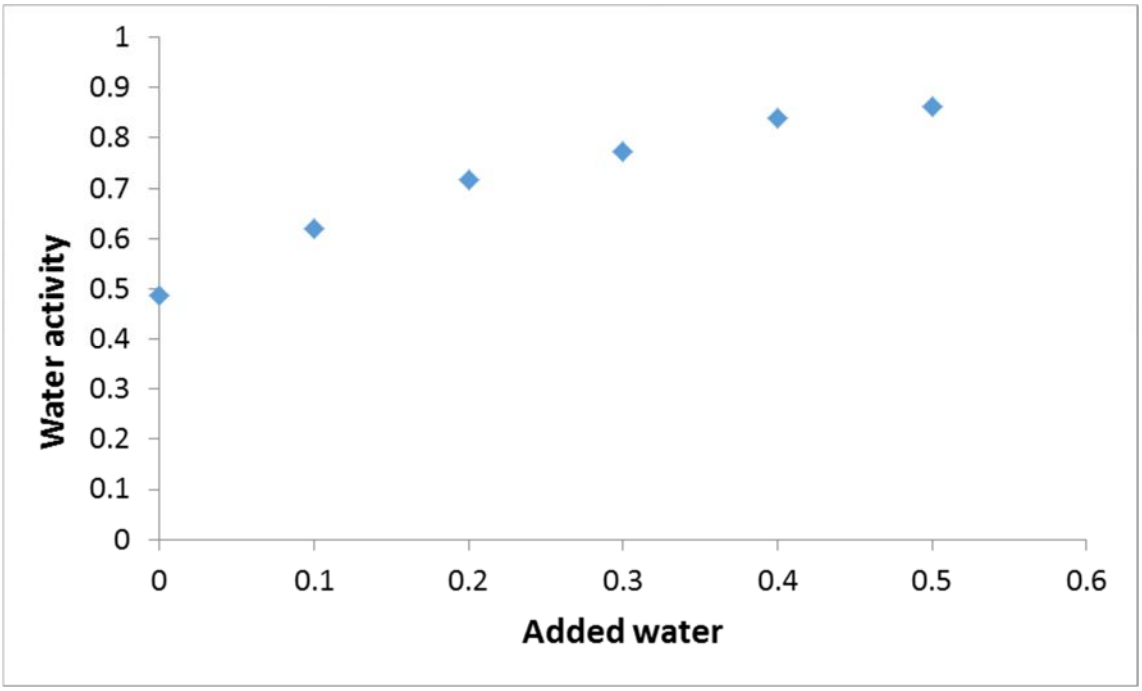
# APPENDIX A




Appendix Figure 1. Water activities of salted pistachio after addition of different amounts of water (ml/5g) per pistachio nut samples