CRANFIELD UNIVERSITY

AHMED ABDEL-HADI

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

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Supervisors: Prof. Naresh Magan
Dr. David Carter
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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<sub>1</sub> (AFB<sub>1</sub>) production with optimum ranges of water activity ($a_w$) and temperature for AFB<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> (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 $a_w \times$ 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<sub>1</sub> production. Highest amounts of AFB<sub>1</sub> 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<sub>1</sub> 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 AFB₁ on YES, however, it produced higher AFB₁ 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 AFB₁ contamination in stored peanuts over periods of 6 weeks under different a_w treatments at 25°C. 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 AFB₁ 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 AFB₁ x log CFUs, log AFB₁ x 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 AFB₁ 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 AFB₁ production in protoplasts of A. flavus EGP9 and AFG₁ production by A. parasiticus NRRL 13005. Changes in AFB₁ 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

ABSTRACT .................................................................................................................. I
ACKNOWLEDGEMENTS ............................................................................................. III
TABLE OF CONTENTS ................................................................................................. IV
LIST OF FIGURES ........................................................................................................ VII
LIST OF TABLES ........................................................................................................... XI
LIST OF PLATES .......................................................................................................... XIII
ABBREVIATIONS .......................................................................................................... XIV

CHAPTER 1 .................................................................................................................... 1
  1 LITERATURE REVIEW .............................................................................................. 1
    1.1 General introduction ............................................................................................ 1
    1.2 Mycotoxins ........................................................................................................ 3
    1.3 Aflatoxins ........................................................................................................... 5
      1.3.1 Worldwide limits for aflatoxins in food .......................................................... 6
      1.3.2 Aflatoxins and Peanuts ............................................................................... 8
      1.3.3 Aflatoxin biosynthesis .............................................................................. 9
    1.4 Environmental factors, fungal growth and mycotoxin production ................. 12
      1.4.1 Water relations of fungi .............................................................................. 12
      1.4.2 Temperature ................................................................................................ 13
    1.5 Analytical methods for the detection of mycotoxins ......................................... 17
    1.6 Molecular methods ............................................................................................. 19
      1.6.1 Polymerase Chain Reaction (PCR) ............................................................... 19
      1.6.2 Reverse transcription-Polymerase chain reaction (RT–PCR) ....................... 20
      1.6.3 PCR in the detection of fungi ...................................................................... 23
    1.7 Real time PCR ..................................................................................................... 25
    1.8 Microarrays .......................................................................................................... 31
    1.9 RNA interference (RNAi) .................................................................................. 32
      1.9.1 RNAi mechanism ...................................................................................... 34
      1.9.2 siRNA delivery .......................................................................................... 34
    1.10 OBJECTIVES ..................................................................................................... 37

CHAPTER 2 .................................................................................................................... 40
  2 ECOLOGY OF ASPERGILLUS FLAVUS IN RELATION TO AFLATOXIN
    GENE CLUSTER EXPRESSION ............................................................................... 40
  2.1 INTRODUCTION ................................................................................................... 40
  2.2 MATERIALS AND METHODS ............................................................................. 42
    2.2.1 Fungal strain .................................................................................................. 42
    2.2.2 Media ............................................................................................................. 42
    2.2.3 Adjustment water activity of media for ecophysiological studies ................. 43
    2.2.4 Effect of water activity x temperature interactions on growth, sporulation,
      aflatoxin B₁ production and expression of aflatoxin cluster gene expression ....... 43
    2.2.4 (a) Measurement of growth ............................................................................ 43
    2.2.4 (b) Determination of aflatoxin B₁ .................................................................... 43
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.4 (c) Spore production in relation to environmental factors</td>
<td>44</td>
</tr>
<tr>
<td>2.2.5 Partitioning and distribution of Aflatoxin B1 in spores, biomass and medium</td>
<td>46</td>
</tr>
<tr>
<td>2.2.6 Extraction of RNA and Molecular analyses</td>
<td>46</td>
</tr>
<tr>
<td>2.2.6 (a) Isolation of RNA from samples</td>
<td>46</td>
</tr>
<tr>
<td>2.2.6 (b) Microarray experiments</td>
<td>48</td>
</tr>
<tr>
<td>2.2.7 Statistical analysis</td>
<td>48</td>
</tr>
<tr>
<td>2.3 RESULTS</td>
<td>50</td>
</tr>
<tr>
<td>2.3.1 Effects of water activity and temperature on growth</td>
<td>50</td>
</tr>
<tr>
<td>2.3.2 Effect of time, temperature, and water activity on aflatoxin B1 production by A. flavus</td>
<td>50</td>
</tr>
<tr>
<td>2.3.3 Effects of water activity, and temperature on asexual sporulation</td>
<td>50</td>
</tr>
<tr>
<td>2.3.4 Partitioning of aflatoxin B1 in spores, biomass and medium</td>
<td>56</td>
</tr>
<tr>
<td>2.3.5 Microarray analysis of aflatoxingene cluster expression in relation to environmental factors</td>
<td>56</td>
</tr>
<tr>
<td>2.3.6 Expression of specific genes</td>
<td>59</td>
</tr>
<tr>
<td>2.4 DISCUSSION</td>
<td>59</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>67</td>
</tr>
<tr>
<td>3 DETECTION OF AFLATOXIGENIC STRAINS BY PCR</td>
<td>67</td>
</tr>
<tr>
<td>3.1 INTRODUCTION</td>
<td>67</td>
</tr>
<tr>
<td>3.2 MATERIALS AND METHODS</td>
<td>68</td>
</tr>
<tr>
<td>3.2.1. Strains, Media and growth conditions</td>
<td>68</td>
</tr>
<tr>
<td>3.2.2. Screening for aflatoxin production using coconut cream agar medium</td>
<td>68</td>
</tr>
<tr>
<td>3.2.3. Aflatoxin B1 extraction and HPLC analysis</td>
<td>68</td>
</tr>
<tr>
<td>3.2.4 Molecular methods</td>
<td>68</td>
</tr>
<tr>
<td>3.2.4 (a) Isolation of RNA from the samples</td>
<td>68</td>
</tr>
<tr>
<td>3.2.4 (b) Aflatoxin genes expression</td>
<td>69</td>
</tr>
<tr>
<td>3.2.4 (c) DNA isolation and amplification</td>
<td>69</td>
</tr>
<tr>
<td>3.2.5 Studies on peanuts</td>
<td>73</td>
</tr>
<tr>
<td>3.2.5 (a) Aflatoxin B1 extraction analysis</td>
<td>73</td>
</tr>
<tr>
<td>3.2.5 (b) RNA extraction and RT-PCR for peanut samples</td>
<td>75</td>
</tr>
<tr>
<td>3.3 RESULTS</td>
<td>75</td>
</tr>
<tr>
<td>3.3.1 Screening the ability of the strains for aflatoxin production</td>
<td>75</td>
</tr>
<tr>
<td>3.3.2 Analysis of aflatoxin gene transcription</td>
<td>75</td>
</tr>
<tr>
<td>3.3.3 Studies on peanuts</td>
<td>77</td>
</tr>
<tr>
<td>3.4 DISCUSSION</td>
<td>83</td>
</tr>
<tr>
<td>CHAPTER 4</td>
<td>87</td>
</tr>
<tr>
<td>4 MONITORING OF NOR-1 (AFLD) GENE OF ASPERGILLUS FLAVUS IN PEANUTS</td>
<td>87</td>
</tr>
<tr>
<td>4.1 INTRODUCTION</td>
<td>87</td>
</tr>
<tr>
<td>4.2 MATERIALS AND METHODS</td>
<td>89</td>
</tr>
<tr>
<td>4.2.1 Fungal strain and growth conditions</td>
<td>89</td>
</tr>
<tr>
<td>4.2.2 Inoculation of peanut samples</td>
<td>89</td>
</tr>
<tr>
<td>4.2.3 Determination of colony forming units (CFUs)</td>
<td>90</td>
</tr>
</tbody>
</table>
4.2.4 Aflatoxin extraction and HPLC analysis ................................................................. 90
4.2.6 Isolation of RNA from the samples and RT-PCR ...................................................... 90
4.2.7 TaqMan probes and primer design ........................................................................... 91
4.2.8 Real-time PCR conditions ....................................................................................... 91
4.3 RESULTS .................................................................................................................... 94
4.3.1 Effect of water activity on populations of A. flavus on stored peanuts ...................... 94
4.3.2 Effect of water activity on aflatoxin production ......................................................... 94
4.3.3 RQ-PCR of nor-1 in relation to water activity ............................................................ 94
4.3.4 Analysis of aflatoxin genes transcription in relation to water activity ....................... 97
4.3.5 Correlation co-efficients for comparing different factors .......................................... 97
4.4 DISCUSSION ............................................................................................................. 102

CHAPTER 5 ................................................................................................................... 105
5 CONTROL OF AFLATOXIN PRODUCTION USING RNA

INTEREFERENCE ........................................................................................................... 105
5.1 INTRODUCTION ....................................................................................................... 105
5.2 MATERIALS AND METHODS .................................................................................. 106
5.2.1. Fungal strain and growth conditions .................................................................... 106
5.2.2. Preparation of protoplast ...................................................................................... 106
5.2.3. siRNA design ...................................................................................................... 107
5.2.4. Delivery of siRNA to protoplast .......................................................................... 107
5.2.5 Aflatoxin extraction and HPLC analysis ................................................................. 109
5.2.6 Isolation of RNA from the samples and RT-PCR .................................................... 109
5.2.7 TaqMan probes and primer design ......................................................................... 109
5.2.8 Real-time PCR conditions ..................................................................................... 111
5.2.9 Statistical analysis ................................................................................................. 111
5.3 RESULTS .................................................................................................................. 111
5.3.1. Treatment of Aspergillus flavus NRRL3357 with siRNA ........................................ 111
5.3.2. Effect of siRNA concentrations on A. flavus NRRL3357 ...................................... 113
5.3.3. Treatment of A. flavus and A. parasiticus with siRNA ........................................... 113
5.4 DISCUSSION ............................................................................................................ 118

CHAPTER 6 .................................................................................................................... 121
6 CONCLUSIONS AND FUTURE WORK ....................................................................... 121
6.1 Conclusion ............................................................................................................... 121
6.2 Suggestions for future work ..................................................................................... 123

CHAPTER 7 .................................................................................................................... 125
7 REFERENCES .............................................................................................................. 125

APPENDIX .................................................................................................................... 151
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Frequency distribution of specific regulatory limits for AFB&lt;sub&gt;1&lt;/sub&gt; in food in 56 countries, in 2002.</td>
<td>7</td>
</tr>
<tr>
<td>1.2</td>
<td>Frequency distribution of specific regulatory limits for the sum of AFB&lt;sub&gt;1&lt;/sub&gt;, AFB&lt;sub&gt;2&lt;/sub&gt;, AFG&lt;sub&gt;1&lt;/sub&gt;, and AFG&lt;sub&gt;2&lt;/sub&gt; in food in 75 countries, in 2002.</td>
<td>7</td>
</tr>
<tr>
<td>1.3</td>
<td>Clustered genes (A) and the aflatoxin biosynthetic pathway (B).</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>The corresponding genes and their enzymes involved in each bioconversion step are shown in panel A.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The vertical line represents the 82-kb aflatoxin biosynthetic pathway gene cluster and sugar utilization gene cluster in <em>A. parasiticus</em> and <em>A. flavus</em>.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>The new gene names are given on the left of the vertical line and the old gene names are given on the right.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NOR, norsolorinic acid; AVN, averantin; HAVN, 5-hydroxyaverantin; OAVN, oxoaverantin; AVNN, averufanin; AVF, averufin; VHA, versical hemiacetal acetate; VAL, versical; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHDMST, dihydrodemethylsterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMAST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB&lt;sub&gt;1&lt;/sub&gt;, aflatoxin B&lt;sub&gt;1&lt;/sub&gt;; AFB&lt;sub&gt;2&lt;/sub&gt;, aflatoxin B&lt;sub&gt;2&lt;/sub&gt;; AFG&lt;sub&gt;1&lt;/sub&gt;, aflatoxin G&lt;sub&gt;1&lt;/sub&gt;; AFG&lt;sub&gt;2&lt;/sub&gt;, aflatoxin G&lt;sub&gt;2&lt;/sub&gt;.</td>
<td>11</td>
</tr>
<tr>
<td>1.4</td>
<td>Minimum water activity (a&lt;sub&gt;ω&lt;/sub&gt;), ranges for the growth of microorganisms in foods and examples of various food materials having a&lt;sub&gt;ω&lt;/sub&gt; within the minimum range.</td>
<td>14</td>
</tr>
<tr>
<td>1.5</td>
<td>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.</td>
<td>21</td>
</tr>
<tr>
<td>1.6</td>
<td>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.</td>
<td>22</td>
</tr>
<tr>
<td>1.7</td>
<td>Reverse transcription polymerase chain reaction (RT-PCR) to obtain a cDNA copy of mRNA. The single strand of DNA produced is then used as a template for the synthesis of a second DNA strand, and then for amplification by PCR.</td>
<td>24</td>
</tr>
<tr>
<td>1.8</td>
<td>Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR.</td>
<td>28</td>
</tr>
</tbody>
</table>
1.9 TaqMan real-time PCR quantitation. 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.

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) labeled 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.

1.11 Mechanism of RNA interference (RNAi). Where (ds) is double stranded, (RISC) is RNA-induced silencing complex.

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

2.1 Calibration curve obtained of aflatoxin B₁ (ng ml⁻¹) versus peak area using HPLC.

2.2 Flowchart of RNA Extraction using RNeasy Kit provided by the manufacturer.

2.3 Mycotoxin gene cluster microarray analyses. Different 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.

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

2.5 Effect of incubation time on aflatoxins B₁ produced by A. flavus at 25 °C and 0.99 water activity using YES medium. Bars indicate standard error of the mean.

2.6 Effect of water activity (a_w) x temperature on aflatoxin B₁ production by A. flavus on a YES medium. Vertical bars denote standard errors.

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 mean.

2.8 Effect of water activity on partitioning of aflatoxin B₁ into medium biomass and conidia at 25°C using A. flavus strain NRRL3357. Bars indicate standard error of the mean.

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 (+). ............................................................... 58

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................................. 60

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................. 60

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................................. 61

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................................. 61

3.1 Thermal cycler PTC-200 used for amplification of genes.......................... 70

3.2 UV transilluminator used for visualization of PCR products separated on a 2% agarose gel, stained with ethidium bromide................................. 71

3.3 Moisture adsorption curve for the relationship between amounts of added water and water activity at 25°C................................................................. 74

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................................................................. 78

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........ 79

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........... 80

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................................. 82

4.1 Bio Rad real-time PCR used for quantification of the expression of required genes........................................................................................................ 92
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, R² value: correlation coefficient ......................................................................................................................... 93

4.3 CFU values, Aflatoxin B₁ production and RQ-PCR of nor-1 gene of A. flavus in peanut at different a_w times and different incubation intervals at 25 °C. Vertical bar indicates 95% confidence limits .......................................................................................................................... 95

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 ................................................................................................................. 99

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 ......................................................................................................................... 100

5.1 Standard curves from real-time PCR by plotting the threshold cycle (Ct) vs log₁₀ 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, R² value: correlation coefficient ......................................................................................................................... 110

5.2 Effect of siRNA for silencing aflD target gene on Aflatoxin B₁ 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 unrelated siRNA as a negative control) ......................................................................................................................... 112

5.3 Effect of different concentrations of siRNA (Nor-Ib) on Aflatoxin B₁ production, gene expression of aflD and aflR by using real-time PCR of Aspergillus flavus NRRL3357. Vertical bar indicates standard error ......................................................................................................................... 115
LIST OF TABLES

Table No. | Page
---|---
1.1 Fungal genera and species of major significance and their associated mycotoxins | 4
1.2 Examples of aflatoxin legal limits found in groundnut from various countries | 10
1.3 Water activity, equilibrium relative humidity and water potentials at 25°C | 15
1.4 Relationship between moisture content (wet weight basis, %) and water activity (a_w) for some key cereals and nuts at 25 °C | 15
1.5 Advantages and disadvantages of classical methods for mycotoxin | 18
1.6 Genes used for the identification of aflatoxigenic fungi by PCR and RT-PCR | 26
2.1 Analysis of variance of the effect of water activity (a_w), temperature (°C) and their interactions on growth rate of A. flavus | 51
2.2 Analysis of variance of the effect of water activity (a_w), temperature (°C) and their interactions on aflatoxin B_1 production by A. flavus | 53
2.3 Analysis of variance of the effect of water activity (a_w), temperature (°C) and their interactions on conidial production by A. flavus | 54
2.4 Analysis of variance of the effect of water activity (a_w) on aflatoxin B_1 partitioning into biomass, medium and spores of cultures of A. flavus on a conducive YES medium | 57
2.5 Ratio of aflS/aflR in relation to aflatoxin B_1 biosynthesis and different combinations of temperature and water activity (a_w) | 62
3.1 Details of primer sequences, target gene, annealing temperature and expected PCR/RT-PCR product length in base pairs (bp) | 72
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. Where each strain was assigned a name in the format EGPN, where EG means Egypt, P represents peanuts, and n is the isolate number | 76
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 | 81
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 | 98
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 |
different a\textsubscript{w} levels for up to 6 weeks storage........................................ 101

5.1 Details of siRNA sequences used in this study.......................................................... 108

5.2 (a) Analysis of Variance of the effect of siRNA for silencing of aflD target gene on AFB\textsubscript{1} production, expression of aflD gene and aflR gene, and (b) Effect of different concentrations of siRNA (Nor-Ib) on log AFB\textsubscript{1} production, log quantification of aflD gene and aflR gene........................................ 114

5.3 Statistical correlations between aflD gene, aflR gene and AFB\textsubscript{1} production of A. flavus NRRL3357 treated with siRNA (Nor-1b)......................................................... 115

5.4 Aflatoxin B\textsubscript{1} (AFB\textsubscript{1}), Aflatoxin G\textsubscript{1} (AFG\textsubscript{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.................. 116

5.5 (a) Analysis of Variance of the effect of siRNA (Nor-Ib) for silencing aflD target gene on aflatoxin B\textsubscript{1}, aflatoxin G\textsubscript{1} expression of aflD and aflR genes of three aflatoxigenic strains (a) A. flavus EPG9, (b) A. parasiticusNRRL13005, and (c) A. parasiticus SSWT2999................................................................. 117
# LIST OF PLATES

<table>
<thead>
<tr>
<th>Plate No.</th>
<th>Plate Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Culture plates of <em>A. flavus</em> on YES medium overlaid with cellophane after 7 days of incubation at different temperatures and water activity (a&lt;sub&gt;w&lt;/sub&gt;) treatments. Single colonies were used at higher a&lt;sub&gt;w&lt;/sub&gt; levels and three colonies were used under marginal conditions for growth to have more biomass for extraction purposes.</td>
<td>55</td>
</tr>
<tr>
<td>4.1</td>
<td><em>A. flavus</em> EGP9 growing on peanuts over a period of 6 weeks at three water activity (a&lt;sub&gt;w&lt;/sub&gt;) levels (0.95, 0.90, and 0.85) and at 25°C.</td>
<td>96</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td><em>Aspergillus</em></td>
</tr>
<tr>
<td>AFAs</td>
<td>Aflatoxins</td>
</tr>
<tr>
<td>AFB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Aflatoxin B&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Aflatoxin B&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>AFG&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Aflatoxin G&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>AFG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Aflatoxin G&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>a&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Water activity</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CAM</td>
<td>Coconut cream agar medium</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxy ribonucleic acid</td>
</tr>
<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2'-Deoxynucleoside-5'-triphosphate</td>
</tr>
<tr>
<td>ERH</td>
<td>Equilibrium relative humidity</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HACCP</td>
<td>Hazard Analysis Critical Control Point</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>µ</td>
<td>Micro (10&lt;sup&gt;-6&lt;/sup&gt;)</td>
</tr>
<tr>
<td>m</td>
<td>Milli (10&lt;sup&gt;-3&lt;/sup&gt;)</td>
</tr>
<tr>
<td>M</td>
<td>Molar (mol/L)</td>
</tr>
<tr>
<td>MEA</td>
<td>Malt Extract Agar</td>
</tr>
<tr>
<td>Min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>n</td>
<td>Nano (10&lt;sup&gt;-9&lt;/sup&gt;)</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>Rnase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase - Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Relative Quanification - Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short-interfering double-stranded RNA</td>
</tr>
<tr>
<td>TFA</td>
<td>Triflouroacetic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>V</td>
<td>Volt</td>
</tr>
<tr>
<td>YES</td>
<td>Yeast Extract Sucrose</td>
</tr>
</tbody>
</table>
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, diacetoxyisocirpenol, 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).

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Mycotoxins</th>
</tr>
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<tbody>
<tr>
<td><em>Aspergillus</em> species (e.g. <em>A. flavus</em>, <em>A. parasiticus</em> and <em>A. nomius</em>)</td>
<td>Aflatoxins, predominantly <em>B</em>&lt;sub&gt;1&lt;/sub&gt; and <em>B</em>&lt;sub&gt;2&lt;/sub&gt; (A. <em>flavus</em>) and, in addition, <em>G</em>&lt;sub&gt;1&lt;/sub&gt; and <em>G</em>&lt;sub&gt;2&lt;/sub&gt; (<em>A. parasiticus</em>). <em>B</em>&lt;sub&gt;1&lt;/sub&gt; is metabolized to <em>M</em>&lt;sub&gt;1&lt;/sub&gt; which is excreted in milk of humans and animals</td>
</tr>
<tr>
<td><em>Aspergillus</em> species (e.g. <em>A. flavus</em>, <em>A. parasiticus</em>, <em>A. nidulans</em>)</td>
<td>Sterigmatocystin</td>
</tr>
<tr>
<td><em>Fusarium</em> species (e.g. <em>F. graminearum</em> (Gibberella <em>zeae</em>), <em>F. culmorum</em>, <em>F. sporotrichioides</em>, <em>F. langsethiae</em>, <em>F. Poae</em> and <em>F. cerealis</em>)</td>
<td>Trichothecenes: Type A (e.g. T-2, HT-2, diacetoxyarsenol (DAS), neosolaniol) produced by <em>F. sporotrichioides</em>, <em>F. poae</em> and/or <em>F. equiseti</em> Type B (e.g. nivalenol (NIV), deoxynivalenol (DON) and acetylated derivatives) produced by <em>F. graminearum</em>, <em>F. culmorum</em> and/or <em>F. cerealis</em></td>
</tr>
<tr>
<td><em>Fusarium proliferatum</em> and <em>F. verticillioides</em></td>
<td>Fumonisins</td>
</tr>
<tr>
<td><em>Fusarium</em> species (e.g. <em>F. proliferatum</em> and <em>F. avenaceum</em>)</td>
<td>Enniatins and beauvericin</td>
</tr>
<tr>
<td><em>Penicillium</em> and <em>Aspergillus</em> species (e.g. <em>P. verrucosum</em>, <em>A. ochraceus</em> and <em>A. carbonarius</em>)</td>
<td>Ochratoxin A</td>
</tr>
<tr>
<td><em>Penicillium</em> species (e.g. <em>P. expansum</em>)</td>
<td>Patulin</td>
</tr>
<tr>
<td><em>Penicillium</em> species</td>
<td>Citrinin and roquefortine</td>
</tr>
<tr>
<td><em>Claviceps purpurea</em>, <em>Aspergillus</em>, <em>Penicillium</em> and <em>Acremonium</em> species</td>
<td>Ergot alkaloids</td>
</tr>
<tr>
<td><em>Alternaria</em> species</td>
<td>Alternariol, altenuene, altertoxin, tenuazonic acid</td>
</tr>
</tbody>
</table>
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 B₁ (AFB₁) and B₂ (AFB₂) whereas *A. parasiticus* produces AFB₁, AFB₂, aflatoxin G₁ (AFG₁) and G₂ (AFG₂). 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). AFB₁ 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 AFB₁ 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 AFB₁ 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 AFB₁ and the sum of the AFB₁, B₂, G₁ and G₂ 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 AFB₁. Many countries regulated AFAs in food with limits for the sum of the AFB₁, AFB₂, AFG₁, and AFG₂, occasionally in combination with a specific limit for AFB₁. 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 AFB₁ 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 \( \text{AFB}_1 \) 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 \( \text{AFB}_1, \text{AFB}_2, \text{AFG}_1 \) and \( \text{AFG}_2 \) 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 AFB1 (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 (Figure 1.3). 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 AFB1, B2, G1 and G2, (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 from various countries (from Freeman et al., 1999)

<table>
<thead>
<tr>
<th>Countries</th>
<th>Aflatoxin Legal Limit (a) (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AFB(_1)</td>
</tr>
<tr>
<td>Egypt</td>
<td>20</td>
</tr>
<tr>
<td>UK</td>
<td>4</td>
</tr>
<tr>
<td>USA</td>
<td>20</td>
</tr>
<tr>
<td>Sweden</td>
<td>5</td>
</tr>
<tr>
<td>Germany</td>
<td>2</td>
</tr>
<tr>
<td>Belgium</td>
<td>5</td>
</tr>
<tr>
<td>The Netherlands</td>
<td>0</td>
</tr>
<tr>
<td>Ireland</td>
<td>5</td>
</tr>
<tr>
<td>Italy</td>
<td>5</td>
</tr>
</tbody>
</table>

\(a\) 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, o xoaverantin; AVNN, averufanin; AVF, averufin; VHA, versiconal hemiacetal acetate; VAL, versiconal; VERB, versicolorin B; VERA, versicolorin A; DMST, demethylsterigmatocystin; DHD MST, dihydrodemethyl sterigmatocystin; ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, O-methylsterigmatocystin; DHOMST, dihydro-O-methylsterigmatocystin; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; AFB<sub>2</sub>, aflatoxin B<sub>2</sub>; AFG<sub>1</sub>, aflatoxin G<sub>1</sub>; AFG<sub>2</sub>, aflatoxin G<sub>2</sub> (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 ($\Psi$), 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 \( \Psi \) 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 \( \Psi \), 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 \textit{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).

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

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

<table>
<thead>
<tr>
<th>$a_w$</th>
<th>Groundnuts</th>
<th>Wheat</th>
<th>Sorghum</th>
<th>Rice</th>
<th>Maize</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.98</td>
<td>16–17</td>
<td>30–34</td>
<td>31–32</td>
<td>26–28</td>
<td>30–32</td>
</tr>
<tr>
<td>0.95</td>
<td>14.5–15</td>
<td>26–28</td>
<td>26–27</td>
<td>23–24</td>
<td>26–27</td>
</tr>
<tr>
<td>0.90</td>
<td>12.5–13.5</td>
<td>21–22</td>
<td>22.5–23</td>
<td>20–21</td>
<td>23–24</td>
</tr>
<tr>
<td>0.80</td>
<td>9–10</td>
<td>16–17</td>
<td>18–19</td>
<td>17–18</td>
<td>16–17</td>
</tr>
<tr>
<td>0.70</td>
<td>7–8</td>
<td>14–14.5</td>
<td>16–17</td>
<td>14–14.5</td>
<td>15–16</td>
</tr>
</tbody>
</table>
Previously, the effect of a wide range of temperatures (2-52°C) on two aflatoxin-producing strains of *A. flavus* was studied by Schindler et al. (1967). They found that the highest amount of AFB₁ was produced at 24°C and AFA production was not related to growth rate of *A. flavus*. No aflatoxins were produced at temperatures <18°C or >35°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 et 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 aₜ, when water stress was imposed at 0.94 aₜ, 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 AFB₁ production of *A. parasiticus* NRRL 2999 grown on maize grain (whole and crushed). They found that, the highest amount of AFB₁ 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, aₜ, 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 aₜ 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.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>Simple, inexpensive and rapid Can be used for screening simultaneous analysis of multiple mycotoxins sensitive for aflatoxins &amp; ochratoxin A</td>
<td>Poor sensitivity (for some mycotoxins) Poor precision Adequate separation may require two dimensional analysis Quantitative only when used with densitometer</td>
</tr>
<tr>
<td>GC</td>
<td>Simultaneous analysis of multiple mycotoxins Good sensitivity May be automated (autosampler) provides confirmation (MS detector)</td>
<td>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 &amp; repeatability</td>
</tr>
<tr>
<td>HPLC</td>
<td>Good sensitivity Good selectivity Good repeatability May be automated (autosampler) Short analysis times Official methods available</td>
<td>Expensive equipment Specialist expertise required May require derivatization</td>
</tr>
<tr>
<td>LC/MS</td>
<td>Simultaneous analysis of multiple mycotoxins Good sensitivity (LC/MS/MS) Provides confirmation No derivatization required</td>
<td>Very expensive Specialist expertise requested Sensitivity relies on ionization technique Matrix assisted calibration curve (for quantitative analysis)</td>
</tr>
<tr>
<td>ELISA</td>
<td>Simple sample preparation Inexpensive equipment High sensitivity Simultaneous analysis of multiple samples Suitable for screening Limited use of organic solvents Visual assessment</td>
<td>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)</td>
</tr>
<tr>
<td>Rapid Tests</td>
<td>Simple and fast (5-10 min) No expensive equipment required Limited use of organic solvents suitable for screening purposes can be used in situ</td>
<td>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</td>
</tr>
</tbody>
</table>
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°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 2ⁿ (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 aflatoxinogenic genes (aflR, aflD, aflM and aflP) to discriminate between aflatoxinogenic strains and non-aflatoxinogenic 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 Tri5 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Step in AFAs biosynthesis pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>aflD</em></td>
<td>Reductase</td>
<td>Norsolorinic acid to averantin</td>
</tr>
<tr>
<td><em>aflI</em></td>
<td>Oxidase</td>
<td>Averufin to versiconal hemiacetal acetate</td>
</tr>
<tr>
<td><em>aflM</em></td>
<td>Dehydrogenase</td>
<td>Versicolorin A to demethylstrigmatocystin</td>
</tr>
<tr>
<td><em>aflO</em></td>
<td><em>O</em>-methyltransferase B</td>
<td>Demethylstrigmatocystin to strigmatocystin</td>
</tr>
<tr>
<td><em>aflP</em></td>
<td><em>O</em>-methyltransferase A</td>
<td>Strigmatocystin to <em>O</em>-methylstrigmatocystin</td>
</tr>
<tr>
<td><em>AflQ</em></td>
<td>Oxidoreductase</td>
<td><em>O</em>-methylstrigmatocystin to aflatoxin B₂</td>
</tr>
<tr>
<td><em>aflR</em></td>
<td>Transcription factor containing a zinc cluster DNA binding motif</td>
<td>Positive regulator of AFB₂ biosynthesis</td>
</tr>
<tr>
<td><em>aflS</em></td>
<td>Transcription factor</td>
<td>Positive regulator of AFB₂ biosynthesis</td>
</tr>
</tbody>
</table>
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 use 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 \( R_n \) : relative fluorescence 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 mycotoxicogenic 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, trichothecenes 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 AFB1 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 degraded 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.
Phase I

Ecophysiological studies

Ecology of *Aspergillus flavus*
Study the effect of combinations of water activity and temperature on
- Growth rate and sporulation.
- Partitioning and distribution of aflatoxins in spores, biomass and medium
- Aflatoxin production.

Phase II

Molecular studies

- PCR and RT-PCR analysis to discriminate between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section *Flavi* contaminating Egyptian peanuts.
- 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*.
- Kinetics of Real Time PCR for monitoring the *aflD* (nor-1) gene of *A. flavus* in peanuts under different water activities.

Phase III

Control of aflatoxin production by knockdown *aflD* gene using RNA interference (RNAi)

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, aw, 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 aflR and ord1 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 aw, 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)**
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

*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°C). 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 (Filt enborg 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 (Trifluoroacetic 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) ($\lambda_{exc}$ 333 nm, $\lambda_{em}$ 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.
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

\[
y = 414.04x + 41.864 \\
R^2 = 0.9992
\]

**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 cm² 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 B₁ in spores, biomass and medium

Other preliminary experiments were made to study the effect of various a_w levels on partitioning of AFB₁ 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, AFB₁ 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).
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

**Figure 2.3** Mycotoxin gene cluster microarray analyses. Different 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 B₁ production by A. flavus

Initially the effect of time of incubation on AFB₁ production was examined. Figure 2.5 shows that the maximum AFB₁ 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 AFB₁ production.

AFB₁ 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 AFB₁ 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 AFB₁ 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.
Effect of water activity ($a_w$) and temperature on growth rate of *A. flavus* on YES medium. Vertical bars denote standard errors.

**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*.

<table>
<thead>
<tr>
<th>Effect</th>
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<th>MS</th>
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<td>18250.4</td>
<td>0.00 *</td>
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<tr>
<td>$a_w$</td>
<td>2</td>
<td>0.648756</td>
<td>46431.9</td>
<td>0.00 *</td>
</tr>
<tr>
<td>Temperature x $a_w$</td>
<td>6</td>
<td>0.038084</td>
<td>2725.7</td>
<td>0.00 *</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>0.000014</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %
Figure 2.5 Effect of incubation time on aflatoxins B₁ produced by *A. flavus* at 25 °C and 0.99 water activity using YES medium. Bars indicate standard error of the mean.
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

Table 2.2 Analysis of variance of the effect of water activity ($a_w$), temperature ($^\circ$C) and their interactions on aflatoxin B$_1$ production by *A. flavus*.

<table>
<thead>
<tr>
<th>Effect</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>3</td>
<td>1.66534</td>
<td>10.6863</td>
<td>0.000119 *</td>
</tr>
<tr>
<td>$a_w$</td>
<td>2</td>
<td>14.15582</td>
<td>90.8368</td>
<td>0.000000 *</td>
</tr>
<tr>
<td>Temperature x $a_w$</td>
<td>6</td>
<td>0.66497</td>
<td>4.2671</td>
<td>0.004605 *</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>0.15584</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %
Effects of Aspergillus flavus in relation to aflatoxin gene cluster expression

Figure 2.7 Effect of 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 mean.

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

<table>
<thead>
<tr>
<th>Effect</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>3</td>
<td>4.718994E+13</td>
<td>6.7619</td>
<td>0.001827 *</td>
</tr>
<tr>
<td>$a_w$</td>
<td>2</td>
<td>1.359068E+14</td>
<td>19.4744</td>
<td>0.000009 *</td>
</tr>
<tr>
<td>Temperature x $a_w$</td>
<td>6</td>
<td>3.863007E+13</td>
<td>5.5354</td>
<td>0.001017 *</td>
</tr>
<tr>
<td>Error</td>
<td>24</td>
<td>6.978756E+12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 B₁ in spores, biomass and medium

The partitioning of AFB₁ produced by A. flavus colonies when grown on a conducive YES medium at four aₜ levels showed that with freely available water (0.99 aₜ) about 50% of the AFB₁ 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 AFB₁ into the spores, especially at 0.95 and 0.93 aₜ levels. Statistical analysis of data sets showed that effect of aₜ on AFB₁ production into biomass, medium and spores was highly significant (Table 2.4).

2.3.5 Microarray analysis of aflatoxin gene cluster expression in relation 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 AFB₁ 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 aₜ to 25°C/0.90 aₜ. In the moderate zone where usually expression is lower (temperatures of 25-35°C), there were two additional peaks (30°C/0.90 aₜ and 35°C/0.90 aₜ). These could be due to the low aₜ value of 0.90 aₜ.

It should be noted that at 0.90 aₜ, 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 AFB₁ phenotypic production. One of the data sets fitted very well, e.g., at 20 ºC/0.99 aₜ, but at intermediate combinations of temperature × aₜ (25°C/0.95 and 0.99; 30°C/0.95 and 0.99; 35°C/0.95 and 0.99 aₜ) a high production of AFB₁ 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 B₁ 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 B₁ partitioning into biomass, medium and spores of cultures of A. flavus on a conducive YES medium.

<table>
<thead>
<tr>
<th>Effect</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>spores</td>
<td>3</td>
<td>8149142</td>
<td>68.8709</td>
<td>0.000672 *</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>118325</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomass</td>
<td>3</td>
<td>7884539</td>
<td>304.478</td>
<td>0.000036 *</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>25895</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>3</td>
<td>9333468</td>
<td>267.626</td>
<td>0.000046 *</td>
</tr>
<tr>
<td>Error</td>
<td>4</td>
<td>34875</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MS: mean square, P: Probability, DF: Degree of freedom, * Significant < 0.05 %
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

**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 \(\text{aflD and aflF}\) and two regulatory genes \(\text{aflR and aflS}\) 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 \(20^\circ\text{C}\), 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 \(\text{aflS}\) gene. The \(\text{aflS}\) (formerly \(\text{aflJ}\)) gene is a regulatory gene which interacts and activates \(\text{aflR}\), the major regulatory gene, which then activates the aflatoxin structural genes (Chang, 2003). At certain parameter combinations at \(20, 25\) and \(30\ ^\circ\text{C}\) the expression of the \(\text{aflS}\) gene was increased when compared to the other genes of the cluster. This was often accompanied by an increased biosynthesis of \(\text{AFB}_1\) compared to the neighbouring conditions.

At \(20^\circ\text{C}/0.99 \ a_w\) the \(\text{aflS}\) gene was very highly expressed relative to the other genes and accompanied by a similar activation of the \(\text{aflJ}\) (formerly \(\text{estA}\)) gene. Under these conditions growth was moderate and \(\text{AFB}_1\) biosynthesis high. At all analysed conditions the \(\text{aflR}\) 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 \(\text{aflS}\) and \(\text{aflR}\) 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 \(\text{AFB}_1\) was produced. At moderate conditions the ratio is >1. At these conditions high amounts of \(\text{AFB}_1\) 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;
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

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

**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 B₁ biosynthesis and different combinations of temperature and water activity (a$_w$).

<table>
<thead>
<tr>
<th>Environmental factors</th>
<th>Ratio <em>aflS/aflR</em></th>
<th>AFB₁ ng g$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C/0.90 a$_w$</td>
<td>0.5</td>
<td>3.66</td>
</tr>
<tr>
<td>35°C/0.90</td>
<td>0.3</td>
<td>4.67</td>
</tr>
<tr>
<td>25°C/0.95</td>
<td>7.4</td>
<td>830.24</td>
</tr>
<tr>
<td>30°C/0.95</td>
<td>7.1</td>
<td>3016.94</td>
</tr>
<tr>
<td>25°C/0.99</td>
<td>1.5</td>
<td>1957.25</td>
</tr>
<tr>
<td>30°C/0.99</td>
<td>2.7</td>
<td>2758.74</td>
</tr>
</tbody>
</table>
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. parasititious* 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\textsubscript{w} level, whereas with freely available water (0.99 a\textsubscript{w}) about 50% of the AFB\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{1} or related compounds (Kale et al. 1994).

The relative expression of the regulatory gene afl\textit{R} had a similar pattern as spore production, where at 0.99 a\textsubscript{w}, there was low expression of afl\textit{R} and low number of spores and at 0.90 a\textsubscript{w}, there was very high expression of afl\textit{R} 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 afl\textit{R} 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\textsubscript{w}) on the activation of the aflatoxin biosynthesis pathway genes showed that temperature and a\textsubscript{w} have a strong influence on the expression patterns of these genes. Only a few studies have systematically investigated the influence of a\textsubscript{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\textsubscript{1}. Under mild stress conditions...
Ecology of *Aspergillus flavus* in relation to aflatoxin gene cluster expression

(20°C/0.99 aw) several of the cluster genes, in particular *aflS* and *aflJ*, were very highly induced concomitant with high levels of phenotypic AFB₁ 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-Heydt et al., 2008). A similar basal level of expression was found at between 25 and 35°C for *A. flavus*, especially when aw 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 AFB₁ were produced when the *aflS* expression was elevated. These data indicate that the failure of *A. flavus* to produce AFB₁ at 0.90 aw 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 aw and low ratios at 25°C and 30°C at 0.90 aw. This is in agreement with the AFB₁ 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 AFB₁ 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 B₁ 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 AFB₁ 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 AFB₁ or AFG₁ production by *A. parasiticus* at various combinations of aw and temperature, where high *aflS/aflR* ratios at
17-30°C related to AFG\textsubscript{1} production and low ratio above 30°C corresponded with AFB\textsubscript{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 AFB1 based on (a) a qualitative medium-based assay, (b) HPLC, and (c) use of different biosynthetic and regulatory genes as indicators of AFB1 production, and (d) to confirm the
ability of AFB₁ producing and non-toxin producing strains after colonisation of Egyptian peanuts under specific temperature/aw 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 AFB₁ 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 B₁ extraction and HPLC analysis

AFB₁ 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 H₂O (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 H₂O (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).

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Gene</th>
<th>Primer sequence (5′- 3′)</th>
<th>Optimal Annealing Temp. (°C)</th>
<th>PCR product size (bp)</th>
<th>RT-PCR product size(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tub1-F</td>
<td>Tub 1</td>
<td>GTCCGGTGCTGGTAACACAAGCT</td>
<td>65</td>
<td>902</td>
<td>837</td>
</tr>
<tr>
<td>Tub1-R</td>
<td></td>
<td>GGAGGTGGAGTTTCCAATGAAAA</td>
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<tr>
<td>NOR1-F</td>
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<td>400</td>
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<tr>
<td>NOR1-R</td>
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<tr>
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<tr>
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<tr>
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<td>AATCCTCGCCACCATACTA</td>
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</tbody>
</table>
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 AFB1 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 AFB1 and AFB2, while A. parasiticus 2747 also produced AFG1 and AFG2. The AFB1 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 AFB1; 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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>PCR</th>
<th></th>
<th></th>
<th></th>
<th>RT-PCR</th>
<th></th>
<th></th>
<th>CAM fluorescence</th>
<th>HPLC Aflatoxin B$_1$ µg$^{-1}$</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>β-tub</td>
<td>aflR aflD aflP aflM</td>
<td>β-tub aflR aflD aflP aflM</td>
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<td></td>
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</tr>
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<td></td>
<td></td>
<td>+ + +</td>
<td></td>
<td></td>
<td>+</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
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</tr>
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<td>+ - +</td>
<td></td>
<td></td>
<td>+</td>
<td>50.63</td>
</tr>
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<td>+ + +</td>
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<td></td>
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<td></td>
<td>+ + +</td>
<td></td>
<td></td>
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<td>+ - +</td>
<td></td>
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<td></td>
<td></td>
<td>-</td>
<td>0</td>
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</table>
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 AFB1 when grown on peanuts. Interestingly, the non-producing strains EGP14 and EGP15 synthesised AFB1; 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.
Detection of aflatoxigenic strains by PCR

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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Aflatoxin B₁ in YES µg/g</th>
<th>Aflatoxin B₁ in Peanuts µg/g</th>
<th>RT-PCR in peanuts</th>
<th>RT-PCR on YES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>β-tub  aflR aflD aflP aflM</td>
<td>β-tub aflR aflD aflP aflM</td>
</tr>
<tr>
<td><em>A. flavus</em> EGP3</td>
<td>21.33</td>
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</tr>
<tr>
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<td>0.94</td>
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<td>+ - + - + - +</td>
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<tr>
<td><em>A. flavus</em> EGP16</td>
<td>0</td>
<td>0</td>
<td>+ - - - - -</td>
<td>+ + + - + - +</td>
</tr>
</tbody>
</table>
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 fluorescence 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
Detection of aflatoxigenic strains by PCR

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 AFB1 and AFB2 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-variants 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 \textit{aflR} and \textit{aflJ}.

The fact that \textit{aflD} transcription became detectable when a non-producing strain (on YES media) was grown on peanuts and produced aflatoxins suggests that \textit{aflD} transcription is a good marker for aflatoxin production, and suggests that \textit{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 \textit{A. flavus} EGP3 did not produce any aflatoxin on YES. However, it produced higher AFB$_1$ than \textit{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 \textit{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 \textit{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 \textit{Aspergillus flavus} in peanuts

4.1 Introduction

The study in Chapter 3 showed the potential use of \textit{aflD} transcription as a good marker to discriminate between aflatoxigenic and non-aflatoxigenic strains contaminating peanuts. Further ecophysiological studies on \textit{aflD} expression in stored peanuts was thus carried out to determine the correlations between \textit{aflD} expression and phenotypic AFB\textsubscript{1} production in relation to changes in \textit{a}_w and storage time for controlling \textit{A. flavus} in stored peanut 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 (\textit{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 \textit{A. flavus} infection of peanuts stored for up to 6 months. Highest population counts of \textit{A. flavus} were found in peanuts stored at 13.5 \% moisture content (approx. 0.90 \textit{a}_w) at 15°C for 1 month. Recently, a survey of Egyptian peanuts by Sultan and Magan (2010) showed that \textit{Aspergillus} section \textit{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 biosynthesis 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 aw, 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 aw 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 AFB1 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 AFB₁ 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) AFB₁ 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 B₁ and B₂ 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 °C for 20 min. After cooling, the water was added and after equilibration the peanut samples were inoculated with 1 ml of 10⁶ 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 AFB₁ 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\(^{\circ}\)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 millilitres 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 (Trifluoroacetic 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) (\(\lambda_{\text{exc}}\) 360 nm; \(\lambda_{\text{em}}\) 440 nm) and a C\(_{18}\) column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 \(\mu\)m). 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 (\(\beta\)-tubulin) was used as a control (see Section 3.2.4b).
Monitoring of nor-1 (aflD) gene of Aspergillus flavus in peanuts

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'-GTCCAAGCAACAGGCAAGT-3'; nortaq-2 5'-TCGTGCATGTTGGTGATGGT-3'; norprobe 6FAM TGTCTTGATCGG GCCCG-BHQ2; bentaq-1 5'-CTTGTGACCAGGGTGTGGAT-3'; bentaq-2 5'-GTCGCAGCCCTAGCGCT-3', benprobe CY5-CGATGTGGTCCGAGGTGCT-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 95°C for 10 min, and all 40 cycles at 95°C for 15 s, 55°C for 20 s and 72°C 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-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.
Monitoring of nor-1 (aflD) gene of *Aspergillus flavus* in peanuts

**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, Tulas, OK) software for interaction between factors \( (a_w \times \text{time}) \).

### 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 \( (a_w, \text{time}) \) 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 \( a_w \times \text{time} \) 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 \( \beta \)-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 B₁ production and RQ-PCR of nor-1 gene of *A. flavus* in peanut at different *a*<sub>w</sub> 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\textsubscript{w} and the highest expression was after 3 weeks and then decreased further. At 0.85 a\textsubscript{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\textsubscript{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\textsubscript{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, \textit{aflD} (nor-1), \textit{aflM} (ver-1), \textit{aflP} (omt A) and \textit{aflR} in the biosynthetic pathway for AFA production were assessed (Figure 4.5). The expression of the housekeeping gene (\(\beta\)-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\textsubscript{w}, all four genes were transcripted from the beginning to the end of the storage period. At 0.90 a\textsubscript{w}, \textit{aflD} was expressed from the start of the experiments while \textit{aflR} only after 4 weeks onwards, the genes \textit{aflP} and \textit{aflM} were expressed from the 2\textsuperscript{nd} week onwards. In the driest conditions tested (0.85 a\textsubscript{w}) only two genes were transcribed (\textit{aflD} and \textit{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 \textit{A. flavus} CFUs and a\textsubscript{w} (R= 0.75; P= 0.00), AFB\textsubscript{1} correlated significantly with a\textsubscript{w} (R= 0.68; P= 0.00) and AFB\textsubscript{1} x log CFUs (r=0.85). However, for other factors there were no significant correlations.
Monitoring of nor-1 \textit{(aflD)} gene of \textit{Aspergillus flavus} in peanuts

Table 4.1 (a) Analysis of Variance of the effect of \( a_w \), time and their interactions on CFUs of \textit{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 \textit{A. flavus} in peanuts.

(a)

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(b)

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(c)

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</tbody>
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DF: Degree of freedom, MS: mean square, P: Probability * Significant < 0.05 %
Monitoring of nor-1 (aflD) gene of *Aspergillus flavus* in peanuts

**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 (aw) 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 B₁ 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.

<table>
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<td>log aflatoxin &amp; RQ-PCR</td>
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<td>0.000 *</td>
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<td>log CFUs &amp; water activity</td>
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<td>78.22</td>
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</table>

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 (*otapksPv*) in wheat stored at three water contents (14, 19 and 24%) for up to three months. They showed good correlations between the *otapksPv* expression, phenotypic OTA production and in some cases that this was paralleled by CFUs of *P. verrucosum*. 
Monitoring of nor-1 (aflD) gene of *Aspergillus flavus* in peanuts

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 transcribed 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 \textit{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, \textit{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 \textit{aflD} expression which may occur in relation to changes in water activity levels in stored peanuts. Often a high expression of \textit{aflD} resulted in high AFB$_1$ production. Further silencing studies on \textit{aflD} expression were carried out to confirm the role of \textit{aflD} expression in AFB$_1$ 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 \textit{CAP59} and \textit{ADE2} genes by double-stranded RNA homologous to these genes in the basidiomycetous yeast \textit{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 \textit{Aspergillus}
Control of aflatoxin production using RNA interference

นิคูลันส์. ดูแลมันที่มีการผลิต RNA (dsRNA) ถูกส่งเสริมโดยตรงไปยังโปรทีสแห้งที่ 
Phytophthora infestans, ซึ่งอยู่ในสกุลสัณฐาน Omymes (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 H2O, 2 ml of 0.2 M NaPO4 [pH 5.8], 0.4 ml of 1.0 M CaCl2, 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
Control of aflatoxin production using RNA interference

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 CaCl₂, 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 x 10⁵ 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 x 10⁵) 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

<table>
<thead>
<tr>
<th>siRNA name</th>
<th>siRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nor-Ia</td>
<td>Sense strand: CAUGUAUGCUCCCCGUCCUAUU</td>
</tr>
<tr>
<td></td>
<td>Antisense strand : UAGGACGGGAGCAUACAUUU</td>
</tr>
<tr>
<td>Nor-Ib</td>
<td>Sense strand: GCAACAGGCCAAGUUGUUGCUU</td>
</tr>
<tr>
<td></td>
<td>Antisense strand : GACAAACUUUGCGCGUGUGCUU</td>
</tr>
<tr>
<td>Nor-Ic</td>
<td>Sense strand: CAGGCAAGUUGUCUUGAUU</td>
</tr>
<tr>
<td></td>
<td>Antisense strand : UCAAGAACACUUGGCCUGUU</td>
</tr>
</tbody>
</table>
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 (Trifluoroacetic 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'-GTCCAAGCAACAGGCAAGT-3' ; nortaq-2 5'-TCGTCATGTGCTGATGTG-3; norprobe 6FAM TGTCTTGATCGCGCCCG- BHQ2 (Mayer et al., 2003); AflRtaq-1 5'-TCGTCCTTATCGTTCTCAAGG-3; AflRtaq-2 5'-ACTGTTGCTACAGCTGCCACT-3, AflRprobe 6FAM AGCAGGCACCACCGTACCTCAAC-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'-CGAGTTGTGCCAGTCAAAAA-3; AflR2, 5'-AATCCTCGGCCACCATACTA-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-Ia 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).
Control of aflatoxin production using RNA interference

**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 unrelated 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 AFB₁ production, expression of aflD gene and aflR gene, and (b) Effect of different concentrations of siRNA (Nor-Ib) on log AFB₁ production, log quantification of aflD gene and aflR gene.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aflD copy numbers</td>
<td>4</td>
<td>8.58E+08</td>
<td>42.47</td>
<td>0.000003 *</td>
</tr>
<tr>
<td>AFB₁</td>
<td>4</td>
<td>1.24E+06</td>
<td>18.74</td>
<td>0.0001 *</td>
</tr>
<tr>
<td>aflR copy numbers</td>
<td>4</td>
<td>8.16E+09</td>
<td>8.63</td>
<td>0.0027 *</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log aflD</td>
<td>5</td>
<td>1.87</td>
<td>10.95</td>
<td>0.0003 *</td>
</tr>
<tr>
<td>Log AFB₁</td>
<td>5</td>
<td>0.41</td>
<td>199.13</td>
<td>0.00000 *</td>
</tr>
<tr>
<td>Log aflR</td>
<td>5</td>
<td>2.4659</td>
<td>6.05</td>
<td>0.005 *</td>
</tr>
</tbody>
</table>

DF: Degree of freedom, MS: mean square, P: Probability * Significant < 0.05 %
Table 5.3 Statistical correlations between aflD gene, aflR gene and AFB1 production of A. flavus NRRL3357 treated with siRNA (Nor-1b).

<table>
<thead>
<tr>
<th>Correlations</th>
<th>R value</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>aflD &amp; aflR</td>
<td>0.82</td>
<td>28.41</td>
<td>0.0001 *</td>
</tr>
<tr>
<td>aflD &amp; AFB1</td>
<td>0.88</td>
<td>47.26</td>
<td>0.00001 *</td>
</tr>
<tr>
<td>aflR &amp; AFB1</td>
<td>0.66</td>
<td>10.039</td>
<td>0.0074 *</td>
</tr>
<tr>
<td>log aflD &amp; siRNA conc.</td>
<td>0.86</td>
<td>46.31</td>
<td>0.000 *</td>
</tr>
<tr>
<td>log AFB1 &amp; siRNA conc.</td>
<td>0.91</td>
<td>77.75</td>
<td>0.000 *</td>
</tr>
<tr>
<td>log aflR &amp; siRNA conc.</td>
<td>0.45</td>
<td>4.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Strains</th>
<th>AFB(_1) (µg ml(^{-1}))</th>
<th>AFG(_1) (µg ml(^{-1}))</th>
<th>AflD copy numbers x10(^3)</th>
<th>AflR copy numbers x 10(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>Treated</td>
<td>Untreated</td>
<td>Treated</td>
</tr>
<tr>
<td>A. flavus EPG9</td>
<td>0.7 ± 0.05</td>
<td>0.088 ± 0.003</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. parasiticus- NRRL13005</td>
<td>0.15±0.04</td>
<td>0.05 ± 0.01</td>
<td>2.7 ± 0.5</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>A. parasiticus- SSWT2999</td>
<td>1.48±0.37</td>
<td>1.1±0.4</td>
<td>7.8 ± 1.9</td>
<td>5.5± 3.0</td>
</tr>
</tbody>
</table>
Table 5.5 (a) Analysis of Variance of the effect of siRNA (Nor-Ib) for silencing *aflD* target gene on aflatoxin B₁, aflatoxin G₁ expression of *aflD* and *aflR* genes of three aflatoxigenic strains (a) *A. flavus* EPG9, (b) *A. parasiticus* NRRL13005, and (c) *A. parasiticus* SSWT2999.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. flavus</em> EPG9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>aflD</em> copy numbers</td>
<td>1</td>
<td>5.7 E+10</td>
<td>11.71</td>
<td>0.026*</td>
</tr>
<tr>
<td>AFB₁</td>
<td>1</td>
<td>7.4 E+05</td>
<td>163.06</td>
<td>0.0002*</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>A. parasiticus</em> NRRL13005</td>
<td></td>
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<td>Factor</td>
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<tr>
<td><em>aflD</em> copy numbers</td>
<td>1</td>
<td>6.3 E+08</td>
<td>17.07</td>
<td>0.01*</td>
</tr>
<tr>
<td>AFG1</td>
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<td>6.9 E+06</td>
<td>12.34</td>
<td>0.02*</td>
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<tr>
<td>(c)</td>
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<tr>
<td><em>A. parasiticus</em> SSWT2999</td>
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<tr>
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<tr>
<td><em>aflD</em> copy numbers</td>
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<tr>
<td>AFG1</td>
<td>1</td>
<td>8.3 E+06</td>
<td>0.42</td>
<td>0.54</td>
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</table>

* 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 AFB1 production. Changes in AFB1 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 aflID showed a good correlation (r=0.82; P =0.0001). This suggests the functional role of aflID gene in aflatoxin biosynthesis and may suggest that reduction in the expression of structural gene (aflD) can affect on the regulatory gene (aflIR). 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. Using siRNA to target aflID (nor-1) gene expression, an early enzymatic step in the aflatoxin biosynthetic pathway could be an 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 quantitative PCR (Abdelhadi et al., 2010). Thus disruption or deletion of the aflID (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 aflID 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 aflIR 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 aflID or aflIR, suggesting that the results observed with aflID-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 aflID (targeting gene) of all three strains and an inhibition
of AFB\textsubscript{1} production by \textit{A. flavus} EGP9 and AFG\textsubscript{1} production by \textit{Aspergillus parasiticus} NRRL 13005. However, there was no significant decrease in AFG\textsubscript{1} by \textit{Aspergillus parasiticus} SSWT 2999. This suggests that the biosynthesis of aflatoxins may be slightly different in \textit{A. flavus} and \textit{A. parasiticus}. Wilkinson et al., (2007) reported that the regulatory mechanism or mechanisms that control aflatoxin production in \textit{A. flavus} and \textit{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\textsubscript{1} and AFB\textsubscript{2} production for \textit{A. flavus}. However, for \textit{A. parasiticus}, an increase in expression profile and AFB\textsubscript{1} and AFG\textsubscript{1} production were observed.

The present study suggests that the $aflD$ gene has a role in monitoring the biosynthetic direction of aflatoxin biosynthesis in \textit{A. flavus} and \textit{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 water 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 conidia 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 (\textit{aflID}, \textit{aflM}, \textit{aflP} and \textit{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 \textit{aflID} transcription can be used as a marker to discriminate between aflatoxin and non-aflatoxin producers, while \textit{aflM}, \textit{aflP} and \textit{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 \textit{A. flavus} EGP3 did not produce any aflatoxin on YES, however, it produced higher aflatoxin than \textit{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 \textit{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\textsubscript{10} CFUs found. No statistical correlation between \textit{A. flavus} CFUs and the quantified \textit{aflID} 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 \textit{a}_w and 0.95 \textit{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\textsubscript{1} production (r= 0.488; p=0.000).

This poor correlation is probably due to the \textit{aflID} expression being initiated very early, prior to phenotypic aflatoxin production being synthesised. Water activity had a significant effect on \textit{aflIR} transcription, especially at 0.90 \textit{a}_w, where it was transcripted from the 4\textsuperscript{th} week, while during the initial 3 weeks there was high expression of \textit{aflID} gene, transcription of the structural genes and high AFB\textsubscript{1} production. This suggests that \textit{aflIR} may not have a role in regulation of structural gene expression in food matrices such as peanuts.
All three siRNAs designed to target \textit{aflD} gene gave excellent levels of silencing. The decrease in mRNA expression of \textit{aflD} level caused subsequent decrease in mRNA abundance \textit{AflR} level and AFB$_1$ production (98\%, 97\% and 97\% of the control level) in \textit{A. flavus} SRRC, respectively. Changes in AFB$_1$ production in relation to mRNA level of \textit{AflD} showed a good correlation ($r$=0.88; $P$ =0.00001), and changing in \textit{AflR} mRNA level in relation to mRNA level of \textit{aflD} showed a good correlation ($r$=0.82; $P$ =0.0001). There was a significant decrease in \textit{aflD} (targeting gene) of all three strains and an inhibition of AFB$_1$ production by \textit{A. flavus} EGP9 and AFG$_1$ production by \textit{Aspergillus parasiticus} NRRL 13005. This study suggests that the \textit{aflD} gene has a role in monitoring the biosynthetic direction of aflatoxin biosynthesis in \textit{A. flavus} and \textit{A. parasiticus}. This could thus be a good 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 \textit{aflD} gene in aflatoxin biosynthesis of \textit{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 \textit{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 \textit{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 \textit{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


3- Markus Schmidt-Heydt, Corinna E. Rüfer, Ahmed Abdel-Hadi, Naresh Magan and Rolf Geisen (2010). The production of aflatoxin B<sub>1</sub> or G<sub>1</sub> by *Aspergillus parasiticus* at various combinations of temperature and water activity is related to the ratio of *aflS* to *aflIR* expression. Mycotoxin Research 4, 241-246.


II. POSTER PRESENTATION


Ahmed Abdel-Hadi, David Carter and Naresh Magan. Diversity and molecular discrimination between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavi* group
Appendix
Complex regulation of the aflatoxin biosynthesis gene cluster of *Aspergillus flavus* in relation to various combinations of water activity and temperature

Markus Schmidt-Heydt, Ahmed Abdel-Hadi, Naresh Magan, Rolf Geisen

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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 aw 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 B1 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 aqR/aqF expression was correlated with increased aflatoxin biosynthesis.

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1. Introduction

Aflatoxins are produced by *Aspergillus flavus* 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* (Bhamagar 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 B1 and B2. 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; Vaunande et al., 2003). Most strains of this species are able to produce the aflatoxins B1, B2, G1, and G2.

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 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 *afr* and *agf* gene of the aflatoxin pathway in relation to various nutritional media. Xu et al. (2000) monitored the expression of the *agfD* gene after growth of *A. parasiticus* on peanut pods by a reporter gene approach. Mayer et al. (2003) showed that expression of the *agfD* gene and the biosynthesis of aflatoxin in wheat are directly coupled. Recently Price et al. (2005) used a whole genome microarray approach to analyze 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
Temporal monitoring of the nor-1 (afID) gene of Aspergillus flavus in relation to aflatoxin B₁ production during storage of peanuts under different water activity levels

A. Abdel-Hadi, D. Carter* and N. Magan

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 (=afID) gene of Aspergillus flavus using real-time PCR in relation to phenotypic aflatoxin B₁ (AFB₁) production and populations of A. flavus in stored peanuts at three water activity levels (aw: 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 (=afID), ver-1 (=afU2), and amtA (=afEP), and the regulatory gene aFR of the aflatoxin biosynthetic pathway were also assayed. There were significant differences between nor-1 gene expression at the three aw levels: higher expression at 0.90 aw in weeks 1–3, when compared to 0.95. In contrast, in the driest treatment (0.85 aw) 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 aw. Highest AFB₁ production was at 0.90 and 0.95 aw from weeks 3–6. A aw had a significant effect on aflatoxin transcription at 0.95 aw over the 6-week period, while at 0.90 aw only in the last 2 weeks.

Conclusions: Correlations between different factors showed that log AFB₁ α log CFUs, log AFB₁ α aw, and log CFUs × aw were statistically significant, while log CFUs × RQ-PCR and RQ-PCR × aw were not. The AFR 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 aw levels could be helpful to predict potential risk of aflatoxin production during storage of this hygroscopic food product and minimize contamination with the AFB₁.

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. 1992; Costy 1997; Payne and Brown 1968; 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 (aw) and temperature (Belli et al. 2004; Hope et al. 2005).
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

Markus Schmidt-Heydt · Corinna E. Rüfer · Ahmed Abdel-Hadi · Naresh Magan · Ralf Geisen

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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 37 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 those species occur.

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

Introduction

Aflatoxins are toxic metabolites formed predominantly 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...
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 (ornt 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 B₁ and B₂ 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, 1996). 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 (Youssel et al., 2008).

The major mycotoxins found in Egyptian peanuts are aflatoxins (El-Magraby 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 B₁ (AFB₁) 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; Shapiira et al., 1996).
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

<table>
<thead>
<tr>
<th>Gene</th>
<th>20 °C 0.99</th>
<th>25 °C 0.90</th>
<th>25 °C 0.95</th>
<th>25 °C 0.99</th>
<th>30 °C 0.90</th>
<th>30 °C 0.95</th>
<th>30 °C 0.99</th>
<th>35 °C 0.90</th>
<th>35 °C 0.95</th>
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<td>223</td>
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<td>281</td>
<td>305</td>
<td>1481</td>
<td>205</td>
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<td>4143</td>
<td>273</td>
<td>300</td>
<td>3781</td>
<td>416</td>
<td>274</td>
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<td>371</td>
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<td>296</td>
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<tr>
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<td>217</td>
<td>505</td>
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<td>266</td>
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<td>305</td>
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