CRANFIELD UNIVERSITY

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MOLECULAR ECOLOGY OF *ASPERGILLUS* SECTION *FLAVI* SPECIES: APPROACHES TO UNDERSTAND THE ROLE OF AFLATOXIN GENES IN AFLATOXIN BIOSYNTHESIS

CRANFIELD HEALTH
APPLIED MYCOLOGY GROUP

PhD Thesis
Academic year: 2007-2011

Supervisors: Prof. Naresh Magan
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ABSTRACT

This is the first study to integrate and correlate the effect of ecophysiological factors on the life cycle of *Aspergillus flavus* by carrying out complementary work on gene expression of the aflatoxin gene cluster, with growth, sporulation and phenotypic toxin production. This information was used to understand the role of ecological factors on key biosynthetic genes and examine the use of such information for control of aflatoxin production using RNA interference.

Ecological studies showed the profiles for growth, sporulation and aflatoxin B_1 (AFB_1) production with optimum ranges of water activity (a_w) and temperature for AFB_1 production being identified. *A. flavus* grew faster at 0.99 a_w at all temperatures, but optimally at 30-35°C. The highest amount of asexual conidia was produced at 0.95 a_w followed by 0.90 a_w and then 0.99 a_w at all temperatures examined. Interestingly, the partitioning of AFB_1 into biomass, medium and spores showed that at 0.99 a_w, about 50% of the mycotoxin was present in the biomass and the medium, with very little present in the spores. However, as water stress was imposed there was a switch to a significantly higher channelling of AFB_1 (about 45%) into the spores, especially at 0.95 and 0.93 a_w levels.

A microarray analysis was used to examine the effect of a_w x temperature interactions on the relative expression of the aflatoxin gene cluster for the first time using *A. flavus* NRRL 3357. This showed that under mild stress conditions (20°C/0.99 a_w) several of the cluster genes, in particular *aflS* and *aflJ*, were highly induced concomitant with high levels of phenotypic AFB_1 production. Highest amounts of AFB_1 were produced in all conditions where *aflS* expression was elevated. When the ratio between the normalised expression data of the *aflS*/*aflR* genes was generated, high ratios were obtained at 25°C and 30°C at 0.99 and 0.95 a_w and low ratios at 25°C and 30°C at 0.90 a_w. This is in agreement with the AFB_1 production profile.

Studies were then conducted to discriminate between aflatoxin and non-aflatoxin strains of *Aspergillus* section *Flavi* isolated from Egyptian peanuts using PCR and RT-PCR. This was done by examining whether strains had some key aflatoxin genes (*aflD*, *aflM*, *aflP* and *aflR*). The transcription of *aflD* was used as a marker to discriminate between aflatoxin and non-

aflatoxin producers, while *aflM*, *aflP* and *aflR* failed to differentiate between aflatoxigenic and non-aflatoxigenic strains. In this study *A. flavus* EGP3 did not produce any AFB_1 on YES, however, it produced higher AFB_1 than *A. flavus* EPG14 (an aflatoxin producer on YES) in stored peanuts.

Quantitative real-time PCR was developed for the *aflD* gene and used to examine the temporal relationship between expression and AFB_1 contamination in stored peanuts over periods of 6 weeks under different a_w treatments at 25^oC. There were significant differences between *aflD* gene expression at the three a_w levels; higher expression at 0.90 a_w in weeks 1–3, when compared to 0.95. In contrast, in the driest treatment (0.85 a_w) none or very low *aflD* expression occurred. The populations of *A. flavus* (CFUs g^{-1}) increased over time with the highest at 0.95 a_w. Highest AFB_1 production was at 0.90 and 0.95 a_w after 3–6 weeks storage. A_w had a significant effect on *aflR* transcription at 0.95 a_w over the 6-week period, while at 0.90 a_w, only in the last 2 weeks. Correlations between different factors showed that log AFB_1xlog CFUs, log AFB_1x a_w, and log CFUs x a_w were statistically significant, while log CFUs x RQ-PCR and RQ-PCR x a_w were not. The *aflR* gene may not have an important role in the regulation of *aflD* expression in food matrices (e.g. peanuts).

RNA silencing technique was employed to target the mRNA sequence of the *aflD* gene, to control aflatoxin production. A decrease in *aflD* mRNA, *aflR* mRNA abundance, and AFB_1 production (98%, 97% and 97% of the control level) in *A. flavus* NRRL3357 when treated with siRNA, respectively. There was a significant inhibition of *aflD* and AFB_1 production in protoplasts of *A. flavus* EGP9 and AFG_1 production by *A. parasiticus* NRRL 13005. Changes in AFB_1 production in relation to mRNA levels of *aflD* showed a good correlation (r=0.88; P =0.00001); changes in *aflR* mRNA levels in relation to mRNA level of *aflD* also showed a good correlation (r=0.82; P =0.0001).

These detailed studies suggest that stress applied by external abiotic factors has a complex role in the activation of the genes and that their activation may be some kind of stress response leading perhaps to an increased fitness of the producing fungus to withstand the imposed stress conditions. Also, these studies have found a rapid molecular assay for the detection of aflatoxin-producing species in contaminated peanuts, and a good target gene for inactivation, to develop efficient means of aflatoxin control by using RNA silencing technology.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PLATES

ABBREVIATIONS

A.	*Aspergillus*
AFAs	Aflatoxins
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
a_w	water activity
bp	Base pair
CAM	Coconut cream agar medium
cDNA	Complementry deoxy ribonucleic acid
C_t	Threshold cycle
DNA	Deoxy ribonucleic acid
DNase	Deoxyribonuclease
dNTPs	2.-Deoxynucleoside-5´-triphosphate
ERH	Equilibrium relative humidity
h	Hour
HACPP	Hazard Analysis Critical Control Point
HPLC	High Pressure Liquid Chromatography
L	Litre
µ	Micro (10^{-6})
m	Milli (10^{-3})
M	Molar (mol/L)
MEA	Malt Extract Agar
Min	Minute
mRNA	Messenger Ribonucleic Acid
n	Nano (10^{-9})
OD	Optical density
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
Rnase	Ribonuclease

RNAi	RNA interference
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
RQ-PCR	Relative Quanification- Rolymerase Chain Reaction
Sec	Second
siRNA	Short-interfering double-stranded RNA
TFA	Triflouroacetic acid
Tris	Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid
U	Unit
V	Volt
YES	Yeast Extract Sucrose

Chapter 1

Literature Review

1 LITERATURE REVIEW

1.1 General introduction

Mycotoxigenic filamentous fungi have the ability to contaminate a wide range of food and animal feed with one or more mycotoxins. Approximately 25 to 50 % of the crops harvested worldwide are contaminated with mycotoxins. This percentage is highest in tropical regions where up to 80% of the crops are reported to contain significant amounts of mycotoxins (Konietzny and Greiner, 2003). Food and feeds, especially in warm climates, are susceptible to invasion by aflatoxigenic *Aspergillus* species. The production of aflatoxins (AFAs) can occur pre-harvest, during drying, processing, or storage. Because the toxicity and carcinogenicity of mycotoxins, contaminated commodities destined for human or animal consumption pose a serious health hazard and are, therefore, closely monitored and regulated.

The key environmental determinants pre- and post-harvest contamination with mycotoxins are water availability, temperature and intergranular gas composition (Magan et al., 2003; Magan and Aldred, 2007; Magan et al., 2010). Depending on the particular combination of external growth parameters, the biosynthesis of AFAs can either be completely inhibited, even though normal growth is possible, or the biosynthesis pathway can be fully activated. Knowledge about these relationships enable the assessment of which abiotic factor combinations enable AFA biosynthesis and which combinations may control contamination from a food safety point of view.

Peanuts are an important agricultural crop in Egypt. The major mycotoxins found in Egyptian peanuts are AFAs (El-Maghraby and El-Maraghy, 1987). Contamination of peanuts with AFAs is one of the main factors that compromise their quality. There is thus interest in understand the regional contamination of peanuts in Egypt and the major species responsible for AFA contamination (Sultan and Magan, 2010).

Recently, several multiplex polymerase chain reaction (PCR) systems for aflatoxin-producing fungi were developed, capable of detecting several genes coding for enzymes at different

stages of the aflatoxin biosynthesis pathway (Geisen, 1996; Shapira et al., 1996; Criseo et al., 2001; Chen et al., 2002). However, there is currently no agreement on which single genes, in terms of expression, can be used to fully discriminate between aflatoxin and non-aflatoxin producers (Scherm et al., 2005; Degola et al., 2007; Rodrigues et al., 2009).

Real-time RT-PCR is highly sensitive and has the ability to quantify rare transcripts and any small changes in gene expression. Using real-time PCR to assess and monitor the ability of the fungus to activate mycotoxin biosynthesis genes under different environmental conditions is useful for an early indication of contamination in a specific food chain. Previously, it has been reported that the biosynthetic mycotoxin genes are induced and not expressed constitutively (Peplow et al., 2003; Price et al., 2005). Their induction can be determined some time before the detection of mycotoxin by analytical methods (Xu et al., 2000; Mayer et al., 2003). The signalling processes that switch on aflatoxin biosynthesis during ripening or in poorly stored crops are still not well understood. In order to state whether the biosynthesis of mycotoxin may be possible under certain environmental conditions in a food sample, monitoring of the whole pathway of genes would therefore be useful.

It is now more important to prevent AFA contamination of food raw materials and animal feeds than subsequent cure. So, Hazard Analysis Critical Control Point (HACCP) approaches are being improved to investigate the critical control points (CCPs) at which aflatoxigenic moulds and AFAs may enter a range of food chains. Studies have been done to control or even lower AFA contamination, but, these have often not worked consistently. Thus, there is a need to understand the ecophysiology of mycotoxin production at a genetic level and to identify control systems which can minimise contamination of staple foods.

One approach could be the use of RNA interference (RNAi) technology which has received much attention in biology in recent years. The reason for this enthusiasm is that RNAi rapidly ablates specific messenger RNA (mRNA) species by inducing their degradation via a cellular protein machinery collectively called the RNA-induced silencing complex or RISC (Ketting et al., 2001). For the first time it is now possible to synthesize small RNA species, as double-stranded, and introduce these molecules through common transfection methods into cells. They serve to guide the RNA degradation machinery to the selected target gene. RNAi is an

effective tool to investigate gene function, and may be a useful tool to quench the expression of undesirable gene products.

1.2 Mycotoxins

Mycotoxins are a group of toxic chemical secondary metabolites produced by strains of some fungal species when they grow under favourable conditions on a wide range of foods and feeds (CAST, 2003). They appear to have no role in the normal metabolism involving growth of the fungus. Mycotoxins affect several agricultural products, including cereals, oilseeds, pulses, nuts, root crops, dried fruits, and coffee beans which form the agricultural economic backbone of most developing countries in Africa. Contamination of agricultural products occurs as a result of infection by toxigenic fungi under favourable environmental conditions in the field and may occur at various stages in the food chain, e.g., pre-harvest, during harvest, drying, and storage. Whether fungi will grow and produce toxins depends on the environmental conditions and the specific temperature and a_w requirements of the particular fungal species (Sanchis and Magan, 2004).

Mycotoxin contamination is a world-wide problem and is not confined to any one geographical area or country. Countries within West and Central Africa contain a wide variation in climatic conditions. Generally, the conditions of temperature and humidity found in most of these countries are favourable for the growth of toxigenic fungi and mycotoxin production. Miller (1995) reported that, there are three main genera of fungi that produce mycotoxins: *Aspergillus*, *Fusarium*, and *Penicillium*. Of the mycotoxins, five types are of major agricultural and human health significance: (i) aflatoxins, (ii) fumonisins, (iii) ochratoxin A, (iv) zearalenone and (v) the trichothecenes, e.g., T-2/HT-2 toxins, diacetoxyscirpenol, deoxynivalenol and nivalenol (Tables 1.1).

Mycotoxins generally are of concern in human health, food safety and trade because of their acute and chronic effects on humans and domesticated animals. The presence of excessive mycotoxins can cause grain shipments to be rejected by importing countries resulting in a loss

Table 1.1 Fungal genera and species of major significance and their associated mycotoxins (from Nicholson, 2004).

Fungi	Mycotoxins
Aspergillus species (e.g. *A. flavus, A. parasiticus* and *A. nomius*)	Aflatoxins, predominantly B_1 and B_2 (*A. flavus*) and, in addition, G_1 and G_2 (*A. parasiticus*). B_1 is metabolized to M_1 which is excreted in milk of humans and animals
Aspergillus species (e.g. *A. flavus, A. parasiticus, A nidulans*)	Sterigmatocystin
Fusarium species (e.g. *F. graminearum* (*Gibberella zeae*), *F. culmorum, F. sporotrichioides, F. langsethiae, F. Poae* and *F. cerealis*)	Trichothecenes: Type A (e.g. T-2, HT-2, diacetoxyscirpenol (DAS), neosolaniol) produced by *F.sporotrichioides, F. poae* and/or *F. equiseti* Type B (e.g. nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives) produced by *F. graminearum, F. culmorum* and/or *F. cerealis*
Fusarium proliferatum and *F. verticillioides*	Fumonisins
Fusarium species (e.g. *F. proliferatum* and *F. avenaceum*)	Enniatins and beauvericin
Penicillium and *Aspergillus* species (e.g. *P. verrucosum, A. ochraceus* and *A. carbonarius*)	Ochratoxin A
Penicillium species (e.g. *P. expansum*)	Patulin
Penicillium species	Citrinin and roquefortine
Claviceps purpurea, Aspergillus, Penicillium and *Acremonium* species	Ergot alkaloids
Alternaria species	Alternariol, altenuene, altertoxin, tenuazonic acid

in consumer confidence in the importing country and severe economic losses for the exporting country. A major potential danger of mycotoxins in the human diet, therefore, resides in the inability to detect them biologically.

All of the mycotoxins mentioned above are considered important because of their increasing abundance and their toxicity to plants, animals or humans (Pitt, 2000). Mycotoxins can be classified into four kinds of toxicity: as hepatotoxins, nephrotoxins, neurotoxins, and immunotoxins. Cell biologists put them into generic groups such as teratogens, mutagens, carcinogens, and allergens. Deterioration of the liver or kidney function has been described as the most common effect of acute mycotoxin poisoning, which in extreme cases may lead to death. Some of mycotoxins can interfere with synthesis of protein, and result in effects fluctuating from skin sensitivity or necrosis to extreme immunodeficiency. Others are neurotoxins, which at low doses, can lead to continuous trembling in animals, but at high doses result in a brain damage or death. The primary chronic effect of many mycotoxins is the induction of cancer, especially of the liver. Some mycotoxins inhibit DNA and RNA replication, and hence can cause mutagenic or teratogenic effects (Rodncks et al., 1977; Ueno, 1983; Smith and Moss, 1985).

1.3 Aflatoxins (AFAs)

AFAs are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species. AFAs were isolated and characterized from mould-contaminated peanut meal after the death of more than 100,000 turkey poults (turkey X disease; Blout**,** 1961). AFAs are produced by five species of Aspergilli: *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii* and *A. bombycis* (Samson, 2001; Varga et al., 2003). Richard and Payne (2003) reported that only two of these species, *A. flavus* and *A. parasiticus*, are of agronomic importance and cause annual losses of approximately $270M. Lewis et al. (2005) reported that the danger of consuming foodstuffs contaminated with aflatoxin at levels above the regulatory limit was again demonstrated in 2004 in Kenya where 125 people died following the consumption of homegrown maize containing high levels of aflatoxin.

AFAs are produced by species of *A. flavus* and *A. parasiticus* in various commodities including maize, rice, barley, wheat, sorghum, peanuts, pistachios and copra. *A. flavus* produces only aflatoxins B_1 (AFB_1) and B_2 (AFB_2) whereas *A. parasiticus* produces AFB_1, AFB_2, aflatoxin G_1 (AFG_1) and G_2 (AFG_2). The toxicological effects of aflatoxins are dose-dependent, at high doses they are lethal if consumed, causing liver, myocardial and kidney tissue damage. At sub-lethal doses aflatoxins cause chronic toxicity, e.g. liver cirrhosis, and at low-level exposure, they are potent human hepatocellular carcinogens (Wild and Turner, 2002).

AFAs are also mutagenic and teratogenic and can depress cell-mediated immunity (Williams et al., 2004). AFB_1 acts synergistically with hepatitis B virus infection to increase its carcinogenic potency thirty-fold in hepatitis B surface antigen positive populations. The International Agency for Research on Cancer (IARC) has evaluated AFB_1 as a Group 1 carcinogen producing liver cancer in humans (IARC, 1993).

1.3.1 Worldwide limits for aflatoxins in food

The range of maximum tolerated levels for AFB_1 in food in 2002 has narrowed a little (1–20 µg/kg) and the median is 2 µg/kg, a limit which is now in force in 27 countries (Figure 1.1). Most of these countries belong to the EU (where, since 1998, harmonized limits for AFB_1 and the sum of the AFB_1, B_2, G_1 and G_2 have been in force), to EFTA (European Free Trade Association) and candidate EU countries. Another major limit of 5 µg/kg, has been set in 19 countries, spread over Africa, Asia/Oceania, Latin America and Europe. The USA and Canada do not have a single set limit for AFB_1. Many countries regulated AFAs in food with limits for the sum of the AFB_1, AFB_2, AFG_1, and AFG_2, occasionally in combination with a specific limit for AFB_1. The limits vary from 1–35 µg/kg, while the most often existing limit (Figure 1.2) is set at 4 µg/kg (used by 28 countries). This limit is established as part of the regulations in the EU, EFTA and candidate EU countries, where both limits for AFB_1 and for total AFAs are compulsory. An additional major peak occurs at 20 µg/kg, which is used by 18 countries, with half of them in Latin America, and several in Africa.

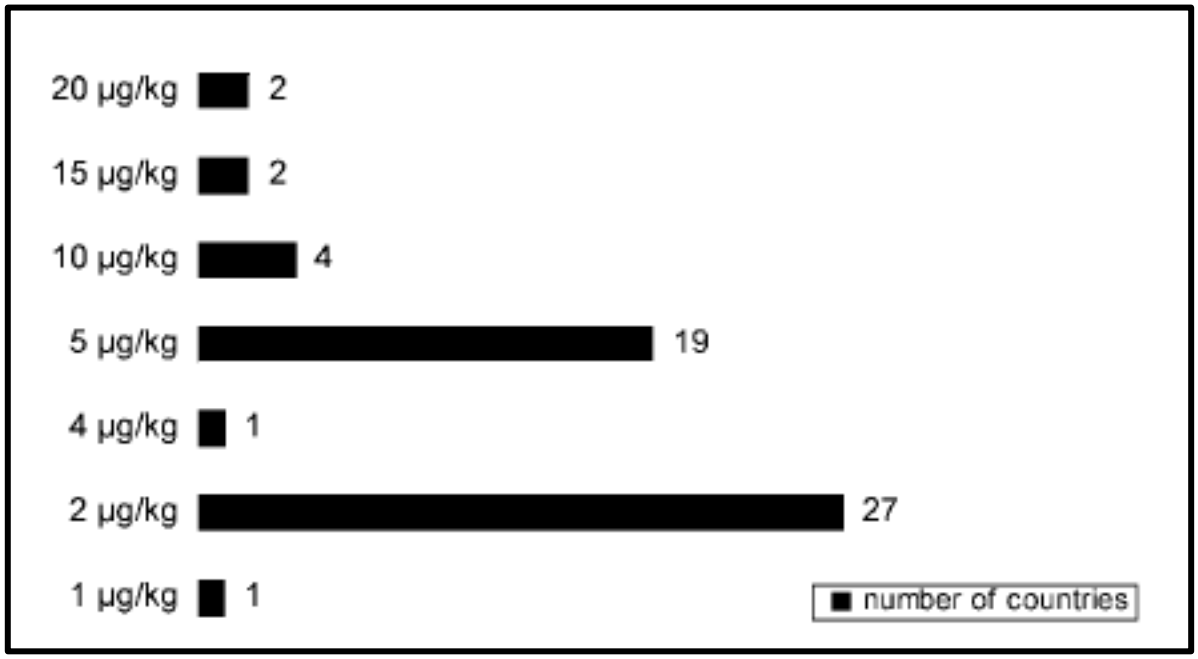

Figure 1.1 Frequency distribution of specific regulatory limits for AFB_1 in food in 56 countries, in 2002. (from Van Egmond and Jonker, 2004).

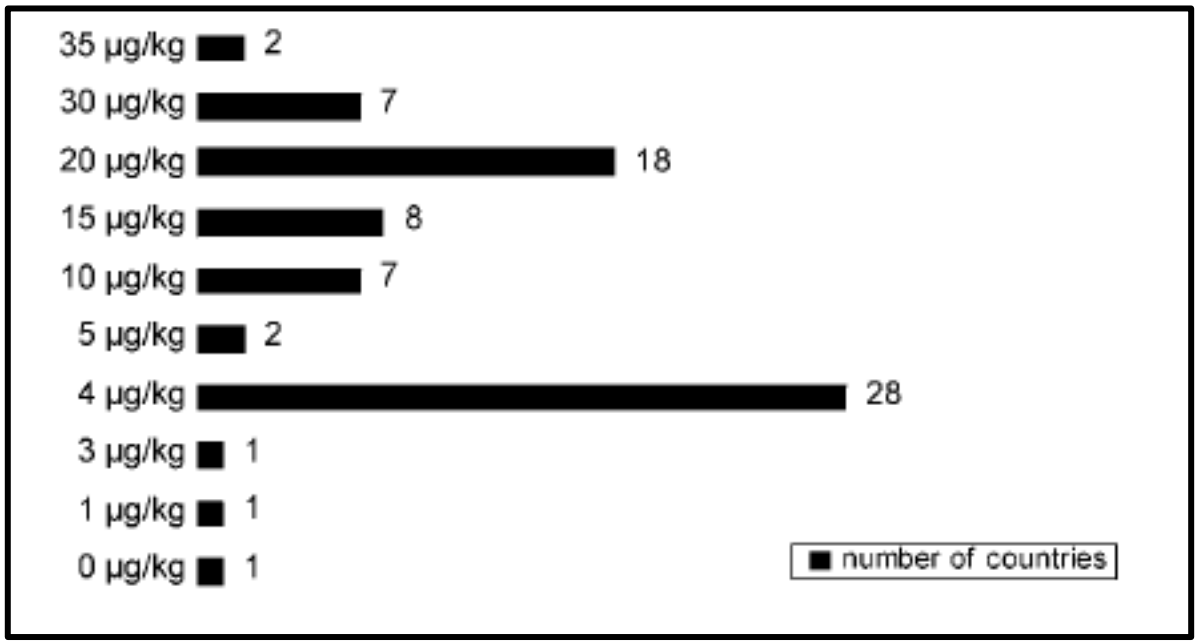

Figure 1.2 Frequency distribution of specific regulatory limits for the sum of AFB_1, AFB_2, AFG_1 and AFG_2 in food in 75 countries, in 2002. (from Van Egmond and Jonker, 2004).

1.3.2 Aflatoxins and Peanuts

Peanuts are employed in the manufacturing of sweets, candies and pastes and as well as a raw material in oil production. About 60% of the peanuts world production is used in the extraction of oil, with peanut oil being the fifth most common type consumed (Santos, 2000). The peanut seed possesses a high nutritional and commercial value due to the presence of proteins, fatty acids, carbohydrates, and fibres, in addition to vitamins, calcium and phosphorus (Camara, 1998). Contamination of peanuts with AFAs is one of the main factor that compromises the quality of the product. The inability to conserve peanuts at safe moisture contents during the storage period result in contamination of stored peanuts with mycotoxigenic moulds. Inappropriate kernel moisture during storage can proceed from leaky roofs, condensation because of improper ventilation in the warehouse, high-moisture foreign material associated with stored peanuts, and high-moisture peanuts initially going into storage (Davidson et al., 1982).

Peanuts are important agricultural crops for Egypt. The main growing areas are in the north of the country; they include the areas of reclaimed desert to the east and west of the Nile Delta, north of Cairo. Egypt has a large number of peanut producers, with many farming small fields of < 2.5 ha (WTO, 1998). *Aspergillus* section *Flavi* have the ability to infect peanuts under both pre-harvest and post-harvest conditions (Barros et al., 2003). It has recently been shown that in Egypt peanut contamination is dominated by *A. flavus* strains (Sultan and Magan, 2010). Usually, AFA contamination pre-harvest occurs under conditions of heat and drought stress during the latter stages of the growing season (Dorner and Cole, 2002).

In May, 1999 the European Commission suspended the import of peanuts from Egypt due to the presence of AFAs in concentrations in excess of the maximum levels specified in the EU regulations. For raw peanuts entering the EU, the level must be <4 µg kg total AFAs and have not more than 2 µg kg AFB_1 (European Commission, 2006). Recently, a FAO/WHO survey reported that about 100 countries have a regulation for aflatoxin and other mycotoxins in foods and animal feeds (Food and Agriculture Organization, 2003). Nevertheless, the maximum limits vary widely from country to country due to the absence of correspondence on what constitutes a safe maximum level for humans. Some of the maximum limits found in the

FAO/WHO survey for aflatoxin are shown below in (Table 1.2). Egypt is a major peanut exporting country and the European markets account for 68% of its peanut exports.

1.3.3 Aflatoxin biosynthesis

AFAs are polyketide-derived secondary metabolites produced via the following conversion path: acetate to polyketide to anthraquinones to xanthones to aflatoxins (Yu et al., 2002; Bhatnagar et al., 2003). Generally, the aflatoxin gene cluster in *A. flavus* and *A. parasiticus* contains 25 genes reaching about 70 kb (Figure1.3). Among the 25 genes identified in the aflatoxin biosynthetic pathway gene cluster, the functions of 19 in aflatoxin biosynthesis have been assigned and the functions of six are unassigned. Among the genes assigned to the pathway steps, *aflR*, which represents a positive regulatory gene coding for a sequence-specific, is a zinc finger DNA-binding protein. *AflR* is required for transcriptional activation of most, if not all, of the AFA structural genes (Bhatnagar et al., 2003). *aflS* (*aflJ*) is close to and differently transcribed from the *aflR* gene. It has not shown significant homology with any other genes/proteins present in the databases.

Although the exact function of *aflS* (*aflJ*) is not clear at this time, it has been shown to be necessary for expression of other genes in the AFA cluster (Chang, 2003). Nor-1, Ver-1 and Omt-1 are critical genes in the pathway of AFAs biosynthesis. The conversion can either be from NOR to averantin (AVN) involving a dehydrogenase, encoded by the gene nor-1 (Chang et al., 1992; Trail et al., 1994), or can also be catalyzed by the dehydrogenase encoded by norA. Two genes – ver-1 (encoding a ketoreductase; Skory et al., 1992) and verA (encoding a cytochrome P-450 monooxygenase) – are required for the conversion of versicolorin A (VERA) to demethylsterigmatocystin (DMST). The final step in the formation of AFAs is the conversion of O-methylsterigmatocystin (OMST) or dihydro-O-methylsterigmatocystin (DHOMST) to AFB_1, B_2, G_1 and G_2, (Yu et al., 1998; Prieto and Woloshuk, 1997). For consistency and uniformity with the functions of the genes in the AFAs biosynthetic pathway the gene naming in *Aspergillus* has been modified. The three-letter code "*afl*" is used to represent AFA pathway genes. A capital letter in alphabetical order from "A" to "Y" represents each individual gene confirmed to be or potentially involved in AFA biosynthesis, e.g., *aflA* to *aflY* for all of the 25 genes (Figure 1.3, Yu et al., 2004).

Table 1.2 Examples of aflatoxin legal limits found in groundnut fromvarious countries (from Freeman et al., 1999)

Countries	Aflatoxin Legal Limit [a] (ng/g)	
	AFB_1	**Total Aflatoxins**
Egypt		20
UK		4
USA		20
Sweden		5
Germany	2	
Belgium	5	
The Netherlands	0	
Ireland	5	
Italy	5	

a AFB_1 = Aflatoxin B_1 & Total Aflatoxins = AF(B 1+B 2+ G 1+ G 2)

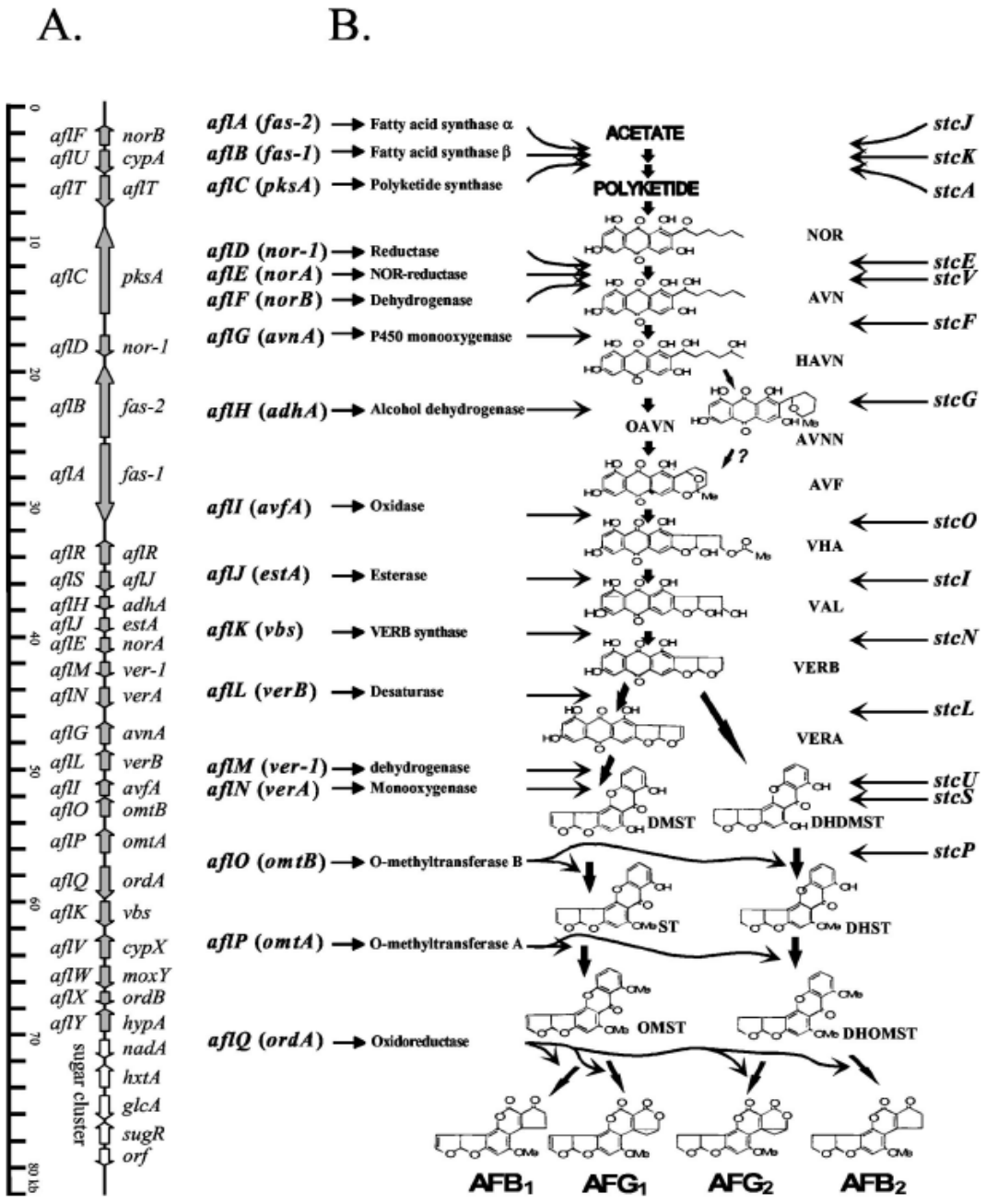

Figure 1.3 Clustered genes (A) and the aflatoxin biosynthetic pathway (B). The corresponding genes and their enzymes involved in each bioconversion step are shown in panel A. The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in *A. parasiticus* and *A. flavus*. The new gene names are given on the left of the vertical line and the old gene names are given on the right. NOR, norsolorinic acid; AVN, averantin; HAVN, 5-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethyl sterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmato cystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2 ; AFG_1 , aflatoxin G_1 ; AFG_2 , aflatoxin G_2 (from Yu et al., 2004).

1.4 Environmental factors, fungal growth and mycotoxin production

Aflatoxin production is the consequence of a combination of species, substrate, and environment. The factors affecting AFA production can be divided into three categories: physical, nutritional, and biological. Physical factors include temperature, pH, moisture, light, aeration and level of atmospheric gases. Here the key factors of water activity and temperature are discussed.

1.4.1 Water relations of fungi

The importance of water to all life is well recognized, as well as the role of water in controlling the growth of microorganisms in foods and other biological systems (Labuza, 1968; Chirife and Buera, 1996). All food ingredients and systems contain water ranging from small amounts, such as in the case of crystalline sucrose, with an average 0.04% of moisture content on a wet weight basis, to very high amounts, as in fresh fruits and vegetables, many of which have a moisture content >90% wet weight basis (Chirife and Fontan 1982). Chemical reactions, enzymatic changes, and microbial growth may occur readily in foods with high water contents when their occurrence is not restricted by environmental factors such as pH or temperature.

The theory of water availability: In 1957, Scott was the first to determine the significance of water availability and attempted to correlate this to the total water content of substrates. He proposed that water activity (a_w) would best describe the water availability for microbial activity. So, a_w represents the ratio between the vapour pressure of water in a substrate (P) and the vapour pressure of pure water (Po) at the same temperature and pressure; thus a_w= P/Po. The a_w of pure water is 1. A substrate containing no free water has a smaller vapour pressure than pure water and it's a_w is consequently less. An alternative measure to a_w is that of water potential (Ψ), which is commonly used in soil microbiology and measured in Pascals (Pa). This is the sum of the osmotic, matric and turgor potentials and is related directly to a_w by the following formula:

$$\text{Water potential } (\Psi) = RT/V \log_n a_w (+P),$$

Where R is the ideal gas constant, T the absolute temperature, P the atmospheric pressure and V is the volume of 1 mol of water.

The relation between a_w and Ψ is shown in Table 1.3. A_w and equilibrium relative humidity (ERH) are the same numerically except that a_w is a decimal fraction of one and ERH is the relationship between a_w and Ψ, which is expressed as a percentage (%) (Magan, 2007). Water activity (a_w) is considered as one of various hurdles that can be varied to provide stability and safety in foods (Leistner, 1994). The minimum requirement for microbial growth is 0.62 a_w, which allows growth of xerophilic yeasts (Chirife and Buera 1994). An increasing a_w allows the growth of moulds, other yeasts, and finally bacteria under the wettest conditions. The most important a_w value for the safety of food materials is probably 0.86 which allows the growth of *Staphylococcus aureus*, a well-known bacterial pathogen. The a_w limits for growth of various microorganisms is shown in Figure 1.4). Although this is slightly dependent on growth media, they are well established and successfully used in food development and manufacturing (Christian, 1981). Pitt and Hocking (1985) reported that Ascomycetes comprise most of the organisms capable of growing at <0.90 a_w. On studying the production of AFB_1 in peanuts, Diener and Davis (1967) reported an optimum value of 0.95 a_w whereas no significant quantity of AFAs was found at 0.85 a_w. A minimal value of 0.84 a_w for AFA production in corn was reported by Hunter (1969) but a lower a_w value than 0.84 suppressed AFA production.

A_w is a more useful parameter than water content since it reflects the availability of water for metabolic processes. Moisture sorption isotherms are used to portray the relationship between a_w and total water content in different materials (Table 1.4). Different products with the same water activity may thus have very different water contents. For example, oilseeds have a greater a_w at a given water content than starchy cereal seeds (Pixton, 1967).

1.4.2 Temperature

Temperature is one of the most important environmental factors influencing growth and toxin production by toxigenic fungi. Except for a few studies (Schroeder and Hein, 1968; West, et al. 1973; Stutz and Krumperman, 1976), reports on the relationship of aflatoxin production to temperature have been conducted at constant temperatures (Schindler, et al. 1967; Schroeder and Hein, 1967; Sorenson, et al. 1967; Davis and Diener, 1970; Shih and Marth, 1974).

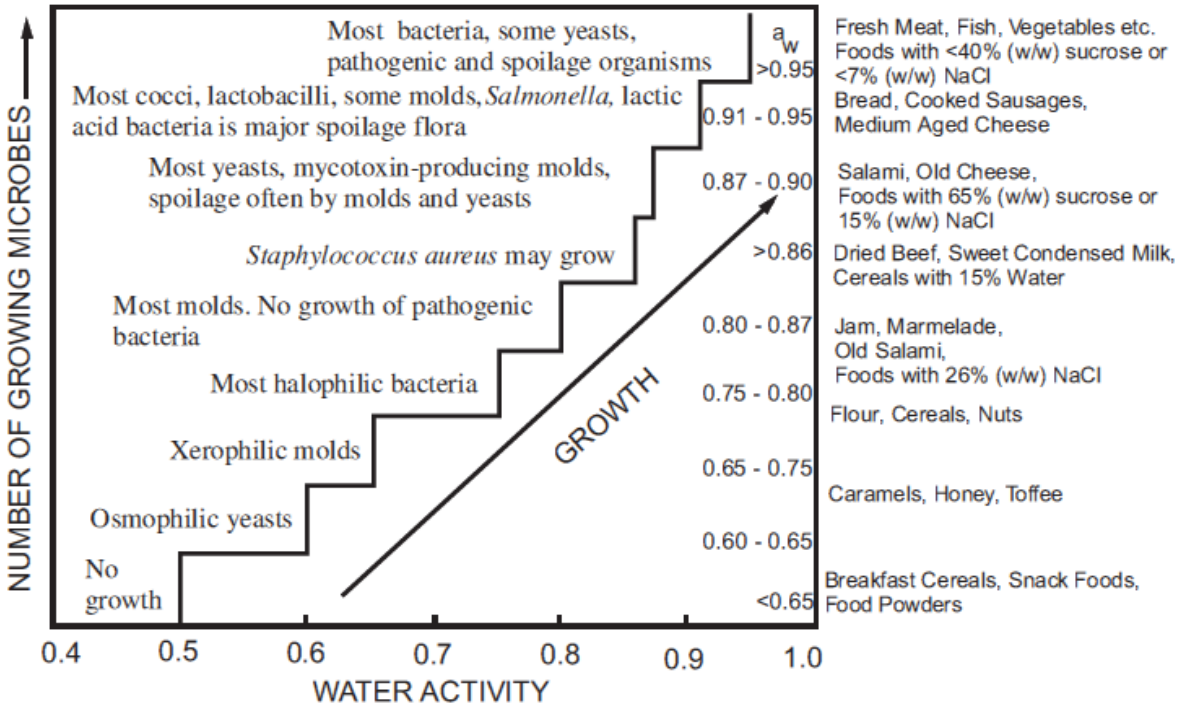

Figure 1.4 Minimum water activity (a_w), ranges for the growth of microorganisms in foods and examples of various food materials having a_w within the minimum range (from Roos, 2007).

Table 1.3 Water activity, equilibrium relative humidity and water potentials at 25°C (from Magan, 2007).

Water activity	E.R.H %	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.65	65	59.30
0.60	60	70.30

Table 1.4 Relationship between moisture content (wet weight basis, %) and water activity (a_w) for some key cereals and nuts at 25 ºC (from Sanchis and Magan, 2004).

	Moisture content				
a_w	**Groundnuts**	**Wheat**	**Sorghum**	**Rice**	**Maize**
0.98	16–17	30–34	31–32	26–28	30–32
0.95	14.5–15	26–28	26–27	23–24	26–27
0.90	12.5–13.5	21–22	22.5–23	20–21	23–24
0.80	9–10	16–17	18–19	17–18	16–17
0.70	7–8	14–14.5	16–17	14–14.5	15–16

Previously, the effect of a wide range of temperatures (2-52^{o}C) on two aflatoxin-producing strains of *A. flavus* was studied by Schindler et al. (1967). They found that the highest amount of AFB_1 was produced at 24°C and AFA production was not related to growth rate of *A. flavus*. No aflatoxins were produced at temperatures <18^{o}C or >35^{o}C.

The optimal range for AFA production was reported at 20–35°C, with only small amounts of toxin produced at 10 or 40°C, when four of *Aspergillus* section *Flavi* isolates (two *A. flavus* and two *A. parasiticus*) were grown on substrates such as cottonseed, shelled peanuts, and rice (Schroeder and Hein, 1967).

West at al. (1973) reported that the increasing the temperature of rice fermentation of *A. parasiticus* NRRL 2999 from 15 to 21°C after 24 h incubation and then to 28°C after 48 h led to about a four times increase in total AFAs than that kept constant at 28°C for 6 days. Shih and Marth (1974) reported maximum AFAs production at 25°C, whereas maximal fungal growth occurred at 35°C. Using *A. parasiticus* NRRL 2999 grown in liquid culture, AFA production was high at temperatures between 13°C and 32°C at 0.99 a_w, when water stress was imposed at 0.94 a_w, the maximum AFAs production occurred at 24°C (Northolt et al., 1976). Durakovic et al. (1987) studied the effect of a range of temperatures (15, 20, 30, and 40°C) on AFB_1 production of *A. parasiticus* NRRL 2999 grown on maize grain (whole and crushed). They found that, the highest amount of AFB_1 were produced at 30°C followed by 40°C with 47 µg g^{-1} dry weight mycelium and then 20°C.

Barrios-Gonzalez et al. (1990) found that *A. parasiticus* can grow and produce AFAs at 35°C in solid-state fermentation of cassava. However, the operation temperature of the protein enrichment process (35°C) drastically reduced potential toxin production. As reported previously by Feng and Leonard (1998), no AFAs was detected when *A. parasiticus* was cultured at 37°C, but high levels were produced at 27°C and only marginal amounts were produced at 33°C. Gqaleni et al. (1997) studied the effects of and interactions among temperature, a_w, incubation period, and substrate on AFA production and cyclopiazonic acid (CPA) by an isolate of *Aspergillus flavus*. They reported that the optimum temperatures for the production of AF and CPA were 30 and 25°C, respectively. No AFAs were produced in either yeast extract agar or Czapek yeast autolysate agar medium at 0.90 a_w and 20 or 37°C after 15 days. From all the previous studies, it can be concluded that the optimum

temperature for AFAs production is probably between 24 and 30°C, with some variation due to strains and nutritional matrices.

1.5 Analytical methods for the detection of mycotoxins

There are a number of classical analytical methods for mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC); gas chromatography (GC) and mass spectrometry (MS). Most of these techniques have been coupled with immunoaffinity techniques to simplify extraction and improve mycotoxin recovery and measurement from foodstuffs. Commercial immunological techniques for mycotoxins are based on specific monoclonal and polyclonal antibodies produced against the toxin, and divided broadly into immunoaffinity (IAC) column-based analysis and enzyme-linked immunosorbent assays (ELISA). A range of biosensors has been reported for mycotoxin analysis, including those based on optical (e.g. surface plasmon resonance, SPR, and evanescent wave fibre optic) and surface acoustic wave (e.g. quartz crystal microbalance) principles (Stroke et al., 2000).

Because conventional methods for the detection of mycotoxigenic fungi are time consuming, labour-intensive, costly and require substantial mycological expertise and facilities, alternative methods have been sought. There is thus a need to develop methodologies that are relatively rapid, highly specific and an alternative to the existing methods. Table 1.5 summarises the main methods which can be used, their advantages and disadvantages.

Lin et al. (1998) used conventional methods to distinguish between toxigenic and non-toxigenic isolates in the *A. flavus* group by culturing on suitable inducing media, extracting AFAs with organic solvents, and monitoring their presence by chromatographic techniques. Other studies have used media to induce AFA production to try and differentiate between aflatoxin-producing and non-producing strains of the *A. flavus* group (Davis et al., 1987; Abarca et al., 1988; Bennett and Papa, 1988).

Recently, molecular techniques have been developed to overcome the conventional methods problems. These techniques depend on using PCR which can decrease the time required for detection of mycotoxigenic fungi from several days to several hours.

Table 1.5 Advantages and disadvantages of classical methods for mycotoxin analysis (from Pascale and Visconti, 2008). Key: TLC = Thin Layer Chromatography; GC = Gas Chromatography; HPLC = High Performance Liquid Chromatography; LC/MS = Liquid Chromatography/Mass Spectrometry; ELISA = Enzyme-Linked Immunosorbent Assay; Rapid tests = membrane-based card test; antibody-coated tube; immunodot cup test.

Method	Advantages	Disadvantages
TLC	Simple, inexpensive and rapid Can be used for screening simultaneous analysis of multiple mycotoxins sensitive for aflatoxins & ochratoxin A	Poor sensitivity (for some mycotoxins) Poor precision Adequate separation may require two dimensional analysis Quantitative only when used with adensitometer
GC	Simultaneous analysis of multiple mycotoxins Good sensitivity May be automated (autosampler) provides confirmation (MS detector)	Expensive equipment Specialist expertise required Derivatization required Matrix interference problems Non-linear calibration curve Drifting response Carry-over effects from previous sample Variation in reproducibility & repeatability
HPLC	Good sensitivity Good selectivity Good repeatability May be automated (autosampler) Short analysis times Official methods available	Expensive equipment Specialist expertise required May require derivatization
LC/MS	Simultaneous analysis of multiple mycotoxins Good sensitivity (LC/MS/MS) Provides confirmation No derivatization required	Very expensive Specialist expertise requested Sensitivity relies on ionization technique Matrix assisted calibration curve (for quantitative analysis)
ELISA	Simple sample preparation Inexpensive equipment High sensitivity Simultaneous analysis of multiple samples Suitable for screening Limited use of organic solvents Visual assessment	Cross-reactivity with related mycotoxins Matrix interference problems Possible false positive/negative results Confirmatory LC analysis required Critical quantitation near regulatory limits Semi-quantitative (visual assessment)
Rapid Tests	Simple and fast (5-10 min) No expensive equipment required Limited use of organic solvents suitable for screening purposes can be used *in situ*	Qualitative or semi-quantitative (cut off level) Possible false positive/negative results Cross-reactivity with related mycotoxins Matrix interference problems Lack of sensitivity near regulatory limits

1.6 Molecular methods

1.6.1 Polymerase Chain Reaction (PCR)

PCR is based on enzymatic amplification of a target DNA region defined by two oligonucleotides, called primers, which bind opposite to complementary DNA strands (Knippers, 1997). PCR was developed in 1986 by Mullis and is widely applied in laboratories today. PCR utilises a DNA extension enzyme (DNA polymerase) that can add nucleotide bases once a template is provided. There are three basic steps in PCR:

1. *Denaturation*: The two strands of the target DNA molecule are separated into its component strands by heating. DNA can be reversibly denatured by a cycle of heating and cooling. This step is most often performed at 94^{o}C.

2. *Annealing*: By decreasing the temperature of the reaction mix to the annealing temperature which is usually range between 50°C and 65°C (dependent on the design of the oligonucleotide sequences used as primers), the specific oligonucleotide primers hybridizes to the two target strands.

3. *Extension:* After the primer hybridisation step, the temperature is raised to approximately 72°C, (an optimal temperature for thermostable DNA polymerase mediated DNA strand replication) in the presence of Taq DNA polymerase, PCR buffer, dNTP's and magnesium ($Mg\ 2^{+}$) molecules. After each cycle of replication, each newly synthesised double stranded DNA molecule (known as an amplimer or amplicon) as illustrated in Figure (1.5). After one cycle, each of the single-stranded DNA target segments has become double-stranded through the polymerase's activities. The cycle is then repeated, and each time a new target segment of DNA is synthesised. Theoretically, the number of templates produced equals 2^{n} (Van Pelt-Verkuil et al., 2008).

There are many ways to detect the amplified PCR products; agarose gel electrophoresis followed by ethidium bromide staining represents the easiest and most common way to analyze PCR products (Sambrook et al., 1989).

Ethidium bromide is a fluorescent dye that intercalates between the stacked bases of DNA causing the DNA to fluoresce when exposed to UV light at 260 nm. Ethidium bromide can be either added to an agarose gel solution prior to pouring the gel or it can be used as a solution to stain the gel following electrophoresis (Figure 1.6).

DNA products are visualized and can be photographed under UV light. Identification of the PCR product is based on the appearance of a DNA band of the expected length. Sizing of the DNA bands is achieved by running the PCR products next to DNA markers (mixture of DNA molecules of known size). A 2 % agarose gel is adequate for the analysis of PCR products from 150 to 1000 bp, because the majority of PCR products are between 100 and 500 bp in length. Although many laboratories use standard electrophoresis grade agarose, agaroses specially designed for the separation of small DNA fragments are available.

The major disadvantage in the use of ethidium bromide as a sole method for PCR product detection is that the dye can only detect bands that contain ~ 5 ng or more of DNA (Sharp et al., 1973). For some experiments, this may be a more than adequate level of detection, whereas for others it is too insensitive and therefore not appropriate. A second disadvantage of using ethidium bromide is that all of the DNA products (both specific and nonspecific) produced during the PCR reactions will be stained. In cases where the nonspecific bands are in excess of the specific product and/or are very close to the same size as the expected DNA product, the use of ethidium bromide to detect the PCR products can lead to faulty interpretations of the results.

1.6.2 Reverse transcription-Polymerase chain reaction (RT–PCR)

Reverse transcription - polymerase chain reaction (RT-PCR) has been devised as a method of RNA amplification and quantification after its conversion to cDNA. RT-PCR can be used for cloning, cDNA library construction and probe synthesis. The technique consists of two parts (Figure 1.7), the synthesis of cDNA from RNA by reverse transcription (RT) and the subsequent amplification of a specific DNA molecule by polymerase chain reaction (PCR). The RT reaction uses an RNA template (typically either total RNA or polyA + RNA), a primer (random hexamer oligos or oligo dT primers), dNTPs, buffer and a reverse transcriptase enzyme to generate a single-stranded DNA molecule complementary to the RNA (cDNA). After that, the cDNA then serves as a template in the PCR reaction.

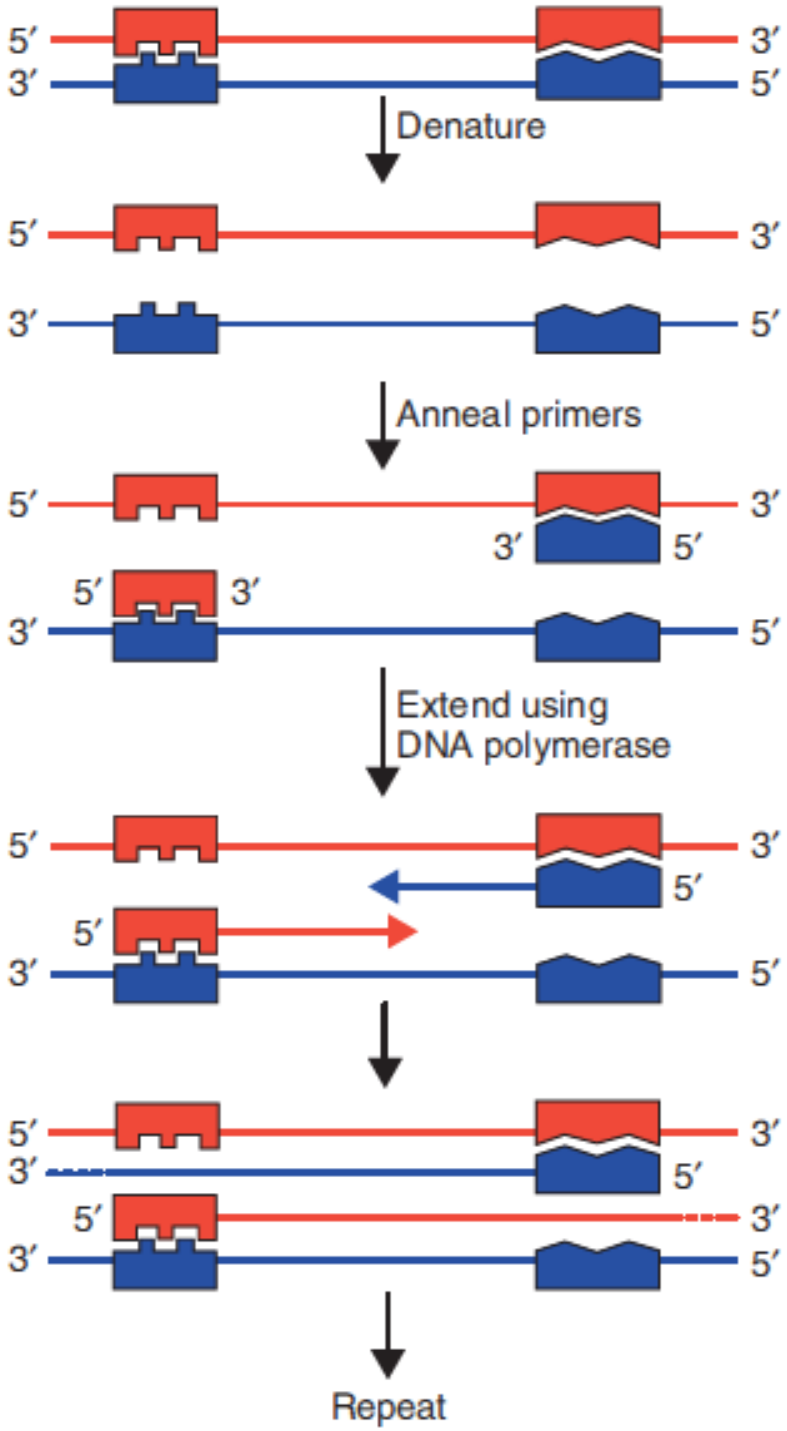

Figure 1.5 The steps of a PCR experiment. The two DNA strands of the target DNA molecule, shown in red and blue to differentiate them, are denatured, or separated, by heating. The boxed regions depict unique sequences within the target DNA to which the oligonucleotide primers will bind (from Reece, 2003).

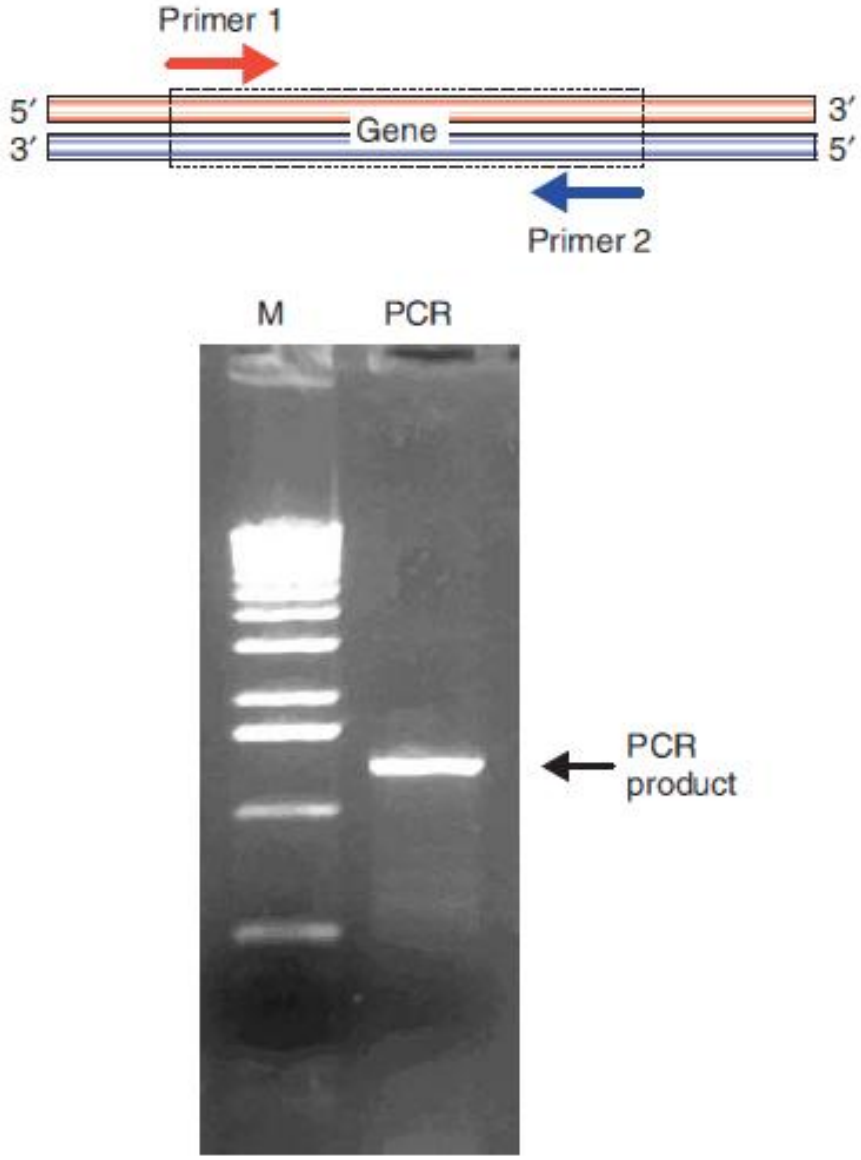

Figure 1.6 PCR amplification of a gene from genomic DNA. Two oligonucleotides primers were designed to flank the gene in the human genome. A PCR reaction was performed for 25 cycles, and one-10th of the total reaction was run on an agarose gel adjacent to a series of DNA size standards (M). The gel was stained with ethidium bromide and photographed under UV light (from Reece, 2003).

Like other methods of mRNA analysis, such as northern blots and nuclease protection assays, RT-PCR can be used to quantify the amount of mRNA that was contained in the original sample. This type of analysis is particularly important for monitoring changes in gene expression. However, because PCR amplification is exponential, small sample-to-sample concentration and loading differences are amplified as well. Even large differences in target concentration (100-fold or more) may produce the same intensity of band after 25 or 30 PCR cycles. Therefore, RT-PCR requires careful optimization when used for quantitative mRNA analysis.

1.6.3 PCR in the detection of fungi

Because of its specificity and sensitivity, PCR is an attractive method for the detection of fungi. There are already many examples of PCR-based assays developed for the detection of fungi in both medical and plant pathology. PCR can be used to detect groups of strains, pathotypes, species or higher taxa, provided that specific oligonucleotide primers for these taxa are available. Thus, the development of PCR-based detection procedures requires knowledge of sequences of at least a part of the target DNA region in order to design specific primers (Dieffenbach et al., 1993; Atkins and Clark, 2004).

DNA sequences which are polymorphic between fungal species, such as internal transcribed spacer (ITS) regions, are good candidates for the detection of a species to the exclusion of all other species. For example, differences in ITS sequences have been used to develop PCR-based assays for the detection of many phytopathogenic fungal species in host plants without previous isolation of the fungi (Moukhamedov et al., 1994; Beck and Ligon, 1995). PCR amplification methods with specific fungal primers are powerful tools not only in diagnostics but also in ecological studies for monitoring fungi in natural environments, such as water, soil, plant or clinical samples. Furthermore, the development of specific primers has greatly facilitated studies on obligate parasites and symbionts (Di Bonito et al., 1995). Previously, a biomolecular technique (PCR) have been applied by using a set of primers of aflatoxigenic genes (*aflR*, *aflD*, *aflM* and *aflP*) to discriminate between aflatoxigenic strains and non-aflatoxigenic strain *of A. flavus* and *A. parasiticus* contaminating food and feed (Woloshuk et al., 1994; Shapira et al., 1996; Geisen, 1996; Chen, 2002; Criseo et al., 2001). Edwards et al. (2001) developed a PCR-based assay to quantify trichothecene-producing *Fusarium* based on primers derived from *Tri*5 gene that encodes trichodiene synthase gene.

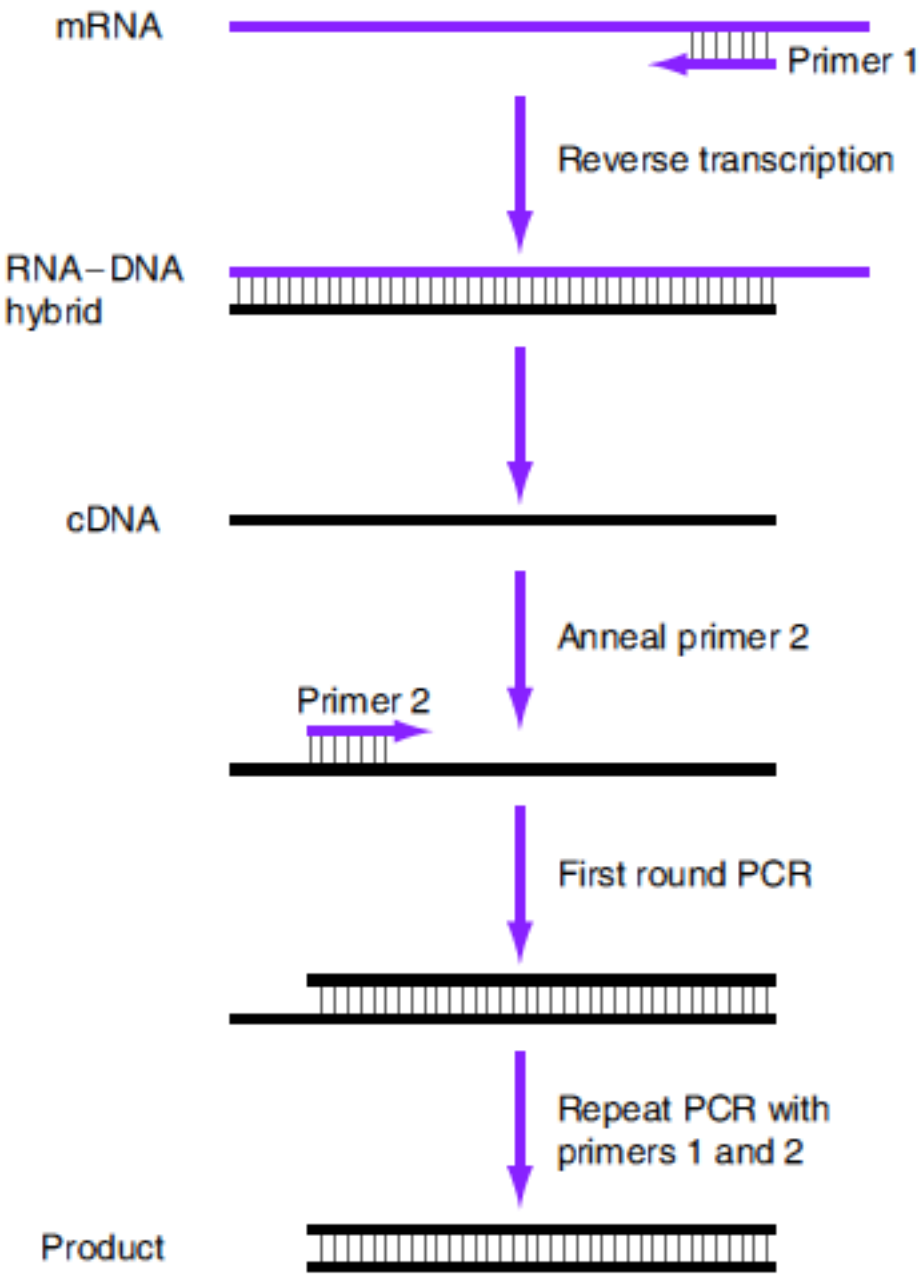

Figure 1.7 Reverse transcription polymerase chain reaction (RT-PCR) to obtain a cDNA copy of mRNA. The single strand of cDNA produced is then used as a template for the synthesis of a second DNA strand, and then for amplification by PCR (from Dale and Schantz 2002).

Recently, Glynn and Edwards (2010) tested the efficacy of competitive PCR assays for quantifying seed-borne *Microdochium* and *Fusarium* seedling blight pathogen DNA contaminating wheat and to determine test and year repeatability and sources of variability. Research groups have tested the expression [reverse transcription polymerase chain reaction (RT-PCR)] for the aflatoxin biosynthetic genes. The presence of the aflatoxigenic strain and of the AF biosynthetic enzymes can be detected by RT-PCR. Previously, some studies used multiplex RT-PCR containing a set of primer of aflatoxigenic genes of *A. flavus* [*aflD*, *aflO, aflP*, *aflQ*, *aflR* and *aflS* (*aflJ*)] to monitor the expression of aflatoxin genes (Sweeney et al., 2000; Scherm et al., 2005; Degola et al., 2007; Rodrigues et al., 2009). Table 1.6 shows the genes, their enzyme products and their functions in the AFAs biosynthetic pathway. Non-aflatoxigenic producers have a mutation in one or more AF biosynthesis genes (Shapira et al., 1996) and their mRNA products (Degola et al., 2007).

1.7 Real time PCR

Real-time PCR has the ability to measure the amplified PCR product at each cycle throughout the PCR reaction. In practice, a light sensors records the light emitted by a fluorochrome incorporated into the newly synthesized PCR product. Real-time quantitative PCR assists in the continuous collection of fluorescent signal from one or more polymerase chain reactions over a range of cycles, and is considered as the most sensitive and reproducible form of PCR-based quantification.

The increase in the number of amplifiers during PCR amplification based on the proportional increase in fluorescence intensity can be detected by Real-time PCR machines. Using these machines, any change in amplified product is indicated in a change in the fluorescence intensity measured, a process that is largely independent of the amplifier size. In the early cycles of PCR, there is no detectable signal and the amplification is below the level of detection of the real time instrument. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline reflects the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter C_t (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold.

Table 1.6 Genes used for the identification of aflatoxigenic fungi by PCR and RT-PCR.

Gene	Enzyme	Step in AFAs biosynthesis pathway
aflD	Reductase	Norsolorinic acid to averantin
aflI	Oxidase	Averufin to versiconal hemiacetal acetate
aflM	Dehydrogenase	Versicolorin A to demethylstrigmatocystin
aflO	*O*-methyltransferase B	Demethylstrigmatocystin to strigmatocystin
aflP	*O*-methyltransferase A	Strigmatocystin to *O*-methylstrigmatocystin
AflQ	Oxidoreductase	*O*-methylstrigmatocystin to aflatoxin B_2
aflR	Transcription factor containing a zinc cluster DNA binding motif	Positive regulator of AFB_2 biosynthesis
aflS	Transcription factor	Positive regulator of AFB_2 biosynthesis

A plot of the log of initial target copy number for a set of standards versus C_t is a straight line (Figure 1.8). The detection of RNA or DNA targets by real-time PCR can be measured by one of three basic methodologies based on the using of the fluorescent dyes. In each case, a low initial fluorescent signal is increased proportionally during each succeeding PCR cycle in tandem with the exponential increase in the DNA product(s) formed. One of the simplest assay system includes the incorporation of a free dye into the newly formed double-stranded DNA product. The common used dye for this purpose in real-time PCR is SYBR® Green I. Another category of signaling systems for real-time PCR are those involving Hydrolysis probes, exemplified by the TaqMan chemistry, also known as 5′nuclease assay, fluoresce upon probe hydrolysis to detect PCR product accumulation (Figure 1.9).

The sequence-specific probe is labelled with a reporter dye on the 5′end and a quencher dye on the 3′end, which permits the quencher to decrease the reporter fluorescence intensity by fluorescence resonance energy transfer (FRET) when the probe is complete (Clegg, 1992). During hydrolysis and hybridization probes depend on FRET to change the intensity of fluorescence emission, the energy transfer works in opposite manners in these two chemistries. When the probe anneals to its complementary target sequence, the two dyes are maximally separated and quenched probe will be degraded by the DNA polymerase's 5′ nuclease ability during the extension step of the PCR, then the reporter signal detected by the instrument. Probe degradation separates the reporter from the quencher dye, resulting in increased fluorescence emission (Heid et al., 1996; Gibson et al., 1996).

A further advantage of a probe-based assay is extraneous signals from primer dimers that will be detected by free dye or dye-primer-based assays are not detected by probe-based assays. Extra-assay DNA products larger than primer dimers will also not be detected. The only PCR amplicon that can be detected by a probe-based assay are those to which the primers and the probe are both able to bind simultaneously.

Quantification usually takes one of two forms – relative or absolute.

• Relative quantification determines the changes in mRNA levels of a gene across multiple samples and expresses it relative to the level of an internal control (Bustin, 2004).

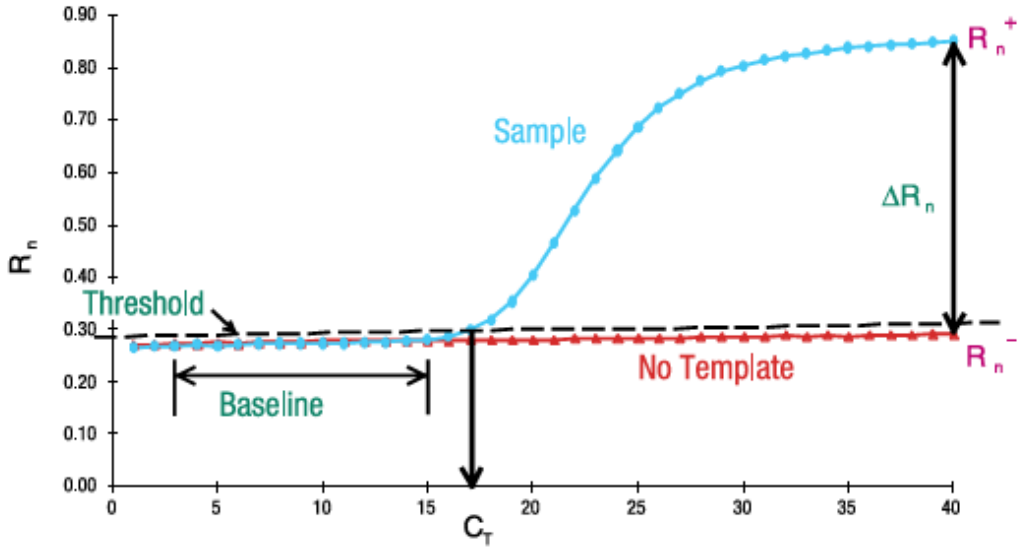

Figure 1.8 Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR (Applied Biosystem DNA/RNA Real-time PCR Quantification PCR bulletin) where Rn : relative flourscence reporter.

This reference gene is often a housekeeping gene that is widely expressed in abundance and is usually employed for normalization in real-time PCR with the assumption of 'constant expression'. Currently, checking which housekeeping genes are suitable for the target cell or tissue is important, and then to use more than one of them in normalization.

By using a reference, the results are expressed as a target/reference ratio. To calculate the mean normalized gene expression from relative quantification assays, there are numerous mathematical models available. On the basis of the employed method, these can yield different results and thus inconsistent measures of standard error (Liu and Saint, 2002). The comparative C_t method is a mathematical model that calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample.

• Absolute quantification uses serially diluted standards of known concentrations to generate a standard curve. The standard curve is generated from a linear relationship between C_t and initial amounts of total RNA or cDNA. This can be used to determine the unknowns concentration based on their C_t values (Heid et al., 1996). This method assumes all standards and samples have approximately equal amplification efficiencies (Souaze et al., 1996). In addition, the concentration of serial dilutions should encompass the levels in the experimental samples and stay within the range of accurately quantifiable and detectable levels specific for both the real-time PCR machine and assay.

Real time PCR detection of the *Tri5* gene in *Fusarium* species by LightCycler®-PCR using SYBR Green I for fluorescence monitoring was developed by Schnerr et al. (2001). Voetz and Rath (2002) developed a real time PCR based assay for the identification and quantification of ochratoxin synthesizing fungi on cereals. The principles of real time PCR are already applied in fungal diagnostics, for instance by detecting airborne filamentous fungi (Wu et al., 2003). Reverse transcription (RT)-PCR and real time RT-PCR assays have been developed to detect and quantify of viable yeasts and moulds contaminating yoghurts and pasteurised food products by Bleve et al. (2003).

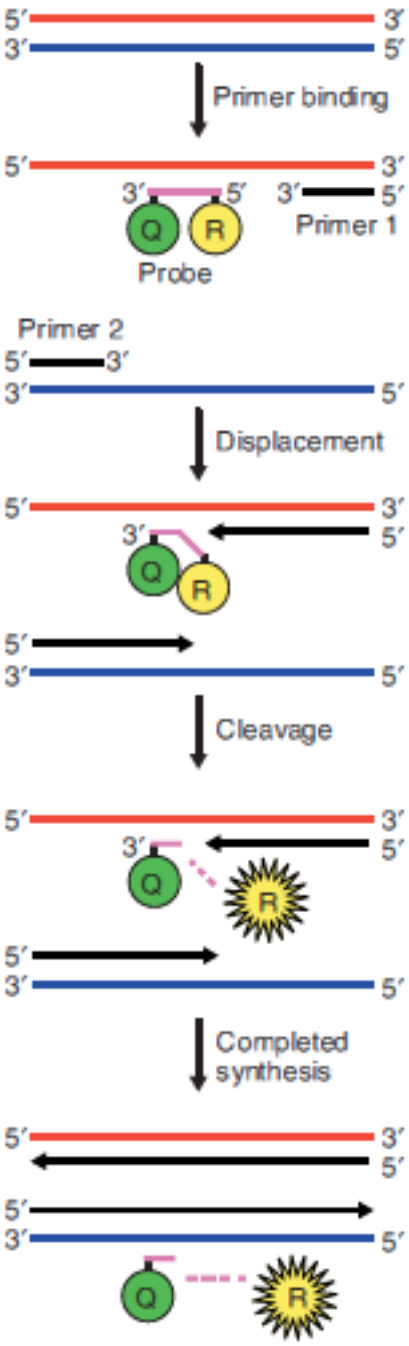

Figure 1.9 TaqMan real-time PCR quantification. Three primers are used during the PCR process – two of these (primers 1 and 2) dictate the beginning of DNA replication on each DNA strand, and the third (the probe) binds to one strand in between. The probe contains two modified bases – a fluorescent reporter (R) at its 5´-end and a fluorescence quencher (Q) at its 3´-end (from Reece, 2003).

Jurado, et al. (2008) reported that using quantitative RT-PCR methods is a useful tool to investigate the ecophysiological basis for fumonisin gene expression of *Fusarium verticillioides* and ultimately may result in more effective control strategies for this important mycotoxigenic pathogen. Water stress caused an increase in *FUM1* transcript levels, especially, when stress was imposed by a non-ionic solute.

1.8 Microarrays

Microarrays are a part of the technology that allows one to screen for gene(s), sequence(s) or specific mRNA among many of possible sequences or genes in a single test (Freeman et al., 2000). There are two different formats of microarray based on the target nucleic acid components, i.e., the oligonucleotide array and the cDNA microarray. DNA microarrays are becoming most frequent because this technology allows investigators with the opportunity to explore simultaneously the interactions among all genes in an organism's genome. cDNA microarray analysis has rapidly become an invaluable tool for defining regulatory networks in cells (Alizadeh et al., 2000; Welcsh et al., 2002).

The basic principle of microarray technology is based on the hybridization RNA to cDNA or oligonucleotides immobilized on a glass chip or, in increasingly rare cases, on a nylon membrane to identify specific genes that are expressed differentially. In microarray technologies, hundreds to thousands of immobilized DNA (targets) spots on solid surface, such as a glass slide, can be simultaneously hybridized with two samples (probes) labelled with different fluorescent dyes (Figure 1.10). The detection of fluorescent signals of two probes bound to individual spots are recorded with a con-focal laser scanner. The separately scanned images from each of the two probes are then combined and pseudocoloured by means of computer software and the normalized expression ratios of two probes are calculated. Based on these systematic procedures, microarrays make possible large-scale gene expression monitoring in a parallel fashion.

Hughes et al. (2000) explained this power by clustering expression data from 300 different array experiments using the yeast *Saccharomyces cerevisiae* and identifying previously unknown ergosterol biosynthesis genes. Currently, Luo et al. (2005) studied the gene expression profiles in a peanut genotype which was drought tolerant and resistant to pre-harvest aflatoxin contamination, using a cDNA microarray containing 384 unigenes selected

from two expressed sequenced tag (EST) cDNA libraries challenged by biotic and abiotic stresses. Wilkinson et al. (2007) reported that microarray analysis of RNA extracted from fungi grown in aflatoxin inducing media (YES) with 50 mM tryptophan showed 77 genes that are expressed significantly differently between *A. flavus* and *A. parasiticus*, including the aflatoxin biosynthetic genes *aflD* (nor-1), *aflE* (norA), and *aflO* (omtB).

A microarray had been developed for the first time by Schmidt-Heydt and Geisen (2007), which includes sub-arrays for the major relevant mycotoxin gene clusters involved in aflatoxin, ochratoxin, trichothcenes and patulin production. The differences in mycotoxin pathway gene expressions after growth on various media for trichothecene and ochratoxin A biosynthesis was demonstrated by using this mycotoxin microarray. The microarray was used further to study and compare the expression kinetics of the trichothecene biosynthesis genes of *Fusarium* species on different trichothecene supporting media. An expression pattern indicative of trichothecene biosynthesis could be identified (Schmidt-Heydt and Geisen, 2007). However, little detailed knowledge is available on the impact that changing environmental factors may have on relative gene expression of aflatoxin related structural and regulatory gene clusters and relate this to phenotypic AFB_1 production.

1.9 RNA interference (RNAi)

Since the advent of DNA sequencing and polymerase chain reaction (PCR), there has been rarely one emerging technology that has received as much attention as the use of RNA interference (RNAi). The story of RNAi in fungi began with an interesting finding reported by Romano and Machino in 1992, whereby gene expression was shown to be interfered with by transformation with homologous sequences in the fungus Neurospora crassa (Romano and Machino, 1992). The gene inactivation was spontaneously reversible and involved the silencing of both transgenes and endogenous genes. This phenomenon was termed ‘quelling’. An interesting feature of quelling in fungi is that it is resulted from targeting of post-transcriptional events; moreover, they found that the effector molecule was cytoplasmic, and thus most likely RNA (Cogoni et al., 1996).

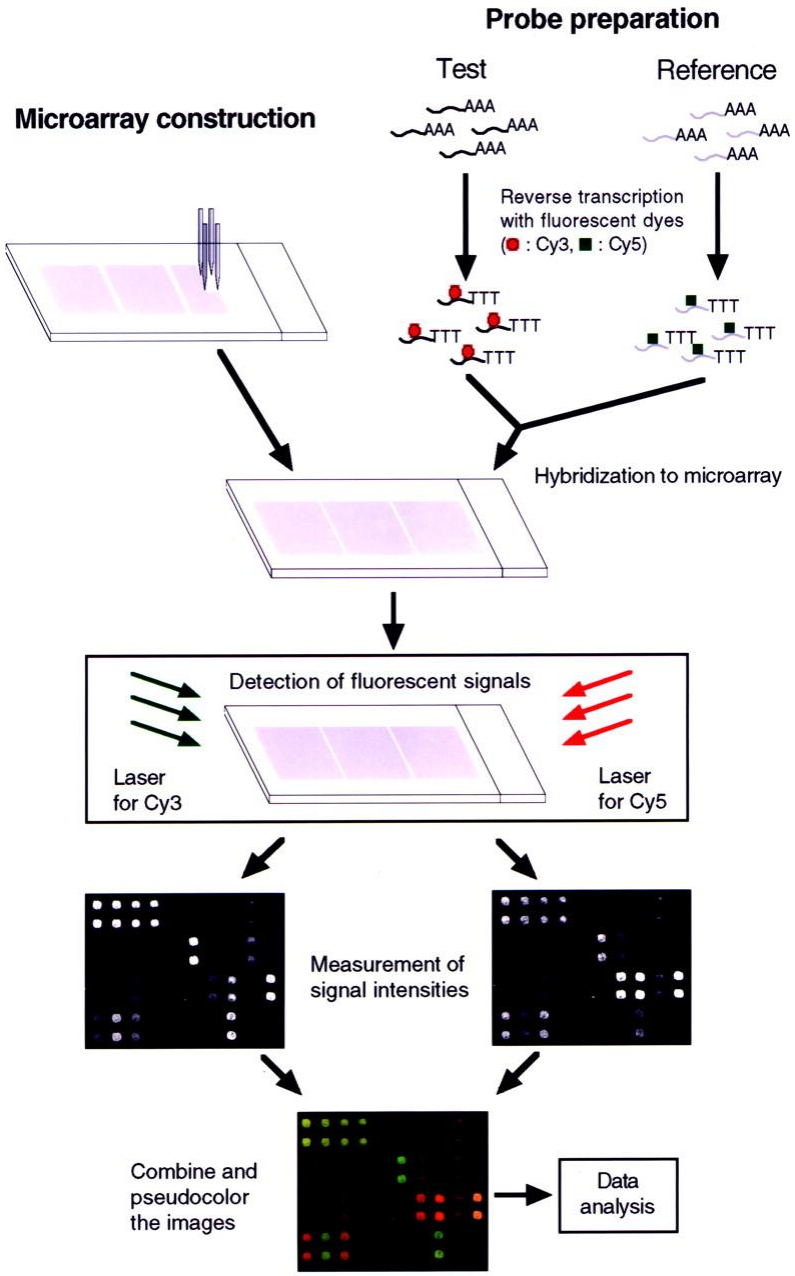

Figure 1.10 Principle of the cDNA microarray analysis system. Target cDNAs are cloned, and amplified by PCR. Purified PCR products are printed onto glass microscope slides with a robotic microarrayer. cDNA probes (test or reference) labelled with different fluorescent dyes (Cy3-dUTP and Cy5-dUTP) are synthesized from total RNA or mRNA derived from test and reference samples. Pooled probes are hybridized to the microarray. Hybridized fluorescent signals are detected with a dual-wavelength laser scanner. Separately scanned images are combined and pseudocolored by means of specialized computer software. Normalized ratios of Cy3/Cy5 are calculated for individual target genes (from Iida and Nishimura 2002).

1.9.1 RNAi mechanism

RNAi molecular mechanism remained unclear until the discovery of a potent gene silencing effect in late 1990s by Fire et al. (1998), when elegant work in the nematode *Caenorhabditis elegans* showed that injecting purified dsRNA directly into adult *Caenorhabditis elegans* resulted in gene silencing. Similar post-transcriptional gene silencing by double-stranded RNA was soon confirmed in a range of organisms: plants, *Neurospora*, *Drosophila*, *C. elegans*, and mammals (Tijsterman et al., 2002).

The dsRNAs are introduced into the cell either using a short oligo (Short Interfering RNA) siRNA or a DNA plasmid from which a short hairpin RNA (shRNA) can be transcribed. The cell has a specific enzyme (it is called Dicer) that is responsible for cleaving double stranded molecules, whether derived from endogenous miRNAs or from replicating viruses, into small RNA duplexes of 19–25 base pairs (bp) (Bernstein et al., 2001). These short RNA fragments (siRNA) serve as guides to carry nuclease machinery to the target mRNA: each siRNA is integrated into a protein complex called RNA-induced silencing complex (RISC). Within this RISC complex, the double stranded is unwound to allow the antisense strand and the complementary-/-target mRNA to hybridize in the cell. RNAi enzymatic machinery within the activated RISC, leading to degradation of target mRNA. The degradated mRNA cannot be translated into protein. This means the protein cannot be expressed, resulting in knockout of the protein (Figure 1.11).

1.9.2 siRNA delivery

The successful knockdown using RNAi depends on the delivery system for cells and this should be chosen carefully to allow the right amount of duplexes to enter the cells. Low transfection efficiency and low cell viability are in fact some of the most frequent causes of unsuccessful gene silencing experiments. Efficient delivery of RNAi probes can be achieved by several methods, including (i) direct transfection of siRNAs molecules or (ii) introduction of short hairpin RNA (shRNA) expressing plasmids. Even though the direct delivery of synthetic siRNA to cultured cells is a common method to introduce RNAi in mammalian systems.

Recently, direct delivery has been very rarely reported in fungi. Whisson et al., (2005) examined the applicability of the direct delivery of synthetic siRNA into protoplasts in *Phytophthora infestans*, which belongs to the fungus-like Oomycetes. A marker gene, GFP, and two *Phytophthora infestans* genes, *inf1* and *cdc14* were transiently silenced by Lipofectin mediated transfection of protoplasts with *invitro* synthesized Cy3-labeled dsRNAs (150–300 bp in size). In *Aspergillus nidulans*, ornithine decarboxylase (ODC), a key polyamine biosynthesis gene, was specifically silenced by treating germinating spores with synthetic 23 nucleotide siRNA duplex (Khatri and Rajam, 2007).

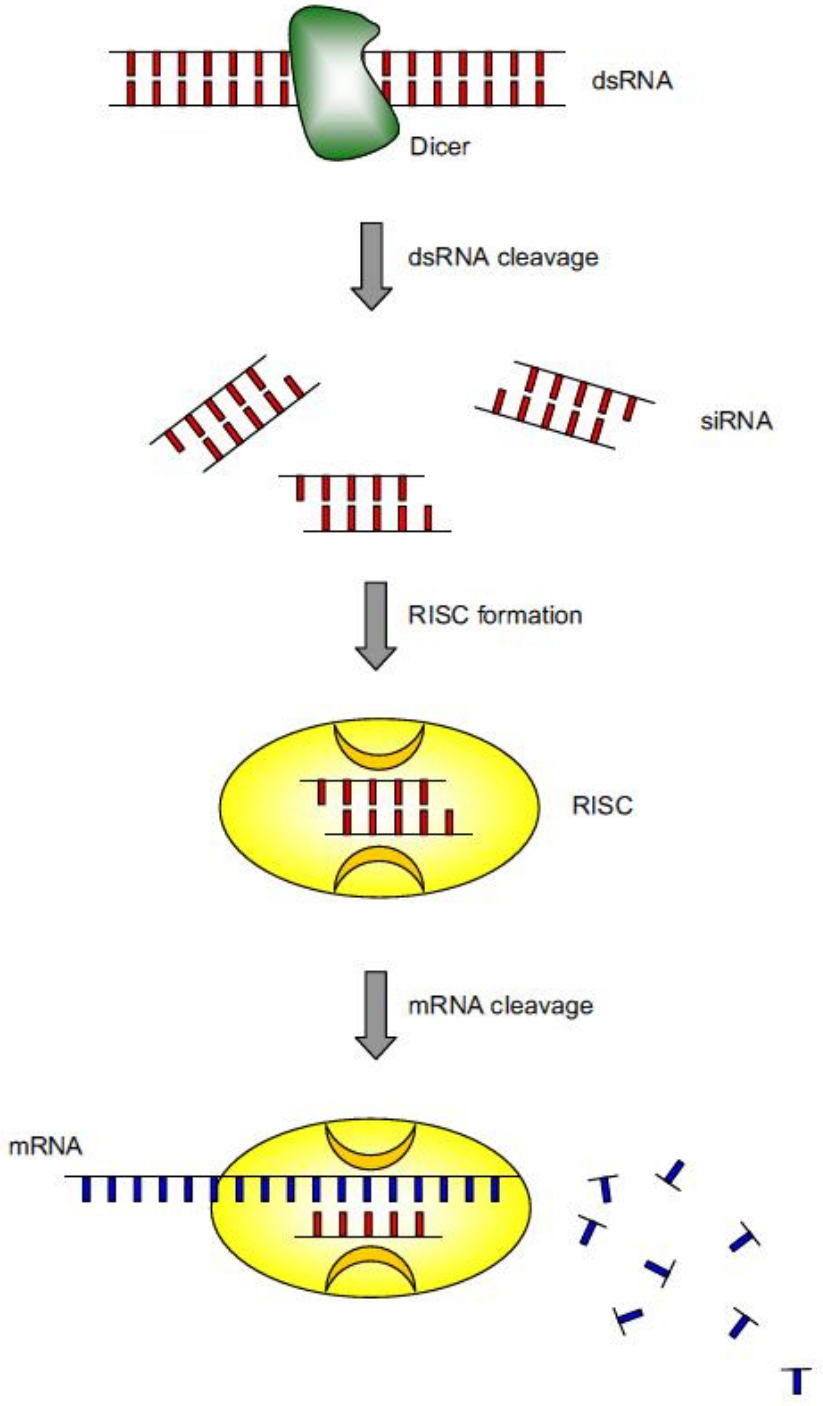

Figure 1.11 Mechanism of RNA interference (RNAi). Where (ds) is double stranded, (RISC) is RNA-induced silencing complex (from Mocellin and Provenzano 2004).

1.10 OBJECTIVES

The main objective of this study was to determine and understand how environmental factors influence growth and the regulation of AFB_1 production, and to gain knowledge of the correlation of ecophysiological conditions with toxin gene expression of the aflatoxin genes in relation to phenotypic production.

In this study the following studies have been carried out:

- Effect of combinations of water activity x temperature interactions on growth, and sporulation, and partitioning of AFB_1 into spores, mycelium and medium using *A. flavus* NRRL 3357.

- Use of a microarray and real-timePCR to examine the effects of a_w x temperature interactions on a conducive YES and to examine the relationship between the expression of the genes and phenotypic aflatoxin production

- A wide range of *Aspergillus* section *Flavi* strains were isolated from Egyptian peanut samples. 18 of these strains were compared with two type strains (*A. flavus* NRRL 3357, *A. parasiticus* 2747) for aflatoxin production based on (a) qualitative fluorescence using a Coconut cream Agar Medium (CAM), and (b) aflatoxin B_1 production on a conducive YES medium using HPLC. These results were validated by using molecular approaches (the structural genes, *aflD* (nor-1), *aflM* (ver-1) and *aflP* (omt A) and the regulatory gene *aflR* to discriminate between aflatoxigenic and non-aflatoxigenic strains of the *Aspergillus* section *Flavi* group *invitro* and on peanuts

- A relative quantification system (RQ-PCR) was used to monitor and correlate the temporal activity of the *aflD* gene *of Aspergillus flavus* using real-time PCR in relation to phenotypic AFB_1 production and populations of *A. flavus* in stored peanuts at three water activity levels (0.95, 0.90 and 0.85 a_w) for six weeks.

- To examine the potential of three designed siRNA sequences (Nor-Ia, Nor-Ib, Nor-Ic) to target the mRNA sequence of the *aflD* gene for using RNA silencing technology to

control aflatoxin production. Thus, the effect of siRNAs targeting of two key genes in the aflatoxin biosynthetic pathway, *aflD* (structural) and *aflR* (regulatory gene) and AFB_1, and AFG_1 production were examined. Figure 1.12 shows the different components of this research project and the links between the different phases. The work is presented in self-contained Chapters with each including a short Introduction, Materials and methods, Results and Discussion section (Chapters 2-5). Chapter 6 contains a final list of Conclusions and Future work.

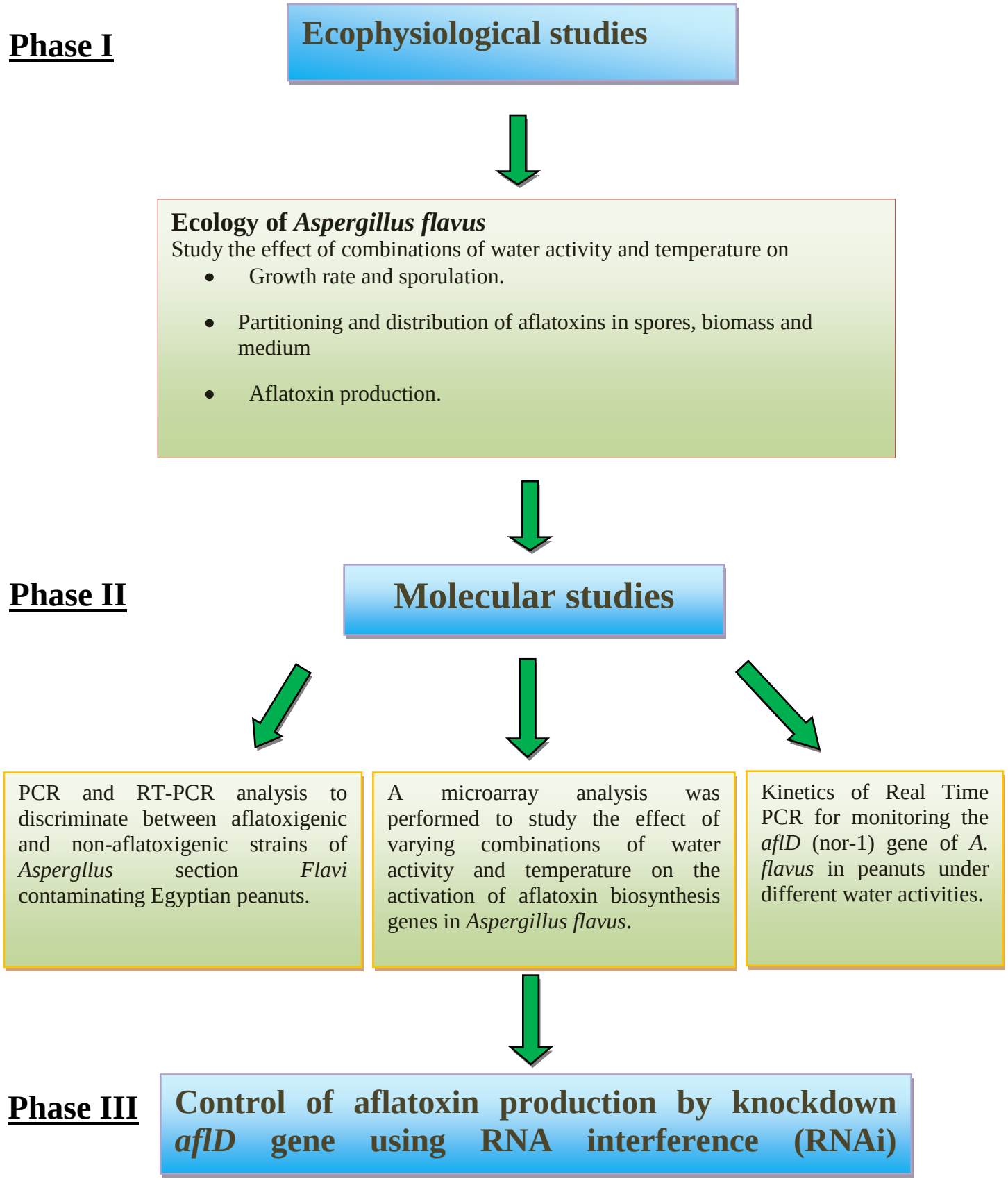

Figure 1.12 Flow diagram of the experimental work carried out in this thesis.

Chapter 2

Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

2 Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

2.1 Introduction

The aflatoxins (AFAs) are toxic secondary metabolites produced by certain *Aspergillus* species and are thought to be some of the most carcinogenic natural substances known. Economically and biologically the most important fungal species able to produce AFAs are *A. flavus* and *A. parasiticus* (Bhatnagar et al., 2002). It has previously been shown that a_w and temperature are important criteria for understanding the ecology of spoilage fungi, especially mycotoxigenic species. A significant amount of information is now available on the growth and mycotoxin profiles for many of these species (Sanchis and Magan, 2004). While there is now detailed knowledge of the relationship between these factors, germination and growth, less information is available on effects on AFAs production. Indeed, it has been suggested that AFAs production is linked to asexual sporulation capacity. However, few studies have attempted to integrate studies of the impact of ecophysiological conditions on expression of the gene clusters involved in the biosynthetic pathway of AFAs and the phenotypic toxin quantified analytically.

Fungal developmental processes are associated with secondary metabolism such as sporulation and sclerotial formation (Bennett et al., 1986; Calvo et al., 2002). It has been suggested that there is a strong relationship between conidial development and secondary metabolite formation. However, there is little detailed information on the partitioning of AFAs into spores, biomass and medium. In a study of the relationship between sporulation and aflatoxin production, isolates inhibited from sporulation with diaminobutanone, and a non-sporulating mutant, as well as evidence from a sectoring mutant, indicated that aflatoxin biosynthesis was correlated with sporulation (Guzman-de-Pena and Ruiz-Herrara, 1997). The relationship between mycotoxin production and sporulation was also found by Mostafa et al. (2005) who demonstrated that most of the toxins were produced after the fungus had completed its initial growth phase and began the developmental stage, represented by sporulation, and for *A. flavus* sclerotial formation. Studies by Atoui et al. (2007) on *Aspergillus carbonarius* strains suggests that a significant percentage of the mycotoxin ochratoxin A, was channelled into the conidia, and this varied with environmental stress.

The AFAs biosynthetic pathway elucidation has received a lot of attention in the last decade and this resulted in the whole genome being elucidated. The major biochemical pathway steps have been determined and the chemical structures of AFAs intermediates characterized (Payne and Brown, 1998; Yu et al., 2004). At least 23 enzymatic reactions are involved in aflatoxin formation. No less than 15 structurally-defined aflatoxin intermediates have been identified in the aflatoxin/ST (sterigmatocystin) biosynthetic pathway (Yu et al., 2004). The structural genes are regulated by the transcriptional factor *aflR* and the accessory regulatory gene *aflS* (Chang, 2003).

Biotic factors, especially nutritional matrices including C:N ratio, amino acids, lipids, and trace elements or abiotic factors such as pH, a_w , temperature or modified atmospheres, are known to affect AFAs biosynthesis in *Aspergillus* section *Flavi* species, despite the molecular mechanisms for these effects still being unclear (Ellis et al., 1993; Luchese and Harrigan, 1993; Payne and Brown, 1998; Molina and Gianuzzi, 2002; Guo et al., 2005; Ribeiro et al., 2006; Giorni et al., 2008).

Despite this, few studies have tried to link the impact of environmental factors on gene expression and relate this to phenotypic AFAs production. A mathematical model to describe the relationship between pH, propionic acid concentration and temperature on aflatoxin production by *A. parasiticus* was described by Molina and Giannuzzi (2002). Sweeney et al. (2000) developed a reverse transcription polymerase chain reaction to analyse the expression of the *afl*R and *ord*1 gene of the AFAs pathway in relation to various nutritional media. Xu et al. (2000) monitored the expression of the *nor*-1 gene after growth of *A. parasiticus* on peanut pods by a reporter gene approach. Mayer et al. (2003) showed that the expression of the *nor*-1 gene in wheat was correlated to conventional colony forming unit (CFU) data obtained from the same set of samples.

Recently Price et al. (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of AFA biosynthesis genes. With a pathway specific microarray and by real-time PCR, the influence of physical parameters like a_w, temperature and pH on the expression of ochratoxin A, trichothecene and aflatoxin biosynthesis genes have been analysed and a common expression profile was found (Schmidt-Heydt and Geisen, 2007). The published data show a correlation between external parameters and gene expression; however no comprehensive systematic analysis of this in

relation to aflatoxin production has been performed. Recently, Georgianna and Payne (2009) summarized the current knowledge of the regulation of aflatoxin biosynthesis in relation to external factors.

The objective of this study was to investigate the influence of a_w, temperature and their interactions on (a) growth rate, (b) sporulation, (c) AFB_1 production and partitioning of AFB_1 into spores, mycelium and medium, and (d) the regulation of the AFA biosynthesis genes of *A. flavus* using a microarray containing the toxin gene clusters for the most important mycotoxins (Schmidt-Heydt and Geisen, 2007).

2.2 Material and Methods

2.2.1 Fungal strain

Aspergillus flavus NRRL3357, isolated from maize, was kindly provided by Dr. D. Bhatnagar (USDA, New Orleans, USA) was used in this study.

2.2.2 Media

The following media were used in these studies

(a) Malt Extract Agar (MEA)

This medium was used for growing up the strain for inoculation of treatments (20.0 g Malt extract (Difco, Oxford, UK), 2.0 g Peptone (Difco), 15.0 g Agar (Sigma Aldrich, Dorset, UK) for 7 days at 25°C in the dark. The spores were gently removed from the colony surface into suspensions of 10 ml sterile distilled water containing 0.1 % Tween-80 in 25 ml Universal bottles. The spore concentration was determined using a haemocytometer and adjusted to 10^6 spores ml^{-1}.

(b) Yeast Extract Sucrose (YES)

A. flavus NRRL 3357 was inoculated on Yeast Extract Sucrose (YES) medium which is an aflatoxin-induced medium (20.0 g l^{-1} Yeast extract, 150.0 g l^{-1} Sucrose, 15 g l^{-1} Agar) (Davis, et al. 1966).

2.2.3 Adjustment water activity of media for ecophysiological studies.

Glycerol water solutions were made up in the range 0.99 to 0.90 a_w and substituted for water to modify the a_w to the required target levels. Glycerol was used because of its temperature stability over a wide range (5-40^oC). The media were sterilized by autoclaving at 121°C for 20 min. Molten cooled media were poured into 9 cm sterile Petri dishes (approx. 15 ml per plate). The same a_w treatments were kept in separate polyethylene bags and the a_w checked using an AQUALAB ® 3TE (Decagon, Pullman, WA, USA) for confirmation of actual levels. These were within 0.005 a_w of the target treatments.

2.2.4 Effect of water activity x temperature interactions on growth, sporulation, aflatoxin B_1 production and expression of aflatoxin cluster gene expression

Petri plates modified to 0.99, 0.95, and 0.90 a_w were centrally inoculated with 5 µl of the spore suspension and incubated at 20, 25, 30, and 35°C. The experiments were carried out with at least nine replicates per treatment and some treatments repeated. Three replicates were used for growth measurement and AFB_1 determination, three replicates for quantification of spore production and three replicates for RNA extraction for subsequent microarray analyses.

(a) Measurement of growth

Colony diameters of replicate plates were measured in two directions at right angles to each other. Measurements were recorded daily during growth for 8 days (Aldred et al., 1999). The growth rate was calculated by plotting the radial extension against time and the slope of the linear growth phase used to determine the radial growth rates (Cm day^{-1}, Patriarca et al., 2001).

(b) Determination of aflatoxin B_1

Preliminary experiments were made to monitor the temporal production of AFB_1 by this *A. flavus* strain. *A. flavus* was inoculated on YES and incubated for up to 16 days. Every two days three plugs (3 mm diameter) were cut across the colony surface one in the centre and two on both sides using a sterile cork borer and placed in 2 ml Eppendorf tubes, and weighed. $AFB_1$was extracted by adding 1 ml chloroform and shaking for 1 hour (Filtenborg et al., 1983). The biomass was discarded after centrifugation and chloroform was evaporated to dryness. The residue was derivatized according to the AOAC method (2000). Two hundred microlitres HPLC grade hexane was added to the residue in the 2 ml Eppendorf tube, then 50 µl TFA (Triflouroacetic acid) followed by vortexing for 30 seconds. Eppendorf tubes were left for 5 minutes after which 950 µl water: acetonitrile (9:1) was added and then the mixture vortexed for 30 seconds. The Eppendorph tubes were left for 10 mins for separation of the layers. The upper layer was discarded and the extracts were filtered through Nylon 13 mm, 0.2 µm filter (SMI- Lab Hut LTD, UK) directly into amber HPLC vials (Jaytee Biosciences LTD, UK).

Aflatoxin B_1 analysis by HPLC: A stock solution of 200 µl of aflatoxins mixture (B_1, B_2, G_1, G_2) (Sigma Aldrish Supelco) with a concentration of 0.2 µg ml^{-1} for AFB_1 and AFG_1; 0.06 µg ml^{-1} for AFB_2 and AFG_2, was evaporated and derivatized as described previously and diluted to give a range of 10, 20, 30, 40 and 50 ng ml^{-1} for AFB_1 and AFG_1; 3, 6, 9, 12, 15 ng ml^{-1} for AFB_2 and AFG_2. Samples were analyzed by HPLC using a 470 fluorescence detector (Millipore Waters, Corp., Milford, MA, USA) (λ_{exc} 333 nm, λ_{em} 460 nm) and a C_{18} column (Luna Spherisorb ODS2 150 x 4.6 mm, 5 µm; Water Corp.) all under the control of Waters Millenium ^{32} software (Water Corp.). The analysis was performed at a flow rate 1 ml min^{-1} with a mobile phase of 30% Methanol: 60 % Water: 10 % Acetonitrile and the run time was 25 minutes. The liner regressions for AFB_1 concentrations against peak area were plotted (R^2= 0.999) as shown in Figure 2.1. The limit of detection for AFB_1 was 0.5 ng ml^{-1}. AFB_1 for all treatments were extracted using the agar plug technique at the end of the incubation period (8 days) and determined by HPLC as described above.

(c) Spore production in relation to environmental factors

The *A. flavus* strain was centrally inoculated onto YES medium previously overlaid with sterile cellophane sheets (8.5 cm, P400, Cannings Ltd., Bristol, UK); this enabled the entire mycelial colony to be removed later.

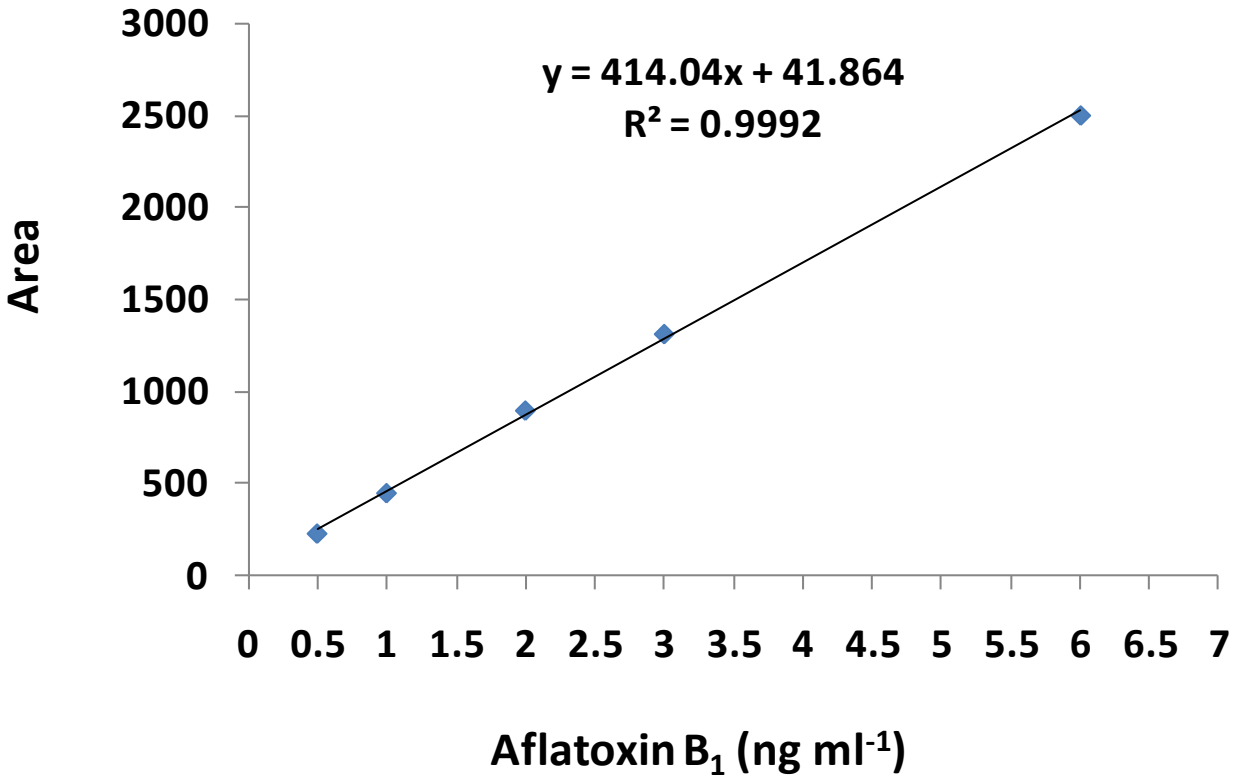

Figure 2.1 Calibration curve obtained of aflatoxin B_1 (ng ml^{-1}) versus peak area using HPLC.

The treatments were harvested after 8 days incubation with each colony suspended in 10 ml of sterile water containing a wetting agent (Tween 80, 0.1%) to wet the spores (Ramos et al., 1999). Spores were collected by filtering through sterile glass wool, and the filtrate was centrifuged to obtain a spore pellet. The number of spores was determined in defined volume and calculations used to give spores per cm^2 of colony area by using a haemocytometer and a binocular microscope connected to a video imaging unit (Parra et al., 2004).

2.2.5 Partitioning and distribution of aflatoxin B_1 in spores, biomass and medium

Other preliminary experiments were made to study the effect of various a_w levels on partitioning of AFB_1 into biomass, medium and conidia. As mentioned in Section 2.2.4c, the spores were removed at the end of the incubation period. The spore pellets, mycelial biomass and medium were weighed. After separation of the spores, biomass and medium, AFB_1 was extracted from spores and biomass by adding 1 ml of 1 mol l^{-1} HCl and extracted three times with 3 ml chloroform, and evaporated to dryness. The extracts were immediately derivatized (Stormer et al., 1998). The extracts were filtered directly into amber HPLC vials.

2.2.6 Extraction of RNA and molecular analyses

(a) Isolation of RNA from samples

To perform microarray and real-time PCR experiments RNA was isolated using the RNAeasy plant mini kit (Qiagen, Hilden, Germany). After 8 days of incubation on cellophane overlaid YES medium, the entire mycelial colony was removed together with the cellophane layer. The mycelia were quickly frozen in liquid nitrogen and stored at -80 °C until extracted. An amount of 0.5-1.0 g of the mycelium was ground in a mortar with a pestle in the presence of liquid nitrogen. About 250 µg of the mycelial powder was then used for isolation of total RNA. The 250 µg mycelial powder was resuspended in 750 µl lysis buffer, mixed with 7.5 µl β-mercaptoethanol and about 100 µg glass beads with a diameter of 1 mm in a 2 ml RNase free microreaction tube. RNA extraction from the ground mycelia was accomplished with the RNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer (Figure 2.2). After RNA extraction, residual DNA in the samples was digested with a DNase I (RNase free DNase I, Amplification Grade, Sigma) according to the following protocol:

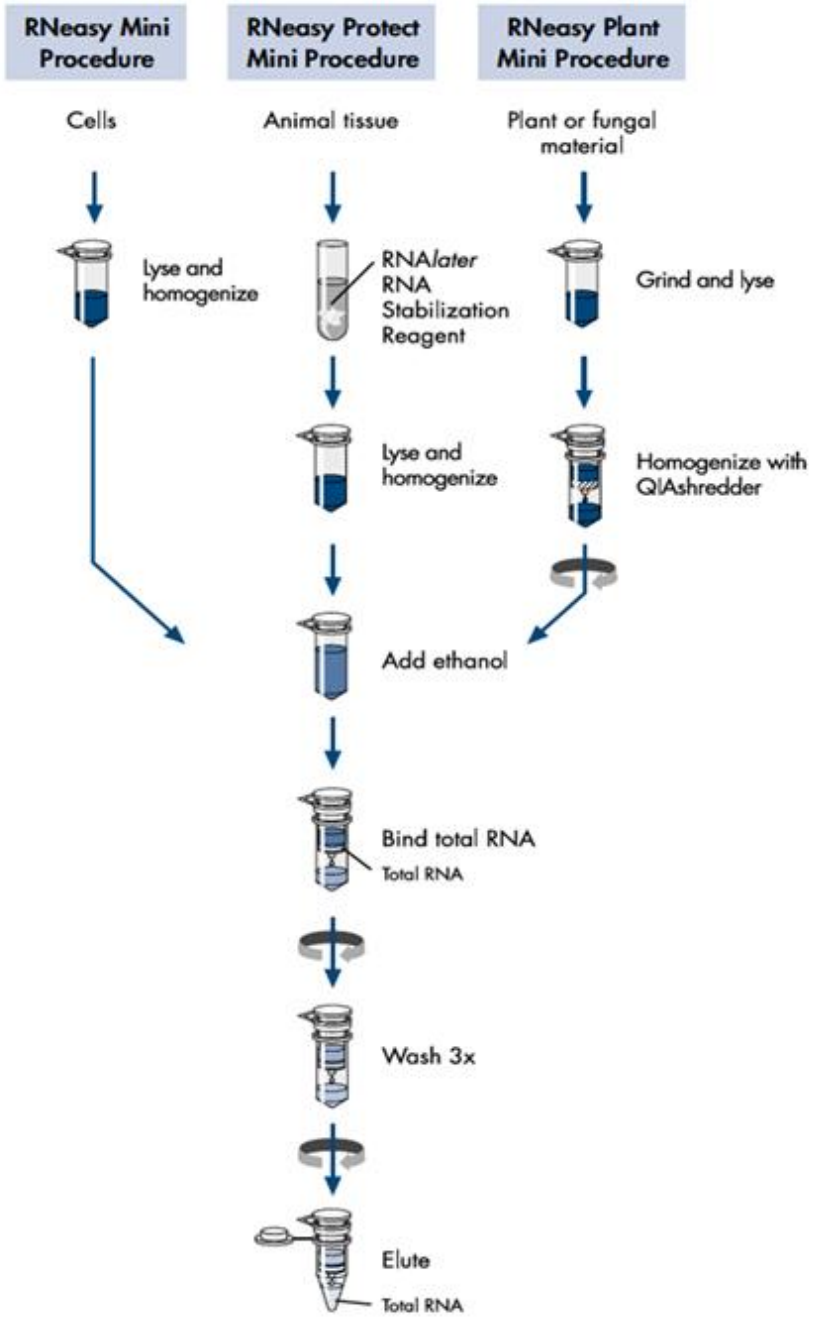

Figure 2.2 Flowchart of RNA Extraction using RNeasy Kit provided by the manufacturer (Qiagen GmbH, Hilden, Germany).

RNA in 8 µl water, 1 µl of 10X Reaction Buffer and 1 µl of Amplification Grade DNase I, 1 unit/µl were added to an RNase-free PCR tube. The tube was mixed gently and incubated for 15 mins at room temperature and, 1 µl of Stop Solution was added to inactivate the DNase I. The tube was heated at 70 °C for 10 minutes to denature both the DNase I and the RNA.

(b) Microarray experiments

The microarray analysis was performed by Dr M. Schmidt-Heydt & Prof. R. Geisen at the Max-Rubner Institute, Karlsruhe, Germany using the following protocol: For labeling of cDNA an amount of 10 - 50 µg of the DNase I treated total RNA was used according to the specifications of the manufacturer of the Micromax cDNA direct labeling kit (PerkinElmer Life And Analytical Sciences, Inc. Boston, USA). After cDNA synthesis and labelling, the cDNA was purified with a QiaQuick MinElute-Kit (Qiagen, Hilden, Germany). The labelled and purified cDNA was brought to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, USA), re-suspended in 60 µl hybridization buffer (Scienion, Berlin, Germany), heated for 2 min. at 95 °C and hybridized for 18 h at 42 °C to the microarray by using an automatic hybridization station (Perkin Elmer, Boston, USA). After hybridization the array was scanned with a confocal laser system (Scanarray lite, Perkin Elmer) at a resolution of 5 µm. The analysis of the results was performed using the Scanarray software (Perkin Elmer, Boston, USA). The results were normalized using the Lowes algorithm (locally weighted scatter plot smoothing) and the subtraction of the background signal intensity. As control, the constitutive expressed β- tubulin gene was used. Mayer et al. (2003) reported that the β-tubulin gene was constitutively expressed and constant during the subsequent growth phases when compared with the expression of nor-1 gene of *A. flavus.* The microarray was developed by Schmidt-Heydt & Geisen (2007) and comments of sub-array for all the major clusters of the genes for mycotoxigenic fungi (Figure 2.3)

2.2.7 Statistical analysis

All experiments were carried out with 3-4 replicates and repeated twice with similar results. Statistical tests were performed using Statistica version 8 (StatSoft, Inc, 1984-2007, Statsoft, Tulasa, OK) for three-way ANOVA and LSD Fisher was determined at the 95% confidence limits for interacting factor (a_w x temperature).

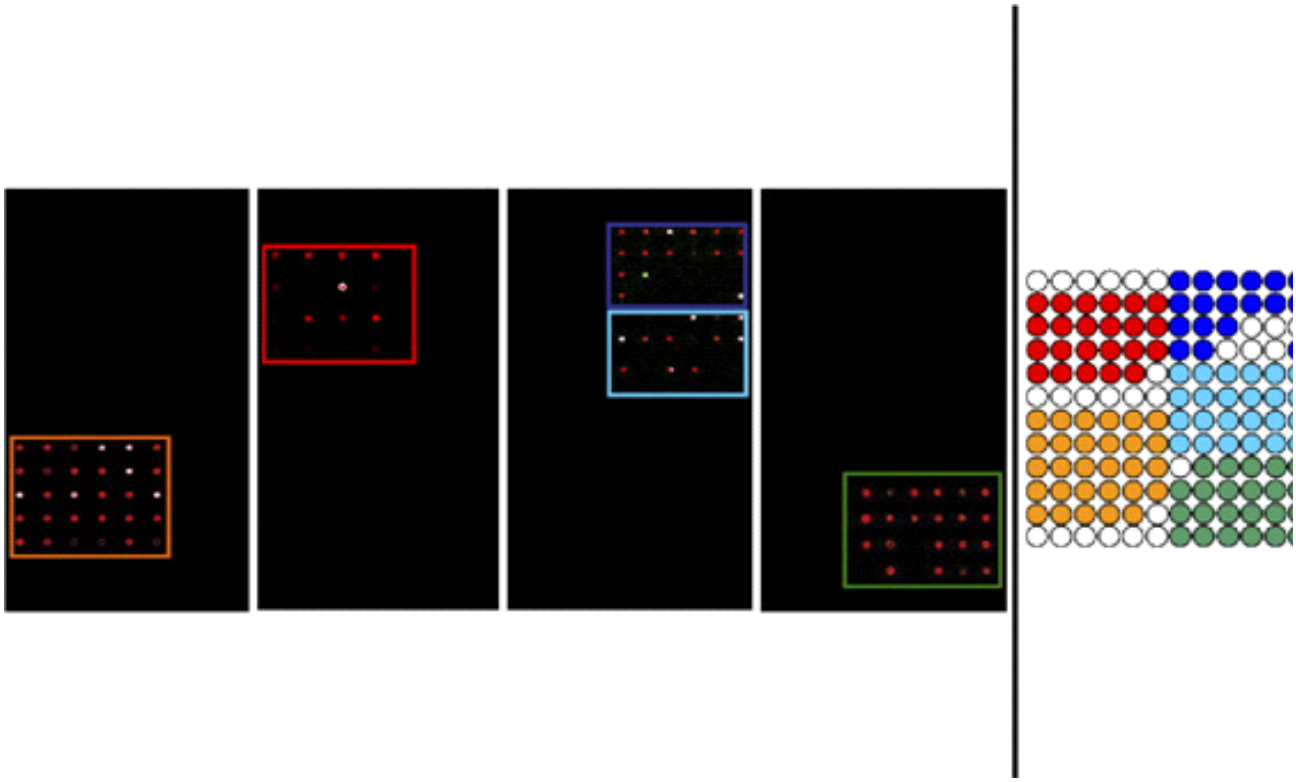

Figure 2.3 Mycotoxin gene cluster microarray analyses. Diffrent subarrays are indicated by different colours (red = ochratoxin A, light brown = aflatoxin, dark blue = trichothecenes (type A), dark green =fumonisins), which correspond to the colours of the frames surrounding the hybridized spots after microarray analysis (from Schmidt-Heydt & Geisen, 2007).

2.3 Results

2.3.1 Effects of water activity and temperature on growth

Figure 2.4 shows the effect of changes in a_w x temperature combinations on growth of *A. flavus* grown for 8 days on YES medium. At each temperature tested mycelial growth increased as a_w was increased from 0.90 to 0.99 a_w. No growth was observed at 0.90 a_w and 20°C. The fastest growth was registered at 0.99 a_w and 35°C. Statistical analysis of variance (ANOVA) showed that the effect of temperature, a_w and their interactions significantly influenced growth ($P< 0.05$; Table 2.1).

2.3.2 Effect of time, temperature and water activity on aflatoxin B_1 production by *A. flavus*

Initially the effect of time of incubation on AFB_1 production was examined. Figure 2.5 shows that the maximum AFB_1 production by *A. flavus* was observed after 8 days incubation followed by a slow decrease. Subsequently, in subsequent studies 8 days was used to examine AFB_1 production.

AFB_1 production was highest at 0.99 a_w followed by 0.95 a_w at all temperatures. Only a low amount was produced at 0.90 a_w. This paralleled the growth patterns. The highest amount of AFB_1 was produced at between 25-30°C (Figure 2.6). Statistical analysis of the data showed that interactions between temperature and a_w was highly significant for AFB_1 production (Table 2.2).

2.3.3 Effects of water activity, and temperature on asexual sporulation

Figure 2.7 shows the effect of interactions between a_w and temperature on conidial spore production by colonies of *A. flavus*. This shows that at 0.95 a_w, the highest amount of spores was produced at all temperatures followed by 0.90 a_w and 0.99 a_w levels. Statistically significant effects of single factors, temperature and a_w, and their interaction are shown in Table 2.3. Plate 2.1 shows an example of the effect of a range of conditions on sporulation of colonies.

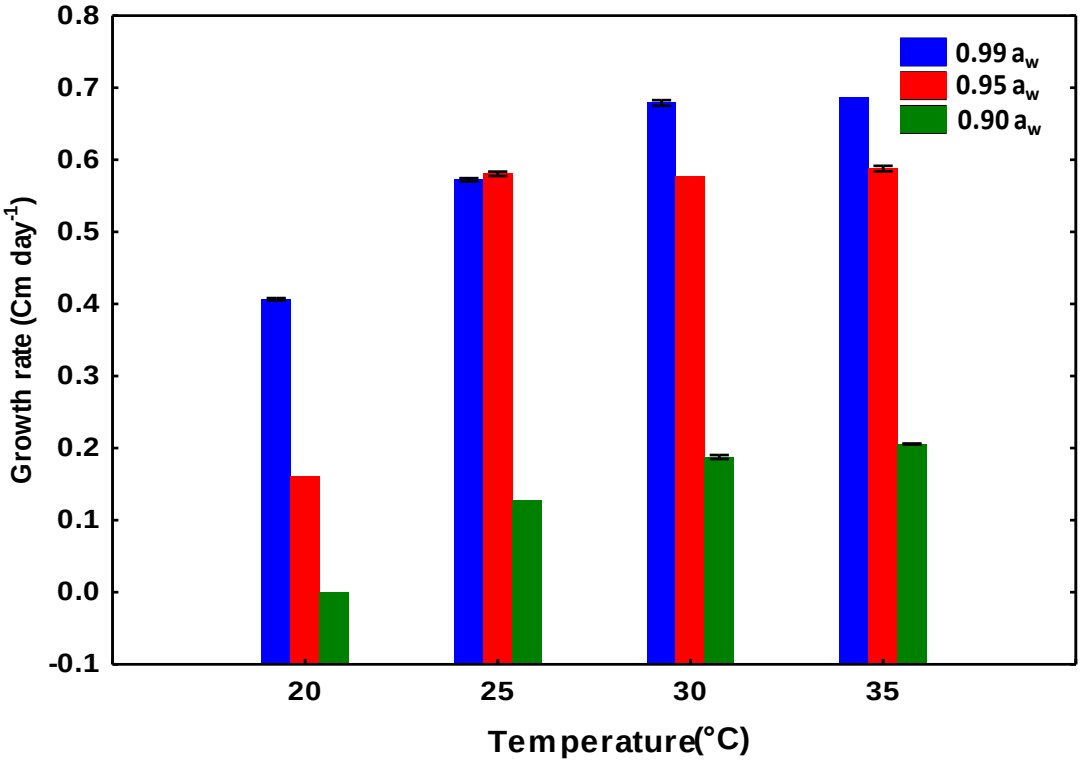

Figure 2.4 Effect of water activity (a_w) and temperature on growth rate of *A. flavus* on *YES* medium. Vertical bars denote standard errors.

Table 2.1 Analysis of variance of the effect of water activity (a_w), temperature (°C) and their interactions on growth rate of *A. flavus*.

Effect	DF	MS	F	P
Factor				
Temperature	3	0.254998	18250.4	0.00 *
a_w	2	0.648756	46431.9	0.00 *
Temperature x a_w	6	0.038084	2725.7	0.00 *
Error	24	0.000014		

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %

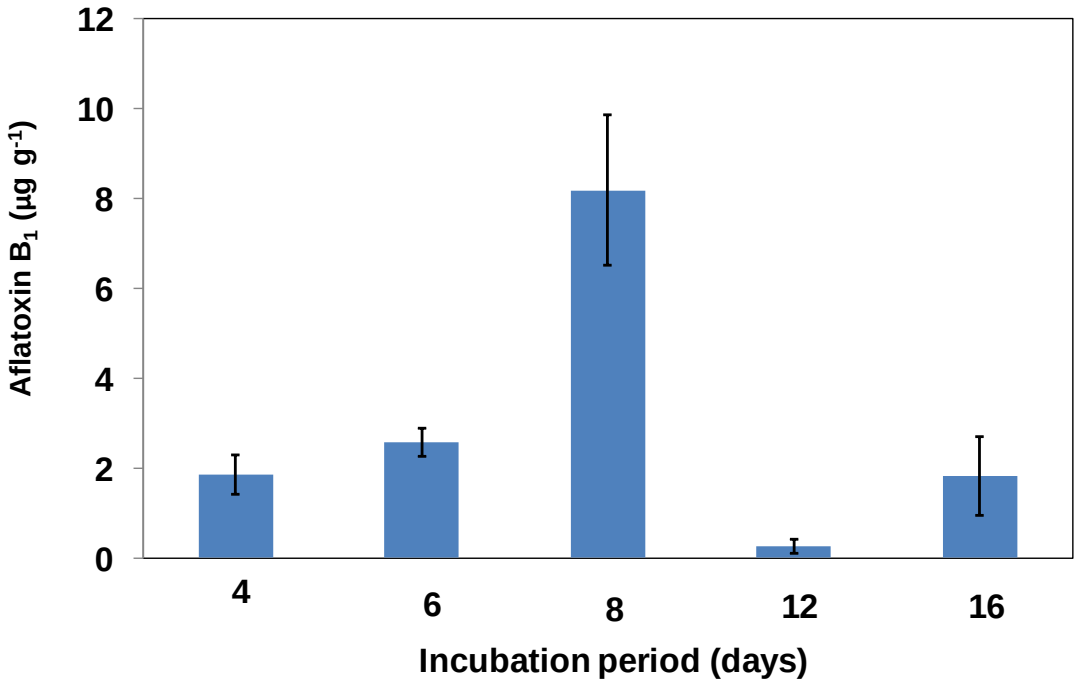

Figure 2.5 Effect of incubation time on aflatoxins B_1 produced by *A. flavus* at 25 °C and 0.99 water activity using YES medium. Bars indicate standard error of the mean.

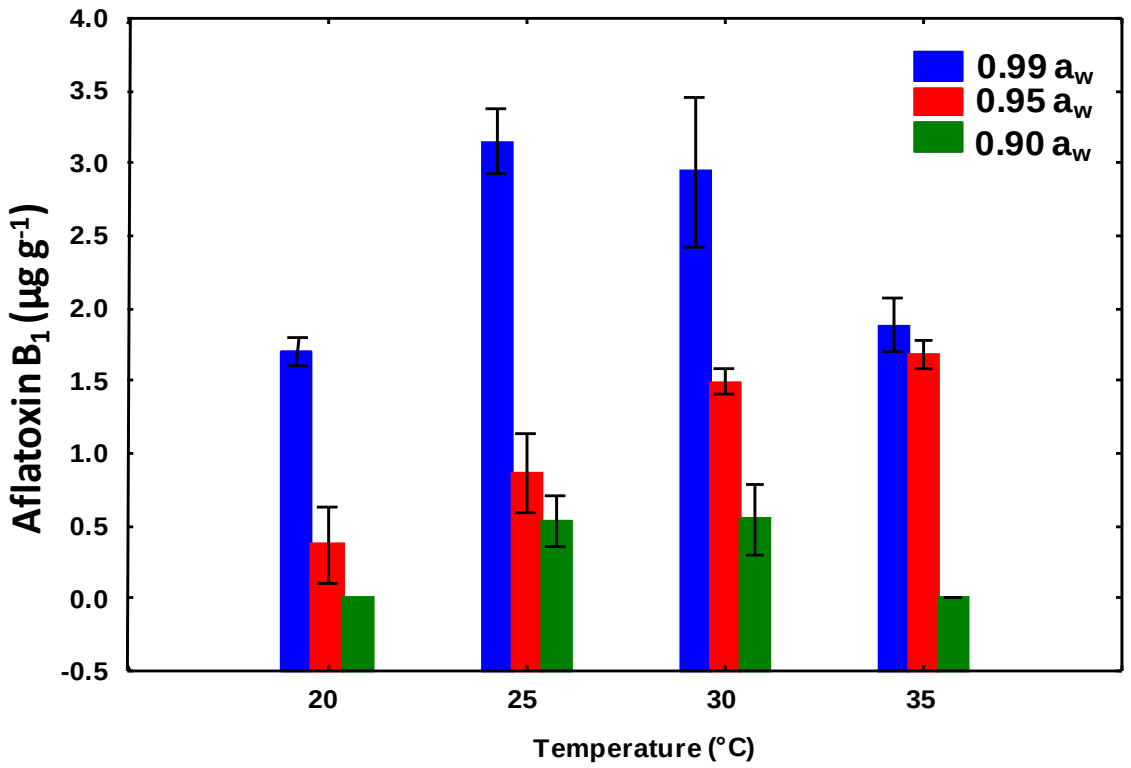

Figure 2.6 Effect of water activity (a_w) x temperature on aflatoxin B_1 production by *A. flavus* on a YES medium. Vertical bars denote standard errors.

Table 2.2 Analysis of variance of the effect of water activity (a_w), temperature (^oC) and their interactions on aflatoxin B_1 production by *A. flavus*.

Effect	DF	MS	F	P
Factors				
Temperature	3	1.66534	10.6863	0.000119 *
a_w	2	14.15582	90.8368	0.000000 *
Temperature x a_w	6	0.66497	4.2671	0.004605 *
Error	24	0.15584		

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %

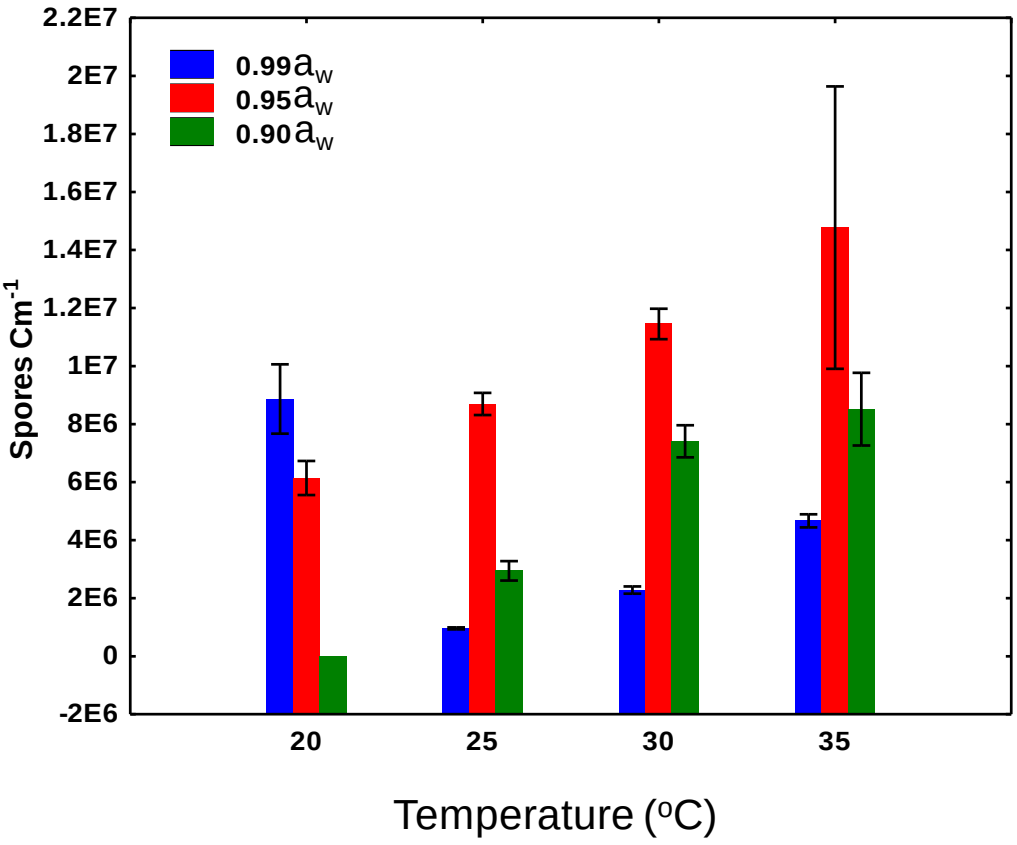

Figure 2.7 Effect water activity (a_w) and temperature on amounts of conidia produced by *A. flavus* when grown on a YES medium at different temperatures. Vertical bars indicate standard error of the

Table 2.3 Analysis of variance of the effect of water activity (a_w), temperature (^oC) and their interactions on conidial production by *A. flavus*.

Effect	DF	MS	F	P
Factors				
Temperature	3	4.718994E+13	6.7619	0.001827 *
a_w	2	1.359068E+14	19.4744	0.000009 *
Temperature x a_w	6	3.863007E+13	5.5354	0.001017 *
Error	24	6.978756E+12		

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %

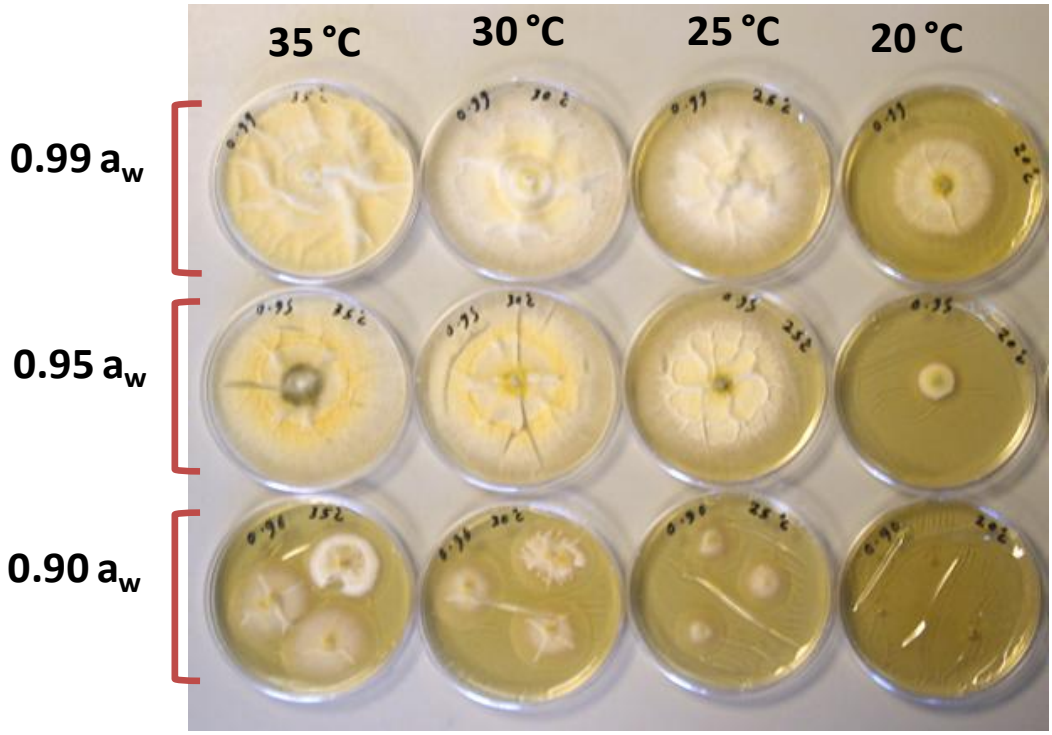

Plate 2.1 Culture plates of *A. flavus* on YES medium overlaid with cellophane after 7 days incubation at different temperatures and water activity (a_w) treatments. Single colonies were used at higher a_w levels and three colonies were used under parginal conditions for growth to have more biomass for extraction purposes.

2.3.4 Partitioning of aflatoxin B_1 in spores, biomass and medium

The partitioning of AFB_1 produced by *A. flavus* colonies when grown on a conducive YES medium at four a_w levels showed that with freely available water (0.99 a_w) about 50% of the AFB_1 was present in the biomass and the medium, with very little present in the spores (Figure 2.8). However, as water stress was imposed there was a switch to a significantly higher channelling of AFB_1 into the spores, especially at 0.95 and 0.93 a_w levels. Statistical analysis of data sets showed that effect of a_w on AFB_1 production into biomass, medium and spores was highly significant (Table 2.4).

2.3.5 Microarray analysis of aflatoxin gene cluster expression in realtion to environmental factors

The microarray analysis showed that the expression profiles of the aflatoxin cluster genes were changed in response to environmental conditions and were partly inversely related to the growth rate and also to AFB_1 biosynthesis. Figure 2.9 shows the plot of the complete data set for the strain of *A. flavus* examined. Reference data set of microarray is shown in appendix I. There is a peak of expression for a number of genes at 20°C/0.99 a_w to 25°C/0.90 a_w. In the moderate zone where usually expression is lower (temperatures of 25-35°C), there were two additional peaks (30°C/0.90 a_w and 35°C/0.90 a_w). These could be due to the low a_w value of 0.90 a_w.

It should be noted that at 0.90 a_w, conditions where growth was reduced when compared to that at freely available water, and all temperature conditions, except for 20°C, there was a clear induction of expression of the aflatoxin genes. However, this was not paralleled by AFB_1 phenotypic production. One of the data sets fitted very well, e.g., at 20 °C/0.99 a_w, but at intermediate combinations of temperature × a_w (25°C/0.95 and 0.99; 30°C/0.95 and 0.99; 35°C/0.95 and 0.99 a_w) a high production of AFB_1 was observed despite the fact that the cluster genes are transcribed only to a certain basal level. Interestingly most of the cluster genes are activated nearly to the same level for all these combinations except for some single genes which were expressed at a significantly higher level.

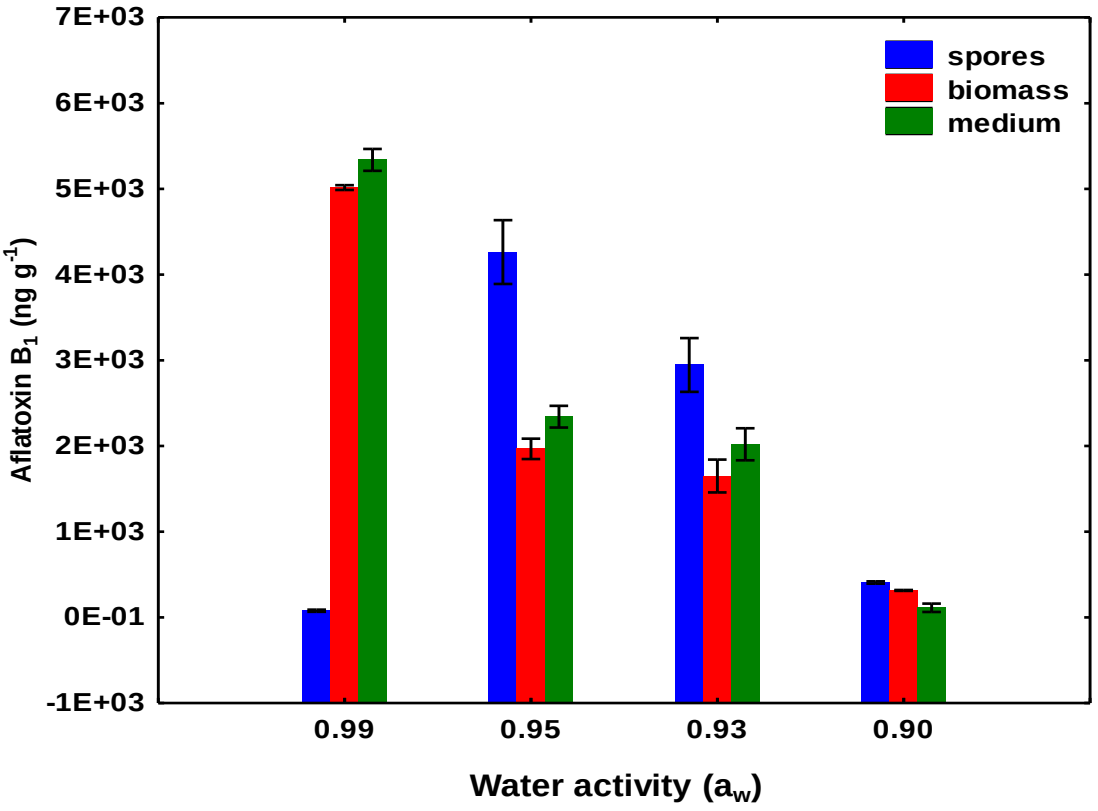

Figure 2.8 Effect of water activity on partitioning of aflatoxin B_1 into medium biomass and conidia at 25°C using *A. flavus* strain NRRL 3357. Bars indicate standard error of the mean.

Table 2.4. Analysis of variance of the effect of water activity on aflatoxin B_1 partitioning into biomass, medium and spores of cultures of *A. flavus* on a conducive YES medium.

Effect	DF	MS	F	P
Factor				
spores	3	8149142	68.8709	0.000672 *
Error	4	118325		
Biomass	3	7884539	304.478	0.000036 *
Error	4	25895		
Medium	3	9333468	267.626	0.000046 *
Error	4	34875		

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %

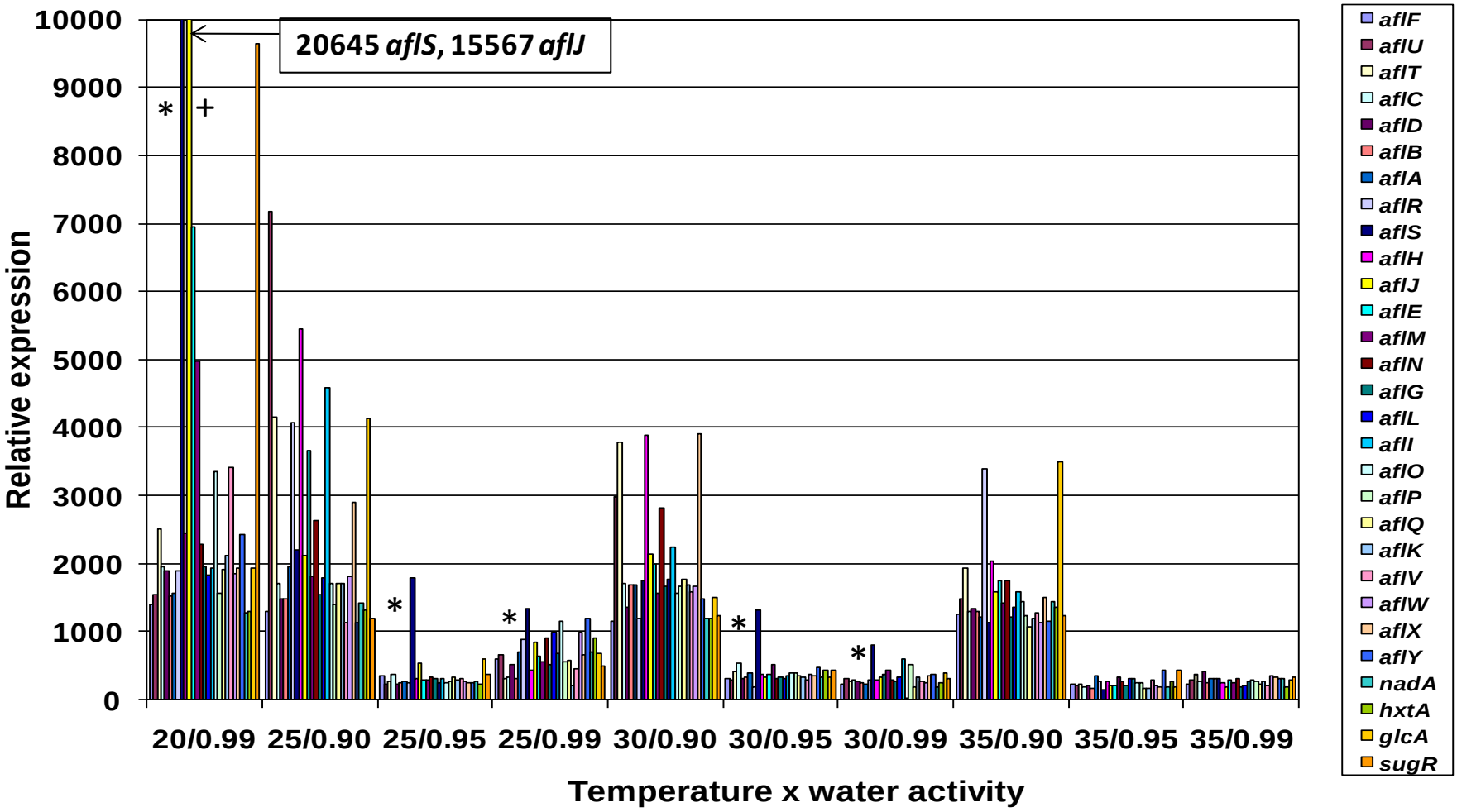

Figure 2.9 Relative cluster gene expression determined by microarray of *A. flavus* grown for 8 days on YES medium. The various combinations of temperature × water activity are indicated at the bottom. The numbers in the upper row indicate the relative expression levels of the highly expressed genes *aflS* and *aflJ* at the combinations where expression exceeds the level of the y axis. Increased *aflS* expression is indicated by an asterisk (*), increased *aflJ* expression is indicated by a cross (+).

2.3.6 Expression of specific genes

Four aflatoxin genes, i.e., two structural genes (*aflD* and *aflF*) and two regulatory genes (*aflR* and *aflS*) were selected from the microarray expression data to analyse the influence of a_w and temperature. Overall, the expression patterns of the two structural genes were very similar, whereas under stress condition (0.90 a_w), both the genes were highly expressed at all temperatures examined except 20°C, where there was higher gene expression at 0.99 a_w (Figures 2.10-2.11).

The expression of some genes was much higher than the general cluster gene expression level under certain parameter combinations. This was especially true for the *afl*S gene. The *afl*S (formerly *afl*J) gene is a regulatory gene which interacts and activates *afl*R, the major regulatory gene, which then activates the aflatoxin structural genes (Chang, 2003). At certain parameter combinations at 20, 25 and 30 °C the expression of the *afl*S gene was increased when compared to the other genes of the cluster. This was often accompanied by an increased biosynthesis of AFB_1 compared to the neighbouring conditions.

At 20°C/0.99 a_w the *afl*S gene was very highly expressed relative to the other genes and accompanied by a similar activation of the *aflJ* (formerly *est*A) gene. Under these conditions growth was moderate and AFB_1 biosynthesis high. At all analysed conditions the *afl*R gene was not prominently expressed and followed the general expression of the cluster genes (Figures 2.12 and 2.13). However, the ratio of expression between the *afl*S and *afl*R genes seemed to be critical for aflatoxin biosynthesis (Table 2.5). At conditions with low a_w (0.90) the ratio was far below 1. At these conditions low amounts of AFB_1 was produced. At moderate conditions the ratio is >1. At these conditions high amounts of AFB_1 was produced.

2.4 Discussion

The interactions between a_w and temperature are the most critical determinants of fungal growth and mycotoxin production. Conditions for mycotoxins production are generally more restrictive than those for growth and can differ between different mycotoxins produced by the same species and between fungi producing the same mycotoxin (Frisvad and Samson, 1991;

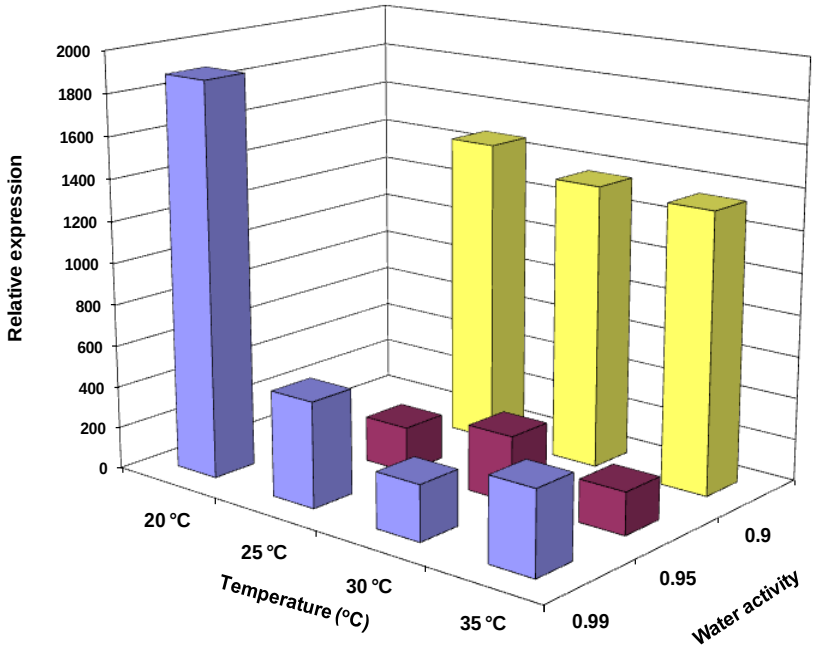

Figure 2.10 Selected microarray expression data of the single gene *aflD* expression in relation to the interacting conditions of temperature and water activity. Gene expression is relative to the control β-tubulin gene.

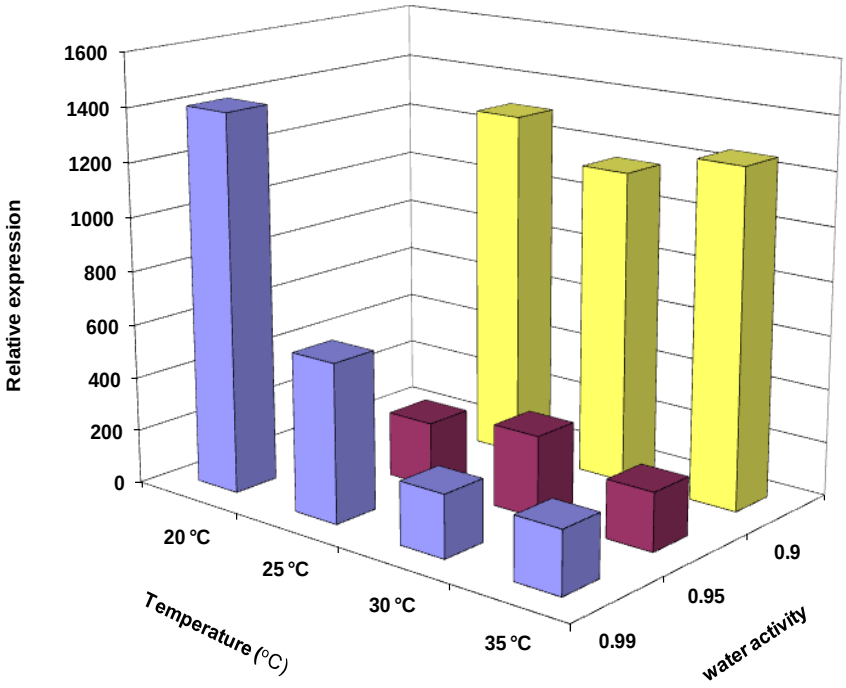

Figure 2.11 Effect of temperature and water activity interactions on expression of the *aflF* gene. Gene expression is relative to the control β-tubulin gene.

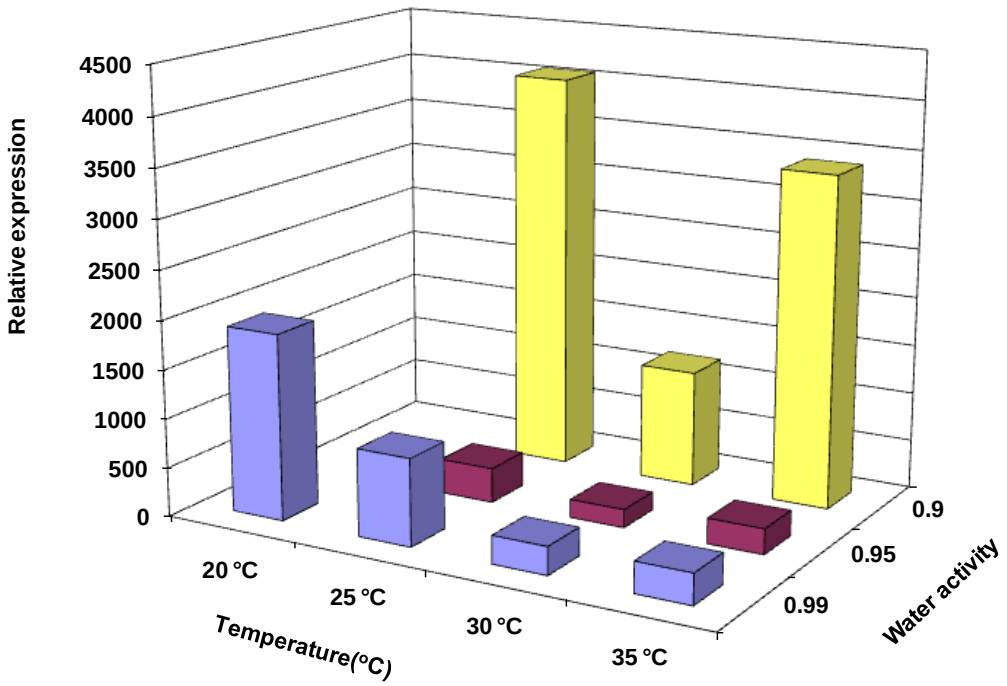

Figure 2.12 The effect of temperature and water activity on expression of the *aflR* gene. Gene expression is relative to the control β-tubulin gene.

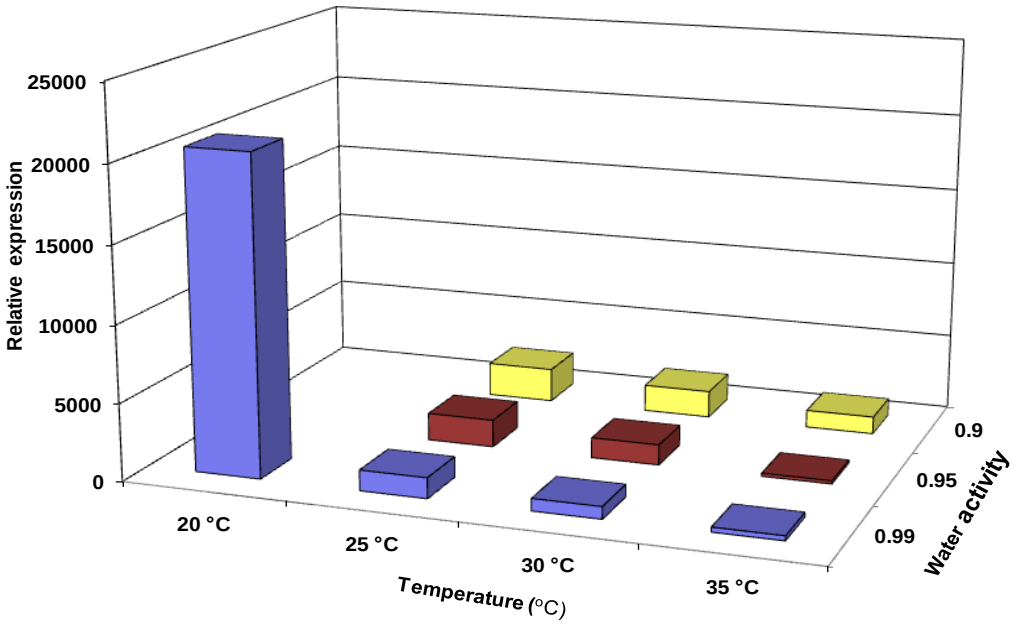

Figure 2.13 The effect of temperature and water activity on expression of the *aflS* gene. Gene expression is relative to the control β-tubulin gene.

Table 2.5 Ratio of *aflS*/*aflR* in relation to aflatoxin B_1 biosynthesis and different combinations of temperature and water activity (a_w).

Environmental factors	**Ratio *aflS*/*aflR***	**AFB_1 ng g^{-1}**
25°C/0.90 a_w	0.5	3.66
35°C/0.90	0.3	4.67
25°C/0.95	7.4	830.24
30°C/0.95	7.1	3016.94
25°C/0.99	1.5	1957.25
30°C/0.99	2.7	2758.74

Magan et al., 2002; Sanchis and Magan, 2004). This study has provided detailed profiles on growth, sporulation, toxin partitioning, AFB_1 production and aflatoxin gene cluster expression in relation to temperatures x water activity interactions.

The results showed that the influence of a_w and temperature on growth rate and AFB_1 was significant. AFB_1 production followed the growth rate pattern and was higher at 0.99 a_w at all temperatures and the highest amount was at 25-30°C. Similar results were obtained by O' Brian et al. (2007) who found that their strain of *A. flavus* produced aflatoxins optimally at 28-30°C, with production decreasing as temperatures approach 37°C and no production above 37°C. *A. flavus* F2R4FP1-5 produced the highest levels of AFB_1 at 0.996 a_w and 30°C, the highest level tested in a study by Gqaleni et al. (1997). Faraj et al. (1991) studied the effects of a_w (0.90, 0.95, 0.98) and temperature (25, 30 and 35°C) on AFB_1 production by *A. flavus* and *A. parasiticus* grown on irradiated maize seeds, with the highest AFB_1 produced by *A. parasitious* at 25°C and 0.98 a_w and by *A. flavus* at 30°C at 0.95 and 0.98 a_w. At 0.90 a_w toxin production was consistently low for both species at all temperatures. Maximum aflatoxin production was found at 30°C for one strain each of *A. flavus* and *A. parasiticus* (Ogundero, 1987). The growth rate patterns showed that maximum growth was observed at 0.99 a_w and 25-30°C. Our results support those obtained by Giorni et al. (2008) for Italian strains of *A. flavus*. They demonstrated that the higher fungal growth of three *A. flavus* strains was at 30°C and -1.4 and -2.8 MPa (0.98-0.99 a_w),

In this study the influence of a_w and temperature on sporulation was shown to be significant. The highest amount of conidia produced was at 0.95 a_w followed by 0.90 a_w and 0.99 a_w at all temperatures examined. Giorni et al. (2008) found that maximum number of spores by Italian strains of *A. flavus* was produced at 4.8 MPa (0.96 a_w). Previous studies by Gervais and Molin (2003) with *Penicillium roqueforti* strains from cheese grew optimally at 0.97-0.98 a_w, while maximum spore production was at 0.96 a_w. Parra et al. (2004) showed that highest amount of spores produced by a genetically engineered *Aspergillus niger* strain was at 0.95 a_w when this was modified by glycerol at 35°C.

The present study showed that there was no direct relationship between sporulation and AFB_1 production in relation to a_w and temperature. The highest amount of AFB_1 was at 0.99 a_w at all temperatures and the lowest numbers of spores was produced at this a_w at all temperatures except 20°C. This may be due to the distribution of AFB_1 between spores, biomass and

medium which plays a functional role at this a_w level, whereas with freely available water (0.99 a_w) about 50% of the AFB_1 was present in the biomass and the medium, with very little actually channelled into the spores. Previous studies have demonstrated that *A. carbonarius* spores can contain 60-70% of the ochratoxin A content relative to the biomass (Atoui et al., 2007). Reib (1982) added different sporulation inhibitors to *A. parasiticus* NRRL 2999 and found that even without any reduction in mycelia growth, a reduction in sporulation was correlated with AFB_1 production. Other observations support the hypothesis that microbial secondary metabolite production and asexual sporulation are intimately associated (Adams and Yu, 1998). Similarly, earlier observations suggested that the ability to complete wild type asexual sporulation could be a prerequisite for ST/AF biosynthesis in the genus *Aspergillus* generally (Kale et al., 1994; 1996). For example, Guzman-de-Pena and Ruiz-Herrara (1997) reported that there was a relationship between sporulation and AFB_1 production, whereas isolates inhibited from sporulation with diaminobutanone, and a non-sporulating mutant, as well as evidence from a sectoring mutant, indicated that AFB_1 biosynthesis was correlated with sporulation. Serial transfer of *A. parasiticus* SU-1 and five mutants also produced poorly sporulating isolates unable to produce AFB_1 or related compounds (Kale et al. 1994).

The relative expression of the regulatory gene *aflR* had a similar pattern as spore production, where at 0.99 a_w, there was low expression of *aflR* and low number of spores and at 0.90 a_w, there was very high expression of *aflR* and high numbers of conidia produced. Previous studies by Kale et al. (1996, 2003) showed that the genes for secondary metabolite production were still present, but expression of the regulatory gene *aflR* was five to ten times lower in poorly sporulating isolates that did not produce aflatoxins or related compounds.

The microarray for the analysis of the influence of environmental parameters (temperature and a_w) on the activation of the aflatoxin biosynthesis pathway genes showed that temperature and a_w have a strong influence on the expression patterns of these genes. Only a few studies have systematically investigated the influence of a_w and temperature on the expression of mycotoxin biosynthesis genes (Geisen, 2004; O Callaghan et al., 2006; Schmidt-Heydt & Geisen, 2007; Schmidt-Heydt et al., 2007; Jurado et al., 2008; Schmidt-Heydt et al., 2010).

In conditions favourable for growth of *A. flavus*, the cluster genes are expressed at a basal level with the strain producing high amounts of AFB_1. Under mild stress conditions

(20°C/0.99 a_w) several of the cluster genes, in particular *aflS* and *aflJ*, were very highly induced concomitant with high levels of phenotypic AFB_1 production. It was shown previously that mild stress conditions can lead to mycotoxin gene activation (Baert et al., 2007; Schmidt- Heydt et al., 2008). This combination probably imposes stress on the fungus because of the low temperature and the fact that growth was reduced. This effect was also observed with other mycotoxin producing fungi like the ochratoxin A producing *P. verrucosum* or the trichothecene producing *F. culmorum* (Schmidt-Hyedt et al., 2008). A similar basal level of expression was found at between 25 and 35°C for *A. flavus*, especially when a_w was decreased to 0.90. This suggests that such mild stress may activate the genes involved in secondary metabolite production at these sub-optimal temperatures.

A slight increased expression of the *aflS* gene was also found at certain moderate parameter combinations at 30, 25 and 20°C where the cluster is expressed at the basal level. Interestingly, at all these combinations, high amounts of AFB_1 were produced when the *aflS* expression was elevated. These data indicate that the failure of *A. flavus* to produce AFB_1 at 0.90 a_w is not due to lack of transcription of *aflR* or *aflS*. When a ratio between the normalised expression data of the *aflS*/*aflR* genes was generated, high ratios could be found at 25°C and 30°C at 0.99 and 0.95 a_w and low ratios at 25°C and 30°C at 0.90 a_w. This is in agreement with the AFB_1 production profile. The products of both the *aflR* and *aflS* genes are regulatory proteins. *AFLR* is a transcription factor which directly binds to a consensus sequence in front of the AFB_1 structural genes (Ehrlich and Cary 1995; Ehrlich et al. 1999). The activity of the *aflS* gene on the other hand is not absolutely clear. It is not a DNA binding protein; however, according to Chang (2003), it interacts with *aflR* and somehow supports DNA binding by the latter. Ehrlich (2009) recently discussed that *aflS* may interact with the global secondary metabolite regulatory factor *laeA*. According to a recent analysis of Du et al. (2007), *aflS* appears to modulate the activity of *aflR*. This activity would coincide with the results reported in the present study. Assuming that the expression of the regulatory genes *aflR* and *aflS* is directly correlated to the amount of gene products, the regulation of aflatoxin B_1 seems to be dependent on the ratio of the transcription of the regulatory genes. The *aflS*/*aflR* ratio is high under conditions which favour high AFB_1 biosynthesis. As a modulator, *aflS* may influence the activity of *aflR*. Schmidt-Heydt et al. (2010) reported that the ratio of the expression data between *aflS*/*aflR* was corresponded to the AFB_1 or AFG_1 production by *A. parasiticus* at various combinations of a_w and temperature, where high *aflS*/*aflR* ratios at

17-30°C related to AFG_1 production and low ratio above 30°C corresponded with AFB_1 production.

The data in this study suggest that stress applied by external abiotic factors has a complex role in the activation of the genes and that their activation may be some kind of stress response leading perhaps to an increased fitness of the producing fungus to withstand the imposed stress conditions.

Chapter 3

Detection of aflatoxigenic strains by PCR

3 Detection of aflatoxigenic strains by PCR

3.1 Introduction

The study in Chapter 2 showed that both regulatory and structural AFA genes have a role in AFAs biosynthesis. This information was used to inform the molecular diagnostic studies on *Aspergillus* section *Flavi* contaminating Egyptian peanuts in this chapter by using a set of regulatory and structural AFA genes. Peanuts are an important agricultural crop in Egypt. The country has a large number of peanut producers, with many farming small fields of 2.5 ha (WTO 1998). Egypt is a major peanut exporting country and the European market accounts for 68% of its exports. The USA is also a major consumer of Egyptian nuts, importing over $11 million worth of nuts annually (Youssef et al., 2008). The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Maghraby and El-Maraghy 1987). Contamination of peanuts with AFAs is one of the main factors that compromises their quality. A high incidence of human liver cancer in central Africa and parts of Southeast Asia has been linked with aflatoxins (Groopman, and Kensler, 1996; Montesano, et al., 1997). In the last few years, an increase in the number of human liver diseases in Egypt has been reported. This initiated a multidisciplinary study to investigate the role of AFAs in high incidence of hepatotoxicity in Egypt (Badria 1996).

Recently, several multiplex polymerase chain reaction (PCR) systems for aflatoxin-producing fungi have been developed, capable of detecting several genes coding for enzymes at different stages of the AFAs biosynthesis pathway (Geisen, 1996; Shapira et al., 1996; Criseo et al., 2001; Chen, et al., 2002). The use of reverse transcription polymerase chain reaction (RT-PCR) to assess AFAs production potential is attractive because it is based on the activity of genes rather than their presence (Sweeney et al., 2000). Several studies have measured the expression of genes involved in the AFAs biosynthesis pathway to distinguish between AFAs producers and non-producers (Scherm et al., 2005; Degola et al., 2007; Rodrigues et al., 2009). However, there is currently no agreement on single genes whose expression can be used to fully discriminate between aflatoxin and non-aflatoxin producers. The objectives of this study were to isolate a range of *Aspergillus* section *Flavi* strains from peanuts from different regions of Egypt and to examine the ability of 18 such strains to produce AFB_1 based on (a) a qualitative medium-based assay, (b) HPLC, and (c) use of different biosynthetic and regulatory genes as indicators of AFB_1 production, and (d) to confirm the

ability of AFB_1 producing and non-toxin producing strains after colonisation of Egyptian peanuts under specific temperature/a_w regimes.

3.2 Material and methods

3.2.1. Strains, media and growth conditions

In this study, 18 strains of *Aspergillus* section *Flavi* group were isolated from Egyptian peanuts from peanut growing regions (Alexandria, El-Behiera, El-Sharqiya and El-Daqahlia) on MEA (Sultan and Magan, 2010), and they were identified according to morphological and microscopic characteristic. In addition, 2 type aflatoxigenic strains, *Aspergillus flavus* NRRL 3357 (supplied by Dr. D. Bhatnagar, USDA, New Orleans, U.S.A), and *A. parasiticus* PRR-2747 were used for comparison. Each strain was assigned a name in the format EGPn, where EG means Egypt, P represents peanuts, and n is the isolate number. Fungal strains were sub-cultured before examination on MEA for 7 days at 25°C in the dark.

3.2.2. Screening for aflatoxin production using coconut cream agar medium

A preliminary screening for AFB_1 production by the strains was performed by growing them on coconut cream agar medium (CAM) based on fluorescence under long-wave UV light (365 nm). To prepare CAM, 50% coconut cream was homogenized for 5 min with 1000 ml of 100°C distilled water. The homogenate was filtered through four layers of cheese cloth. Agar was added (20 g l^{-1}), and the mixture heated to boiling point and cooled to about 50°C. The mixture was then autoclaved, mixed and poured into sterile 9 cm Petri dishes (Davis et al., 1987). All strains were inoculated centrally into agar plates and then incubated at 25°C for 8 days in the dark on a conducive YES medium.

3.2.3. Aflatoxin B_1 extraction and HPLC analysis

AFB_1 extraction was performed as mentioned in Section 2.2.4b

3.2.4 Molecular methods

(a) Isolation of RNA from the samples

RNA extraction was performed as mentioned in Section 2.2.6a

(b) Aflatoxin gene expression

The expression of three structural genes, *aflD* (nor-1), *aflM* (ver-1), and *aflP* (omt A), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway was assayed for all the strains. The expression of the housekeeping gene (β-tubulin) was used as a control.

Reverse transcriptase PCR: RT was performed using Qiagen sensiscript ® kit (Qiagen, UK) using oligo-dT primers to amplify mRNA. The reaction was assembled in a 20 µl tube as follows: 1 µM Oligo(dT) primer, 1 x reaction buffer, 4U sensiscript Reverse Transcriptase, 2 µM dNTPs, 10 U RNase inhibitor , and 40 ng RNA sample in 12 µL H_2O (RNase free). The mixtures was incubated at 37°C for 60 min followed by 93°C for 5 min in a thermal cycler (Peltier Thermal cycler PTC-200 MJ Research; Genetic Research Instrumentation Ltd, Braintree, UK, Figure 3.1), followed by rapid cooling on ice. Each 25 µl PCR reaction contained 800 µM dNTP, 1 x reaction buffer , 1.25 U Taq DNA polymerase I, 0.2 µM of each primer, 1 µl cDNA mixture, 12 µL H_2O (RNase and DNase free). PCR conditions were an initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 60 s at 65°C and 90 s at 72°C, with a final extension at 72°C for 7 min (Scherm et al., 2005). Primer sequences are detailed in Table 3.1. PCR products were visualized on a UV transilluminator (Gene Genius Bio Imaging system, SynGene, UK, Figure 3.2).

(c) DNA isolation and amplification

Total DNA was purified from all tested strains grown on cellophane-overlaid YES medium. Half gram of the mycelium was ground in a mortar with a pestle in the presence of liquid nitrogen. Approximately 100 mg of the mycelial powder was then used for isolation of total DNA. DNA extraction from the ground mycelia was accomplished with the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the instructions provided by the manufacturer. PCR was performed as described previously, using the same set of primers designed for the RT-PCR.

Figure 3.1 Thermal cycler PTC-200 used for amplification of genes.

Figure 3.2 UV transilluminator used for visualization of PCR products separated on a 2% agarose gel, stained with ethidium bromide.

Table 3.1 Details of primer sequences, target gene, annealing temperature and expected PCR/RT-PCR product length in base pairs (bp).

Primer pair	Gene	Primer sequence (5'- 3')	Optimal Annealing Temp. (°C)	PCR product size (bp)	RT-PCR product size(bp)
Tub1-F	**Tub 1**	GTCCGGTGCTGGTAACAACT	65	902	837
Tub1-R		GGAGGTGGAGTTTCCAATGA			
NOR1-F	**aflD**	ACCGCTACGCCGGCACTCTCGGCAC	65	400	400
NOR1-R		GTTGGCCGCCAGCTTCGACACTCCG			
VER1-F	**aflM**	GCCGCAGGCCGCGGAGAAAGTGGT	65	537	487
VER1-R		GGGGATATACTCCCGCGACACAGCC			
OmtA-F	**aflP**	GTGGACGGACCTAGTCCGACATCAC	65	797	624
OmtA-R		GTCGGCGCCACGCACTGGGTTGGGG			
AflR-F	**aflR**	CGAGTTGTGCCAGTTCAAAA	55	999	999
AflR-R		AATCCTCGCCCACCATACTA			

3.2.5 Studies on peanuts

Inoculation and culture conditions: An aflatoxin producing strain (*A. flavus* EGP3) and three non-aflatoxigenic strains (*A. flavus* EGP14, *A. flavus* EGP15 and *A. flavus* EGP16) were grown on peanuts modified with sterile water to 0.95 a_w. A moisture adsorption curve was prepared for peanuts in order to accurately determine the amount of water required to add to obtain the target a_w level (Figure 3.3). This curve was obtained by adding different quantities of water to peanuts and calculating the moisture content as the difference in weight before and after drying at 130°C overnight and plotting against the a_w level measured with an AQUALAB ® 3TE, (Decagon, Pullman, WA, USA).

20 g sub-samples of peanuts (three replicates per treatment) were put in glass jars covered with lids containing a microporous membrane and autoclaved. Peanut samples were inoculated with 0.2 ml of 10^6 spore suspension and vigorously shaken to cover the peanuts with spores and incubated at 25°C for one week in polyethylene sandwich boxes containing glycerol/water solutions to maintain the equilibrium relative humidity conditions.

(a) Aflatoxin B_1 extraction and analysis

10 g of each treatment and replicate was taken for clean up and AFB_1 extraction using an immunoaffinity column (Easi-Extract® Aflatoxin, R-Biopharm, Darmstadt, Germany). The extraction was performed as follow: 10 g of peanut sample was placed into blender (Waring ® Commercial Blender 8010E) and then 20 ml of 80% methanol/water (v/v) were added. The mixture was stirred for 2 min. at high speed. The extract was filtered through a Whatman No. 4 filter paper. Cleaning up based on removal of the substances, which may interfere with the detection of the analyte. Two millilitres of the final extract, corresponding to 1 g of the original material was diluted with 14 ml of phosphate buffered saline (PBS, pH 7.4) to give a solvent concentration of 10 % or less (in order to protect the antibodies in immunoaffinity columns).

The mixture was allowed to pass through column by gravity or at a flow rate of 5 ml min^{-1}. The column contains monoclonal antibodies to aflatoxins bound to a solid support. By passing the diluted extract through column any aflatoxins present in the sample are bound to the antibody within the column. The column was then washed with 20 ml of PBS. The elution of aflatoxins was done with 1 ml of methanol. AFB_1 was analysed by HPLC as described previously.

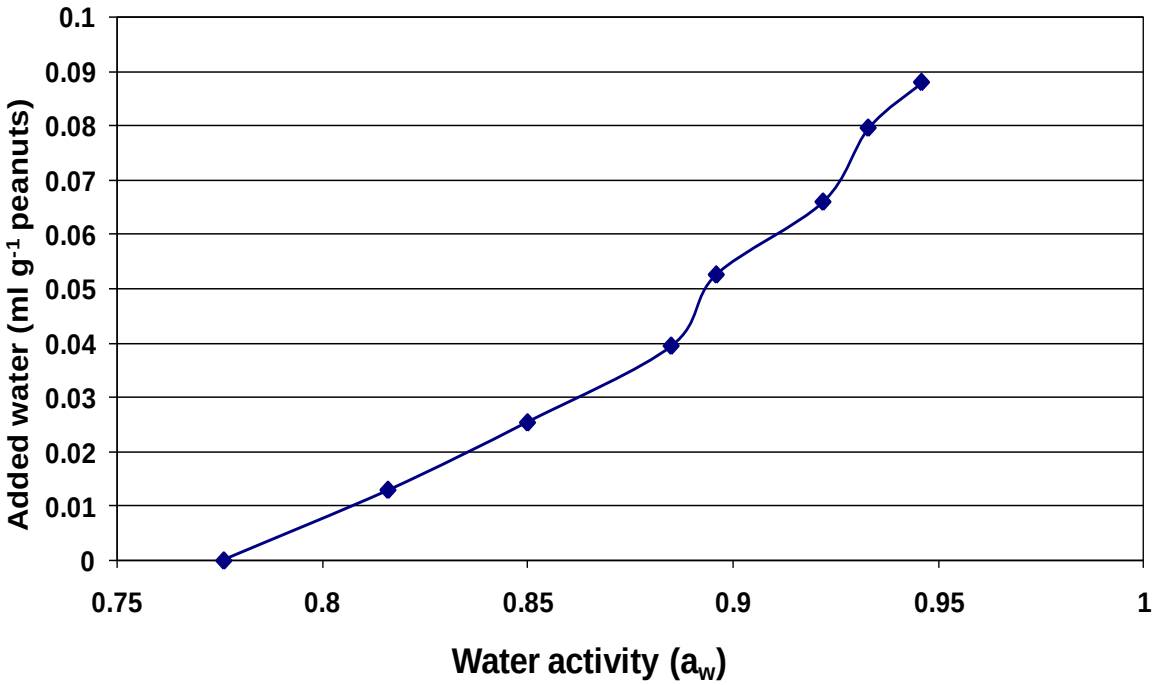

Figure 3.3 Moisture adsorption curve for the relationship between amounts of added water and water activity at 25°C.

(b) RNA extraction and RT-PCR for peanut samples

Total RNA was extracted from inoculated peanuts after the one week incubation using the RNeasy and Plant Mini Kit (Qiagen GmbH, Hilden, Germany) with minor modifications. An amount of 1 g of peanuts was ground in a mortar with a pestle in the presence of liquid nitrogen, 500 µl of lysis buffer from RNeasy kits and 0.5 g of polyvinylpolypyrrolidone (PVPP). Insoluble PVPP binds to both polysaccharide and phenolic compounds and prevents the undesirable binding between nucleic acids and these compounds (Chen et al., 2000). RNA extraction was then performed according to the instructions provided by the manufacturer. RNA was treated with DNase I (RNase free DNase I, Amplification Grade, Sigma) to digest residual DNA in the samples. RT-PCR was performed as described above, using the same set of primers designed for RT-PCR.

3.3 Results

3.3.1 Screening of the ability of the strains for aflatoxin production

A total of 18 strains of *A. flavus* isolated from Egyptian peanuts (EGP1-EGP18) were assessed for AFB_1 production during growth on YES medium using HPLC (Table 3.2). Thirteen of these strains, and two control strains tested (*A. flavus* NRRL 3357 and *A. parasiticus* 2747) produced AFB_1 and AFB_2, while *A. parasiticus* 2747 also produced AFG_1 and AFG_2. The AFB_1 was in the range 1.27- 213.35 µg g^{-1} medium; one strain was a very high producer (213.35 µg g^{-1}), nine strains were high producers (15.92-50.63 µg g^{-1}) and five strains were low producers (0.15-6.59 µg g^{-1}).

Five of the strains showed no detectable levels of aflatoxin. Strains were grown on CAM and fluorescence was used to assess AFB_1; the results showed a good correlation between fluorescence and levels of aflatoxin as measured by HPLC.

3.3.2 Analysis of aflatoxin gene transcription

Several genes code for proteins involved in the aflatoxin biosynthesis pathway. Measuring their transcriptional status should reveal if the strain is capable of producing aflatoxins. In this study we analysed the presence and transcription of four genes, *aflD* (nor-1), *aflM* (ver-1), *aflP* (omt A) and *aflR* in strains of *Aspergillus* section *Flavi*. The expression of the house keeping gene (β-tubulin) was used as a control.

Table 3.2 Diagnostic results for 18 *Aspergillus* section *Flavi* isolates and two type isolates obtained by PCR, RT-PCR and conventional methods. Key: CAM, fluorescence on coconut cream agar medium; HPLC, High Pressure Liquid Chromatography.

Strains	PCR					RT-PCR					CAM	HPLC
	ß-tub	***aflR***	***aflD***	***aflP***	***aflM***	***ß-tub***	***aflR***	***aflD***	***aflP***	***aflM***	**fluorescence**	**Aflatoxin B_1 μg^{-1}**
A. flavus NRRL 3357	+	+	+	+	+	+	+	+	-	+	+	1.42
A. parasiticus 2747	+	+	+	+	+	+	+	+	+	+	+	0.15
A. flavi EGP1	+	+	+	+	+	+	-	+	-	+	+	50.63
A. flavi EGP2	+	+	+	+	+	+	+	+	-	+	+	34.28
A. flavi EGP3	+	+	+	+	+	+	-	+	-	+	+	21.33
A. flavi EGP4	+	+	+	+	+	+	-	+	+	+	+	6.59
A. flavi EGP5	+	+	+	+	+	+	+	+	+	+	+	15.92
A. flavi EGP6	+	+	+	+	+	+	+	+	+	+	+	1.48
A. flavi EGP7	+	+	+	+	+	+	-	+	-	+	+	1.27
A. flavi EGP8	+	+	+	+	+	+	+	+	+	+	+	44.83
A. flavi EGP9	+	+	+	+	+	+	+	+	+	+	+	213.35
A. flavi EGP10	+	+	+	+	+	+	-	+	-	-	+	19.74
A. flavi EGP11	+	+	+	+	+	+	+	+	-	+	+	19.38
A. flavi EGP12	+	+	+	+	+	+	+	+	+	+	+	33.65
A. flavi EGP13	+	+	+	+	+	+	+	+	+	+	+	36.49
A. flavi EGP14	+	+	+	+	+	+	-	-	-	-	-	0
A. flavi EGP15	+	+	+	+	+	+	-	-	-	-	-	0
A. flavi EGP16	+	+	+	+	+	+	+	-	-	-	-	0
A. flavi EGP17	+	+	+	+	+	+	-	-	+	-	-	0
A. flavi EGP18	+	+	+	+	+	+	-	-	-	-	-	0

PCR revealed that all strains tested contained all four genes (Figure 3.4), therefore the ability to produce aflatoxin was not directly related to the presence or absence of these genes (see Table 3.2). Transcription of the genes was subsequently assayed by RT-PCR. To ensure there was no DNA contamination in the RNA from each strain, PCR was performed following an RT reaction in the presence (+RT) or absence (-RT) of the reverse transcriptase enzyme. An additional no-template control PCR reaction for each strain confirmed that results were not due to contamination of PCR products.

Table 3.2 summarizes the results obtained by RT-PCR for all four gene examined. Electrophoresis patterns showed that of all the aflatoxin-producing strains (15 strains), five were negative for transcription of *aflR*, seven were negative for *aflP*, and one did not transcribe *aflM* (Figure 3.5 A and B). Furthermore, of five non-aflatoxigenic strains, two transcribed *aflR* and *aflP* (Figure 3.6). Thus, transcription of these three genes does not fully discriminate between aflatoxin-producing and non-producing strains. However, transcription of the structural gene, *aflD*, gave positive results with all aflatoxin producing strains (see Figure 3.5 A and B) and gave negative results with all non-aflatoxin strains (Figure 3.6). So the transcription of this gene was correlated with the ability to produce aflatoxins in all strains tested.

3.3.3 Studies on peanuts

The production of aflatoxins was tested during colonisation by selected strains on peanuts (Table 3.3). The aflatoxin-producing strain EFGP3 also produced AFB_1 when grown on peanuts. Interestingly, the non-producing strains EGP14 and EGP15 synthesised AFB_1; this was accompanied by the detection of *aflD* and *aflM* transcription (Figure 3.7). It is notable that EGP15 which produced no detectable levels when grown on YES media produced higher amounts than the producing strain (EGP3) when grown on peanuts. The strain EGP16 was confirmed to produce no aflatoxins on either substrate, which was consistent with a lack of *aflD* and *aflM* transcription.

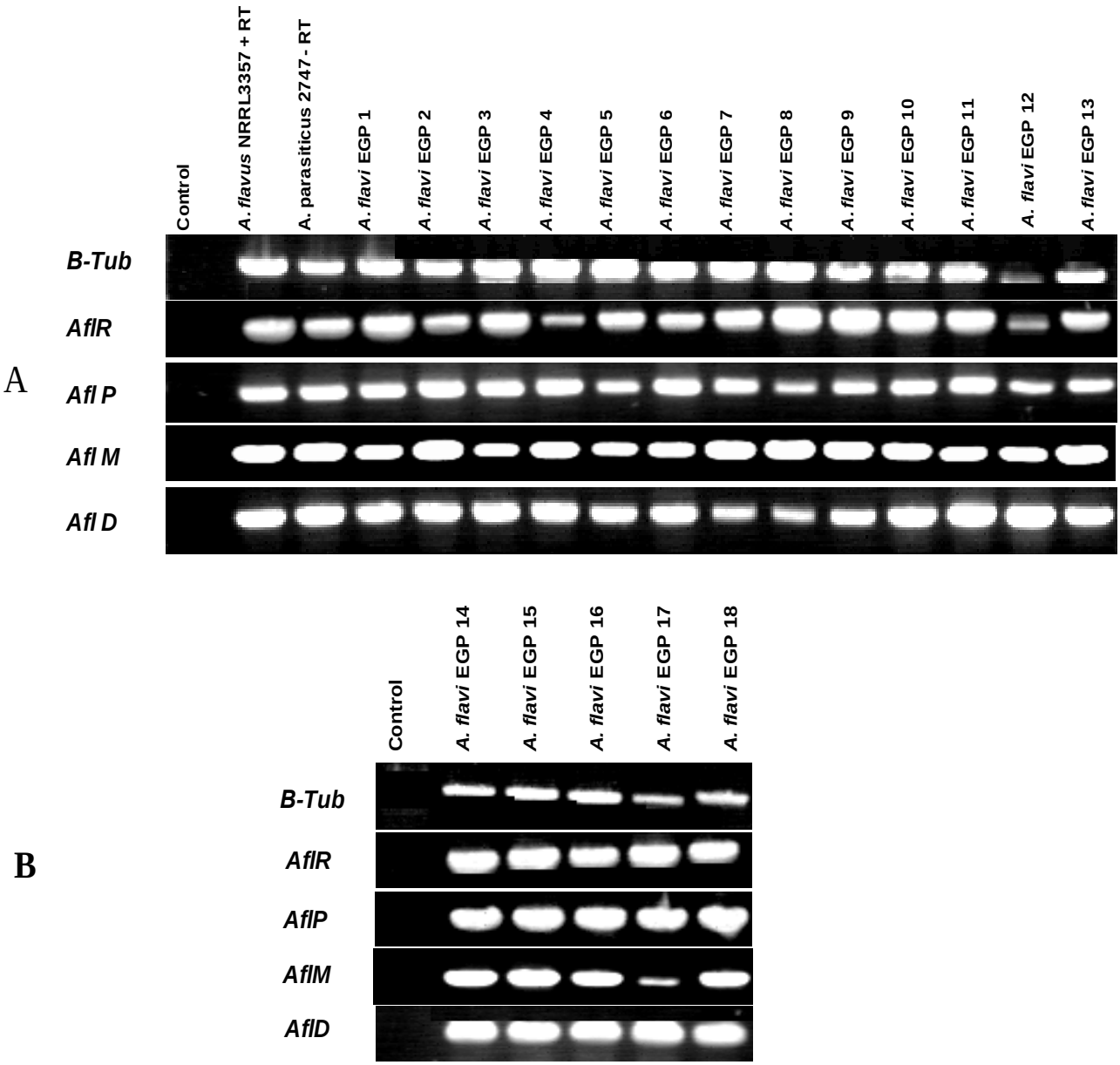

Figure 3.4 Amplification of primer set (β- tubulin, *aflD*, *aflM*, *aflP* and *aflR*) detected by PCR in A) 15 aflatoxigenic strains and B) 5 non-aflatoxigenic strains. DNA from each strain was amplified by PCR. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.

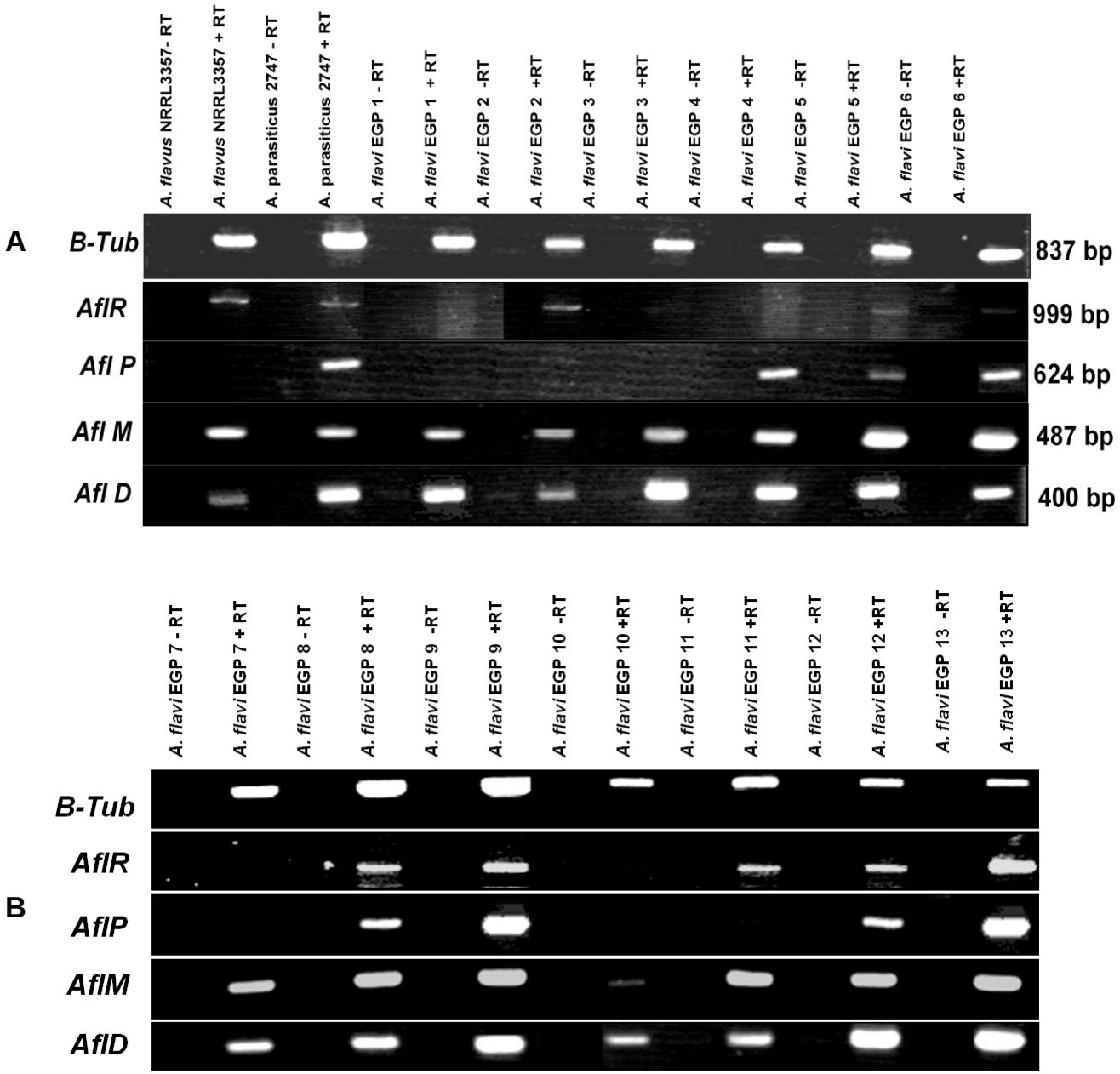

Figure 3.5 A and B; Transcription of primer set (β- tubulin, *aflD*, *aflM*, *aflP* and *aflR*) detected by RT-PCR in 15 aflatoxigenic strains. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.

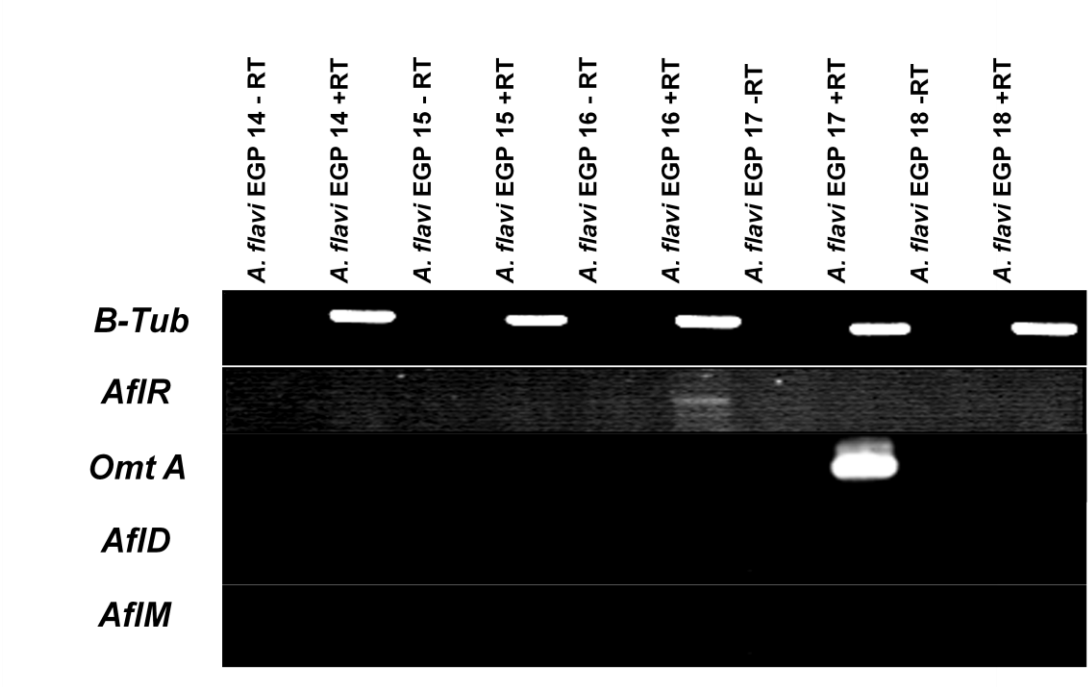

Figure 3.6 Transcription of primer set (β- tubulin, *aflD*, *aflM*, *aflP* and *aflR*) detected by RT-PCR in 5 Non-aflatoxigenic strains. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2 % agarose gel, stained with ethidium bromide and visualized under UV.

Table 3.3 Comparison the results obtained by RT-PCR and HPLC between 4 strains grown on YES medium and peanuts. Where each strain was assigned a name in the format EGPn, where EG means Egypt, P represents peanuts, and n is the isolate number.

Sample	Aflatoxin B_1 in	Aflatoxin B_1 in	RT-PCR in peanuts					RT-PCR on YES				
	YES µg/g	Peanuts µg/g	ß-tub	*aflR*	*aflD*	*aflP*	*aflM*	ß-tub	*aflR*	*aflD*	*aflP*	*aflM*
A. flavi EGP3	21.33	0.67	+	-	+	-	+	+	-	+	-	+
A. flavi EGP14	0	0.94	+	-	+	-	+	+	-	-	-	-
A. flavi EGP15	0	0.01	+	-	+	-	+	+	-	-	-	-
A. flavi EGP16	0	0	+	-	-	-	-	+	+	-	-	-

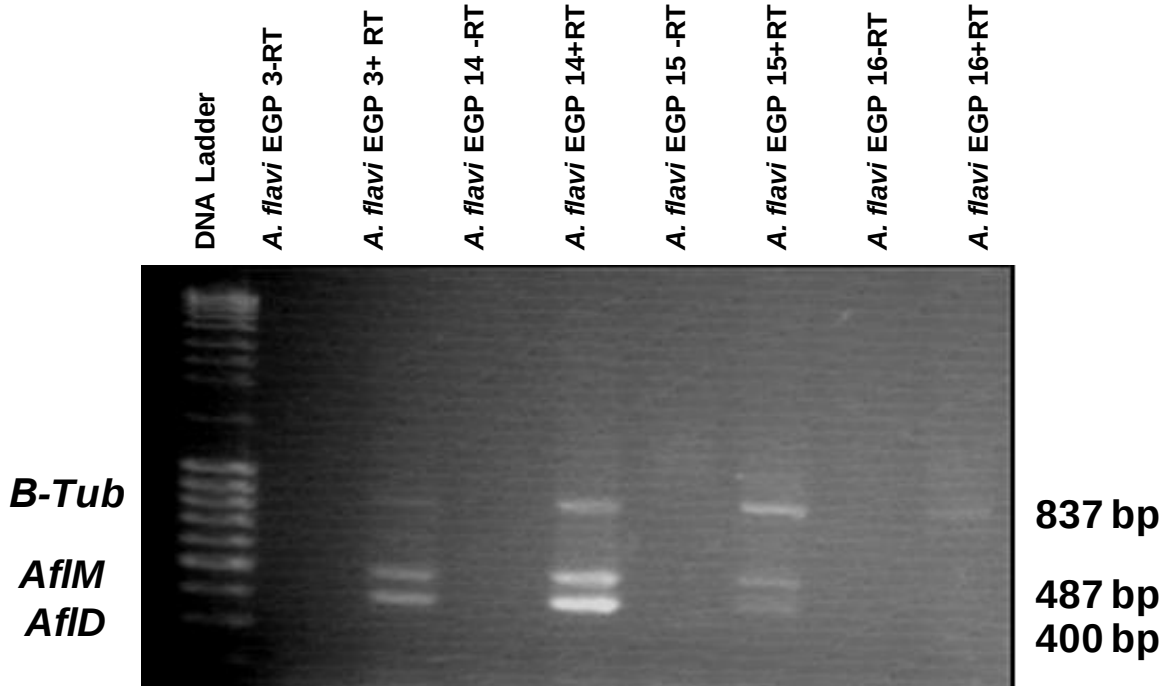

Figure 3.7 Transcription of β- tubulin, *aflD, aflM, aflP* and *aflR* detected by RT-PCR in 4 strains grown on peanuts. RNA from each strain was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV.

3.4. Discussion

In this study, we aimed to establish a molecular marker capable of detecting aflatoxin expression in *Aspergillus* section *Flavi* species contaminating Egyptian peanuts, confirming the relationship between molecular tools and conventional analytical methods (fluorescence and HPLC) and to determine the behaviour of aflatoxigenic and non-aflatoxigenic strains when grown on peanuts. This study was carried out because very recently *A. flavus* has been found to be the most common species isolated from in shell peanuts and from seeds directly, and was the most common mycotoxigenic component of the mycobiota in two seasons in Egypt (Sultan and Magan, 2010). Studies from the USA suggest a similar importance of *A. flavus* from other agricultural commodities (Cotty, 1997). Furthermore, potentially toxigenic species of *A. flavus* have been previously isolated from Egyptian peanuts (El-Maghraby and El-.Maraghy 1987, 1988; Youssef et al., 2008). However, no attempt has been made to use molecular approaches to detect and differentiate between aflatoxin producing and non-producing species in Egyptian strains.

This study revealed that both qualitative (fluorescence) and quantitative levels of aflatoxin measured by HPLC gave similar results. This is in contrast to previous studies which reported that fluorescense on CAM was not always correlated with aflatoxin analysis by HPLC (Scherm et al., 2005; Giorni et al., 2007).

Indeed, Scherm et al. (2005) suggested that detection of aflatoxin production by fluorescence developed by colonies grown on coconut agar (CAM) was not a reliable indicator of aflatoxin production. Using the conventional methods as a tool to differentiate between aflatoxin-producing and non-producing strains of the *A. flavus* group are based on culture on natural and/or artificial media which permits the release of aflatoxins have been studied previously (Davis et al., 1987; Abarca et al., 1988; Bennett and Papa 1988). These methods have drawbacks: time-consuming, laborious and need the expertise of mycologists to avoid misidentification.

Aflatoxins are the end product of a biosynthetic pathway comprising many enzymatic steps. Measuring the level of expression of genes coding for these enzymes should provide a marker indicating whether a strain is capable of producing aflatoxins. However, despite previous work there is currently no agreement on a suitable marker for aflatoxin production, other than

the measurement of aflatoxin production itself (Geisen1996; Shapira et al., 1996; Farber et al., 1997; Criseo et al., 2001; Scherm et al., 2005; Degola et al., 2007; Rodrigues et al., 2009).

PCR showed that all aflatoxin and non-aflatoxin producers harbour the four genes (*aflD*, *aflM*, *aflP* and *aflR*) examined. This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. This is supported by Chang et al. (2005) who reported that the loss of production of AFB_1 and AFB_2 in many non-aflatoxigenic *A. flavus* isolates is not caused by large deletions or a complete loss of the aflatoxin gene cluster, but probably results from point mutations.

RT-PCR results in this study have shown that *aflD* transcription may be used as a marker to discriminate between aflatoxin and non-aflatoxin producers, while *aflM*, *aflP* and *aflR* failed to differentiate between aflatoxigenic and non-aflatoxigenic strains. Similarly Scherm et al. (2005) reported that *aflD* (nor-1), gave the best correlation of aflatoxin production and gene expression on inducing (YES) and non-inducing (YEP) media. The results also showed that RT-PCR technique gave a positive correlation between aflatoxin production and aflatoxin gene transcription of *Aspergillus parasiticus* strain examined. This indicates that RT-PCR assay is valid to use for this species. Previously, Scherm et al. (2005) tested a RT-PCR assay with a set of aflatoxin gene primers against three aflatoxigenic strains of *A. parasiticus*. All of them gave a positive result with the four genes used.

The results contrast with those of Rodrigues et al. (2009), who tested two genes, *aflD* and *aflQ*, in 31 isolates of *Aspergillus* section *Flavi* originating from Portuguese almonds and concluded that *aflD* expression was not a good marker for differentiating between aflatoxigenic and non-aflatoxigenic isolates based on testing 35 isolates (31 almonds isolates and 4 control strains). Only one almond isolate gave a false positive transcription.

Interestingly, *aflR* failed to discriminate between aflatoxin and non-aflatoxin producers, where five of 15 of aflatoxin producers were negative for transcription of *aflR*, and one of five of non-aflatoxin producer was positive for *aflR* transcription. This may be a result of sequence variability which occurs within the entire *aflR* gene of *Aspergillus* section *Flavi*. It was found that particular sequence variability differentiates some species in *Aspergillus* section *Flavi* and can be used to identify non-functionality of the AFLR protein (Lee et al., 2006). Kale et al. (2007) reported that regulation of AFAs production in *A. parasiticus* sec-varients and loss of aflatoxigenicity is not caused by defects in *aflR* or *aflJ* or *laeA* (three

known AF-regulatory genes). It is possible that the sec-strains lack additional positive regulators of secondary metabolic pathways that act independently of or in conjunction with *aflR* and *aflJ*.

The fact that *aflD* transcription became detectable when a non-producing strain (on YES media) was grown on peanuts and produced aflatoxins suggests that *aflD* transcription is a good marker for aflatoxin production, and suggests that *aflD* plays a functional role in the adaptive growth on different media. It is worthwhile noting that the conducive medium (YES medium) is not a definitive assay to confirm whether strains are aflatoxigenic or non-aflatoxigenic per se. It is used for carrying out ecophysiological studies with known aflatoxin producers (Schmidt-Heydt et al., 2009). In the present study *A. flavus* EGP3 did not produce any aflatoxin on YES. However, it produced higher AFB_1 than *A. flavus* EPG14 (an aflatoxin producer on YES) in peanuts.

This suggests that this may be another factor that should be borne in mind as original nutritional substrate (in this case peanuts) may be important. A number of studies have used atoxigenic strains as a biocotrol agent to prevent contamination through competitive exclusion of toxigenic strains during infection (Dorner, 2004; Pitt and Hocking, 2006; Dorner, 2008). Our results indicate that the stability of the aflatoxin- producing phenotype may be an important consideration in selecting strains for use in strategies to prevent aflatoxin contamination through intraspecific competition (Cole and Cotty, 1990; Brown et al., 1991). Cleveland et al. (1990) reported that certain atoxigenic strains of *A. flavus* are known to be unstable and to convert to a highly toxigenic phenotype. Lee (1989) suggested that production of aflatoxin biosynthesis enzymes by an atoxigenic strain is characteristic only of atoxigenic strains generated in the laboratory and that such enzyme-producing strains are not stable and may convert to a toxigenic form on introduction to a crop. Lee (1989) further suggested that these converted strains might cause a net increase in aflatoxin B_1 contamination.

The data suggest that a lipid-rich environment (such as peanuts) represent a type of stress which induces AFB_1 production. Previously, Yu et al. (2003) concluded that substrate-induced lipase gene expression might be indirectly related to aflatoxin formation by providing the basic building block (acetate) for aflatoxin synthesis. Also supplementation of lipid (0.5% soya bean or peanut oil) in the non-aflatoxin-conducive medium (PMS) promoted aflatoxin production of two non-aflatoxigenic *A. flavus* isolates (Yu et al., 2003).

The work presented in this chapter is the first detailed molecular analyses of strains of *Aspergillus* section *Flavi* group from Egyptian peanuts. It also highlights the dynamic nature of aflatoxin production, and the potential use of *aflD* transcription as a marker for aflatoxin. This may be used to develop a more rapid molecular assay for the detection of aflatoxin-producing species in contaminated peanuts and other food matrices.

Chapter 4

Monitoring of *nor-1* (*aflD*) gene of *Aspergillus flavus* in peanuts

4 Monitoring of nor-1 (*aflD*) gene of *Aspergillus flavus* in peanuts

4.1 Introduction

The study in Chapter 3 showed the potential use of *aflD* transcription as a good marker to discriminate between aflatoxigenic and non-aflatoxigenic strains contaminating peanuts. Further ecophysiological studies on *aflD* expression in stored peanuts was thus carried out to determine the correlations between *aflD* expression and phenotypic AFB_1 production in relation to changes in a_w and storage time for controlling *A. flavus* in stored peanust destined for human food, where physical methods are required for safe storage. The key environmental determinants pre- and post-harvest are water availability and temperature (Magan et al., 2003; Magan and Aldred, 2007). The biosynthesis of secondary metabolites, like mycotoxins, is significantly influenced by environmental conditions such as pH, water activity (a_w) and temperature (Belli et al., 2004; Hope et al., 2005).

Previously, Moubasher et al. (1980) examined the effect of different moisture contents (8.5-21% on a dry-weight basis) and temperatures (5-45°C) on *A. flavus* infection of peanuts stored for up to 6 months. Highest population counts of *A. flavus* were found in peanuts stored at 13.5 % moisture content (approx. 0.90 a_w) at 15°C for 1 month. Recently, a survey of Egyptian peanuts by Sultan and Magan (2010) showed that *Aspergillus* section *Flavi* was consistently the most frequent genus in in-shell peanuts and was the dominant mycotoxigenic component of the mycobiota. However, in this 2-year survey, there was no direct correlation between the moisture content of the samples and the fungal populations on peanut seeds from different regions. The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Maghraby and El-Maraghy, 1987).

Molecular techniques have been applied for the detection of aflatoxigenic fungi in food samples (Geisen, 1996; Shapira et al., 1996; Mayer et al., 2003; Somashekar et al., 2004). Traditional methods used to assess the presence of mycotoxigenic fungi in food are dependent on selective media, which are available only for some mycotoxigenic species. However, knowledge of the ability of the fungus to activate mycotoxin biosynthesis genes under different environmental conditions may be a better indicator for determining the risk from specific mycotoxigenic species. Few studies have attempted to relate the expression of

specific mycotoxin biosythesis genes with phenotypic mycotoxin production under different environmental conditions. Some studies have attempted to integrate and correlate ecophysiological data with gene expression of specific key regulatory genes in the biosynthetic pathway and phenotypic toxin production (Schmidt-Heydt et al., 2007; Jurado et al., 2008; Schmidt-Heydt et al., 2009).

It has been reported that at least 25 genes are clustered within a 70-kb DNA region in the chromosome involved in aflatoxin biosynthesis (Yu et al., 2004). One of these is the nor-1 (*aflD*) gene that encodes an enzyme that catalyses the ketoreduction of norsolorinic acid (NA) (the first stable pathway intermediate) to averantin (Chang et al., 1992; Trail et al., 1994). Disruption of this gene in *A. parasiticus* resulted in NA accumulation (Chang et al., 1992), confirming the important function of the nor-1 (*aflD*) in AFA synthesis and suggesting that NA is a substrate for this protein.

Several studies have measured the expression of genes involved in the AFA biosynthetic pathway to distinguish between AFA producers and non-producers (Scherm et al. 2005; Degola et al. 2007; Rodrigues et al. 2009). Real-time RT-PCR is highly sensitive and allows quantification of rare transcripts and small changes in gene expression. Recently, Price *et al*. (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of AFA biosynthesis genes. Yu et al. (2004) described the whole biosynthetic pathway and renamed the genes in the cluster. We have used the new names except for the nor-1 (*aflD*) expression for comparison with previous studies.

Schmidt-Heydt and Geisen (2007) developed and used a mycotoxin gene microarray and real-time PCR to study the influence of physical parameters like a_w, temperature and pH on the expression of ochratoxin A (OTA), trichothecenes and AFA gene clusters. Schmidt-Heydt et al. (2008) studied the effect of temperature and a_w on growth and mycotoxin gene expression of several fungal species, including the AFA cluster of *A. parasiticus*. Recently, the influence of both parameters on AFA gene expression and AFB_1 production by *A. flavus* was analysed (Schmidt-Heydt et al. 2009). The ecology and regulation of AFA biosynthesis by *A. flavus* in relation to external factors have also recently been summarized (Abbas et al., 2009; Georgianna and Payne, 2009). Recently, Passone et al. (2010) applied a real-time PCR system to detect and quantify the nor-1 gene of the aflatoxin biosynthetic pathway based on DNA analyses in relation to *Aspergillus* section *Flavi* populations in stored peanuts.

The objectives of this study were to apply molecular tools and compare this with traditional assessment methods and quantitative AFB_1 analyses in monitoring temporal changes in *A. flavus* colonisation of stored peanuts. Experiments were carried out with *A. flavus*-inoculated peanuts stored at three a_w levels (0.95, 0.90, 0.85) to measure (i) population changes based on CFUs of *A. flavus,* (ii) quantification of nor-1 (*aflD*) gene expression, (iii) AFB_1 production and (iv) transcription of four AFA genes nor-1 (*aflD*), ver-1 (*aflM*), omtA (*aflP*) and *aflR* over a period of 6 weeks storage at 25°C.

4.2 Materials and methods

4.2.1 Fungal strain and growth conditions

In this study, an aflatoxigenic strain *A. flavus* (EGP9) isolated from Egyptian peanuts was used. This was compared with a type strain *Aspergillus flavus* NRRL 3357 provided by Dr. D. Bhatnagar, USDA, and confirmed to be taxonomically similar and an aflatoxin B_1 and B_2 producer. The strain was sub-cultured on Malt Extract Agar for 7 days at 25 °C in the dark prior to use.

4.2.2 Inoculation of peanut samples

A moisture adsorption curve was prepared for shelled peanuts as described previously (see Section 3.2.5). This showed that 90 µl, 53 µl and 25.4 µl of water per gram of peanuts were required to reach the target a_w levels of 0.95, 0.90 and 0.85 respectively. One hundred gram of peanuts (three replicates per treatment) were put in glass jars covered with lids containing a microporous membrane and autoclaved at 121 ºC for 20 min. After cooling, the water was added and after equilibration the peanut samples were inoculated with 1 ml of 10^6 spores ml^{-1} of *A. flavus* and vigorously shaken to coat the peanuts with spores and incubated at 25°C for six weeks in polyethylene sandwich boxes containing glycerol/water solutions to maintain the equilibrium relative humidity conditions. Sub-samples were destructively sampled every 7 days (approx 15 g of contaminated peanuts) and divided into three parts: (a) 10 g for AFB_1 extraction, (b) 1 g for CFU determination and (c) 1 g for RNA extraction followed by RT-PCR and real-time PCR.

4.2.3 Determination of colony forming units (CFUs)

The *A. flavus* populations (CFUs g^{-1}) were determined by serial dilution and spread plating the different dilutions on MEA and incubating for 4-5 days at 25^{o}C before counting numbers of colonies. The dilutions containing 10-100 colonies were counted.

4.2.4 Aflatoxin extraction and HPLC analysis

Ten gram of peanuts was extracted for AFB_1 analyses using an immunoaffinity column (Neogen, Europe Ltd). The extraction was performed as follow: 10 g of peanut sample was placed into a blender (Waring ® Commercial Blender 8010E) and 1 g NaCl was added, then 50 ml of 60% methanol/water (v/v) were added. The mixture was blended for 1 min at high speed. The extract was filtered through a Whatman No. 1 filter paper. Ten milliletres of the final extract equal to 1 g peanut sample diluted 1:1 with phosphate buffered saline (PBS, pH 7.4). The mixture was allowed to pass through the column without drying via a 50 ml glass syringe reservoir attached to the column using a column adaptor at a flow rate of 1.5-2.0 mls min^{-1}. The glass syringe and adaptor were removed and the column completely filled with 25% methanol, then the reservoir/adaptor re-attached. The column was then washed with 20 ml of 25% methanol. The elution of aflatoxins was done with 2 ml of methanol and collected in a 2 ml Eppendorf tube. The residue was derivatized using TFA (Triflouroacetic acid) as described in Section 2.2.4b. Sample extracts were analyzed using an Agilent 1200 series HPLC (Agilent, Berkshire, UK) using a 470 fluorescence detector (FLD, G1321A, Agilent) (λ_{exc} 360 nm; λ_{em} 440 nm) and a C_{18} column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 µm). The analysis was performed using a mobile phase of methanol: water: acetonitrile (30:60:10) at a flow rate of 1 ml/min and a run time of 25 mins.

4.2.6 Isolation of RNA from the samples and RT-PCR

Total RNA was extracted as described in Section 3.2.5b The expression of three structural genes, *aflD* (nor-1), *aflM* (ver-1), and *aflP* (omt A), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway were assayed in all treatments and replicates. The expression of the housekeeping gene (β-tubulin) was used as a control (see Section 3.2.4b).

4.2.7 TaqMan probes and primer design

Real Time RT-PCR was used to amplify the nor-1 gene (target gene) and benA56 (β-tubulin gene) as a control gene (Mayer et al., 2003). The two primers and an internal fluorescence labelled probe used in the reaction were nortaq-1 5 -GTCCAAGCAACAGGCCAAGT-3 ; nortaq-2 5 -TCGTGCATGTTGGTGATGGT-3; norprobe 6FAM TGTCTTGATCGC GCCCG- BHQ2; bentaq-1 5-CTTGTTGACCAGGTTGTGGAT-3; bentaq-2 5-GTCGCAGCCCTCAGCCT-3, benprobe CY5-CGATGTTGTCCGTCGCGAGGCT-BHQ2.

4.2.8 Real-time PCR conditions

Amplification was performed using a total reaction volume of 25 µl in a MicroAmp optical 96-well reaction plate (Applied Biosystems, Warrington, UK). For each reaction 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 2.5 µl cDNA, 3 µl of primer and probe mix (0.5 nM primer and 0.2 nM probe), and 7 µl of free RNases water. Real-time PCR reactions were performed using the Bio Rad CFX96 platform (Bio Rad, Hercules, CA, Figure 4.1) with the following conditions: an initial step at 95ºC for 10 min, and all 40 cycles at 95ºC for 15 s, 55ºC for 20 s and 72ºC for 30 s.

Relative quantification method (RQ-PCR)

The efficiency of PCR (E) was calculated from each linear regression of standard curves of each target and control gene which was calculated from the formula $E= [10^{(-1/slope)} -1] X 100$ (Figure 4.2). This method compares the relative amount of the target gene (nor-1) to control gene (benA56). The target and control amplification were carried out in separate tubes in triplicate. Normalized relative quantity (NRQ) = $E^{Ct\ nor\text{-}1}/E^{Ct\ benA56}$ where E is the PCR efficiency for each target, Ct is the threshold cycle (Pfaffl 2001). Only the linear range was used for quantification.

Contour map of responses

The three dimensional (3D) response contour plot was employed to determine the relationship between RQ-PCR data of *aflD* expression of *A. flavus* and a_w in relation to the temporal storage period.

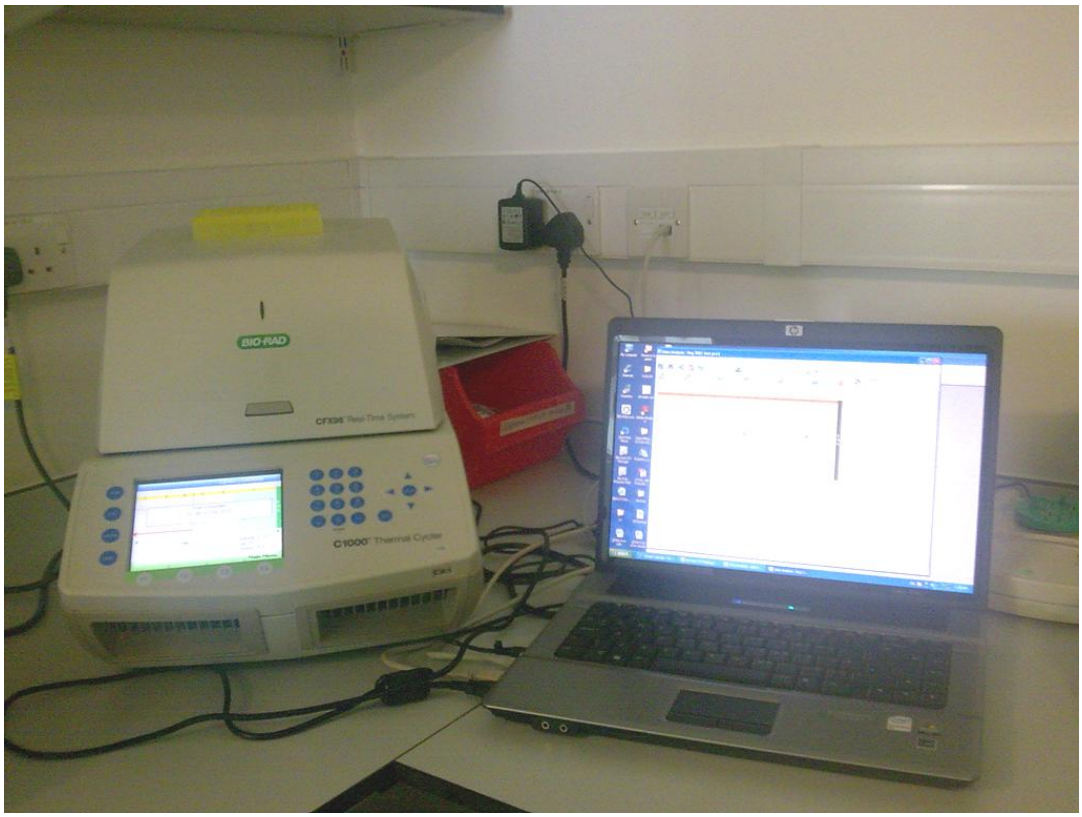

Figure 4.1 Bio Rad real-time PCR used for quantification of the expression of required genes.

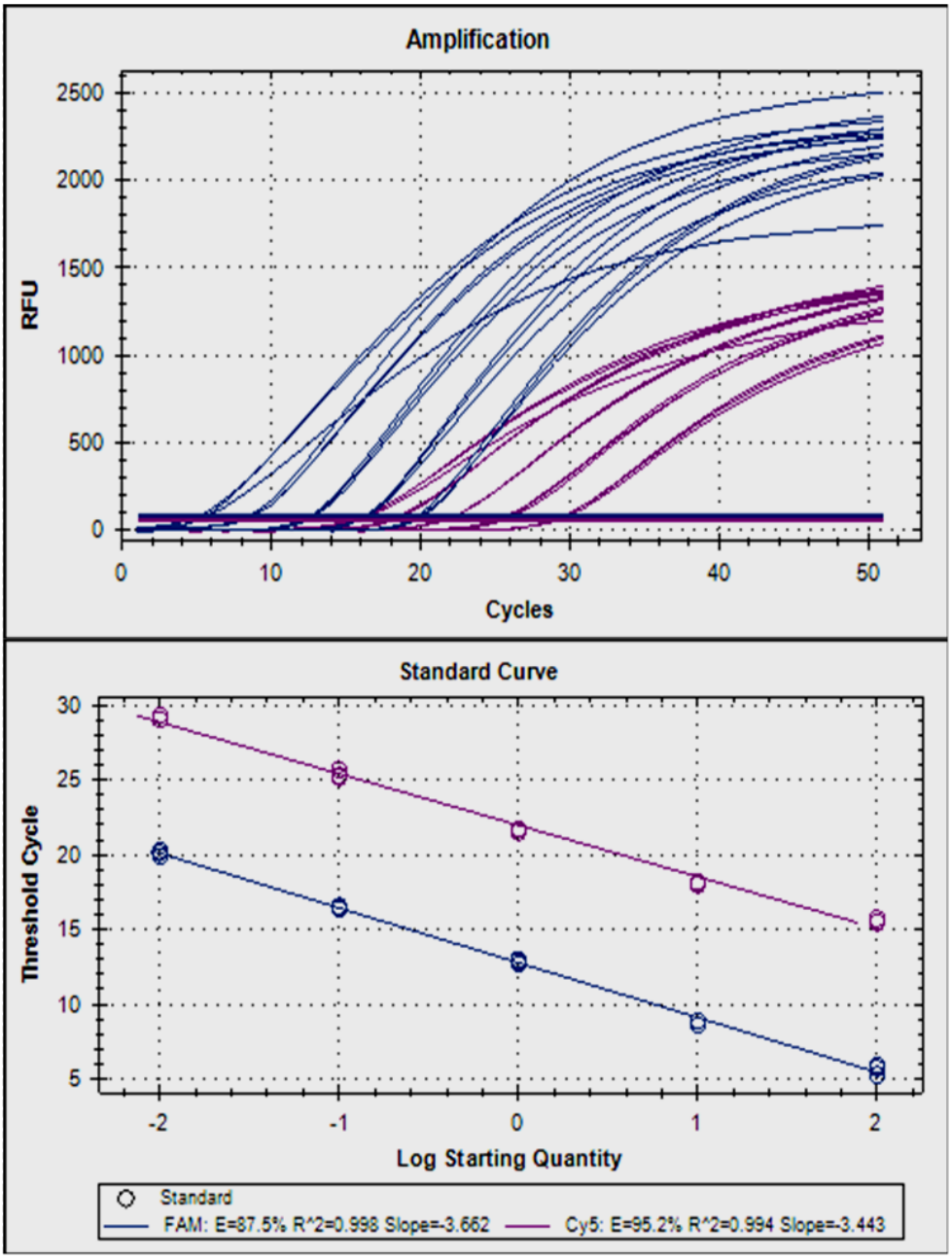

Figure 4.2 Amplification plot and standard curve of nor-1 (target gene) labelled with FAM and β-tubulin gene (control gene) labelled with Cy5. Where (RFU) is Relative fluorescence unit, E: The efficiency of PCR, R^2 value: correlation coefficient.

The data was analysed using Statistica (ANOVA) version 8 (StatSoft, Inc, 1984-2007; Statsoft, Tulasa, OK) software for interaction between factors (a_w x time).

4.3 Results

4.3.1 Effect of water activity on populations of *A. flavus* on stored peanuts

Figure 4.3 shows the temporal changes in *A. flavus* isolated from the three a_w treatments. There was a rapid increase in viable propagules produced at 0.95 a_w reaching a maximum at the end of the experiment. At 0.90 a_w, CFU numbers were less than at 0.95 a_w with a maximum after 4 weeks incubation (Plate 4.1). As water stress was imposed, the populations of *A. flavus* populations isolated were significantly decreased (P=0.05). Statistical analysis of the effect of single factors (a_w, time) and their interactions were statistically significant (Table 4.1a).

4.3.2 Effect of water activity on aflatoxin production

Figure 4.3 also shows the temporal AFB_1 production by *A. flavus* in the stored peanuts under different a_w regimes. Overall, *A. flavus* produced maximum amounts of AFB_1 at 0.90 a_w and 0.95 a_w after 3 weeks storage. The production of AFB_1 was detected after 1 week storage at 0.95 a_w and 0.90 a_w. No increase in AFB_1 production occurred at 0.85 a_w when compared to the control (uninoculated with *A. flavus*) over the storage period. Table 4.1b summarises the statistical significance of the single and two way interaction factors for a_w, time and a_w x time which were all statistically significant.

4.3.3 RQ-PCR of nor-1 in relation to water activity

The normalized relative quantity (NRQ) of nor-1 aflatoxin gene with the β-tubulin gene (housekeeping gene) of *A. flavus* in peanuts was analysed (see Figure 4.3). There was a significant difference between nor-1 expressions at the three a_w levels. There was higher expression at 0.90 a_w especially during weeks 1-3, after which expression decreased.

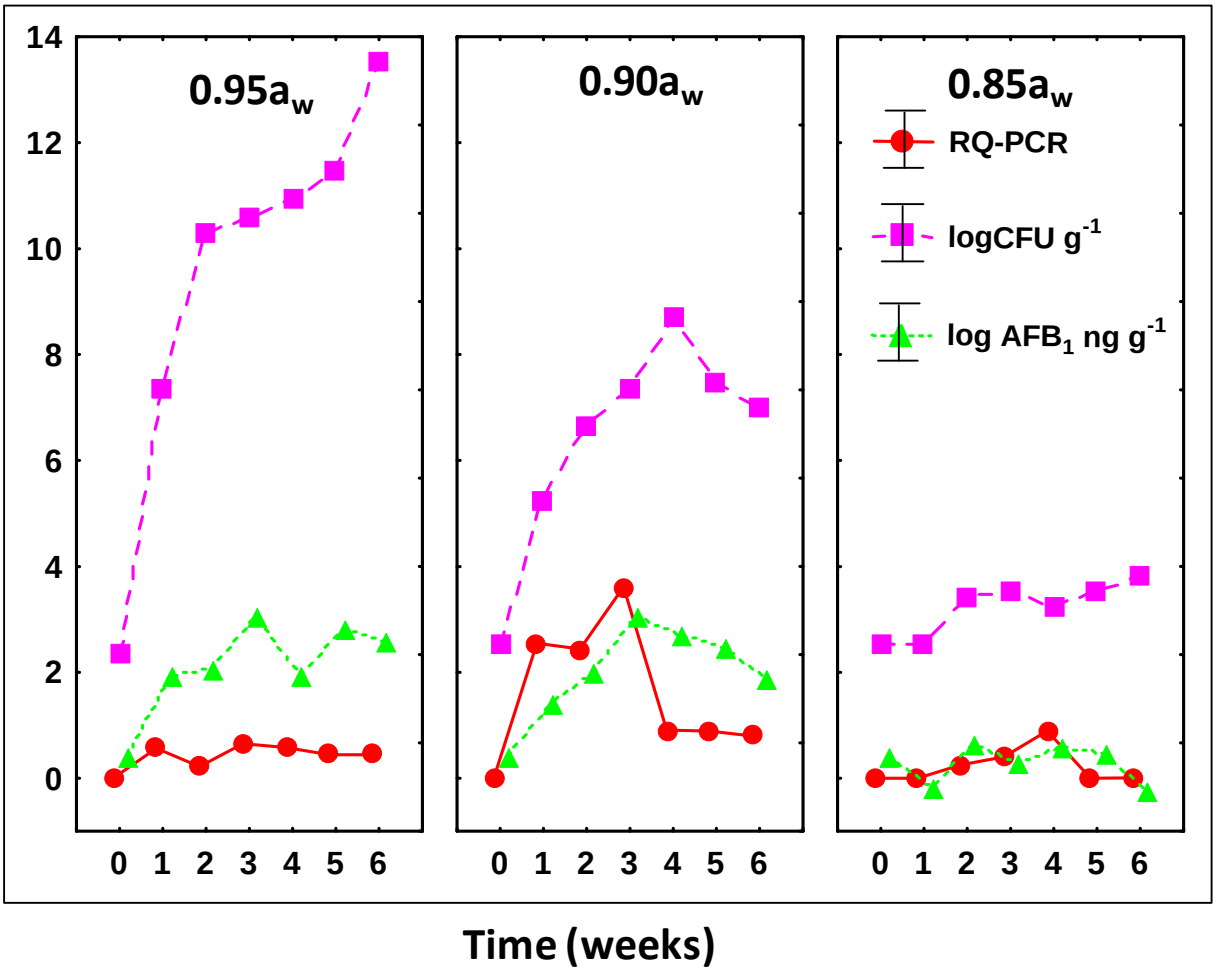

Figure 4.3 CFU values, Aflatoxin B_1 production and RQ-PCR of nor-1 gene of *A. flavus* in peanut at different a_w levels and different incubation times at 25 °C. Vertical bar indicates 95% confidence limits.

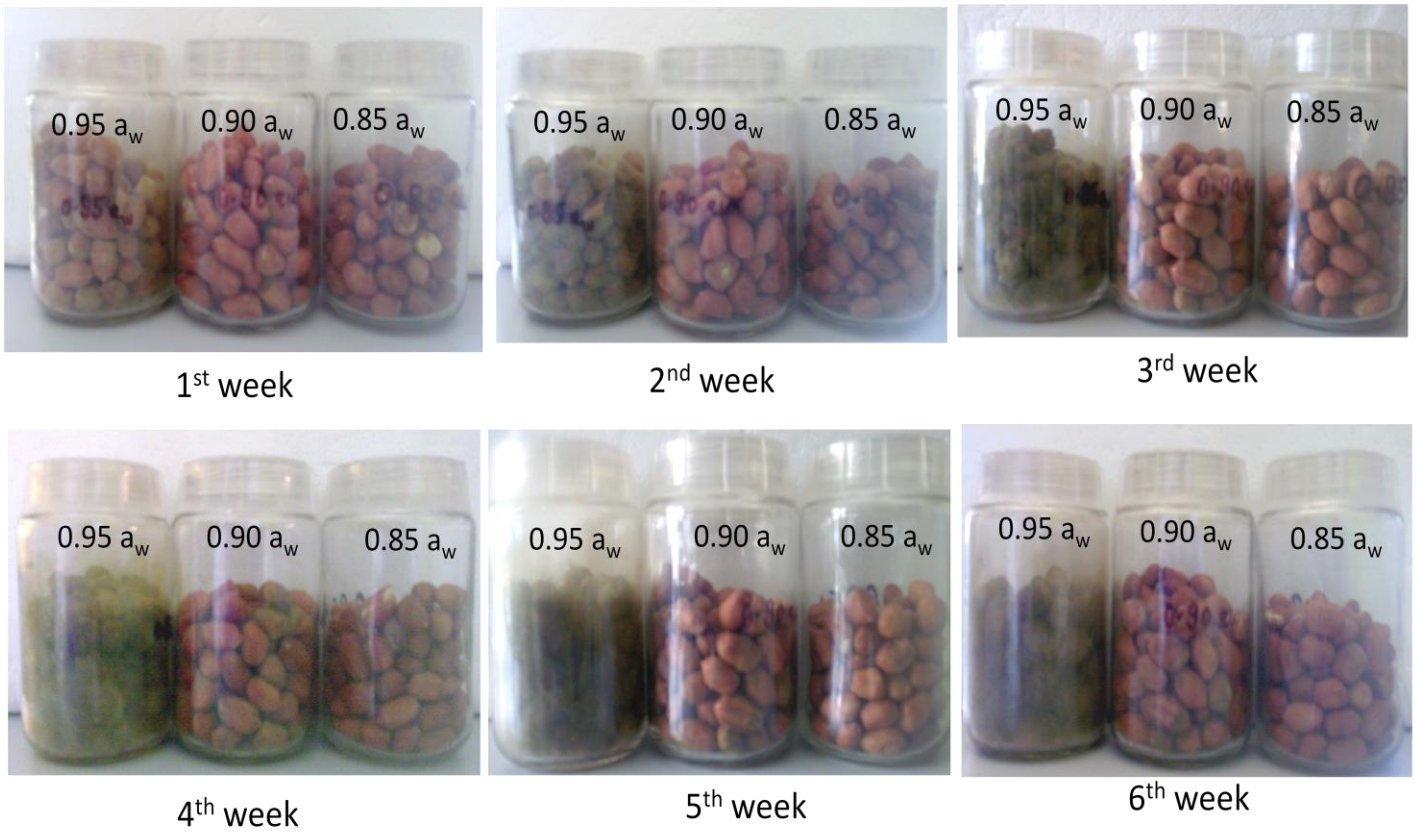

Plate 4.1 *A. flavus* EGP9 growing on peanuts over a period of 6 weeks at three water activity (a_w) levels (0.95, 0.90, 0.85) and at 25°C.

At 0.95, the expression was lower than at 0.90 a_w and the highest expression was after 3 weeks and then decreased further. At 0.85 a_w, there was no nor-1 expression during the first two weeks and very low expression subsequently. Single and interacting factors were all significant (Table 4.1 c).

The data for nor-1 gene relative gene expression was analysed to examine whether there was any pattern to production over time. Figure 4.4 shows the contour map for relative expression of the nor-1 gene at different a_w levels in relation to time. There is a clear optimum of expression at 0.90 during the first 2 weeks of storage, with less expression at 0.95 and 0.85 a_w and over time. This suggests some pattern with regard to relative expression of the nor-1 gene when *A. flavus* colonises peanuts.

4.3.4 Analysis of aflatoxin genes transcription in relation to water activity

In this study the transcription of four genes, *aflD* (nor-1), *aflM* (ver-1), *aflP* (omt A) and *aflR* in the biosynthetic pathway for AFA production were assessed (Figure 4.5). The expression of the house keeping gene (β-tubulin) was used as a control. Transcription of the genes was assayed by RT-PCR. To ensure there was no DNA contamination in the RNA, for each sample PCR was performed following an RT reaction in the presence (+RT) or absence (-RT) of the reverse transcriptase enzyme. RT-PCR results revealed that at 0.95 a_w, all four genes were transcripted from the beginning to the end of the storage period. At 0.90 a_w, *aflD* was expressed from the start of the experiments while *aflR* only after 4 weeks onwards, the genes *aflP* and *aflM* were expressed from the 2nd week onwards. In the driest conditions tested (0.85 a_w) only two genes were transcribed (*aflD* and *aflM*) after 3 weeks.

4.3.5 Correlation co-efficients for comparing different factors

Table 4.2 shows the results from examining the possible correlations between different treatment factors. There was a good correlation between *A. flavus* CFUs and a_w (R= 0.75: P= 0.00), AFB_1 correlated significantly with a_w (R= 0.68; P= 0.00) and AFB_1 x log CFUs (r=0.85). However, for other factors there were no significant correlations.

Table 4.1 (a) Analysis of Variance of the effect of $a_{w,}$ time and their interactions on CFUs of *A. flavus* in stored peanut peanuts; (b) single and two way interactions on aflatoxin production, and (c) single and two way interactions on RQ-PCR of *A. flavus* in peanuts.

(a)

	DF	MS	F	P
Factor				
a_w	2	207.367	1064.18	0.00 *
Time	6	35.263	180.96	0.00 *
Interaction factors				
a_w x Time	12	8.635	44.31	0.00 *
Error	42	0.195		

(b)

	DF	MS	F	P
Factor				
a_w	2	643315	1064.18	0.00000 *
Time	6	895126	180.96	0.00000 *
Interaction factors				
a_w x Time	12	243808	44.31	0.00000 *
Error	42	31469		

(c)

	DF	MS	F	P
Factor				
a_w	2	10.49419	157.7194	0.00000 *
Time	5	1.61667	24.2973	0.00000 *
Interaction factors				
a_w x Time	10	1.4641	22.0044	0.00000 *
Error	36	0.06654		

DF: Degree of freedom, MS: mean square, P: Probability * Significant < 0.05 %

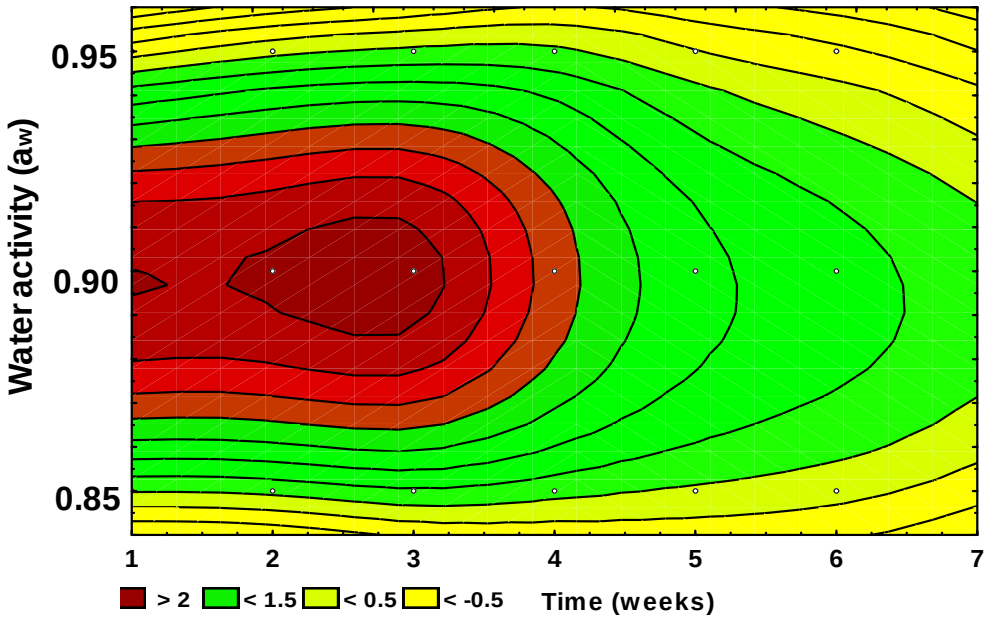

Figure 4.4 3D contour plot of the effect of water activity (a_w) and time on RQ-PCR of nor-1 (*aflD*) gene of *A. flavus* in peanuts at 25 °C.

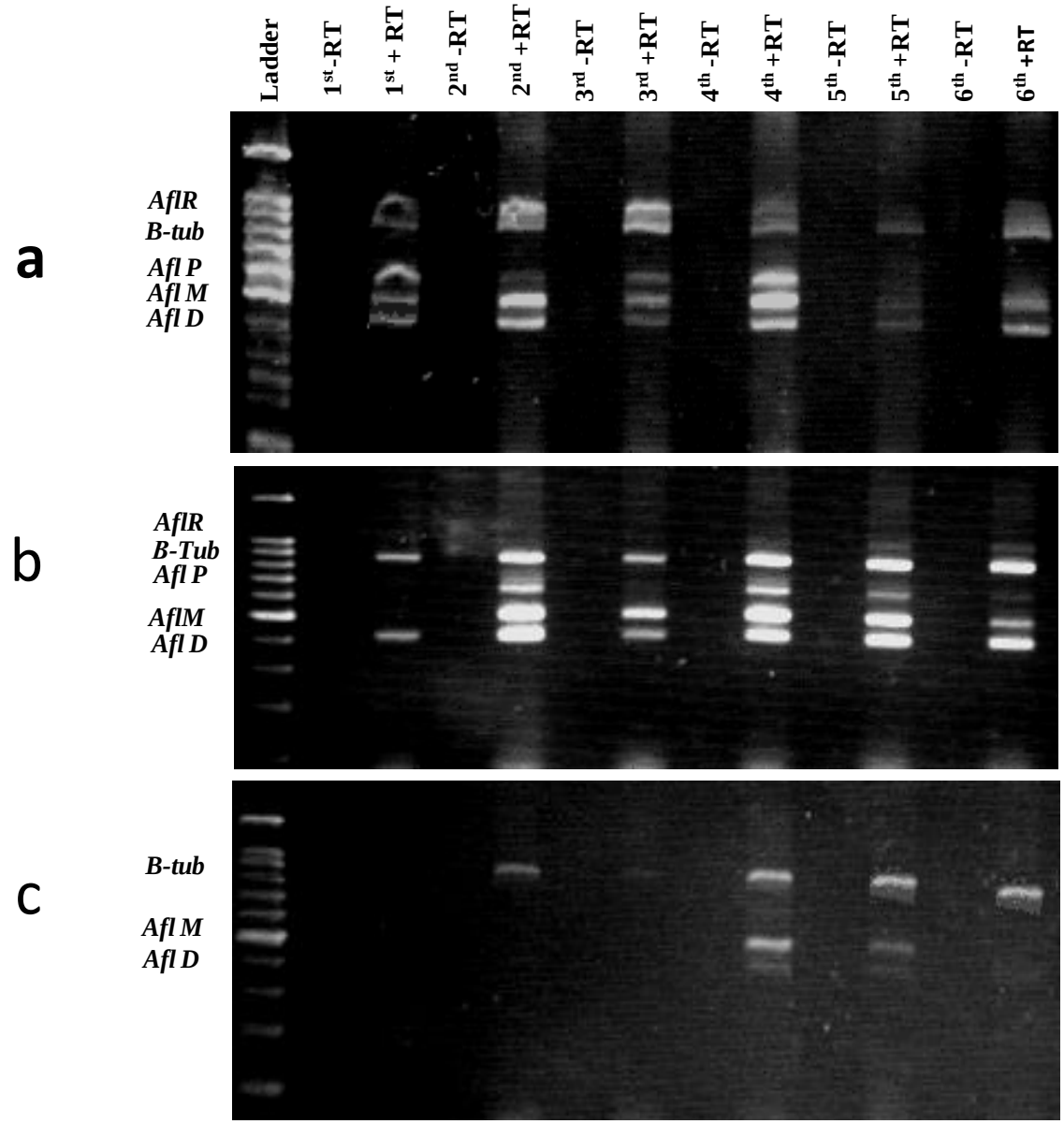

Figure 4.5 Transcription of primer set (β-tubulin, *aflD*, *aflM*, *aflP*, *aflR*) detected by RT-PCR in *A. flavus* EGP9 at three water activity (a_w) levels (a) 0.95, (b) 0.90 and (c) 0.85 for 6 weeks. First lane 100 bp ladder; RNA from each treatment was amplified by PCR following reverse transcription in the absence (-RT) or presence (+RT) of the RT enzyme. PCR products were separated on a 2% agarose gel, stained with ethidium bromide and visualized under UV.

Table 4.2 Statistical correlations between *A. flavus* populations (CFUs), aflatoxin B_1 production, and RQ-PCR of nor-1 gene of *A. flavus* in stored peanuts at different a_w levels for up to 6 weeks storage.

Correlations	R value	F	P
log aflatoxin & log CFUs	0.849	157.44	0.000 *
log aflatoxin & water activity	0.68	78.22	0.000 *
log aflatoxin & RQ-PCR	0.488	19.03	0.000 *
log CFUs & water activity	0.75	78.22	0.000 *
log CFUs & RQ-PCR	0.175	1.919	0.09
RQ-PCR & water activity	0.08	0.416	0.0051 *

R: correlation coefficient. P: Probability * Significant < 0.05 %

4.4 Discussion

This is one of the first studies to compare the influence of a_w on relative quantification of nor-1 gene in relation to CFU, phenotypic aflatoxin production, and aflatoxin gene transcription of *A. flavus* during storage of peanuts. Quantification of the nor-1 gene expression based on the relative expression of this gene versus a reference gene or housekeeping gene (β-tubulin gene) provided useful information to relate molecular changes to ecophysiological parameters. Previously, Mayer et al. (2003) reported that the β-tubulin gene was constitutively expressed and constant during the subsequent growth phases when compared with the expression of nor-1 gene of *A. flavus.*

Our results showed that temporal changes in populations (CFUs) in relation to storage a_w showed a good correlation (r=0.75; P =0.00). In the wettest condition tested more rapid colonization and sporulation occurred reflecting the high $\log_{10}$ CFUs found. No statistical correlation between *A. flavus* CFUs and the quantified nor-1 gene expression levels (r=0.175; P=0.09) was found. This may partially be because of the fact that at lowered a_w levels (e.g., 0.90) there was an increase of nor-1 gene expression although populations (CFUs) of *A. flavus* increased at a slower rate over the 6 week storage period. Recently, Passone et al. (2010) reported a good correlation (r=0.613; P<0.0001) between nor-1 gene quantity and CFUs in naturally stored peanuts over a period of 4 months for *Aspergillus* section *Flavi*. However, this study was based on DNA analyses of the nor-1 gene, not RNA expression. As all propagules contain the gene, the presence of nor-1, per se, may not accurately reflect expression and phenotypic production of aflatoxins.

The only other recent study was that by Schmidt-Heydt et al. (2007) who examined *Penicillium verrucosum* populations and ochratoxin A (OTA) and the OTA polyketide synthesis gene expression (*otapks*Pv) in wheat stored at three water contents (14, 19 and 24%) for up to three months. They showed good correlations between the *otapks*Pv expression, phenotypic OTA production and in some cases that this was paralleled by CFUs of *P. verrucosum*.

Overall, similar results were obtained by RQ- PCR at both 0.90 a_w and 0.95 a_w, with high expression especially during the first 3 weeks, before expression slowed down. The contour map of expression of *nor-1* shows these changes clearly over storage time with the optimum during the first few weeks of storage. There was thus a poor correlation between RQ-PCR data and AFB_1 production (r= 0.488; p=0.000). This poor correlation is probably due to the *nor-1* expression being initiated very early, prior to phenotypic aflatoxin production being synthesised. Thus expression of the *nor-1* transcripts may already be decreasing as the increase in toxin production is detected (Mayer et al., 2003).

The high sensitivity of nor-1 gene expression in relation to changing in water activity levels during storage of peanut can be easily determined by real-time PCR system. This could be a useful tool to improve food safety of peanut and predict the ideal environmental condition that we can use to knockdown nor-1 expression as well as aflatoxin production. Previously, Mayer et al. (2003) used a real-time reverse transcription-PCR system to monitor the expression of nor-1 gene of *Aspergillus flavus* in wheat. They found that the described real-time PCR system is able to completely characterize the mycological status of wheat as a model food matrix.

Several genes code for proteins involved in the aflatoxin biosynthesis pathway. Among the 25 genes involved in aflatoxin biosynthesis, we selected three structural genes *aflD* (early stage), *aflM* (middle stage), *aflP* (late stage) and the regulatory gene *aflR* that plays a role in controlling the level of structural genes expression (Woloshuk et al., 1994; Chang, 2004; Price et al., 2005) to measure their transcriptional status in relation to change in a_w level during peanut storage.

Water activity had a significant effect on *aflR* transcription, especially at 0.90 a_w, where it was transcripted from the 4^{th} week, while during the initial 3 weeks there was high expression of *nor-1* gene, transcription of the structural genes and high aflatoxin production. This suggests that *aflR* may not have a role in regulation of structural gene expression in food matrices such as peanuts. This contrasts with Degola et al. (2007) who reported that structural gene expression follows regulatory genes *aflR* and *aflS* transcription. Incomplete induction of these genes does not permit the detection of the structural gene expression, even by RT- PCR. This may confirm the fact that gene expression may be variable depending on physiological and environmental conditions.

Our results support those obtained by Schmidt-Heydt et al. (2009). They demonstrated that at lowered a_w (0.90) the ratio of *aflS/aflR* was decreased compared to the other genes of the cluster. Thus, although expression was high (including that of *aflD*), low amounts of AFB_1 were produced *in vitro*. In contrast, in peanuts a high amount of AFB_1 was produced. This may partially be because the present study was carried out directly on the food matrix which may give different results from those on a conducive medium. However, *in situ* studies are critical to enable a better understanding of the ecophysiological and functional importance of specific regulatory genes to develop effective control approaches to minimise mycotoxin contamination of a range of important staple food commodities.

Chapter 5

Control of aflatoxin production using RNA interference

5 Control of aflatoxin production using RNA interference

5.1 Introduction

The studies in Chapter 4 showed the changes in *aflD* expression which may occur in relation to changes in water activity levels in stored peanuts. Often a high expression of *aflD* resulted in high AFB_1 production. Further silencing studies on *aflD* expression were carried out to confirm the role of *aflD* expression in AFB1 biosynthesis.

Controlling aflatoxin production is of critical importance. The main traditional control methods including pre-harvest cultural practices, pesticides to control insect damage to ripening corn, and the development of resistant cultivars. However, these have not always been successful in maize and in groundnuts, especially during climatic stress. There is thus interest in exploring alternative means to control or reduce aflatoxin production.

RNA interference technology (RNAi) has received much attention in biology. The reason for this enthusiasm is that RNAi can rapidly ablate specific messenger RNA (mRNA) species by inducing their degradation via cellular protein machinery collectively named the RNA-induced silencing complex (RISC; Ketting et al., 2001). Short-interfering double-stranded RNA (siRNA) is synthesized and introduced through common transfection methods into cells, where they serve to guide the RNA degradation machinery to the selected target gene. RNAi is an effective tool to investigate gene function, and may also be a useful tool to quench the expression of undesirable gene products.

RNA silencing in filamentous fungi has been carried out using plasmid constructs expressing a hairpin dsRNA structure controlled by an inducible or constitutive promoter (Mouyna et al., 2004; Nakayashiki, 2005; Bromley et al., 2006; de Jong et al., 2006). Liu et al. (2002) demonstrated silencing of the cryptococcal *CAP59* and *ADE2* genes by double-stranded RNA homologous to these genes in the basidiomycetous yeast *Cryptococcus neoformans*.

Application of siRNA-mediated RNAi has also been reported in cultured cells from fungi. Katri and Rajam (2007) reported that ornithine decarboxylase (ODC) was specifically silenced by treating germinating spores with synthetic 23 nucleotides siRNA in *Aspergillus*

nidulans. Doubl-stranded RNA (dsRNA) was also delivered directly into protoplasts of *Phytophthora infestans*, which belongs to the fungus-like Oomycetes (Whisson et al., 2005).

In *A. flavus* and *A. parasiticus* the expression of the *aflD* (nor-1), a gene encoding an enzyme that catalyzes the conversion of the first stable aflatoxin biosynthesis intermediate, norsolorinic acid, to averantin (Trial et al., 1994; Zhou and Linz, 1999) is a key structural gene in the biosynthetic pathway. Furthermore, *aflR* is a pathway regulatory gene coding for proteins shown to be involved in transcriptional activation of most of the structural genes (Cary et al., 2000). Recent studies have shown that there may be a relationship between the ratio of *aflR* and *aflS* genes which is influenced by environmental factors (Schmidt-Heydt et al., 2009).

The objectives of this study were to determine (a) potential siRNA for degradation of the target genes and (b) optimization of the best siRNA concentration for inhibition of the target genes and phenotypic aflatoxin control.

5.2 Materials and methods

5.2.1. Fungal strain and growth conditions

In this study, four aflatoxigenic strains (*Aspergillus flavus* NRRL 3357, *Aspergillus flavus* EGP9, *Aspergillus parasiticus* NRRL 13005 and *Aspergillus parasiticus* SSWT 2999) have been used. The strains were sub-cultured on Malt Extract Agar for 7 days at 25 °C in the dark prior to use.

5.2.2. Preparation of protoplast

Protoplasts were prepared from actively growing mycelium. A spore suspension of the strains sub-cultured in 200 ml of Yeast Extract Sucrose broth, and then incubated for 24 h on shaker 200 rpm in dark at 25 °C. The mycelium was harvested by filtration through Miracloth. One gram of mycelia was transferred into 20 ml of filter sterilized enzyme solution (per 20 ml: 17 ml of H_2O, 2 ml of 0.2 M $NaPO_4$ [pH 5.8], 0.4 ml of 1.0 M $CaCl_2$, 1.4 g of NaCl, 0.2 ml of β-glucuronidase [105 U/ml; Sigma], 200 mg of lysing enzyme [Sigma], and 50 mg of

driselase [Sigma]. Mycelia were incubated at 30 °C with shaking (80 rpm) for 3 h. Protoplasts were separated from intact mycelia by passage through Miracloth into a sterile 50 ml tube, and 20 ml of sterile STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl [pH7.5]) was added. Protoplasts were pelleted by low-speed centrifugation (1,000 rpm) at room temperature for 5 mins. The supernatant was carefully removed, and the protoplasts were washed once more in 20 ml of STC and pelleted by centrifugation as described previously. The protoplast pellet was resuspended in 1.0 ml of STC buffer, and the protoplasts were counted on haemocytometer and diluted to 1×10^5 ml^{-1} (Cary et al., 2006).

5.2.3. siRNA design

Three siRNA sequences were designed to target the mRNA sequence of the *aflD* gene of *A. flavus* (accession number EF565463**)** and purchased from Ambion (Applied Biosystem). These siRNA were named as Nor-Ia, Nor-Ib and Nor-Ic (Table 5.1). Annealing of RNA oligonucleotides and purification by HPLC were performed by the company. An siRNA (control-siRNA) with no sequence homology to any *A flavus* genome sequence database was also purchased from Ambion.

5.2.4. Delivery of siRNA to protoplast

All siRNAs were resuspended at a final concentration of 25 nM and tested on *A. flavus* NRRL 3357. In a sterile 1.5 ml micro centrifuge tubes, 10 µl of each siRNA was mixed with an equal volume of Lipofectin reagent (Invitrogen Life Technologies, UK) and allowed to stand for 15 min at 20 °C. Twenty microlitres of protoplasts (1×10^3) were added and mixed gently. The tubes were incubated at 20 °C for 24 h to allow transfection to proceed (Whisson et al., 2005). Then the mixture was inoculated in 10 ml of YES medium with1.2 M of sorbitol for 5 days at 25 °C in the dark. Different dilutions of Nor-Ib (5, 10, 15, 20, 25 nM) were tested on *A. flavus* NRRL 3357. Twenty five nanomoles of Nor-Ib was tested on *Aspergillus flavus* EGP9, *Aspergillus parasiticus* NRRL 13005 and *Aspergillus parasiticus* SSWT 2999. All experiments were carried out using three biological replicates.

Table 5.1 Details of siRNA sequences used in this study

siRNA name	siRNA sequence
Nor-Ia	Sense strand: CAUGUAUGCUCCCGUCCUAUU Antisense strand : UAGGACGGGAGCAUACAUGUU
Nor-Ib	Sense strand: GCAACAGGCCAAGUUUGUCUU Antisense strand : GACAAACUUGGCCUGUUGCUU
Nor-Ic	Sense strand: CAGGCCAAGUUUGUCUUGAUU Antisense strand : UCAAGACAAACUUGGCCUGUU

5.2.5 Aflatoxin extraction and HPLC analysis

Five ml of filtrate was extracted with chloroform, and then the extract was evaporated. The residue was derivatized using TFA (Triflouroacetic acid) and HPLC analysis was performed as described in Section 4.2.4.

5.2.6. Isolation of RNA from the samples and RT-PCR

RNA extraction was performed as mentioned in Section 2.2.6.a

5.2.7 TaqMan probes and primer design

Real Time RT-PCR was used to amplify the *aflD* gene (target gene) and *aflR* gene (regulatory gene). The two primers and an internal fluorescence labelled probe used in the reaction were nortaq-1 5 -GTCCAAGCAACAGGCCAAGT-3 ; nortaq-2 5 -TCG TGCATGTTGGTGATGGT-3; norprobe 6FAM TGTCTTGATCGCGCCCG- BHQ2 (Mayer et al., 2003); AflRtaq-1 5-TCGTCCTTATCGTTCTCAAGG-3; AflRtaq-2 5-ACTGTTGCTACAGCTGCCACT-3, AflRprobe 6FAM AGCAGGCACCCAGTGT ACCTCAAC-BHQ2. To create standard curve, a larger PCR fragment of the *aflD* (nor-1) gene, generated with the primer nor1 and nor2 (Geisen, 1996) (Figure 5.1a). Different dilutions were prepared from stock solution by a factor of 10 and the aliquots of the dilutions were used in standard reactions during each setup of the real-time PCR reaction. The concentration of this standard PCR product was determined in a spectrophotometer (WPA light wave Cambridge, UK) and the number of copies was calculated. The concentration of unknown samples was calculated by the Bio Rad CFX96 system according the generated standard curve. To create standard curve, a larger PCR fragment of the *aflR* gene with the following primers AflR1, 5-CGAGTTGTGCCAGTTCAAAA-3; AflR2, 5-AATCCTCGCCCACCATACTA-3 (Figure 5.1b).

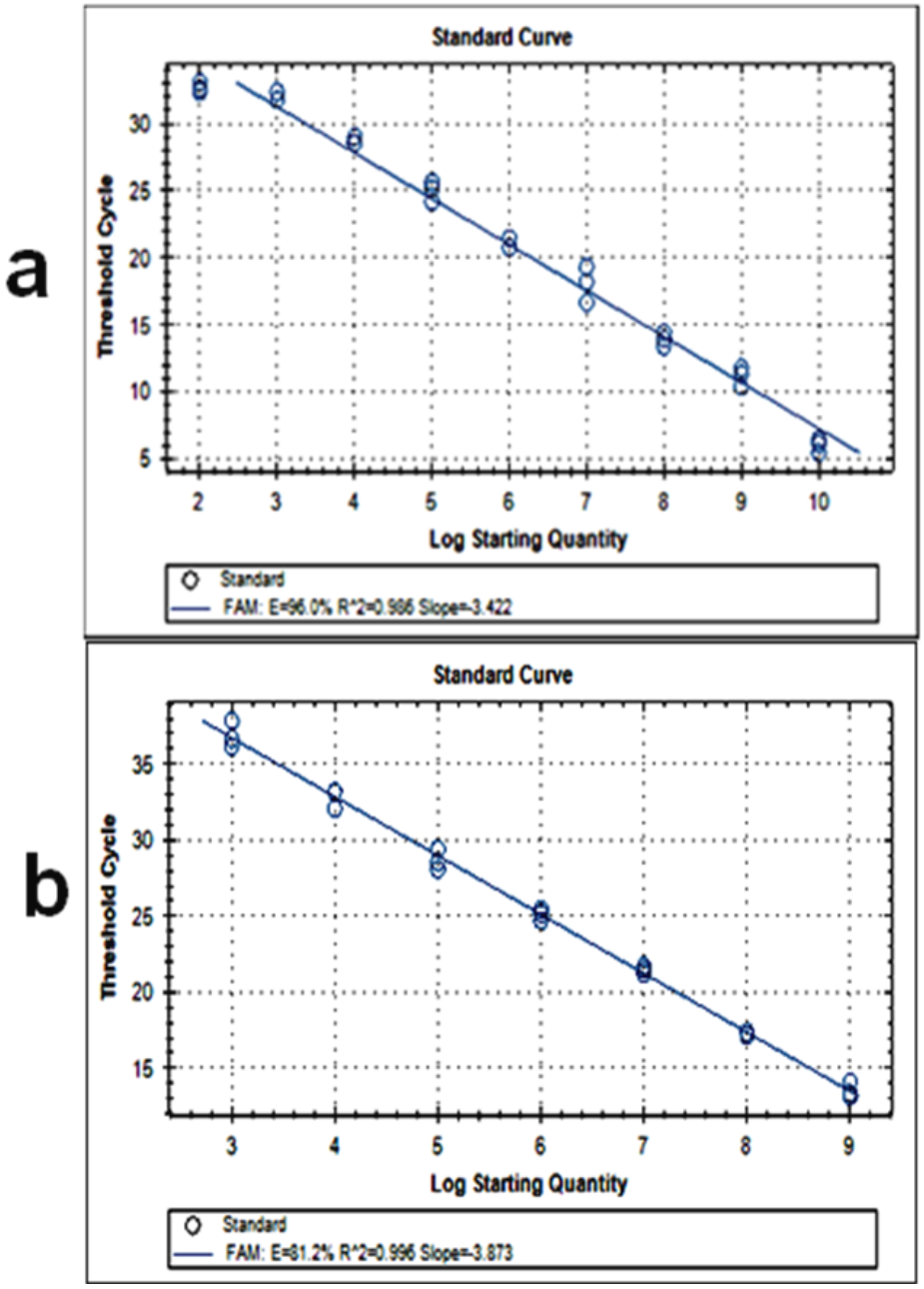

Figure 5.1 Standard curves from real-time PCR by plotting the threshold cycle (Ct) *vs* $\log_{10}$ initial copy numbers of *aflD* gene (a) and *aflR* gene (b) amplified with the primer of labelled with FAM. Where E: The efficiency of PCR, R^2 value: correlation coefficient.

5.2.8 Real-time PCR conditions

Amplification was performed as described in Section 4.2.8.

5.2.9 Statistical analysis

Statistical analysis was performed as described in Section 2.2.7

5.3 Results

5.3.1 Treatment of *Aspergillus flavus* NRRL3357 with siRNA

Figure 5.2 shows the changes in *aflD* mRNA and *aflR* mRNA expression and AFB_1 production by *A. flavus* NRRL 3357 after treatment with control-siRNA or three siRNAs (Nor-Ia, Nor-Ib, Nor-Ic) specific to the *aflD* target gene. Treatment with the control-siRNA had no significant effect on AFB_1 production or *aflD*/*aflR* mRNA copy numbers. There was a significant decrease (95%, 98%, and 91% of the control level) in *aflD* mRNA abundance after treating with Nor-Ia, Nor-Ib and Nor-Ic siRNAs respectively, as assessed by real-time PCR. The lack of any effect using the control-siRNA and the knockdown seen with all three *aflD* siRNAs suggest that the results are not caused by transfection conditions or due to off-target effects.

Interestingly, a decrease (99% (Nor-Ia treatment), 97% (nor-Ib treatment), and 72% (Nor-Ic treatment) of the control level in *aflR* mRNA abundance was also observed following knockdown of *aflD*. Subsequently, a decrease of AFB_1 production as a result of decrease of *aflD* and *aflR* gene expression [79% (Nor-1a treated), 97% (Nor-Ib treated), and 76% (Nor-Ic treated] of the control level. Statistical analysis of the effect of siRNA treatment on *aflD* gene expression, *aflR* gene expression and AFB_1 production were highly significant (Table 5.2a). There was a good correlation in reduction as a result of siRNA treatment between *aflD* and *aflR* expression (R= 0.82: P= 0.0001), *aflD* and AFB_1 (R= 0.88: P= 0.00001) and *aflR* correlated significantly with AFB_1 (R= 0.66; P= 0.0074) (Table 5.3).

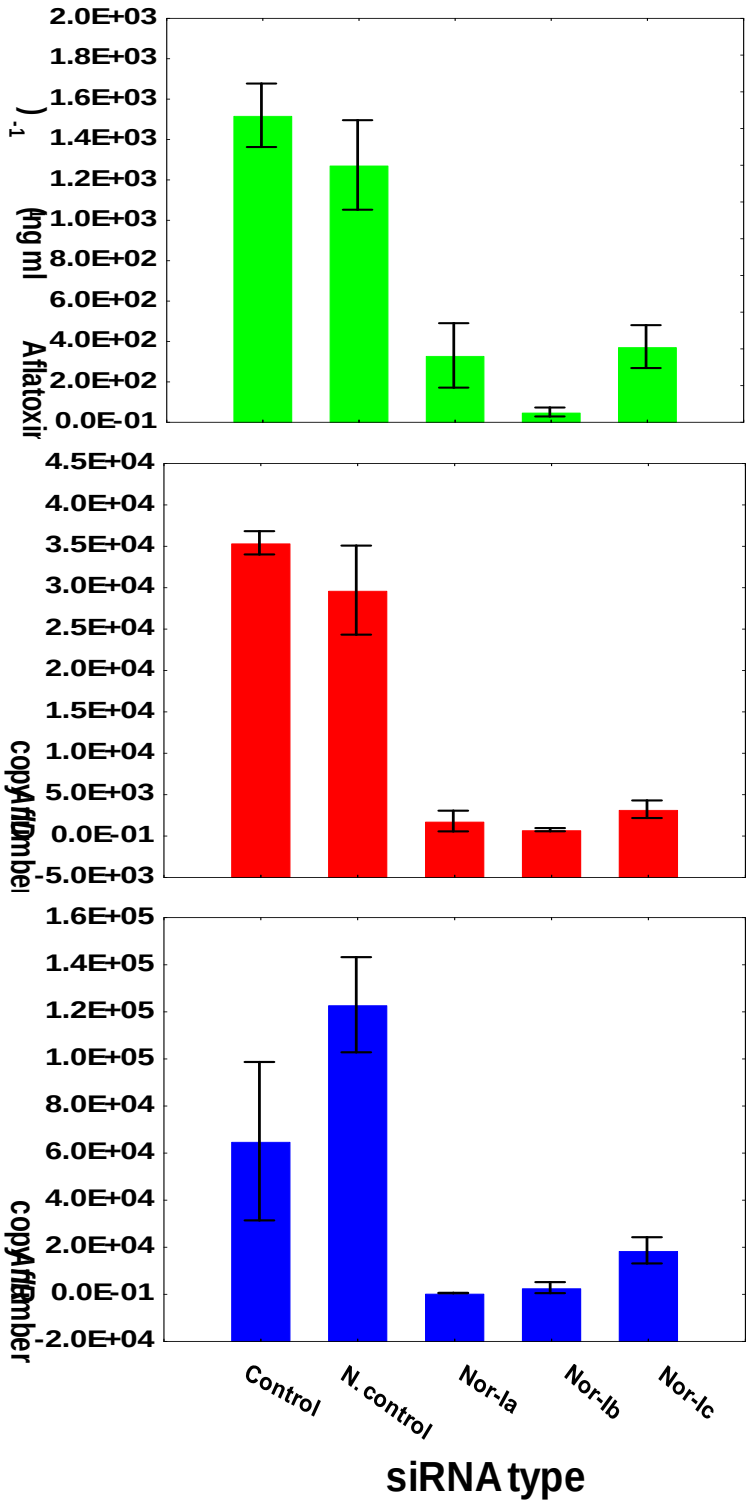

Figure 5.2 Effect of siRNA for silencing *aflD* target gene on Aflatoxin B_1 production, gene expression of *aflD* and *aflR* by using real-time PCR of *Aspergillus flavus* NRRL 3357. Vertical bar indicates standard error, control (untreated with siRNA), and N. Control (treated with unrealated siRNA as a negative control).

5.3.2 Effect of siRNA concentrations on *A. flavus* NRRL 3357

Figure 5.3 compares the effect of different concentrations of siRNA (Nor-Ib) on quantification of *aflD* and *aflR* genes, and AFB_1 production by *A. flavus* NRRL 3357. Overall, the best reduction in *aflD* and *aflR* mRNA abundance and AFB_1 production was at 25 nM siRNA. Statistical analysis of the effect of different concentrations of siRNA treatment on *aflD, aflR* gene expressions and AFB_1 production was statistically significant (Table 5.2b). There was a good correlation in reduction as a result of siRNA treatment between log *aflD* and siRNA concentration (R= 0.86: P= 0.000), log AFB_1 and siRNA concentration (R= 0.91: P= 0.000) and low correlation between log *aflR* and siRNA concentrations (R= 0.45; P= 0.06) (Table 5.3).

5.3.3. Treatment of *A. flavus* and *A. parasiticus* with siRNA

Tables 5.4 and 5.5 show the effect of treating three aflatoxigenic strains with the final chosen concentration of the siRNA (25nM, Nor-Ib). There was a significant effect on *aflD* (targeting gene), and a concomitant decrease in *aflR* mRNA abundance and AFB_1 production by *A. flavus* EGP9 treated with siRNA when compared to the control (99.7%, 83.4%, and 89 %, respectively). Treating *Aspergillus parasiticus* NRRL 13005 with siRNA revealed a reduction in *aflD* (targeting gene) mRNA abundance and AFG_1 production which was statistically significant (89.4 % and 77.2 %, respectively). The data obtained from *Aspergillus parasiticus* SSWT 2999 after treatment with siRNA showed that there was only a significant effect in *aflD* mRNA abundance (92.3%).

Table 5.2 (a) Analysis of Variance of the effect of siRNA for silencing of *aflD* target gene on AFB_1 production, expression of *aflD* gene and *aflR* gene, and (b) Effect of different concentrations of siRNA (Nor-Ib) on log AFB_1 production, log quantification of *aflD* gene and *aflR* gene.

	DF	**MS**	**F**	**P**
(a)				
Factor				
aflD copy numbers	4	8.58E+08	42.47	0.000003 *
AFB_1	4	1.24E+06	18.74	0.0001 *
aflR copy numbers	4	8.16E+09	8.63	0.0027 *
(b)				
Factor				
Log *aflD*	5	1.87	10.95	0.0003 *
Log AFB_1	5	0.41	199.13	0.00000 *
Log *aflR*	5	2.4659	6.05	0.005 *

DF: Degree of freedom, MS: mean square, P: Probability * Significant < 0.05 %

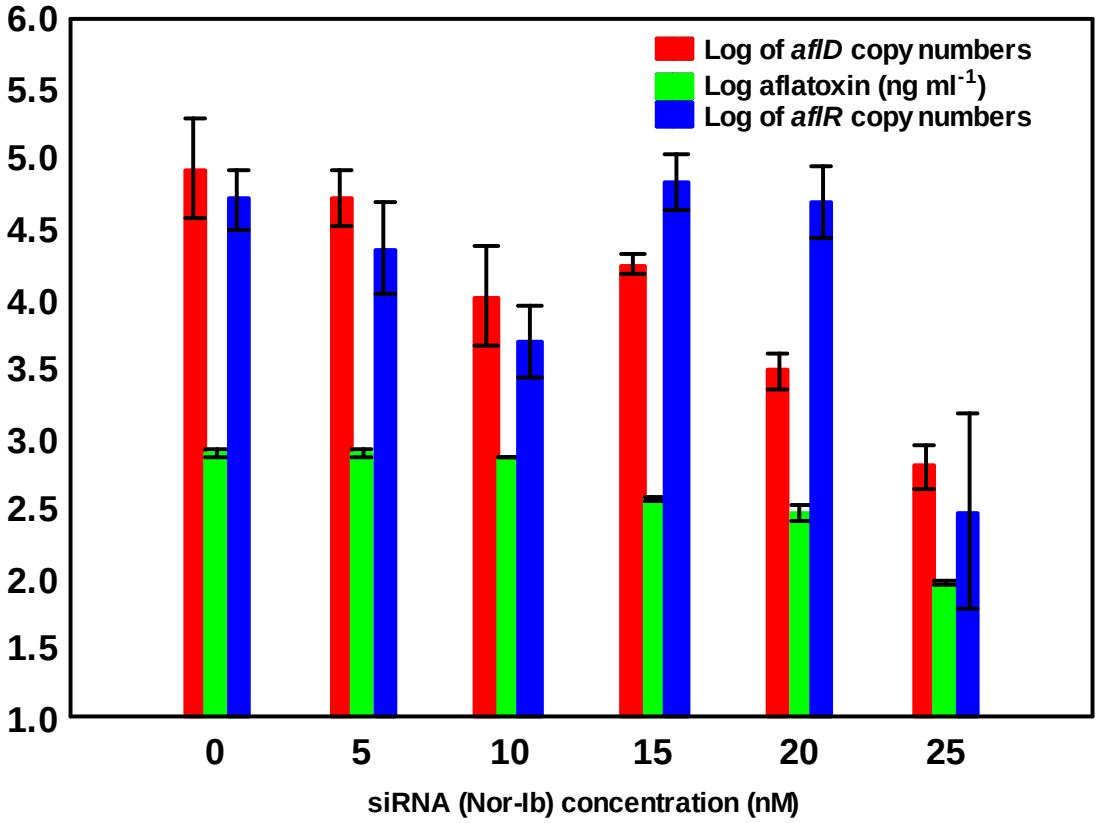

Figure 5.3 Effect of different concentrations of siRNA (Nor-Ib) on Aflatoxin B_1 production, gene expression of *aflD* and *aflR* by using real-time PCR of *Aspergillus flavus* NRRL 3357. Vertical bar indicates standard error.

Table 5.3 Statistical correlations between *aflD* gene, *aflR* gene and AFB_1 production of *A. flavus* NRRL3357 treated with siRNA (Nor-1b).

Correlations	R value	F	P
aflD& *aflR*	0.82	28.41	0.0001 *
aflD & AFB_1	0.88	47.26	0.00001 *
aflR & AFB_1	0.66	10.039	0.0074 *
log *aflD*& siRNA conc.	0.86	46.31	0.000 *
log AFB_1& siRNA conc.	0.91	77.75	0.000 *
log *aflR* & siRNA conc.	0.45	4.07	0.06

R: correlation coefficient. P: Probability * Significant < 0.05 %

Table 5.4 Aflatoxin B_1 (AFB_1), Aflatoxin G_1 (AFG_1), *aflD* and *aflR* expression assayed by control (untreated) and siRNA (Nor-1b) treated of three aflatoxigenic strains. Each value is mean± standard error based on three replicates.

Strains	AFB_1 (µg ml^{-1})		AFG_1(µg ml^{-1})		*AflD* copy numbers x10^3		*AflR* copy numbers x 10^3	
	Un-treated	**Treated**	**Un-treated**	**Treated**	**Un-treated**	**Treated**	**Un-treated**	**Treated**
***A. flavus* EPG9**	0.7 ± 0.05	0.088 ± 0.003	0	0	195.6± 56.9	0.69 ± 0.2	41 ± 10.7	6.8± 0.8
***A. parasiticus*-NRRL13005**	0.15±0.04	0.05 ± 0.01	2.7 ± 0.5	0.6 ± 0.2	22.9 ± 4.7	2.4 ± 1.4	0.6 ± 0.2	0.05 ± 0.007
***A. parasiticus*-SSWT2999**	1.48±0.37	1.1± 0.4	7.8 ± 1.9	5.5± 3.0	499.9 ± 85.	43.6 ± 5.5	0.4 ± 0.1	0.06 ± 0.02

Table 5.5 (a) Analysis of Variance of the effect of siRNA (Nor-Ib) for silencing *aflD* target gene on aflatoxin B_1, aflatoxin G_1 expression of *aflD* and *aflR* genes of three aflatoxigenic strains (a) *A. flavus* EPG9, (b) *A. parasiticus*NRRL13005, and (c) *A. parasiticus* SSWT2999.

	DF	**MS**	**F**	**P**
(a)				
A. flavus EPG9				
Factor				
aflD copy numbers	1	5.7 E+10	11.71	0.026*
AFB_1	1	7.4 E+05	163.06	0.0002*
(b)				
*A. parasiticus*NRRL13005				
Factor				
aflD copy numbers	1	6.3 E+08	17.07	0.01*
AFG1	1	6.9 E+06	12.34	0.02*
(c)				
A. parasiticus SSWT2999				
Factor				
aflD copy numbers	1	3.1 E+11	28.68	0.005*
AFG1	1	8.3 E+06	0.42	0.54

* Significant < 0.05 %

5. 4 Discussion

This is the first study to use RNA interference to silence one of the important structural genes in the aflatoxin biosynthesis pathway (*aflD* gene) in both *A. flavus* and *A. parasiticus* and to try and understand the function of this gene in aflatoxin production. In this study absolute quantification of the *aflD* and *aflR* genes expression have been used. This has been shown that, by using calibration curve provides more accurate, sensitive and highly reproducible data (Bustin, 2000). However, many studies showed that internal standards, mainly housekeeping genes used for the quantification of mRNA expression, could vary with the experimental conditions and could be regulated (Sturzenbaum and Kille, 2001; Radonic et al., 2004). At least two or three housekeeping genes should be used as internal standards because the use of a single gene for normalization could lead to relatively large errors (Thellin et al., 1999; Vandesompele et al., 2002).

The results showed that all three siRNAs designed to target *aflD* gene gave excellent levels of silencing. The transient gene silencing was observed at an early stage after 5 days of protoplast regeneration and hyphal growth; this suggests that protoplasts have the ability to uptake siRNAs from the growth medium during growth. Recently, Khatri and Rajam (2007) reported that germinated spores are capable of taking up siRNAs from the growth medium in the early stages germ of tube extension.

RNA interference was discovered after the injection of dsRNA into the nematode *Caenorhabditis elegans* lead to specific silencing of genes highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). Zamore et al. (2000) reported that using the *Drosophila in vitro* system, dsRNA triggers the specific degradation of homologous RNAs only within the region of identity with dsRNA. The application of direct delivery of synthetic siRNA, have been rarely attempted in fungi (Nakayashiki and Nguyen, 2008). Because of the effect of siRNA in the suppression of particular gene expression, synthetic siRNA duplexes were studied by adding to the culture medium for direct delivery.

The decrease in mRNA expression of *aflD* level caused subsequent decrease in *aflR* mRNA abundance *aflR* and AFB_1 production. Changes in AFB_1 production in relation to mRNA level of *aflD* showed a good correlation ($r=0.88$; $P=0.00001$), and changing in *aflR* mRNA

level in relation to mRNA level of *aflD* showed a good correlation (r=0.82; P =0.0001). This suggests the functional role of *aflD* gene in aflatoxin biosynthesis and may suggest that reduction in the expression of structural gene (*aflD*) can affect on the regulatory gene (*aflR*). In general, the aflatoxin gene cluster in *A. parasiticus* and *A. flavus* consists of 25 genes spanning approximately 70 kb (Skory et al., 1993; Yu et al., 2004). Aflatoxin production could be disrupted if any step in the aflatoxin biosynthetic pathway is completely blocked by a specific inhibitor. Unsing siRNA to traget *aflD* (nor-1) gene expression, an early enzymatic step in the aflatoxin biosynthetic pathway could be appropriate target for inhibiting aflatoxin biosynthesis. Recently, it was shown that there is a pattern of production in relation to changes in environmental factors *in vitro* and *in situ* for *aflD* using quantitive PCR (Abdel-hadi et al., 2010). Thus disruption or deletion of the *aflD* (nor-1) gene leads to the accumulation of a brick-red pigment (norsolorinic acid) in the hyphae and blocks the synthesis of all aflatoxins and their intermediates beyond norsolorinic acid (Bennett, 1981).

Accumulation of norsolorinic acid could be as a result of *aflD* knockdown by siRNA silencing. This may also have an indirect effect on the entire regulatory machinery of aflatoxin biosynthesis. Butchko et al. (1999) described a novel screen for detecting mutants defective in sterigmatocystin (ST) gene cluster activity of *A. nidulans* by use of a genetic block early in the ST biosynthetic pathway that results in the accumulation of the first stable intermediate, norsolorinic acid. They found that sixty of these 83 mutations are linked to the sterigmatocystin gene cluster and are likely defects in *aflR* or known sterigmatocystin gene cluster biosynthetic genes. The biosynthetic and regulatory genes required for ST production in *A. nidulans* are homologous to those required for aflatoxin production in *A. flavus* and *A. parasiticus* (Yabe et al., 1991; Trail et al., 1995).

The control siRNA did not lead to knockdown of *aflD* or *aflR*, suggesting that the results observed with *aflD*-specific siRNAs are not the result of a transfection artifact or an off-target effect. Previous studies by Khatri and Rajam (2007) suggested that siRNA can cause specific silencing effect, with the polyamine biosynthetic pathway without any off-target effects. However, Jackson et al., (2003) demonstrated that siRNAs may cross-react with targets of limited sequence similarity.

To confirm the effect of siRNA silencing, three aflatoxigenic strains were treated with siRNA. There was a significant decrease in *aflD* (targeting gene) of all three strains and an inhibition

of AFB_1 production by *A. flavus* EGP9 and AFG_1 production by *Aspergillus parasiticus* NRRL 13005. However, there was no significant decrease in AFG_1 by *Aspergillus parasiticus* SSWT 2999. This suggests that the biosynthesis of aflatoxins may be slightly different in *A. flavus* and *A. parasiticus*. Wilkinson et al., (2007) reported that the regulatory mechanism or mechanisms that control aflatoxin production in *A. flavus* and *A. parasiticus* are different in response to tryptophan (Trp), where, in the presence of Trp, three aflatoxin biosynthetic pathway genes [*aflE* (norA), *aflD* (nor-1) and *aflO* (omtB)] showed a decrease in expression and a decrease in AFB_1 and AFB_2 production for *A. flavus*. However, for *A. parasiticus*, an increase in expression profile and AFB_1 and AFG_1 production were observed.

The present study suggests that the *aflD* gene has a role in monitoring the biosynthetic direction of aflatoxin biosynthesis in *A. flavus* and *A. parasiticus.* This could thus be a good target gene for inactivation, to develop efficient means of aflatoxin control by using RNA silencing technology. This can be applied, for example, by using mycoviruses as a candidate to mediate and propagate inactivation of the *aflD* gene. It was reported that viruses play a role as vehicles for inter-specific gene transfer, such transfers ensure a continuous uptake of novel sequences through recombination and subsequent dissemination across the species borders, which accelerates species diversification and evolution, particularly among imperfect organisms (Schimdt, 2009).

Chapter 6

Conclusions and future work

6. Conclusions

6.1 Conclusion

Individual ecophysiological factors and their interactions had a significant effect on growth rate, sporulation, partitioning of aflatoxin, aflatoxin production, and aflatoxin genes expression. Ecological studies showed that optimum condition for *A. flavus* NRRL3357 growth was at 35°C and 0.99 a_w, while the highest amount of AFB_1 was between 25-30°C at the same wayer activity.

The present study showed that there was no direct relationship between sporulation and AFB_1 production in relation to a_w and temperature. The highest amount of AFB_1 was at 0.99 a_w at all temperatures and the lowest numbers of spores was produced at this a_w at all temperatures except 20°C. This may be due to the distribution of AFB_1 between spores, biomass and medium which plays a functional role at this a_w level, whereas with freely available water (0.99 a_w) about 50% of the AFB_1 was present in the biomass and the medium, with very little actually channelled into the spores. The expression of the regulatory gene *aflR* had a similar pattern to that of spore production, where at 0.99 a_w, there was low expression of *aflR* and low number of spores and at 0.90 a_w, there was high expression of *aflR* and high numbers of condia produced.

The microarray analysis showed that in conditions favourable for growth of *A. flavus*, the cluster genes are expressed at a basal level with the strain producing high amounts of AFB_1. Under mild stress conditions (20°C/0.99) several of the cluster genes, in particular *aflS* and *aflJ* were very highly induced concomitant with high levels of phenotypic AFB_1 production. Interestingly, at all these combinations, high amounts of AFB_1 were produced when the *aflS* expression was elevated. Examination of the ratio of the normalised expression of the *aflS*/*aflR* genes showed that high ratios occurred at 25°C and 30°C at 0.99 and 0.95 a_w and low ratios at 25°C and 30°C and 0.90 a_w. This is in agreement with the AFB_1 production profile. The data in this study suggests that stress applied by external abiotic factors has a complex role in the activation of the genes and that their activation may be some kind of

response to stress leading perhaps to an increased fitness of the producing fungus to withstand the imposed stress conditions.

PCR showed that all aflatoxin and non-aflatoxin producers harbour the four genes (*aflD*, *aflM*, *aflP* and *aflR*) examined. This discounts the possibility that a lack of aflatoxin production in certain strains is due to loss of the genes from the genome. RT-PCR results showed that *aflD* transcription can be used as a marker to discriminate between aflatoxin and non-aflatoxin producers, while *aflM*, *aflP* and *aflR* failed to differentiate between aflatoxigenic and non-aflatoxigenic strains. It is worthwhile to note that the conducive medium (YES medium) is not definitive assay enough to confirm whether strains are aflatoxigenic or non-aflatoxigenic per se. It is used for carrying out ecophysiological studies with known aflatoxin producers (Schmidt-Heydt et al., 2009). In the present study *A. flavus* EGP3 did not produce any aflatoxin on YES, however, it produced higher aflatoxin than *A. flavus* EPG14 (an aflatoxin producer on YES) in peanut. This suggests that this may be another factor that should be borne in mind as original nutritional substrate (in this case peanuts) may be important.

Temporal changes in populations (CFUs) in relation to storage a_w showed a good correlation ($r=0.75$; $P=0.00$). In the wettest condition tested more rapid colonization and sporulation occurred reflecting the high $\log_{10}$ CFUs found. No statistical correlation between *A. flavus* CFUs and the quantified *aflD* gene expression levels ($r=0.175$; $P=0.09$) was found. RQ-PCR results obtained by real time PCR showed that there were similar results at 0.90 a_w and 0.95 a_w, with high expression especially during the first 3 weeks, before expression slowed down. There was thus a poor correlation between RQ-PCR data and AFB_1 production ($r= 0.488$; $p=0.000$).

This poor correlation is probably due to the *aflD* expression being initiated very early, prior to phenotypic aflatoxin production being synthesised. Water activity had a significant effect on *aflR* transcription, especially at 0.90 a_w, where it was transcripted from the 4^{th} week, while during the initial 3 weeks there was high expression of *aflD* gene, transcription of the structural genes and high AFB_1 production. This suggests that *aflR* may not have a role in regulation of structural gene expression in food matrices such as peanuts.

All three siRNAs designed to target *aflD* gene gave excellent levels of silencing. The decrease in mRNA expression of *aflD* level caused subsequent decrease in mRNA abundance *AflR* level and AFB_1 production (98%, 97% and 97% of the control level) in *A. flavus* SRRC, respectively. Changes in AFB_1 production in relation to mRNA level of *AflD* showed a good correlation (r=0.88; P =0.00001), and changing in *AflR* mRNA level in relation to mRNA level of *aflD* showed a good correlation (r=0.82; P =0.0001). There was a significant decrease in *aflD* (targeting gene) of all three strains and an inhibition of AFB_1 production by *A. flavus* EGP9 and AFG_1 production by *Aspergillus parasiticus* NRRL 13005. This study suggests that the *aflD* gene has a role in monitoring the biosynthetic direction of aflatoxin biosynthesis in *A. flavus* and *A. parasiticus.* This could thus be a good a target gene for inactivation, to develop efficient means of aflatoxin control by using RNA silencing technology.

6.2 Suggestions for future work

Whilst extensive study of the importance of *aflD* gene in aflatoxin biosynthesis of *Aspergillus* was performed in this study, there are still many unanswered questions regarding the relationship of this gene to structural and regulatory genes. Below are some suggestions of possible future avenues of work.

- Molecular studies to check the effect of down regulation of *aflD* transcript on norsolorinic acid (first aflatoxin intermediate) accumulation, and could this in turn have a feed-back inhibition effect on the entire regulatory machinery of aflatoxin biosynthesis.

- The possibility to use inverted repeat transgenes (IRT) containing homologues sequences corresponding to *aflD* gene to regulate this gene by RNA silencing construct. Using mycovirus as a vehicle for interspecific gene transfer to develop efficient means for aflatoxin control on crops.

- Studying factors affecting the stability of non-aflatoxin producers of *A. flavus* such as nutritional, environmental and gene homology sequencing.

- Using a non-aflatoxigenic strain of *A. flavus* that has stability in non-aflatoxin production as a biocontrol agent to prevent aflatoxin contamination in peanuts.

Chapter 7

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Publications

I. PUBLICATIONS

1- Markus Schmidt-Heydt, **Ahmed Abdel-Hadi**, Naresh Magan, Rolf Geisen (2009). Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature. International Journal of Food Microbiology 135, 231–237

2- A. Abdel-Hadi, D. Carter and N. Magan (2010). Temporal monitoring of the nor-1 (*aflD*) gene of *Aspergillus flavus* in relation to aflatoxin B_1 production during storage of peanuts under different water activity levels. Journal of Applied Microbiology 09, 1914–1922

3- Markus Schmidt-Heydt, Corinna E. Rüfer, **Ahmed Abdel-Hadi**, Naresh Magan and Rolf Geisen (2010). The production of aflatoxin B_1 or G_1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *afl*R expression. Mycotoxin Research 4, 241-246.

4- Ahmed Abdel-Hadi, David Carter and Naresh Magan (2011). Discrimination between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *flavi* group contaminating Egyptian peanuts using molecular and analytical techniques.World Mycotoxin Journal 4, 69-77.

5- Ahmed M. Abdel-Hadi, Daniel P. Caley, David. R. F. Carter and Naresh Magan (2011). Control of aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* using RNA silencing technology by targeting *aflD* (nor-1) gene. Submitted to Toxins, Special issue on aflatoxin.

II. POSTER PRESENTATION

Ahmed Abdel-Hadi and Naresh Magan. Effect of environmental factors on sporulation and mycotoxin partitioning in mycotoxigenic fungi. British Mycological society, Annual British Mycological Society Meeting, 9-12 September 2007, Manchester UK.

Ahmed Abdel-Hadi, David Carter and Naresh Magan. Diversity and molecular discrimination between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavi* group

contaminating Egyptian peanuts.ISM, International Society for Mycotoxicology Conference, Tulln, Austria, 9-11 September 2009.

Appendix

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Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature

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ABSTRACT

A microarray analysis was performed to study the effect of varying combinations of water activity and temperature on the activation of aflatoxin biosynthesis genes in *Aspergillus flavus* grown on YES medium. Generally *A. flavus* showed expression of the aflatoxin biosynthetic genes at all parameter combinations tested. Certain combinations of a_w and temperature, especially combinations which imposed stress on the fungus resulted in a significant reduction of the growth rate. At these conditions induction of the whole aflatoxin biosynthesis gene cluster occurred, however the produced aflatoxin B_1 was low. At all other combinations (25 °C/0.95 and 0.99; 30 °C/0.95 and 0.99; 35 °C/0.95 and 0.99) a reduced basal level of cluster gene expression occurred. At these combinations a high growth rate was obtained as well as high aflatoxin production. When single genes were compared, two groups with different expression profiles in relation to water activity/temperature combinations occurred. These two groups were co-ordinately localized within the aflatoxin gene cluster. The ratio of *aflR*/*aflJ* expression was correlated with increased aflatoxin biosynthesis.

1. Introduction

Aflatoxins are produced by *Aspergillus flavi* group species and are thought to be one of the most cancerous natural substances known. Economically and biologically the most important fungal species able to produce the aflatoxins are *Aspergillus flavus* and *Aspergillus parasiticus* (Bhatnagar et al., 2002). The aflatoxin biosynthesis gene cluster of *A. parasiticus* has been completely elucidated (Yu et al., 2004). Moreover the genome of *A. flavus* is known and a whole genome microarray of *A. flavus* is available and has been used to study the regulation of aflatoxin biosynthesis genes (O'Brian et al., 2003). Generally, the aflatoxin biosynthesis genes of *A. flavus* and *A. parasiticus* are highly homologous and the order of the genes within the cluster being the same (Yu et al., 1995). *A. flavus* usually produces only aflatoxin B_1 and B_2. Not all of the strains of *A. flavus* isolated from the natural habitat are able to produce these metabolites at least under laboratory conditions. On the other hand, *A. parasiticus* is a strong and consistent aflatoxin producing species (Bennett and Christensen, 1983; Vaamonde et al., 2003). Most strains of this species are able to produce the aflatoxins B_1, B_2, G_1, and G_2.

The biosynthesis of aflatoxins, as all secondary metabolites, is strongly dependent on growth conditions such as substrate composition (Luchese and Harrigan, 1993) or physical factors like pH, water activity, temperature or modified atmospheres (Ellis et al., 1993; Molina and Giannuzzi, 2002; Ribeiro et al., 2006; Giorni et al., 2008). Depending on the particular combination of external growth parameters the biosynthesis of aflatoxin can either be completely inhibited, albeit normal growth is still possible or the biosynthesis pathway can be fully activated. Knowledge about these relationships enables an assessment of which parameter combinations can control aflatoxin biosynthesis or which are conducive to phenotypic aflatoxin production. A mathematical model which delineates the relationship between pH, propionic acid concentration and temperature on aflatoxin biosynthesis by *A. parasiticus* was described by Molina and Giannuzzi (2002). Sweeney et al. (2000) developed a reverse transcription polymerase chain reaction to analyse the expression of the *aflR* and *aflQ* gene of the aflatoxin pathway in relation to various nutritional media. Xu et al. (2000) monitored the expression of the *aflD* gene after growth of *A. parasiticus* on peanut pods by a reporter gene approach. Mayer et al. (2003) showed that expression of the *aflD* gene and the biosynthesis of aflatoxin in wheat are directly coupled. Recently Price et al. (2005) used a whole genome microarray approach to analyse the influence of substrate composition and pH on the activation of aflatoxin biosynthesis genes. With a pathway specific microarray and by Real Time PCR the influence of physical parameters like water activity, temperature and pH on the expression of ochratoxin A-, trichothecene- and aflatoxin biosynthesis genes have been analysed and a common expression profile was found (Schmidt-Heydt and Geisen, 2007). The published data show a correlation between external parameters and gene expression, however no comprehensive systematic analysis of this in relation to

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ORIGINAL ARTICLE

Temporal monitoring of the *nor-1* (*aflD*) gene of *Aspergillus flavus* in relation to aflatoxin B_1 production during storage of peanuts under different water activity levels

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Keywords
aflatoxin genes, aflatoxins, *Aspergillus flavus*, CFUs, peanuts, real-time PCR.

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Abstract

Aims: A relative quantification system (RQ-PCR) was used to monitor the correlations between the activity of the *nor-1* (=*aflD*) gene of *Aspergillus flavus* using real-time PCR in relation to phenotypic aflatoxin B_1 (AFB_1) production and populations of *A. flavus* in stored peanuts at three water activity levels (a_w, 0·95, 0·90 and 0·85) for 6 weeks.

Methods and Results: Real-time PCR was used to amplify the *nor-1* gene (target gene), and *benA56* (*β*-tubulin gene) used as a control gene. Expression of three structural genes, *nor-1* (=*aflD*), *ver-1* (=*aflM*), and *omtA* (=*aflP*), and the regulatory gene *aflR* of the aflatoxin biosynthetic pathway were also assayed. There were significant differences between *nor-1* gene expression at the three a_w levels; higher expression at 0·90 a_w in weeks 1–3, when compared to 0·95. In contrast, in the driest treatment (0·85 a_w) none or very low *nor-1* expression occurred. The populations of *A. flavus* colony-forming units ($CFUs\ g^{-1}$) increased over time with the highest at 0·95 a_w. Highest AFB_1 production was at 0·90 and 0·95 a_w from weeks 3–6. A_w had a significant effect on *aflR* transcription at 0·95 a_w over the 6-week period, while at 0·90 a_w, only in the last 2 weeks.

Conclusions: Correlations between different factors showed that log AFB_1 × log CFUs, log AFB_1 × a_w, and log CFUs × a_w were statistically significant, while log CFUs × RQ-PCR and RQ-PCR × a_w were not. The *AflR* gene may not have an important role in the regulation of *nor-1* expression in food matrices (e.g. peanuts).

Significance and Impact of the study: Determination of correlations between *nor-1* expression and aflatoxin production by *A. flavus* in raw peanuts under different a_w levels could be helpful to predict potential risk of aflatoxin production during storage of this hygroscopic food product and minimize contamination with the AFB_1.

Introduction

Aflatoxin (AFA) contamination continues to be a serious problem in many parts of the world. *Aspergillus flavus* and *Aspergillus parasiticus* are known as pathogens of cotton, corn, peanuts and other oilseed crops, producing toxins both in the field and during storage under various environmental conditions (Pittet 1988; Llewellyn *et al.* 1982; Cotty 1997; Payne and Brown 1998; Bhatnagar *et al.* 2000; Horn 2007). The key environmental determinants pre- and postharvest are water availability and temperature (Magan *et al.* 2003; Magan and Aldred 2007). The biosynthesis of secondary metabolites, including mycotoxins, is significantly influenced by environmental conditions such as pH, water activity (a_w) and temperature (Belli *et al.* 2004; Hope *et al.* 2005).

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ORIGINAL PAPER

The production of aflatoxin B_1 or G_1 by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflR* expression

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Abstract The influence of varying combinations of water activity (a_w) and temperature on growth, aflatoxin biosynthesis and *aflR*/*aflS* expression of *Aspergillus parasiticus* was analysed in the ranges 17–42°C and 0.90–0.99 a_w. Optimum growth was at 35°C. At each temperature studied, growth increased from 0.90 to 0.99 a_w. Temperatures of 17 and 42°C only supported marginal growth. The external conditions had a differential effect on aflatoxin B_1 or G_1 biosynthesis. The temperature optima of aflatoxin B_1 and G_1 were not at the temperature which supported optimal growth (35°C) but either below (aflatoxin G_1, 20–30°C) or above (aflatoxin B_1, 37°C). Interestingly, the expression of the two regulatory genes *aflR* and *aflS* showed an expression profile which corresponded to the biosynthesis profile of either B_1 (*aflR*) or G_1 (*aflS*). The ratios of the expression data between *aflS*:*aflR* were calculated. High ratios at a range between 17 and 30°C corresponded with the production profile of aflatoxin G_1 biosynthesis. A low ratio was observed at >30°C, which was related to aflatoxin B_1 biosynthesis. The results revealed that the temperature was the key parameter for aflatoxin B_1, whereas it was water activity for G_1 biosynthesis. These differences in regulation may be attributed to variable conditions of the ecological niche in which these species occur.

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Keywords *Aspergillus parasiticus* · Aflatoxin · Biosynthesis · Ecological niche

Introduction

Aflatoxins are toxic metabolites formed predominately by two agronomically important fungi *Aspergillus flavus* and *A. parasiticus*. *A. flavus* produces mainly aflatoxin B_1 and B_2 whereas *A. parasiticus* forms the 4 aflatoxins, B_1, B_2, G_1 and G_2. Generally, *A. parasiticus* produces high concentrations of aflatoxin, and most of the strains isolated from natural habitats (>90%) are able to synthesize aflatoxins. In contrast, only 40–50% of the *A. flavus* isolates are capable of forming the toxin (Bennett and Christensen 1983). The aflatoxin biosynthesis gene cluster has been described (Yu et al. 2004). The structural genes are regulated by the transcription factor *aflR* and the accessory regulatory gene *aflS* (Chang 2003).

The habitats of both species overlap, but distinct differences are obvious. The habitat of *A. flavus* is broader because of being found not only on cereals especially wheat but also on peanuts, tree nuts or high sugar-containing dry fruits. Both species can also be found in soils; however, this is the predominant ecological niche for *A. parasiticus*. Both species occur in subtropical and tropical regions. According to Pitt and Hocking (1999), *A. parasiticus* is endemic in soil-borne peanuts, and they found more *A. parasiticus* than *A. flavus* strains in Australian peanuts. This situation is the opposite in maize, which is more susceptible to *A. flavus* compared to *A. parasiticus*. Pitt and Hocking (1999) also described intriguing differences in the geographical occurrence of both species. According to their data, *A. parasiticus* can only occasionally

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Discrimination between aflatoxigenic and non-aflatoxigenic *Aspergillus* section *Flavi* strains from Egyptian peanuts using molecular and analytical techniques

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Abstract

A wide range of *Aspergillus* section *Flavi* strains were isolated from Egyptian peanut samples. Eighteen of these strains were compared with two type strains (*Aspergillus flavus* SRRC G1907 and *Aspergillus parasiticus* 2747) for aflatoxin production based on (a) qualitative fluorescence using a coconut cream agar medium (CAM), and (b) aflatoxin production on a conducive Yeast Extract-Sucrose (YES) medium using HPLC. These results were validated by using molecular approaches (the structural genes, *aflD* (nor-1), *aflM* (ver-1) and *aflP* (omt A) and the regulatory gene *aflR*) to discriminate between aflatoxigenic and non-aflatoxigenic strains of the *Aspergillus* section *Flavi* group *in vitro* and on peanut seeds. Overall, 13/18 strains producing aflatoxins B_1 and B_2 in the range 1.27-213.35 µg/g medium were identified. In addition, 5 non-aflatoxin producing strains were found. The expression of these four genes was assessed using PCR and RT-PCR. PCR showed that all strains contained the four aflatoxin genes examined, regardless of expression profiles. Our results also showed that *aflD* expression is a reliable marker to discriminate between aflatoxin and non-aflatoxin producers. Interestingly, when an aflatoxin producing strain and three non-aflatoxigenic strains were subsequently grown on peanuts at 0.95 water activity, two of the non-producers were able to initiate aflatoxin biosynthesis. This suggests that growth of strains on the natural food matrix is important for confirming aflatoxigenic production potential.

Keywords: Egyptian peanuts, *Aspergillus* section *Flavi*, aflatoxin, aflatoxin genes

1. Introduction

Peanuts are an important agricultural crop in Egypt. The country has a large number of peanut producers, with many farming small fields of 2.5 ha (WTO, 1998). Egypt is a major peanut exporting country and the European market accounts for 68% of its exports. The US is also a major consumer of Egyptian nuts, importing over $11 million worth of nuts annually (Youssef *et al*., 2008).

The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Maghraby and El-Maraghy, 1987). Contamination of peanuts with aflatoxins is one of the main factors that compromises their quality. Aflatoxins are carcinogenic fungal secondary metabolites produced by *Aspergillus flavus* and other closely related species. Aflatoxin B_1 (AFB_1) has been demonstrated in animal species to be the most potent liver carcinogen known. A high incidence of human liver cancer in central Africa and parts of Southeast Asia has been linked with aflatoxins (Groopman and Kensler, 1996; Montesano *et al*., 1997).

In the last few years, an increase in the number of human liver diseases in Egypt has been reported. This initiated a multidisciplinary study to investigate the role of aflatoxins in the high incidence of hepatotoxicity in Egypt (Badria, 1996). Recently, several multiplex polymerase chain reaction (PCR) systems for aflatoxin-producing fungi were developed, capable of detecting several genes coding for enzymes at different stages of the aflatoxin biosynthesis pathway (Chen *et al*., 2002; Criseo *et al*., 2001; Geisen, 1996; Shapira *et al*., 1996).

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Appendix No. I

Table 1. Effect of water activity x temperature on relative gene expression for all the aflatoxin biosynthesis genes relative to the tubulin gene

	20 °C 0.99	25 °C 0.90	25 °C 0.95	25 °C 0.99	30 °C 0.90	30 °C 0.95	30 °C 0.99	35 °C 0.90	35 °C 0.95	35 °C 0.99
aflF	1106	1301	354	589	1160	303	232	1255	216	233
aflU	1396	7168	223	663	2980	281	305	1481	205	282
aflT	374	4143	273	300	3781	416	274	1930	221	378
aflC	0	1715	371	326	1708	533	296	1287	177	260
aflD	947	1472	217	505	1362	315	266	1344	203	400
aflB	387	1482	235	305	1679	322	238	1296	169	237
alfA	1560	1951	261	693	1681	384	216	1219	345	314
aflR	2036	4063	241	890	1187	186	286	3389	259	313
aflJ	1428	2207	1785	1,335	1751	1,323	793	1134	141	312
aflH	771	5439	315	440	3894	362	294	2035	259	252
aflJ	349	2109	537	835	2138	325	317	1578	202	179
aflE	1243	3654	278	628	1986	363	369	1741	212	295
aflM	1003	1810	288	553	1569	516	429	1418	337	238
aflN	2333	2624	318	896	2806	314	278	1754	265	316
aflG	927	1550	302	520	1673	325	270	1219	213	188
aflL	2250	1794	247	990	1757	312	328	1364	316	213
aflI	1356	4576	302	684	2239	347	594	1579	316	257
aflO	2685	1696	237	1,155	1554	390	1	1436	248	282
aflP	853	1399	269	548	1656	391	509	1241	253	269
aflQ	1211	1705	325	566	1757	340	193	1070	159	230
aflK	17	1707	294	207	1694	323	325	1198	156	260
aflV	574	1128	308	460	1586	295	262	1266	294	213
aflW	2525	1805	261	976	1654	374	245	1139	203	340
aflX	1409	2903	243	659	3895	346	354	1505	180	326
aflY	2925	1139	247	1,199	1486	468	370	1143	436	301
nadA	1603	1410	264	698	1199	330	188	1431	192	309
hxtA	2245	1308	219	906	1197	428	252	1351	271	187
glcA	1391	4135	593	686	1506	325	390	3501	189	291
sugR	492	1193	368	492	1240	423	298	1240	428	328