Mycotoxin production and DNA polymorphism of *Alternaria* species isolated from oilseed rape

by

Michael Ronald Forbes-Smith

A Thesis submitted to University College (University of London) for the degree of Doctor of Philosophy

DEPARTMENT OF BIOLOGY
UNIVERSITY COLLEGE LONDON
GOWER STREET
LONDON WC1E 6BT
UNITED KINGDOM

May, 1994
ABSTRACT

Samples of rapeseed and meal were collected and examined microbiologically in order to determine the occurrence of mycotoxigenic moulds colonising oilseed rape in Great Britain. Up to 19 fungal genera were detected on samples at harvest and during storage; the predominant mould in seed was *Alternaria alternata*. Moulds identified on meal included *Penicillium* and *A. alternata*.

When isolates of *Alternaria* spp were grown on rice, rapeseed and meal, five metabolites were detected - altenuene (ALT), altertoxin-I (ATX-I), alternariol (AOH), tenuazonic acid (TeA) and alternariol methyl ether (AME). These compounds were identified by their UV spectra and thin layer chromatography with standards. In order to quantify these compounds by high performance liquid chromatography, solvent optimisation techniques were adopted. Optimum separation on an octyl column occurred with an isocratic mobile phase consisting of 41.7% aqueous zinc sulphate solution (300mg/L), 30.3% methanol, 18.1% acetonitrile and 9.9% tetrahydrofuran.

Production of mycotoxins was greater on rice than on rapeseed or meal. The most abundant metabolite produced on all substrates was TeA.

Bioassay studies, including those with brine shrimp and cells isolated from rapeseed leaves, were employed to investigate the toxicity of *Alternaria* mycotoxins; TeA was the most toxic of the five metabolites.

Production of TeA by *A. alternata* was quantified on rapeseed and meal under a series of temperature and moisture conditions ranging between 10-30°C and 6.0-55.0% respectively. Higher temperature and moisture levels favoured TeA accumulation but under commercial storage conditions (i.e. ≤9% moisture), none was produced.
A nitrogen-containing compound which was toxic to fungal spores was produced on rice by an isolate of *A. alternata* that did not produce the typical *Alternaria* toxins. The compound was purified using solid phase and preparative TLC.

Analysis of polymerase chain reaction products from genomic DNA of *Alternaria* spp. showed polymorphisms between morphological features and between toxigenic and non-toxigenic isolates of *Alternaria* spp.
ACKNOWLEDGMENTS

I wish to thank Dr Richard N. Strange for his advice and guidance throughout the course of this project. Andrea Johanson and Keith Shawe for their advice on molecular biology techniques, and Michele Solfrizzo for his help with Alternaria mycotoxins. Dr David Carter for his assistance on mass spectrometry, Dr Peter Burns for the use of his computer to write this thesis and Dr Susan Smith for proofreading the manuscript.

This thesis is dedicated to Helen and Jade whose love and understanding made this work possible.

The work was supported by the European Economic Community and Grains Research and Development Corporation, Australia.
# TABLE OF CONTENTS

## CHAPTER 1. INTRODUCTION

### 1.1 Oilseed rape
- **1.1.1** Origin and history 18
- **1.1.2** Species 18
- **1.1.3** Nutritional quality 20
- **1.1.4** Production 32
- **1.1.5** Fungal infection and infestation 22
- **1.1.6** Production of mycotoxins by moulds 25

### 1.2 The *Alternaria*
- **1.2.1** The *Alternaria* mycotoxins 28
- **1.2.2** Natural occurrence 28
- **1.2.3** Toxicity 31
- **1.2.4** Production 32
- **1.2.5** Extraction
  - **1.2.5.1** Liquid-liquid partitioning 33
  - **1.2.5.2** Recrystallisation 34
  - **1.2.5.3** Chromatography 34
  - **1.2.5.4** Bonded phase cartridges 35
- **1.2.6** Quantification
  - **1.2.6.1** Thin layer chromatography 36
  - **1.2.6.2** High performance liquid chromatography 39

### 1.3 Separation of compounds using HPLC solvent optimisation
- **1.3.1** Sequential methods 43
- **1.3.2** Predictive methods 48
- **1.3.3** Iterative methods 50
- **1.3.4** Philips PU6100 solvent optimisation software 51
1.4 Application of random amplified polymorphic DNA for genetic mapping of filamentous moulds

1.4.1 The polymerase chain reaction

1.4.2 Analysis by RAPD

1.5 Objectives

CHAPTER 2. APPRAISAL OF THE MYCOFLORA IN OILSEED RAPE

2.1 Materials and Methods

2.1.1 Source of samples

2.1.1.1 At harvest

2.1.1.2 During storage

2.1.1.3 After conversion into meal

2.1.2 Determination of moulds

2.1.2.1 Isolation

2.1.2.2 Identification

2.1.2.3 Preservation

2.2 Results

2.2.1 At harvest

2.2.2 During storage

2.2.3 After conversion into meal

2.3 Discussion

CHAPTER 3. HPLC SOLVENT OPTIMISATION OF ALTERNARIA TOXINS

3.1 Materials and Methods

3.1.1 Mycotoxins

3.1.2 Chromatography

3.1.3 Solvent optimisation
3.2 Results

3.2.1 Identification of the isoeluotropic plane 73
3.2.2 Assignment of chromascans 78
3.2.3 Optimisation 78

3.3 Discussion 88

CHAPTER 4. PRODUCTION OF MYCOTOXINS BY ALTERNARIA SPP.

4.1 Materials and Methods 94

4.1.1 Biological activity of Alternaria toxins 94
4.1.1.1 Rapeseed germination assay 94
4.1.1.2 Oilseed rape leaf assay 95
4.1.1.3 Oilseed rape leaf cell assay 95
4.1.1.4 Brine shrimp assay 96

4.1.2 Determination of mycotoxigenic Alternaria 97
4.1.2.1 Fungal isolates 97
4.1.2.2 Mycotoxin production 99
4.1.2.3 Extraction of the cultures 99
4.1.2.4 Analysis by TLC 100
4.1.2.5 Analysis by HPLC 100

4.1.3 The effect of temperature, moisture content and incubation period on the production of TeA 101
4.1.3.1 Fungal cultures, mycotoxin production and extraction 101
4.1.3.2 Analysis of mycotoxins 102

4.1.4 Statistical analysis 102
4.2 Results

4.2.1 Biological activity of *Alternaria* toxins

4.2.1.1 Rapeseed germination assay

4.2.1.2 Oilseed rape leaf assay

4.2.1.3 Oilseed rape leaf cell assay

4.2.1.4 Brine shrimp assay

4.2.2 Determination of mycotoxigenic *Alternaria*

4.2.2.1 TLC of mycotoxins

4.2.2.2 HPLC of mycotoxins

4.2.3 The effect of temperature, moisture content and incubation period on the production of TeA

4.2.3.1 Development of an HPLC solvent system for quantifying TeA

4.2.3.2 Variability in moisture content of rapeseed and meal

4.2.3.3 Production of TeA

4.3 Discussion

CHAPTER 5. ISOLATION AND CHARACTERISATION OF AN UNKNOWN, TOXIC COMPOUND FROM *A. ALTERNATA*

5.1 Materials and Methods

5.1.1 Media and growth conditions of *A. alternata*

5.1.2 Extraction

5.1.3 Detection

5.1.4 Purification

5.1.5 Purification studies using centrifugally accelerated TLC

5.1.6 Analysis by TLC

5.1.7 Spectrophotometry

5.1.8 Analysis by HPLC

5.1.9 Analysis by mass spectrometry

5.1.10 Oilseed rape leaf assays
5.2 Results

5.2.1 Detection by antifungal activity

5.2.2 Purification

5.2.3 Purification studies using centrifugally accelerated TLC

5.2.4 Analysis by TLC

5.2.5 Spectrophotometry

5.2.6 Analysis by HPLC

5.2.7 Analysis by spectrometry

5.2.8 Oilseed rape leaf assays

5.3 Discussion

CHAPTER 6.  USE OF RANDOM AMPHIFIED POLYMORPHIC DNA FOR ASSESSMENT OF GENETIC VARIABILITY IN ALTERNARIA SPP.

6.1 Materials and Methods

6.1.1 Growth conditions

6.1.2 Preparation of genomic DNA

6.1.3 Oligonucleotide primers

6.1.4 Amplification of DNA

6.1.5 Electrophoresis

6.1.6 Data analysis

6.2 Results

6.2.1 Preparation of genomic DNA

6.2.2 Development of a reliable PCR method

6.2.3 Variability of the Alternaria using random primers

6.2.4 Differentiation of species

6.2.5 Differentiation of toxin and non-toxin producing isolates

6.3 Discussion

6.3.1 Development of a reliable PCR method

6.3.2 Characterisation of groups using polymorphic DNA

6.3.3 Characterisation of groups by cluster analysis
INDEX TO TABLES, FIGURES AND APPENDICES

TABLES

1.1 Rape grown for oilseed in the United Kingdom (1990) 23
1.2 Natural occurrence of Alternaria mycotoxins 30
1.3 TLC of Alternaria mycotoxins 37
1.4 HPLC of Alternaria mycotoxins 40
2.1 Chemical components of fungal media 60
3.1 Retention times of the five Alternaria toxins in the 10 chromascans 77
4.1 Isolates of Alternaria spp. used in this study 98
4.2 Toxicity of crude Alternaria extracts on the motility of brine shrimp 108
4.3 Production of toxins by Alternaria spp. grown on rice (60% MC) for 3 weeks at 25°C 112
4.4 Production of toxins by Alternaria spp. grown on rapeseed (55% MC) and meal (53% MC) for 3 weeks at 25°C 113
4.5 Production of TeA by Alternaria spp. grown on rapeseed and rapeseed meal after four months incubation 117
5.1 Reagents sprayed on TLC plates for detection and characterisation of compound B 135
6.1 Sequences of oligonucleotide primers 149
6.2 Polymorphic loci for A. brassicae (isolate N), A. brassicicola (F), A. tenuissima (U and W) and HT producing isolates of Alternaria (T, U, V and W) 163
6.3 The total number of DNA fragments for all primers used (18) which are characteristic for individual isolates 164

FIGURES

1.1 Oilseed rape (B. napus) in flower 19
1.2 Molecular structures of Alternaria toxins 29
1.3 Solvent selectivity triangle. Methanol (CH3OH), acetonitrile (CH3CN) and tetrahydrofuran (THF) are represented in groups II, VI and III respectively 44
1.4 Quaternary solvent mixtures, showing the iseluotropic plane 45
1.5 Standard progression of a two-variable simplex optimisation towards the optimum (O) 47
2.1 Incidence of moulds in rapeseed collected at harvest in the UK 62
2.2 Incidence of moulds in rapeseed collected at harvest in Kent 63
2.3 Proportions of (A) *Alternaria* spp. (average incidence in rapeseed; 10.82%), (B) *Penicillium* spp. (2.1%) and (C) *Aspergillus* spp. (0.8%) in rapeseed collected at harvest in the UK

2.4 Postharvest incidence of moulds in rapeseed collected from Camgrain Silo, Cambridge

3.1 Flow diagram of PU6003 solvent optimisation

3.2 HPLC gradient runs (MeOH/zinc sulphate solution; 0-100%) of *Alternaria* toxins

3.3 (A) The iseluotropic plane calculated by the software based on the MeOH/zinc sulphate solution 5 min. gradient. (B) The final iseluotropic plane updated in the light experimental data

3.4 UV spectra of *Alternaria* metabolites.

3.5 The retention surfaces for ALT (A), ATX-I (B), AOH (C), TeA (D) and AME (E) showing the variation in retention time with solvent composition

3.6 Response surface (A) and contour plot (B) showing the effect of solvent composition on the quality of resolution

3.7 Predicted retention times of ALT (stick 1), ATX-1 (2), AOH (3), TeA (4) and AME (5).

3.8 The effect of MeOH as a mobile phase on the resolution of TeA. Chromatograms are displayed as 3 sets of sticks (1 and 7; 2 and 6; 3 and 5) which represent 10%, 25% and 50% absorbance (A280nm) of the TeA peak (stick 4) respectively

3.9 HPLC chromascan (A) and chromatogram (at A245 nm) (B) of ALT (peak 1; RT=2.76 min.), ATX-I (2; 3.52 min.), AOH (3; 4.13 min.), TeA (4; 5.12 min.) and AME (5; 7.08 min.)

3.10 The effect of TeA and AME on rapeseed germination after 60 hours at 20°C

3.11 Toxicity of TeA to a detached leaf of oilseed rape

3.12 (A) Leaf cells of oilseed rape stained with fluorescein diacetate. Live cells fluoresce green under UV light (magnification X200) (B) Toxicity of *Alternaria* metabolites to isolated leaf cells of oilseed rape

3.13 Toxicity of *Alternaria* metabolites to motility of brine shrimp

3.14 TLC of culture extracts of *Alternaria* spp. under (A) long wave (A365 nm) UV light and (B) short wave (A254 nm) UV light

3.15 Standard curves of peak height of ALT (A240 nm; r = 0.999), ATX-I (A260 nm; r = 0.998), AOH (A260 nm; r = 0.999), TeA (A280 nm; 0.999) and AME (A260 nm; r = 0.997), as determined by HPLC analysis
4.7 HPLC chromatogram (A280 nm) of a crude culture extract of *A. tenuissima* (U) grown on rapeseed. The mobile phase was 50.3% MeOH, 18.3% THF and 31.4% aqueous zinc sulphate solution (300 mg/L); RT of TeA = 5.71 min.

4.8 The effect of temperature and moisture content on TeA production by *A. tenuissima* (U) grown on (A) rapeseed and (B) meal for three weeks

5.1 Growth of *Alternaria* spp. (isolates B and G) on rice (60% moisture content) at 25°C for three weeks

5.2 Blueprint of the 'chromatotron' (Model 7924T, Harrison Research, Palo Alto, USA)

5.3 Bioautographic assay (*A. alternata*; isolate B) of MeOH rice culture extracts (isolates B and G)

5.4 Bioautographic assay (*A. alternata*; isolate B) of MeOH rice culture extracts (isolates B and G)

5.5 Thin layer chromatography of the various purification stages of compound B

5.6 TLC plate (solvent system; BAW) of compound B sprayed with various reagents

5.7 Absorption spectrum of compound B in MeOH ('λ' represents maximum at A203.2 nm)

5.8 High performance liquid chromatogram (A207 nm) of a pure preparation containing compound B (RT = 17.83 min.)

5.9 Electron impact mass spectrum of compound B

5.10 Formation of chlorotic lesions on leaves of oilseed rape sprayed with spores produced by isolate B (ca. 1X10^4 spores/ml water)

6.1 Agarose gel of genomic DNA of *Alternaria* spp..

6.2 Spectrum of genomic DNA of *Alternaria* spp. (isolate K)

6.3 Fluorometric quantification of genomic DNA of *Alternaria* spp. (isolate O)

6.4 The effect of different concentrations of *Alternaria* DNA (isolate O) on PCR

6.5 The effect of different ramping rates on PCR banding patterns of *Alternaria* spp. (isolate O)

6.6 The effect of different concentration of Mg^2+ on the PCR of *Alternaria* spp. (isolate O)

6.7 RAPD amplifications of *Alternaria* spp. with primer OP17

6.8 Conidia of *Alternaria* spp. isolated from oilseed rape

6.9 (A) RAPD amplifications of *Alternaria* spp. with primer OX15. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A)
6.10 (A) RAPD amplifications of *Alternaria* spp. with primer OP8. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 169
6.11 (A) RAPD amplifications of *Alternaria* spp. with primer OX1. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 170
6.12 (A) RAPD amplifications of *Alternaria* spp. with primer OP9. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 172
6.13 (A) RAPD amplifications of *Alternaria* spp. with primer OP2. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 174
6.14 (A) RAPD amplifications of *Alternaria* spp. with primer OP4. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 175
6.15 (A) RAPD amplifications of *Alternaria* spp. with primer OP7. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 176
6.16 (A) RAPD amplifications of *Alternaria* spp. with primer OX13. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A) 177
6.17 First and second principal components of RAPD data: (A) OP2 (proportion of total variation; 22.02%); (B) OP4 (31.12%); (C) OP7 (24.01%); (D) OX13 (21.88%) 178
6.18 Cluster dendrograms based on the RAPD products of primer OP7. (A) UPGMA (unweighted pair group arithmetic average method); (B) SL (Single linkage) 184

APPENDICES
1 The effect of Satisfar on the growth of *A. alternata* on rapeseed agar 190
2 The effect of temperature on the growth of *A. alternata* on rapeseed agar 191
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>double-zero</td>
</tr>
<tr>
<td>000</td>
<td>triple-zero</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADM</td>
<td>Archer Daniels Midland</td>
</tr>
<tr>
<td>ALT</td>
<td>altenuene</td>
</tr>
<tr>
<td>AME</td>
<td>alternariol methyl ether</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AOH</td>
<td>alternariol</td>
</tr>
<tr>
<td>AP-PCR</td>
<td>arbitrarily-primed polymerase chain reaction</td>
</tr>
<tr>
<td>ATX-I</td>
<td>altertoxin I</td>
</tr>
<tr>
<td>ATX-II</td>
<td>altertoxin II</td>
</tr>
<tr>
<td>ATX-III</td>
<td>altertoxin III</td>
</tr>
<tr>
<td>AU</td>
<td>absorbance unit</td>
</tr>
<tr>
<td>BAW</td>
<td>n-butanol:acetic acid:water (4:1:1)</td>
</tr>
<tr>
<td>BEOCO</td>
<td>Bibby Edible Oils Company Limited</td>
</tr>
<tr>
<td>C18</td>
<td>octadecyl</td>
</tr>
<tr>
<td>C8</td>
<td>octyl</td>
</tr>
<tr>
<td>cTLC</td>
<td>centrifugal thin layer chromatography</td>
</tr>
<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>CY20S</td>
<td>czapek yeast agar containing 20% sucrose</td>
</tr>
<tr>
<td>CYA</td>
<td>czapek yeast agar</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>DCPA</td>
<td>dichloran chloramphenicol peptone agar</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>2'-deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNTPs</td>
<td>deoxynucleotides</td>
</tr>
<tr>
<td>DRBC</td>
<td>dichloran rose bengal chloramphenicol</td>
</tr>
<tr>
<td>dTTP</td>
<td>2'-deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>G25N</td>
<td>25% glycerol nitrate agar</td>
</tr>
<tr>
<td>GD</td>
<td>genetic distance</td>
</tr>
<tr>
<td>GLC</td>
<td>gas liquid chromatography</td>
</tr>
</tbody>
</table>
HPLC  high performance liquid chromatography
HPTLC  high performance thin layer chromatography
HT  high toxin
ID  internal diameter
IMI  International Mycological Institute
LD  lethal dose
LEAR  low erucic acid rapeseed
LSD  least significance difference
m/z  mass to charge ratio
MC  moisture content
MEA  malt extract agar
MeOH  methanol
NaOCl  sodium hypochlorite
NLT  non/low toxin
PCA  principal components analysis
PCR  polymerase chain reaction
PDA  potato dextrose agar
r  correlation coefficient
RA  rapeseed agar
RAPD  random amplified polymorphic DNA
Rf  retention factor
RFLP  restriction fragment length polymorphism
RNA  ribonucleic acid
RT  retention time
sd  standard deviation
SL  single linkage
TBE  Tris borate EDTA
TE  Tris EDTA
TeA  tenuazonic acid
TEF  toluene-ethyl acetate-formic acid
THF  tetrahydrofuran
TLC  thin layer chromatography
UPGMA  unweighted pair group arithmetic average method
UV  ultra violet
YES  2% yeast extract-4% sucrose broth
CHAPTER 1.

INTRODUCTION

Various studies have reported that potentially mycotoxigenic fungi belonging to the genera *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* contaminate the seed of oilseed rape (Mills et al., 1978; Kanwar and Khanna, 1979; Mills and Abramson, 1986, Chakrabarti and Ghosal, 1987; Madhyastha et al., 1990; Visconti et al., 1992). These fungi are capable of growing and producing mycotoxins on natural substrates including rapeseed (e.g. diacetoxyisicerpenol by *Fusarium oxysporum* Schlecht) (Chakrabarti and Ghosal, 1987) and in Pakistan, the natural occurrence of aflatoxins in rapeseed (Sinha et al., 1988; Sahay and Prasad, 1990; Sahay and Ranjan, 1990) is thought to be a possible cause for the high fatality and low productivity of poultry maintained on the meal or cake after oil extraction (Pakistan Council of Scientific and Industrial Research Laboratory [PCSIR], Karachi, Pakistan, unpublished results).

Consequently, the ability of these mycotoxigenic fungi to contaminate rapeseed has caused concern among oilseed rape producers in the UK and was the incentive for the work detailed in this study. A mycological survey was initiated which lead to the discovery that *Alternaria* was the predominant genus in rapeseed at harvest, during storage and after conversion into meal. In order to assess the risk of *Alternaria* mycotoxins contaminating rapeseed and meal, an isocratic HPLC solvent system was developed to quantitate the compounds (using solvent optimisation techniques). In addition, bioassays were used (e.g. brine shrimp) to estimated their toxicity. As a method for recognising *Alternaria* spp. other than using spore morphology, random amplified polymorphic DNA (RAPD) was generated with the polymerase chain reaction (PCR) to characterised toxigenic and non-toxigenic isolates, and to show genetic diversity of species of *Alternaria*. 
In view of the study presented here, the following literature review is segregated into four appropriate sections; oilseed rape, the Alternaria, separation of mycotoxins using HPLC solvent optimisation and application of RAPD for genetic mapping of filamentous moulds.

1.1 OILSEED RAPE

1.1.1 Origin and history

Oilseed rape is a member of the Brassica family and was originally cultivated four thousand years ago in India, China and probably Japan (Fig. 1.1). Rapeseed was introduced by the Romans to those areas of Europe where oil could not be acquired easily from poppyseeds or olives. By the 18th century, it was well established throughout Great Britain, where rapeseed oil was the preferred commodity for use in oil lamps and the residual meal was used as cattle feed. It was not until after the second world war that rapeseed became favoured as an edible oil. Bunting (1986) and Anon. (1988) predicted that by 1990, 35% of the total UK market for edible oil would be supplied by the crop.

1.1.2 Species

Commercially grown oilseed rape is represented by four species - B. carinata L., B. juncea L., B. campestris L. and B. napus L. For production of oilseed, B. carinata (Ethiopian mustard) is significant only in its country of origin and B. juncea (Oriental mustard) is mostly limited to the Indian sub-continent and China. Brassica napus and B. campestris are the oilseed species grown in Europe, with B. napus being the more widely grown and accounting for approximately 95% of production.

There are autumn planted (winter rape) and spring planted (spring rape) cultivars of B. napus. Broadly speaking, winter rape cultivars have a potential yield some 20% higher than spring rape varieties. In localities where the winter climate is considered too harsh for winter rape, the earlier maturing spring rape may be cultivated (Bunting, 1986). In 1991, roughly 99% of oilseed rape grown in the UK was winter rape; the remainder was spring rape primarily grown in
Fig. 1.1 Oilseed rape (*B. napus*) in flower
Scotland (Anon., 1991A; Department of Agriculture for Northern Ireland, pers. comm.; Scottish Office Agriculture and Fisheries Department, pers. comm.).

1.1.3 Nutritional quality

Rapeseed contains about 40% oil and, after its extraction, the residual meal is potentially valuable for animal feed. However, rapeseed oil, which is used for edible purposes such as margarine spreads and cooking oil, has always been a more valuable commodity on the market than meal. Therefore, primary breeding objectives aimed at developing cvs. which produced seed with high oil content and quality i.e. low (<1%) erucic acid content. Erucic acid is a monosaturated fatty acid which is considered to be a hazard to human health (Kimbler, 1983; Anon., 1988).

Low erucic acid rapeseed (LEAR) varieties are referred to as single zero cultivars. In general, LEAR oil has (i) the lowest level (about 6%) of saturated fat content of any vegetable oil, (ii) low (10%) linolenic acid, which at higher concentrations reduces flavour stability and shelf-life of the product and (iii) high (26%) linoleic acid, commonly known as vitamin F (LaBell, 1987). Nonetheless, a small market still exists for oils high in erucic acid since they have special attributes (e.g. high smoke and flash points) which make them useful in manufacturing (e.g. rubber additives) (Erickson and Bassin, 1990).

The rapeseed meal remaining after removal of the oil is a source of high-quality protein (approximately 40%) but its use as animal feed, until recently, was limited owing to the presence of sulphur containing compounds called glucosinolates. Enzymatic hydrolysis of glucosinolates occurs when the seeds are crushed, producing various breakdown products (e.g. gluconapin). These breakdown chemicals may be bitter tasting, which reduce the palatability of the meal (Thompson and Hughes, 1986) or cause deleterious effects on animals; for example, Greer (1950) stated that oxazolidinethione by-products inhibit the function of the thyroid gland.
Double-zero (00) cvs. or canola*, which contain low quantities of glucosinolates (<30 μmoles/g) as well as low levels of erucic acid, have recently been introduced in the EEC to expand the rapeseed meal market as animal feed. In 1989 and 1990, the percent area of 00 cvs. grown in the UK was 97.4% and 96.0% respectively. Major winter rape 00 cvs. grown in 1990 in descending order of importance included Falcon, Envol, Libravo, Lictor, Tabidor and Samurai (Anon., 1991B).

In 1985, the average inclusion rate of rapeseed meal in cattle feed exceeded 9% in the UK. Some producers had achieved levels of 15% in dairy compound feeds, whilst in West Germany, the average rate was as high as 14% (Anon., 1988). Although this proportion has increased over recent years to 20%, the ratio of rapeseed meal in poultry and pig feeds is lower (roughly 10%) as monogastric animals are more susceptible to glucosinolates (Dalgetys Pty. Ltd., pers. comm.). Rapeseed meal has now replaced soybean meal as the most important single source of vegetable protein in cattle feed (Anon., 1988).

The triple-zero (000) variety already available in Canada as a spring crop is a recent introduction. It has a thinner seed coat and a reduced fibre as well as enhanced protein content and increased palatability of the meal (Bell, 1984).

In addition to animal fodder, meal is a possible source of protein for human consumption and demand in the future may escalate as new uses develop for the commodity (Solsuski et al., 1976).

* the name canola was adopted for 00 cvs. in Canada in 1979 and recognised by the United States Food and Drug Administration (USFDA) in 1985 (Shahidi, 1990)
1.1.4 Production

Production of rapeseed in the UK has quadrupled in the last decade from $3.0 \times 10^5$ tonnes in 1980 to $1.3 \times 10^6$ tonnes in 1990. The value of the crop to the farming sector in 1990 was estimated at £344 million (Anon., 1989). With respect to changes in agricultural land usage, oilseed rape in 1984 accounted for about 6% of the ‘total crops’ area in the UK whereas in 1990, it represented 10.5% and is now the most important arable crop in UK agriculture after wheat and barley (Bunting, 1986; Anon., 1988).

Oilseed rape in the UK is mostly grown in England, followed by Scotland and Northern Ireland (Table 1.1). In 1991, the area allocated to oilseed rape in England was $3.88 \times 10^5$ ha which represented 88.1% of the total rapeseed land usage in the UK. In relation to yield, England produced $1.13 \times 10^6$ tonnes or 87.5% of the total rapeseed harvested in the UK.

On a regional/county basis, oilseed rape was most common in the South East (24.3%), East Midlands (21.9%), Yorkshire & Humberside (13.5%) and East Anglia (12.4%). Undoubtedly, the South East region contained the largest area for rapeseed production with the counties of Essex, Kent and Oxfordshire being major producers (6.0%, 3.6% and 3.6% of the national rapeseed area respectively) (Anon., 1991A).

As a result of rapid expansion in the area of land sown with oilseed rape and the number of facilities constructed for rapeseed and meal storage in recent years, a wide range of biological problems have become more prevalent, including fungal disease and infestation of oilseed rape.

1.1.5 Fungal infection and infestation of oilseed rape

Fungi may cause pre- and postharvest losses in rapeseed, those which are pathogenic being primarily responsible for losses prior to harvest.
<table>
<thead>
<tr>
<th>County and Region</th>
<th>Area (ha)</th>
<th>County and Region</th>
<th>Area (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UNITED KINGDOM</td>
<td><strong>439 672</strong></td>
<td>Hereford &amp; Worcestershire</td>
<td>6 445</td>
</tr>
<tr>
<td>Cleveland</td>
<td>2 900</td>
<td>Shropshire</td>
<td>3 780</td>
</tr>
<tr>
<td>Cumbria</td>
<td>244</td>
<td>Staffordshire</td>
<td>3 849</td>
</tr>
<tr>
<td>Durham</td>
<td>7 575</td>
<td>Warwickshire</td>
<td>11 306</td>
</tr>
<tr>
<td>Northumberland</td>
<td>11 798</td>
<td>West Midlands</td>
<td>535</td>
</tr>
<tr>
<td>Tyne and Wear</td>
<td>1 819</td>
<td><strong>West Midlands Region</strong></td>
<td><strong>25 915</strong></td>
</tr>
<tr>
<td>North Region</td>
<td><strong>24 336</strong></td>
<td>Cheshire</td>
<td>2 072</td>
</tr>
<tr>
<td>Humberside</td>
<td>28 047</td>
<td>Greater Manchester</td>
<td>572</td>
</tr>
<tr>
<td>North Yorkshire</td>
<td>22 555</td>
<td>Lancashire</td>
<td>1 240</td>
</tr>
<tr>
<td>South Yorkshire</td>
<td>5 593</td>
<td>Merseyside</td>
<td>620</td>
</tr>
<tr>
<td>West Yorkshire</td>
<td>3 348</td>
<td><strong>North West Region</strong></td>
<td><strong>4 504</strong></td>
</tr>
<tr>
<td>Yorks &amp; Humberside</td>
<td><strong>59 543</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Derbyshire</td>
<td>28 047</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leicestershire</td>
<td>15 837</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lincolnshire</td>
<td>41 874</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Northamptonshire</td>
<td>20 099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nottinghamshire</td>
<td>14 019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Midlands Region</td>
<td><strong>96 026</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cambridgeshire</td>
<td>20 255</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Norfolk</td>
<td>10 263</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suffolk</td>
<td>17 474</td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Anglia Region</td>
<td><strong>47 992</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bedfordshire</td>
<td>9 318</td>
<td>Shetland</td>
<td>1</td>
</tr>
<tr>
<td>Berkshire</td>
<td>4 138</td>
<td>Orkney</td>
<td>5</td>
</tr>
<tr>
<td>Buckinghamshire</td>
<td>7 976</td>
<td>Western Isles</td>
<td>1</td>
</tr>
<tr>
<td>East Sussex</td>
<td>2 583</td>
<td>Highland</td>
<td>2 052</td>
</tr>
<tr>
<td>Essex</td>
<td>26 292</td>
<td>Grampian</td>
<td>16 756</td>
</tr>
<tr>
<td>Greater London</td>
<td>647</td>
<td>Tayside</td>
<td>12 364</td>
</tr>
<tr>
<td>Hampshire</td>
<td>7 789</td>
<td>Fife</td>
<td>6 781</td>
</tr>
<tr>
<td>Hertfordshire</td>
<td>9 617</td>
<td>Lothian</td>
<td>4 302</td>
</tr>
<tr>
<td>Isle of Wight</td>
<td>1 133</td>
<td>Borders</td>
<td>6 305</td>
</tr>
<tr>
<td>Kent</td>
<td>15 862</td>
<td>Central</td>
<td>902</td>
</tr>
<tr>
<td>Oxfordshire</td>
<td>15 734</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surrey</td>
<td>1 609</td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Sussex</td>
<td>4 060</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South East Region</td>
<td><strong>106 757</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avon</td>
<td>897</td>
<td>Dumfries &amp; Galloway</td>
<td>283</td>
</tr>
<tr>
<td>Cornwall</td>
<td>513</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Devon</td>
<td>1 175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dorset</td>
<td>1 551</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gloucestershire</td>
<td>8 499</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Somerset</td>
<td>1 403</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wiltshire</td>
<td>8 383</td>
<td></td>
<td></td>
</tr>
<tr>
<td>South West Region</td>
<td><strong>22 421</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 86.9% winter rape; 13.1% spring rape

** England and Wales only

Table 1.1 Rape grown for oilseed in the United Kingdom (1990); from Agricultural & Horticultural Census: England & Wales, June, 1991; Scottish Office Agriculture & Fisheries Dep., Economic & Statistics Unit; Department of Agriculture for Northern Ireland, Dundonald House
Darkleaf and pod spot are the major diseases of oilseed rape in the UK caused by *Alternaria brassicae* (Berkshire) Sacc. and to a lesser extent, *A. brassicicola* (Schw.) Wiltshire (Evans and Cox, 1982; Humpherson-Jones, 1985; 1989). Under high disease pressure, dark spots discolour the leaves or pods and reduce the amount of photosynthetic area. This may cause the development of smaller pods which often ripen prematurely and split, resulting in the loss of seed before harvest. Infected material may subsequently rot from secondary invasion of fungal saprophytes or weak pathogens (e.g. *A. alternata* (Fr.) Keissler) (Humpherson-Jones, 1989).

Although the severity of disease depends on climate and cv. type (Sweet et al., 1988), the introduction of low glucosinolate cvs. may have intensified the problem in recent years. Accumulation of glucosinolates in infected oilseed rape tissue may limit the spread of existing fungal infections or inhibit subsequent challenge, especially in younger leaves (Doughty et al., 1991).

Other diseases of oilseed rape cultivars currently grown include blackleg or stem canker (*Leptosphaeria maculans* Desm. (Ces. and de Not)), footrot (*Rhizoctonia solani* Kuhn and *Fusarium roseum* Lk. emend. Snyder and Hansen), downy mildew (*Peronospora parasitica* (Pers. ex Pers. Fr.) and light leaf spot (*Pyrenopeziza brassicae* (Sutton and Rawlinson sp. nov)) (Evans, 1984; Maude and Humpherson-Jones, 1984; Davies, 1986; Gladders, 1987).

Postharvest development of moulds on rapeseed or meal may occur under favourable moisture (>8%) and temperature (10-40°C) conditions (Mills and Sinha, 1980; Anon., 1986; Milton and Pawsey, 1988; White and Jayas, 1989). Moulds may contaminate rapeseed either from infected material in the field or during storage. However, no clear cut division between the categories can really be defined and some cross-over exists.

In the initial stages of mould development, the seed may aggregate, first in small clumps and then into blocks making handling difficult and dangerous.
Serious mould infestation causes discolouration, heating and produces free fatty acids which adversely affect oil quality and may cause corrosion to metal storage containers (Wilken, 1986).

Moulds (e.g. *Alternaria* spp.) growing on rapeseed and other agriculture products may represent a serious risk to human health since repeated inhalation of microbial particles can cause allergic reactions, such as pneumonitis, to certain individuals (Schlueter *et al.*, 1972) and cutaneous alternariosis (Pederson *et al.*, 1976). Fungal contamination of rapeseed may also result in the production of secondary metabolites, known as mycotoxins, which may render meal produced from affected seed unsuitable for animal feed. Mycotoxins are fungal metabolites which evoke pathological changes in man and animals. There are about 300 known mycotoxins which cause a variety of symptoms, including liver damage, haemorrhage, kidney disease, dermatitis, oestrogenism, convulsions, vomiting, gangrene and death.

### 1.1.6 Production of mycotoxins by moulds

Numerous studies have demonstrated that potentially mycotoxigenic fungi from the genera *Alternaria*, *Fusarium*, *Aspergillus* and *Penicillium* colonise oilseed rape in Canada, Italy and India (Mills *et al.*, 1978; Kanwar and Khanna, 1979; Mills and Abramson, 1986; Visconti *et al.*, 1992). In Manitoba, rapeseed stored in farm granaries at 25°C and 12.4% moisture content (MC), *Penicillium* spp. were dominant after 30 days storage and *Aspergillus versicolor* (Vuill.) Tiraboschi after 147 days; at 25°C and 9.7% MC, *Aspergillus glaucus* group species (mainly *A. amstelodami* [Mang.] Thom and Church, *A. repens* [Corda] Sacc. and *A. sejunctus* Bain and Sartory) predominated after 50 days storage (Mills and Sinha, 1980). When storage of meal was extended to 12 months at a MC between 6.3-11.5%, White and Jayas (1989) reported that *Penicillium* spp. was dominant at 10 and 20°C but the *A. glaucus* group species at 40 and 50°C.

There are various natural substrates which support the production of mycotoxins by these genera, including rapeseed. Mills and Abramson (1986)
found that 21 out of 23 isolates of *Aspergillus versicolor*, originally obtained from stored western Canadian rapeseed, produced sterigmatocystin (up to 123.9 μg/g) on ground corn at 39% MC when incubated for 16-22 days at 28°C. Eleven strains of *Alternaria alternata* isolated from oilseed rape grown in Italy all produced mycotoxins, including tenuazonic acid (up to 12 000 mg/kg), alternariol (up to 200 mg/kg), alternariol methyl ether (up to 200 mg/kg) and altertoxin I (2-250 mg/kg) and II (2-70 mg/kg respectively) when cultured on rice (Visconti et al., 1992).

On rapeseed, mycotoxins ochratoxin A and ochratoxin B were produced by *Aspergillus alutaceus* Wilhelm (34 and 19 μg/g rapeseed respectively) and ochratoxin A by *P. verrucosum* Dierckx (3 μg/g) (Madhyastha et al., 1990). Three recognized mycotoxins, diacetoxyscirpenol, T2 toxin and zearalenone, along with fatty acid derivatives of these toxins, were isolated from seeds of *B. campestris* var. sarson cultured with *F. oxysporum* Schlecht (Chakrabarti and Ghosal, 1987).

Natural occurrences of aflatoxins in rapeseed and meal have been detected up to levels of 1420 μg/Kg (Sinha et al., 1988; Sahay and Prasad, 1990A; Sahay and Ranjan, 1990B). Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* which can act as acute toxins, carcinogens, teratogens and mutagens (Elis and Di Paolo, 1967; Wong and Hsieh, 1976; Ong, 1975; Neal et al., 1981; Kurtzman et al., 1987). Research has shown that high mortality and low productivity in poultry sustained on rapeseed meal in Pakistan led to the suggestion that aflatoxins might be responsible. However, no systemic study of mycotoxin levels in oilseed rape was undertaken and therefore it was impossible to establish whether mycotoxins other than aflatoxins could contribute to toxic effects in poultry (PCSIR, Karachi, Pakistan, unpublished results).

Although moulds of the genera *Alternaria* produce a number of toxic metabolites (e.g. tenuazonic acid), there have been no reports of the natural
occurrence of the mycotoxins produced by these species on oilseed rape products, despite the common association of these fungi with the crop (McKenzie et al., 1988; Visconti et al., 1992). The presence of Alternaria spp. suggests that there is the potential for mycotoxin production.

1.2 THE ALTERNARIA

Alternaria spp., saprophytic or pathogenic, are broadly distributed in nature and frequently identified in a number of crops, including sorghum, groundnuts, cereals and oilseed rape (Bruce et al., 1988; El-Magraby and El-Magraby, 1988; Kumar et al., 1992; Nasreen et al., 1988; Visconti et al., 1992). High MC (>28%) is normally required for optimum growth, thus infestation by Alternaria spp. is more often a problem in badly weathered crops and during postharvest storage of fruits and vegetables (Stinson, 1985; Mortimer et al., 1988).

Species of Alternaria are known to produce numerous secondary metabolites which have adverse effects on plants, either functioning as host-specific phytotoxins (e.g. production of ACT-toxin 1b by an opportunistic pathotype of A. alternata infecting tangerines and mandarins induced veinal necrosis of susceptible leaves of mandarins) (Kohmoto et al., 1993) or general phytotoxins (e.g. tentoxin, a cyclic tetrapeptide produced by A. alternata, caused chlorosis of susceptible cotyledon or leaf tissue of Nicotiana suaveolens, cucumber and sorghum) (Duke, 1993). They may also produce compounds which are toxic to animals and may therefore be regarded as mycotoxins.

Contamination of feed and grain by Alternaria spp. has been associated with various illnesses of farm animals* (Gruber-Schley and Thalmann, 1988) and of chickens e.g. diarrhoea, prostration and death with hemorrhagic syndrome

* the type of illnesses and animals were not stated in the study
of poultry (King and Schade, 1984). They have also been associated with human disorders, such as an outbreak of alimentary toxic aleukia in Russia (Joffe, 1960) and oesophageal cancer in areas of China which have a higher incidence of *A. alternata* on cereals than in areas where the incidence is lower (Liu *et al.*, 1988). Mycotoxins produced by *Alternaria* spp. may have been the cause.

### 1.2.1 The *Alternaria* mycotoxins

The most extensively researched mycotoxins of *Alternaria* can be segregated into 3 structural groups; the dibenzopyrone derivatives - altenuene (ALT), alternariol (AOH), alternariol methyl ether (AME) -, the perylenequinone derivatives - altertoxins I, II and III (ATX-I, ATX-II and ATX-III respectively) - and tenuazonic acid (TeA), which is a tetramic acid (Fig. 1.2) (Schade and King, 1984; Seitz, 1984; Stack *et al.*, 1986; Mortimer *et al.*, 1988; Grabarkiewicz-Szczesna *et al.*, 1989; Logrieco *et al.*, 1990; Kostecki *et al.*, 1991).

### 1.2.2 Natural occurrence

*Alternaria* mycotoxins occur as natural contaminants in a number of agricultural commodities, such as rice, sorghum, wheat and sunflower (Umetsu *et al.*, 1974; Sauer *et al.*, 1978; Logrieco *et al.*, 1988; Grabarkiewicz-Szczesna *et al.*, 1989) (Table 1.2). In general, AOH and AME appear to be the most commonly detected toxins, followed by TeA and ALT, but TeA is usually found in greater quantities. The only reports of the natural occurrence of altertoxins were the finding of trace levels of ATX-I in three (out of 12) samples of weathered sorghum (Sauer *et al.*, 1978), and the presence of ATX-I (not quantified) in five (out of eight) samples of apples (Stinson *et al.*, 1981). Despite the prevalence of the mould in rapeseed products (McKenzie *et al.*, 1988; Visconti *et al.*, 1992), there have been no records of contamination of oilseed rape by *Alternaria* mycotoxins.
Fig. 1.2  Molecular structures of Alternaria toxins
<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>ALT</th>
<th>AOH</th>
<th>AME</th>
<th>ATX-I</th>
<th>ATX-II</th>
<th>TeA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>tomatoes</td>
<td>-</td>
<td>0</td>
<td>0.037-</td>
<td></td>
<td></td>
<td>0.024-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.274</td>
<td>0.268</td>
<td></td>
<td></td>
<td></td>
<td>7.21</td>
<td>A</td>
</tr>
<tr>
<td>tomatoes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4-70</td>
<td>B</td>
</tr>
<tr>
<td>tomatoes</td>
<td>0-1.1</td>
<td>0.7-5.3</td>
<td>0.3-0.8</td>
<td></td>
<td></td>
<td>0.1-139</td>
<td>C</td>
</tr>
<tr>
<td>tomato</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>D</td>
</tr>
<tr>
<td>pastes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>olives</td>
<td>1.4</td>
<td>0.101-</td>
<td>0.03-</td>
<td>0</td>
<td>-</td>
<td>0.109-</td>
<td>E</td>
</tr>
<tr>
<td></td>
<td>2.32</td>
<td>2.87</td>
<td></td>
<td></td>
<td></td>
<td>0.263</td>
<td></td>
</tr>
<tr>
<td>apples</td>
<td>0-0.5</td>
<td>0-5.88</td>
<td>0.1-2.3</td>
<td>NQ</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.27-</td>
<td>0.51</td>
<td></td>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wheat</td>
<td>-</td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>barley</td>
<td>-</td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oats</td>
<td>-</td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td>H</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;0.16</td>
<td>&lt;0.16</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rye</td>
<td>0</td>
<td>1.15</td>
<td></td>
<td>0</td>
<td>-</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.15</td>
<td></td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sorghum</td>
<td>-</td>
<td>NQ</td>
<td>NQ</td>
<td></td>
<td>-</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>sorghum</td>
<td>0.1-1.5</td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sorghum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NQ</td>
<td>-</td>
<td></td>
<td>K</td>
</tr>
<tr>
<td>sorghum</td>
<td>-</td>
<td>-</td>
<td>1.2-2.3</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sorghum</td>
<td>0.7</td>
<td>1.8</td>
<td></td>
<td></td>
<td>&lt;6.0</td>
<td></td>
<td>M</td>
</tr>
<tr>
<td>mandarins</td>
<td>-</td>
<td>1-5.2</td>
<td>0.3-1.4-</td>
<td></td>
<td>-</td>
<td>21-173.9</td>
<td>N</td>
</tr>
<tr>
<td>peppers</td>
<td>-</td>
<td>0.6</td>
<td>0.05</td>
<td></td>
<td>-</td>
<td>0.05</td>
<td>N</td>
</tr>
<tr>
<td>melons</td>
<td>-</td>
<td>-</td>
<td>0.55</td>
<td></td>
<td>-</td>
<td>0.08</td>
<td>N</td>
</tr>
<tr>
<td>sunflower</td>
<td>-</td>
<td>0.36-</td>
<td>0.13</td>
<td></td>
<td>-</td>
<td>&lt;6.0</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tobacco</td>
<td>-</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>O</td>
</tr>
<tr>
<td>pecan nut</td>
<td>NQ</td>
<td>NQ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>rice</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>≥379</td>
<td>Q</td>
</tr>
</tbody>
</table>

NQ = not quantified
- = not investigated

A = Visconti et al., 1987
B = Stack et al., 1985
C = Stinson et al., 1981
D = Scott and Kanhere, 1980
E = Visconti et al., 1986
G = Grabarkiewicz-Szczesna et al., 1989
H = Gruber-Schley and Thalmann, 1988
I = Seitz et al., 1975A
J = Sauer et al., 1978
K = Sauer et al., 1978
L = Sydenham et al., 1988
M = Ansari and Shrivastava, 1990
N = Logrieco et al., 1988
O = Lucas et al., 1971
P = Schroeder and Cole, 1977
Q = Umetsu et al., 1974A

Table 1.2 Natural occurrence of Alternaria mycotoxins
1.2.3 Toxicity

It is well established that *Alternaria* cultures or culture extracts are lethal to a wide range of living organisms, including mice, chick embryos, rats and brine shrimp (King and Schade, 1984; Seitz, 1984; Zajkowski et al., 1991). However, it is less evident just which toxins are responsible.

Pero et al. (1973) found that all three groups of *Alternaria* toxins were toxic to human cervical (HeLa) cells, bacteria and mice. AOH and AME seem to be less toxic to mice than TeA or ATX-I and II, although a mixture (1:1) of AOH and AME and AOH alone caused fetotoxic and teratogenic symptoms in mice and were toxic to *Bacillus mycoides*. Bioassays showed AME was weakly mutagenic to *Salmonella typhimurium* (Scott and Stoltz, 1980) and to a greater extent to *Escherichia coli* (An et al., 1989), but no evidence of toxicity was detected in rats or chickens fed with AME (24 µg/g), AOH (39 µg/g) and ALT (10 µg/g) for 21 days (Sauer et al., 1978). Intraperitoneal injections of ALT were mildly toxic to mice (Pero et al., 1973).

Although *Alternaria* spp. produce altertoxins in low concentrations relative to other *Alternaria* mycotoxins, they are still regarded as important (Schade and King, 1984). Scott and Stoltz (1980) reported ATX-I and a yellow pigment that may have been ATX-II were strongly mutagenic to *S. typhimurium*. ATX-III is more mutagenic than ATX-II and ATX-I to *S. typhimurium*, but ATX-III and to a lesser degree ATX-II were unstable in the testing solvent (dimethylsulphoxide) and rapidly broke down to purple coloured compounds (Stack and Prival, 1986).

Tenuazonic acid is often considered to be the most toxic of the *Alternaria* toxins (Meronuck et al., 1972; Harvan and Pero, 1976; Schade and King, 1984). The tetramic acid has antiviral, antibiotic and antitumour qualities (Miller et al., 1963; Gitterman, 1965), can interfere with protein synthesis in mammalian tissue (Shigeura and Gordon, 1963; Carrasco and Vazquez, 1972) and is said to be connected with Onyalai, an acute haematologic disorder among black African
populations south of the Sahara (Rabie et al., 1975; Steyn and Rabie, 1976). TeA has insecticidal activity (Umetsu et al., 1974) and is also regarded as a non-selective phytotoxin (Nishimura and Kohmoto, 1983), causing tobacco brown spot (Mikami et al., 1971) and stunting in rice, mung bean, radish, turnip and oilseed rape seedlings (Umetsu et al., 1974B; Visconti et al., 1992).

1.2.4 Production

For evaluating the mycotoxigenic potential of isolates of Alternaria spp, most workers utilise unsupplemented milled rice under optimum moisture (35-50%) and temperature (20-25°C) conditions (Bruce et al., 1984; Wei and Swartz, 1985; Mortimer et al., 1988; Logrieco et al., 1990; Visconti et al., 1992). Production of toxins by Alternaria spp is greater on rice than on other solid substrates, including wheat, rye, oats, maize, barley, olives, sorghum, tomato and rapeseed (Burroughs et al., 1976; Visconti et al., 1986, 1987; Mortimer et al., 1988; Logrieco et al., 1990; Kostecki et al., 1991; Visconti et al., 1992). Addition of supplements (e.g. yeast extract) to the rice may reduce metabolite production (Burroughs et al., 1976).

Liquid medium is also favourable for production of Alternaria metabolites. An isolate of A. tenuissima (Kunze ex Pers.) Wiltshire grown at 20°C in 2% yeast extract-4% sucrose broth (YES) attained maximum production of TeA (10.2 mg/100 ml) at 42 days (Young et al., 1980). Wei and Swartz (1985) discovered greater toxin production (ALT, AOH and AME) in semi-synthetic broth* (23.9, 535 and 42.4 µg/100 ml respectively) as apposed to synthetic broth** (5, 113

* glucose 50 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 3.0 g, KH<sub>2</sub>PO<sub>4</sub> 10.0 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 2.0 g, Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O 0.7 mg, (NH<sub>4</sub>)MO<sub>2</sub>O<sub>4</sub>·4H<sub>2</sub>O 0.5 mg, Fe(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O 10.0 mg, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.3 mg, MnSO<sub>4</sub>·H<sub>2</sub>O 0.11 mg and ZnSO<sub>4</sub>·7H<sub>2</sub>O 17.6 mg per litre water

** glucose 40 g, yeast extract 1.0 g, KH<sub>2</sub>PO<sub>4</sub> 1.0 g, NaNO<sub>3</sub> 1.0 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5 g, NH<sub>4</sub>Cl 0.25 g, KCl 0.25 g, NaCl 0.25 g, ZnSO<sub>4</sub>·7H<sub>2</sub>O 0.01 g and Fe(SO<sub>4</sub>)<sub>2</sub>·7H<sub>2</sub>O 0.01 g per litre water
and 19 \( \mu g/100 \) ml respectively), although rice culture was generally more efficient for production of large quantities of toxin (ca. 25, 744 and 55 \( \mu g/g \) respectively). Rice was also found by Maas et al. (1981) to be a better substrate for production of *Alternaria* toxins than liquid media, such as modified Czapek Dox broth.

1.2.5 Extraction

Extraction of *Alternaria* toxins is accomplished by blending or shaking the culture with a single or mixed solvent system. Blending with a miscible (polar) solvent, usually methanol (MeOH) or acetone, is preferable as exact aliquots can be taken from the resulting slurry and used as a technique for reducing sample size (Velasco and Morris, 1976; Coker, 1984). If extraction of TeA is required with the dibenzopyrones and perylenequinones, the first extraction may be made after acidifying the culture medium to pH 2. This is approximately two units below the pKa (3.5) of the analyte, thus suppressing the ionic form of TeA. Subsequent solvent extractions may include ethyl acetate, benzene, chloroform and methylene chloride (King and Schade, 1984).

After filtering, the extract requires cleaning up to eliminate unwanted materials which can hamper the purification and quantification of *Alternaria* mycotoxins. Such clean-up procedures may include liquid-liquid partitioning, recrystallisation, chromatography and bonded phase cartridges.

1.2.5.1 Liquid-liquid partitioning

Liquid-liquid partitioning between an aqueous and organic phase may be a useful procedure for:

(i) defatting (e.g. with hexane or n-heptane), which removes undesirable fats (Burroughs et al., 1976; Kostecki et al., 1991), although this may simultaneously allow partial (29%) extraction of AME (Seitz et al., 1975A; Seitz and Mohr, 1976).
(ii) chemical adsorption (e.g. addition of 20% ammonium sulphate), which permits precipitation of solids in a colloidal form (Magan et al., 1984).

(iii) the isolation of TeA, sometimes referred to as a sodium bicarbonate purification. This approach generally consists of extracting TeA into aqueous 5% sodium bicarbonate, acidifying to pH 2.0 and re-extracting the TeA with the same or different organic solvent (Visconti et al., 1987).

1.2.5.2 Recrystallisation

Purification by recrystallisation is feasible if sufficient amounts of toxins are extracted. AME and AOH crystallise from ethanol-water combinations, ALT from cold acetone-hexane combinations (Pero et al., 1971A; 1971B) and the copper salt of TeA is formed by adding Cu(ii) acetate to aqueous MeOH solutions containing TeA (Harwig et al., 1979). Divalent metal ions such as Cu$^{2+}$ form covalent complexes with TeA which provide a stable form of storage for the metabolite (e.g. copper tenuazonate) (Lebrun et al., 1985). On standing TeA for a long duration, heating or treatment with a base, the optical activity of TeA is lost and crystallisation may occur; this is attributed to the formation of iso-TeA (Stickings, 1959).

1.2.5.3 Chromatography

The adsorbents used in chromatographic clean-up include polystyrene based resins, silica, florisil, alumina and kieselguhr.

Flash (macro-column) chromatography has been successfully used in purifying Alternaria toxins but can be time consuming and is more suitable for preparation of large (mg) quantities (Seitz, 1984; Stack and Prival, 1986).

When amounts of Alternaria metabolites are limited, thin layer chromatography (TLC) facilitates sample clean-up, either as a separate stage or part of a quantification procedure. After developing with a suitable solvent system (see reference below), bands of interest are scraped from the plate and
eluted with a solvent (e.g. MeOH) to strip the metabolite from the silica. Harwig et al. (1979) used preparative TLC with toluene-ethyl acetate-formic acid (TEF) (5:4:1) to partially purify AOH and AME from ethyl acetate extracts of cultures of A. alternata.

High performance liquid chromatography (HPLC) is an effective procedure for obtaining large quantities of pure Alternaria toxins, either using semi-preparative or preparative columns (Chu, 1981; Chu and Bennet, 1981).

1.2.5.4 Bonded phase cartridges

Bonded phase cartridges are becoming increasingly favoured for the detection of mycotoxins because they allow rapid and stable purification, and offer an extensive range of interactions for analyte selectivity, owing to the distinct functional groups that can be covalently bonded to the silica. The chromatographic processes available are:

(i) non-polar or reverse phase (e.g. octadecyl - C18), where the retention is facilitated by dissolving the compound in a polar solvent (e.g. water/MeOH mixture) and followed by elution by non-polar solvents. Non-polar adsorption is attributable to Van de Waal's interactions between the carbon/hydrogen bonds of the compound and the operative group.

(ii) polar or normal phase (e.g. diol - 2OH), which is utilised when extracting from organic or non-polar solvents. Polar adsorption results from higher energy interactions, such as hydrogen bonding and dipole-dipole

(iii) ion-exchange (e.g. trimethylaminopropyl - SAX), which is used to remove ionic compounds from aqueous solutions. Ion-exchange adsorption results from the dipolar association between ions of opposite charge.

Although cation exchange resin (e.g. Dowex 50W-X8) minicolumns have been employed for the conversion of copper tenuazonate into TeA (Scott and
Kanhere, 1980; Stack et al., 1985), there are no reports of solid phase procedures for cleaning up culture extracts of *Alternaria* spp. However, Bradburn (1993) has recently investigated bonded phase methods for isolating *Alternaria* toxin standards from rapeseed samples. Using aqueous MeOH extracts of rapeseed spiked with ALT, AOH and AME, he found adequate retention of the metabolites with normal and reverse phase, but with insufficient clean-up. The major problem with this method was an unknown interference specific to AOH which was eventually eluted with AME after passing chloroform through a phenyl cartridge. Secondary elution of AOH and ALT was accomplished using diethyl ether. Good recovery (91.2%) of TeA was obtained from spiked, aqueous MeOH rapeseed extracts (100 μg/g) using a phenyl/diol bonded phase method.

Immunoaffinity columns, which have been used to isolate aflatoxins (Candlish et al., 1991), are not available for *Alternaria* toxins.

### 1.2.6 Quantification

There are several methods available for the quantitative analysis of *Alternaria* toxins, namely gas liquid chromatography (GLC), TLC and HPLC. While GLC has been used with some success to separate ALT, AOH and AME (Pero et al., 1971; Pero and Harvan, 1973) and to analyse TeA quantitatively (Sauer et al., 1978; Harwig et al., 1979; Scott and Kanhere, 1980), TLC and HPLC are preferred as they are usually more rapid and do not require preparation of silyl derivatives.

#### 1.2.6.1 Thin layer chromatography

Solvent systems employed for TLC analysis of *Alternaria* toxins are presented in Table 1.3. The most popular solvent systems were TEF, 5:4:1 or 6:3:1, probably because they gave the best separation.
<table>
<thead>
<tr>
<th>SOLVENT SYSTEM</th>
<th>ALT</th>
<th>AOH</th>
<th>AME</th>
<th>ATX-I</th>
<th>ATX-II</th>
<th>TeA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>i</td>
<td>0.14</td>
<td>0.21</td>
<td>0.39</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>ii</td>
<td>0.13</td>
<td>0.19</td>
<td>0.54</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>iii</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
<td>NS</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>iv</td>
<td>0.29</td>
<td>0.32</td>
<td>0.71</td>
<td>0.20</td>
<td>NS</td>
<td>C, D</td>
<td></td>
</tr>
<tr>
<td>v</td>
<td>0.10</td>
<td>0.39</td>
<td>0.65</td>
<td>-</td>
<td>-</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>vi</td>
<td>0.20</td>
<td>0.44</td>
<td>0.39</td>
<td>0.34</td>
<td>-</td>
<td>0.29</td>
<td>G</td>
</tr>
<tr>
<td>vii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5-0.6</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>viii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5-0.6</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>ix</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>F</td>
</tr>
<tr>
<td>iv</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>iv</td>
<td>0.33</td>
<td>0.65</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>I</td>
</tr>
<tr>
<td>v</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>K</td>
<td></td>
</tr>
<tr>
<td>v</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>v</td>
<td>0.33</td>
<td>0.54</td>
<td>0.74</td>
<td>0.43</td>
<td>0.48</td>
<td>-</td>
<td>T</td>
</tr>
<tr>
<td>viii</td>
<td>0.15</td>
<td>0.32</td>
<td>0.78</td>
<td>0.28</td>
<td>-</td>
<td>0.10</td>
<td>G</td>
</tr>
<tr>
<td>viii</td>
<td>-</td>
<td>0.41</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>ix</td>
<td>0.11</td>
<td>0.54</td>
<td>0.72</td>
<td>-</td>
<td>-</td>
<td>X</td>
<td></td>
</tr>
</tbody>
</table>

NS = not stated
- = not investigated

i = benzene-ethanol (95:5)
ii = chloroform-methanol (95:5)
iii = chloroform-acetone (88:12)
iv = toluene-ethyl acetate-formic acid (6:3:1)
v = toluene-ethyl acetate-formic acid (5:4:1)
vi = benzene-methanol-acetic acid (90:5:5)
vii = chloroform-ethanol-ethyl acetate (90:5:5)
viii = dichloromethane-methanol-water (53:3:3)
ix = tetrahydrofuran-benzene (20:80)
x = toluene-tetrahydrofuran-acetic acid (75:20:5)

A = Perp et al., 1971
B = Reib, 1983;
C = Visconti et al., 1986; Logrieco et al., 1990
D = Visconti et al., 1992
E = Seitz et al., 1975
F = Vinas et al., 1992
G = Grabarkiewicz-Szczesna et al., 1989
H = Young et al., 1980
I = Davis et al., 1977
J = Durackova et al., 1976
K = Bradburn, 1993
L = Wei and Swartz, 1985;
M = Maas et al., 1981;
S = Scott and Stoltz, 1980.
T = Chu, 1981
U = Bruce et al., 1984;
V = Stack and Prival, 1986;
W = An et al., 1989
X = Thomas, 1961

Table 1.3 TLC of Alternaria mycotoxins
Several of the *Alternaria* metabolites fluoresce under long (\(\lambda364\) nm) wave UV radiation, the alternariols, AOH and AME, appear as blue and ALT as yellow fluorescent spots on \(F_{254}\) TLC plates (Schade and King, 1984; Stack and Prival, 1986; Visconti *et al.*, 1986). The minimum amount of these compounds detectable under UV light is reported as 0.1 \(\mu g\) (Pero *et al.*, 1971; Visconti *et al.*, 1986). Fluorescence of dibenzopyrones may be enhanced on spraying with ethanolic aluminium chloride (Grabarkiewicz-Szczesna *et al.*, 1989) or 50% ethanolic sulphuric acid spray followed by heating at 100°C for five minutes (Schroeder and Cole, 1977).

ATX-I exposed to long wave UV radiation radiates as a yellow spot on TLC plates (Visconti *et al.*, 1986). Although numerous workers report ATX-II as a fluorescent yellow-orange spot (Scott and Stolz, 1980; Chu, 1981; Scott and Stolz, 1980), Pero *et al.* (1973) claimed the metabolite to be light quenching (black) under short wave (\(\lambda254\) nm) UV light.

TeA is not fluorescent under long or short wave UV radiation but small quantities (0.5 \(\mu g\)) may be detected by its quenching of fluorescence of \(F_{254}\) TLC plates under short wave UV light (Scott and Kanhere, 1980). The compound fluoresces green under long wave UV light on spraying with acidic anisaldehyde and heating at 130°C for 5-10 minutes (Scott and Kanhere, 1980).

Various spray reagents may be used to detect *Alternaria* metabolites on TLC plates. Exposure to iodine causes ALT, AOH and AME to appear as dark, brown spots, and spraying with sulphanilic acid colours ALT yellow, AOH cherry-red and AME wine-coloured (Pero *et al.*, 1971). ATX-I and ATX-II are distinguished as purple-green spots when sprayed with ferric chloride in MeOH and reddish-brown spots when sprayed with 2,4-dinitrophenyl chloride in ethanol (Chu, 1981).

Although TeA is visible as a tan spot, several sprays enhance the sensitivity of detection under visible light, including ethanolic ferric chloride.
(appears orange-red-brown) (Rosett et al., 1957; Steyn and Rabie, 1976; Davis et al., 1977), 2,4-dinitrophenyl hydrazine (yellow) and 1% cerium sulphate in 6N sulphuric acid (grey-brown) (Steyn and Rabie, 1976). Thin layer chromatograms of *Alternaria* toxins have been evaluated quantitatively by measuring fluorescence with densitometers. Haggerblom and Unestam (1979) demonstrated that AOH and AME display a linear correlation between the fluorescence peak heights and toxin quantity between 0.1 and 1.0 \( \mu g \). Bradburn (1993) using high performance TLC (HPTLC) techniques established the maxima for excitation of the mycotoxins was: ALT, 270-280 nm; AOH and AME, 290-300 nm. After phenyl bonded phase clean up of aqueous MeOH extracts of rapeseed spiked with the metabolites, the limits of detection of ALT, AOH and AME were 17, 12.2 and 17.9 \( \mu g/g \) respectively.

HPTLC is superior to TLC because the stationary phase consists of smaller and more uniform particles, development is over shorter distances, allowing speedier separation, lower detection limits and less solvent consumption (Bradburn, 1993). However, in comparison with HPLC, the separation efficiency of HPTLC is inferior. For instance, the number of theoretical plates on a 20 cm HPLC analytical column is 10 000-15 000 whereas it is 5 000 for a 10 cm HPTLC (Fried and Sherma, 1986).

1.2.6.2 High Performance Liquid Chromatography

The HPLC solvent systems used for quantifying *Alternaria* toxins are presented in Table 1.4. There is no solvent system which separates ALT, AOH, AME, TeA and at least one altertoxin during a single analysis. Palmisano et al. (1989) developed a system which separated five metabolites (ALT, AOH, AME, ATX-I, ATX-II) but this had the disadvantages that it did not include TeA, which is generally regarded as the most important *Alternaria* toxin, owing to its high toxicity and production (King and Schade, 1984), and the mobile phase consisted of a series of gradients.
<table>
<thead>
<tr>
<th>COLUMN</th>
<th>MOBILE PHASE</th>
<th>ALT</th>
<th>AOH</th>
<th>AME</th>
<th>ATX-I</th>
<th>ATX-II</th>
<th>TeA</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP18</td>
<td>i</td>
<td>-</td>
<td>4.25</td>
<td>7.60</td>
<td>-</td>
<td>-</td>
<td>6.90</td>
<td>A</td>
</tr>
<tr>
<td>RP18</td>
<td>ii</td>
<td>5.52</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>A</td>
</tr>
<tr>
<td>C18</td>
<td>iii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.30</td>
<td>B</td>
</tr>
<tr>
<td>C18</td>
<td>iii</td>
<td>-</td>
<td>5.60</td>
<td>7.50</td>
<td>-</td>
<td>-</td>
<td>6.30</td>
<td>C</td>
</tr>
<tr>
<td>silica</td>
<td>iv</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.50</td>
<td>3.00</td>
<td>-</td>
<td>B</td>
</tr>
<tr>
<td>C18</td>
<td>v</td>
<td>-</td>
<td>3.11</td>
<td>5.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>silica</td>
<td>vi</td>
<td>15.8</td>
<td>5.60</td>
<td>7.50</td>
<td>-</td>
<td>-</td>
<td>3.30</td>
<td>E</td>
</tr>
<tr>
<td>C18</td>
<td>vii</td>
<td>-</td>
<td>c.a.5</td>
<td>c.a.4</td>
<td>c.a.7</td>
<td>-</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>C18</td>
<td>vili</td>
<td>5.80</td>
<td>10.8</td>
<td>15.3</td>
<td>9.1</td>
<td>12.4</td>
<td>-</td>
<td>G</td>
</tr>
<tr>
<td>C18</td>
<td>ix</td>
<td>9.3</td>
<td>12.1</td>
<td>17.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>H</td>
</tr>
<tr>
<td>C18</td>
<td>x</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.90</td>
<td>H</td>
</tr>
<tr>
<td>C18</td>
<td>xi</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>I</td>
</tr>
<tr>
<td>anion exchange ligand</td>
<td>xii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.2*</td>
<td>J</td>
</tr>
<tr>
<td>exchange ion pair</td>
<td>xiii</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5.8*</td>
<td>J</td>
</tr>
<tr>
<td></td>
<td>xiv</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.8*</td>
<td>J</td>
</tr>
</tbody>
</table>

NS = not stated

* = k'

i = MeOH-water (80:20) containing 300 mg zinc sulphate
ii = MeOH-water (60:40) containing 300 mg zinc sulphate
iii = MeOH-water (85:15) containing 300 mg zinc sulphate
iv = chloroform-MeOH-acetic acid (98:1:1)
v = MeOH-water-tetrahydrofuran (65:30:5)
vi = chloroform-MeOH (95:5)
vii = 60% MeOH in water containing 0.1 M sodium nitrate, 1 mM nitric acid
viii = MeOH-aqueous phosphoric acid (pH 3) (50 to 70% MeOH in 10 min., 70 to 85% in 0.1 min., 85% for 8 min.)
ix = acetone-water (65:35)
x = MeOH-water (90:10)
xii = 70% MeOH in water containing ammonium acetate (0.2 M) pH 5.9
xiii = 5% ACN in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM)
xiv = 75% MeOH in ammonium acetate buffer (30 mM, pH 6), d_{12}-dien (5 mM), zinc sulphate (5 mM)
xiv = 55% MeOH in phosphate buffer (10 mM, pH 6), ethylenediamine (1 mM), cetrimide (5 mM)

A = Visconti et al., 1986;
B = Stack and Prival, 1986;
C = Stack et al., 1985
D = Maas et al., 1981
E = Ozcelik et al., 1990
F = Visconti et al., 1991
G = Palmisano et al., 1989
H = Heisler et al., 1980
I = Magan et al., 1984
J = Lebrun et al., 1989

Table 1.4 HPLC of Alternaria mycotoxins
Although gradient analysis is advantageous for uniformly separating compounds which are spread over a wide range of polarity, the compatibility of the solvent mixtures with the detector, or miscibility with the sample solution must be considered. To illustrate, the baseline will drift if the UV absorbance of the solvents differ (e.g. water/MeOH). In addition, it is important to run blank gradients to determine contaminating artefacts (e.g. plasticisers), which can accumulate during gradient analyses, as well as to re-equilibrate the column before loading the next sample, by pumping through approximately five column volumes of the initial solvent (Lindsay, 1992). Palmisano et al. (1989) stated that in their gradient system the equilibrium period between two successive runs was 8 minutes. Ozcelik et al. (1990) developed an isocratic system (see Table 1.4) which separated the dibenzopyryrones, together with TeA on a normal phase (silica) column (Microsil, 7.5 μm; 5.0X300 mm). However, normal phase chromatography has several disadvantages as opposed to reverse phase chromatography; it uses more expensive solvents, equilibration of the mobile phase is longer and separation is generally slower and less reproducible.

Ion suppression chromatography of TeA using a reverse phase column (e.g. C18) (Heisler et al., 1980) is more useful than anion exchange chromatography (Lebrun et al., 1989) because it has additional application in separating non-ionic solutes (e.g. AME). Inclusion of a divalent metal complexant such as Zn²⁺ to the mobile phase may improve the peak efficiency of TeA with ion suppression chromatography (Stack et al., 1985).

Ion pairing and ligand-exchange chromatographic systems were also shown by Lebrun et al. (1989) to allow efficient quantification of TeA in rice leaves infected by Pyricularia oryzae.

The problem of developing an isocratic HPLC method usually involves attaining an acceptable degree of separation between all the components of interest in a stipulated (or minimum) time. Although a extensive range of
columns with vastly different selectivities has been developed, it is the composition of the mobile phase which is the most influential variable for the majority of separations. This is especially the case with an isocratic quaternary solvent mixture system, which can often replace gradients in separating complex samples.

1.3 SEPARATION OF COMPOUNDS USING HPLC SOLVENT OPTIMISATION

It has been established that no isocratic HPLC solvent system separates the *Alternaria* mycotoxins ALT, ATX-I, AOH, TeA and AME. One customary method in approaching this problem would be to perform numerous experiments and then select the best. However, this is usually time consuming as a vast number of experiments may be required. A superior strategy to separate these compounds is to adopt solvent optimisation techniques.

The relative distribution of a solute between a stationary and mobile phase is decided by the interactions of the solute species with each phase. The relative strength of these interactions is dependent on the intermolecular forces or polarity of the sample and that of the two phases.

Eluents are arranged according to polarity, the strong ones being polar (e.g. water) and the weak ones non-polar (e.g. hexane). The retention of solutes is usually altered by changing mobile phase polarity.

The relative polarity of solvents can be indexed numerically, either using the solubility parameter (δ), solvatochromic polarity measurement or polarity index (P') (Lindsay, 1992). An improved model of P' is the Synder classification, where each solvent is allocated three selectivity parameters; proton acceptor (xₐ), proton donor (x₈) and strong dipole (x₉) (Snyder, 1978). The classification segregates solvents (81 were studied) into eight categories derived on homogeneity of the x parameters. Solvents with similar overall polarity (as indexed by P') will now be presumed to show analogous behaviour only if they

42
are in the same group. Therefore, when selecting a mobile phase, the solvents should be from groups having selectivity differences that are as far distant from each other as possible on the triangle (Fig. 1.3). However, the actual solvents available are limited by additional properties, such as solubility, viscosity, UV cut-off, volatility and toxicity. In an isocratic reverse phase quaternary system, the solvents which offer the best selectivity, while at the same time give acceptable compatibility, include MeOH, acetonitrile (ACN) and tetrahydrofuran (THF), with water (or buffer) to regulate the polarity (see Fig. 1.3).

A quaternary solvent system may be best represented as a tetrahedron (Fig. 1.4). Each corner of the tetrahedron portrays a single solvent, the edges represent the binary mixtures and each face is one of the four possible ternaries. If a suitable solvent mixture for separation of the compounds of interest exists, it will be located within the volume described by the tetrahedron.

A number of approaches have been developed for determining the optimum mobile phase, namely sequential, predictive and iterative methods (Lindsay, 1992).

1.3.1 Sequential methods

Although there are several sequential procedures for locating an experimental optimum, most are based on the sequential simplex rule, devised by Spendley et al. (1962). The simplex itself is a geometric design with one more vertex than the number of variables being examined. To illustrate, two variables are represented by a triangle and three variables by a tetrahedron, with each vertex describing a set of operating parameters as described previously. More than three sets of operating parameters can be examined but the geometric design occupies more than three dimensions.
Fig. 1.3  Solvent selectivity triangle. Methanol (CH$_3$OH), acetonitrile (CH$_3$CN) and tetrahydrofuran (THF) are represented in groups II, VI and III respectively (Lindsay, 1992)
Fig. 1.4 Quaternary solvent mixtures, showing the isoeeluotropic plane
Optimisation is initialised by running chromatograms at each of these sets of parameters and the quality of separation is evaluated for each set. The set giving the least desirable response is eliminated and is replaced by reflecting the simplex across the plane of the remaining vertices. Repeated application of the procedure advances to the set of operating parameters which provide optimal separation.

For diagrammatic purposes, a standard progression of a 2-variable simplex toward the optimum is described in Fig. 1.5. From the initial experimental conditions designated by points A, B and C, point B had the lowest response, which was then discarded and the simplex reflected to point D. The response at point D was monitored. Point A had the least desirable result and the triangle reflected again, this time to point E. The simplex continued in the direction of the region containing the optimum separation (O).

Near the optimum, reflection of the simplex will occasionally produce a simplex which had previously been appraised, such as MLN, with N giving the worst outcome. Rather than dismissing N and reflecting back to point K, the second worst result (L) was deleted and the triangle reflected towards P. This approach was repeated until R was reached, after which no new computations were proposed by eliminating either the worst or second worst result.

Application of a two variable simplex procedure to a ternary solvent system is relatively simple as two of the three volume proportions in a solvent mixture correspond to the two variables. The third proportion is calculated from the total difference of these two proportions from one.

Modifications to the standard simplex method, including expansion and contraction, are used in addition to reflection to quicken and adapt transfers to a particular response curve (Deming and Parker, 1978).
Fig. 1.5  Standard progression of a two-variable simplex optimisation towards the optimum (O) (see text). The dotted lines depict contours of equal separation quality (Lindsay, 1992)
Although the sequential simplex method is simple and does not use traditional testing of significance, it may run into several problems. A large number of experiments is usually required, and transfers may be misdirected to regions which produce good separation (local optima), in preference to the region of best separation (the global optimum). A global optimum is presumed if simplex transfers originating from different areas centre on the same point, but this further escalates the number of experiments (Lindsay, 1992).

Detection of all analytes is important in the simplex procedure, as also validating peak uniformity. Failure to include all analytes in the response function and to distinguish concealed peaks may result in an incorrect optimum (Wright, 1990).

1.3.2 Predictive methods

While it is possible to collect data to model retention behaviour throughout a whole factor space i.e. the space delimited by the vertices (see 1.3.1), this is generally of limited use because compositions in the region of the water corner would lead to extended analysis times, whereas compositions in the organic solvent corner regions would result in peaks eluting near the solvent front, with inadequate resolution (Wright, 1990).

The initial strategy of predictive solvent optimisation methods is to reduce the factor space to, in the case of a quaternary solvent system, an isoeluotropic plane, which joins points of equal solvent polarity (see Fig. 1.4). With this approach, the number of variables is reduced to two as any point on the plane can be created by combining suitable proportions of the three corner eluents. The corners (i.e. MeOH/water, ACN/water and THF/water) correspond to the binary isoeluotropic mixtures, and any point on this plane will be a quaternary composition isoeluotropic with the binaries. The position of the corners governs the amount of water present, thus eliminating the third variable.
In application, substitution of a mobile phase on a isoeluotropic plane will result in roughly the same analysis time, but the capacity factor ($k'\ast$) of individual components of the solvent mixture will alter, due to differences in the polarity parameters discussed previously.

The most effective way for identifying the isoeluotropic plane is to run a gradient on the sample using 0-100% MeOH/water. From this it is feasible to estimate an isocratic MeOH/water mixture that will elute all analytes within an acceptable $k'$ range between 1 and 10 (Schoenmakers et al., 1981). As this strategy is derived from hypothetical, ideal solutes, this mixture may require a small amount of fine-tuning. The two other isoeluotropic corners (ACN/water and THF/water) are then calculated using imperial transfer rules. Again, these transfer rules are based on theoretical solutes and limited alterations may be needed (Lindsay, 1990).

Once the isoeluotropic plane is found, further chromatograms are run on various points on the plane. From these results, the retention behaviour of individual solutes is modelled by a number of mathematical functions, resulting in a map of retention (retention surface) for each solute over the entire surface of the plane.

One of the most widely used mathematical expressions to calculate a retention surface is the special cubic function, requiring a minimum of seven experimental points. More complex mathematical functions can be employed but these require more points (e.g. a full cubic function needs ten points) (Wright, 1990).

* $k'$ represents the elution of analytes relative to an unretained solute
From the retention surface of each solute, the response surface is created, which describes the chromatographic quality of solutes across the isoeluotropic plane. Overlapping resolution mapping techniques, which utilises the triangular response surface and special cubic function, can conveniently present the response surface as a trilateral contour map. Although this technique does not locate an exact global optimum, it will however indicate regions of different chromatographic quality (Schoenmakers, 1987; Lynch and Measures, 1990).

### 1.3.3 Iterative methods

Predictive optimisation methods often suffer model inaccuracy, whereas sequential optimisation can miss the global optimum. One approach which aims to address both of these limitations is the iterative design (Schoenmakers et al., 1982; Lindsay, 1992).

Iterative methods have been applied to optimise HPLC separations with respect to one or two variables. They involve a basic model conformed to a couple of experimental points to predict an optimum composition, which is then evaluated. If the proposed optimum is unacceptable, the results of the test are employed to improve the model and a true optimum is located.

In the case of a ternary solvent system, a gradient is used to predict the two binary isoeluotropic eluents of each modifier with an aqueous phase. This in turn reduces the number of variables to one. A separation is run in the two eluents to determine the $k'$ values for each component in both chromatograms. A model presuming a linear relationship of $\log k'$ with volume proportion is plotted. Using this assumption, a phase selection diagram is then constructed where a line is drawn connecting the experimental $\log k'$ values for each solute to predict chromatographic quality over the range of mobile phase composition. The response factor employed here ($\pi R$) is the product of all resolution values between adjacent peaks in the chromatogram. An ideal separation approaches one whereas a complete coelution of peaks equals zero.
Therefore, a maximum response on the plot coincides with optimum separation of peaks. A solvent mixture is run under these stipulated optimum conditions and the chromatographic quality is reassessed. If the actual and predicted values differ to an extent, the new $k'$ data is used to update the $nR_s$. This iterative procedure is repeated until there are no further improvements in peak separation.

For two variable optimisation, planar models are adapted to the data. However, a larger number of iterations are required and computation of the response curve takes considerable time (Wright, 1990).

### 1.3.4 Philips PU6100 solvent optimisation software

A major disadvantage of both predictive and iterative optimisation methods is that the retention time (RT) of each peak in each chromatogram must be known. As the proportions of the mobile phase solvents are varied the elution sequence of peaks may alter, or one or more peaks may coelute, or both. If hidden peaks are not recognised, this will lead to a failure in the response factor.

Although running standards at each mobile phase would establish total peak recognition, incorporation of multiple detection wavelengths and chemometric* methods (e.g. iterative target transformation factor analysis used in association with second derivative peak finding) have been found to be a practical method for the peak deconvolution and reconstruction of HPLC chromatograms and spectra (Naish et al., 1989).

* chemometrics describes the application of mathematics and statistical methods to chemistry (Lindsay, 1992)
The Philips PU6100 optimisation package is based upon predictive methods using the triangular isoeutropic plane. Ten experimental points are analysed using the experimental design exercised for a full cubic function and recognition of the peaks is by both spectra and total peak volume integral. Overlapping constituents are handled by implementing chemometric deconvolution procedures (Lynch and Measures, 1990).

1.4 APPLICATION OF RANDOM AMPLIFIED POLYMORPHIC DNA FOR GENETIC MAPPING OF FILAMENTOUS MOULDS

Polymorphic genetic markers have potential application in molecular plant pathology as means for identifying plant pathogenic fungi (e.g. *Mycosphaerella* spp. on banana; Johanson and Jeger, 1993) and segregating isolates of moulds in relation to a particular character, such as geographic origin (e.g. *Rhizoctonia solani* Kuhn. in Australia; Duncan et al., 1993). One established method for constructing polymorphic maps is the use of restriction fragment length polymorphism (RFLP) patterns on agarose gels. This involves using restriction endonuclease enzymes (e.g. *Eco RI*) that recognise specific nucleotide sequences and cleave the template DNA at, or adjacent to, these sites (Beckmann and Soller, 1983). As differently sized DNA fragments are normally produced, subsequent electrophoresis of DNA on an agarose gel results in a continuous smear unless many copies of repetitive DNA are present. Therefore, individual fragments are distinguished by using a suitable probe which is a cloned DNA sequence homologous to a specific DNA segment (or some part of it). This is achieved by capillary blotting the DNA profile from the gel to a solid support, such as a nitrocellulose or nylon filter (i.e. southern blotting; Southern, 1975), and exposure to, for example, a radioactively labelled probe under conditions which promote DNA-DNA hybridisation. Afterwards, unhybridised DNA segments are washed away and the remaining radioactively labelled DNA fragments are then detected by autoradiography.

A more recently introduced method which also reveals polymorphic DNA but has several advantages over proven RFLP methodologies is the random
amplified polymorphic DNA assay (RAPD). The RAPD technique is easier and more rapid than RFLP in generating polymorphic DNA. There is no need for specific probes or DNA libraries, analysis requires very small quantities of template DNA (15-25 ng) and most significantly, no radio-isotopes are required, thus giving a reduction in cost and increase in safety (Rafalski et al., 1991; Foster et al., 1993). The RAPD marker technique is based on the polymerase chain reaction (PCR).

1.4.1 The polymerase chain reaction

The PCR is a newly developed molecular biology technique which permits enzymatic amplification of DNA or RNA. The methodology of PCR consists essentially of three steps; denaturation of the double-stranded DNA, annealing of oligonucleotides to the denatured DNA strands and primer extension by a thermostable DNA polymerase enzyme (Taq polymerase) (Saiki et al., 1988; Remick et al., 1990).

In the first stage of PCR, template DNA is denatured by increasing the temperature to approximately 94°C. The second stage entails lowering the temperature (typically to 35-50°C) to permit hybridisation of primers on opposite strands of the DNA, which flank the segment of the DNA to be amplified. Since the primers have different, non-complementary sequences, they will not anneal to each other but to opposite DNA strands only. Surplus oligonucleotide in the reaction mixture promotes primer-DNA hybridisation, as opposed to re-coupling of template DNA. The last stage of the cycle is the synthesis of complementary DNA (at 72°C), where Taq polymerase binds to the DNA-primer complex and then proceeds to extend the DNA in the 5' to 3' direction.

After the first cycle of PCR, the resulting portion of DNA amplified is referred to as the long product. With subsequent cycles, the Taq polymerase halts at the previous enzyme inception site on the long product and produces a slightly smaller DNA fragment, designated as the short product. It is the short product that then doubles and generates exponentially on further amplification.

53
The long product also continues to be generated but only at one copy per cycle because only the two original single strands of DNA are capable in producing the long product.

In a typical PCR analysis, 30-45 cycles are performed in an automated heating block. As Taq polymerase can withstand high incubation temperatures (up to 98°C), it does not require to be replaced after each cycle of the amplification process. Amplification eventually results in sufficient amounts of DNA to give a band on an agarose gel when visualised by staining with ethidium bromide.

For amplification of specific DNA fragments, some information about the template DNA sequence is required so that synthetic oligonucleotide primers can be selected. However, no prior sequence knowledge is required for polymorphic DNA markers, since they are generated arbitrarily.

1.4.2 Analysis by RAPD

A novel assay for determining DNA polymorphisms by PCR was described simultaneously in two independent studies. Williams et al. (1990), while characterising genetic mapping applications, denoted the new method as the RAPD assay. Welsh and McClelland (1990), who focussed on genome fingerprinting, called their assay arbitrarily-primed PCR (AP-PCR). Both procedures are based upon the principle that a single, short oligonucleotide of randomly chosen DNA sequence (usually 10 nucleotides long; ≥50% GC content), when combined with genomic DNA and Taq polymerase, and subjected to thermal cycling of the PCR, will prime the amplification of DNA segments. The products are then simply run on an agarose gel and visualised by UV radiation following ethidium bromide staining. As a single base alteration in the primer sequence can generate completely different DNA fragment profiles, amplification of genomic DNA with arbitrary sequence primers is a highly sensitive procedure for detecting polymorphisms randomly scattered throughout the genome.
Many studies have shown RAPD analysis to be a useful tool for relating isolates of fungi to specific variables, including geographical origin, host type, pathogenic variability and mating type. For example, Duncan et al. (1993) discovered that RAPD patterns of isolates of R. solani in Australia were more variable among those originating from different geographical locations than among isolates from the same region. Vaillancourt and Hanua (1992) in their studies of Glomerella (Colletotrichum) spp. found that isolates from maize and sorghum, although morphologically identical, could easily be distinguished by RAPD analysis. Goodwin and Annis (1991) and Schafer and Wostemyer (1992) were able to differentiate between virulent and non-virulent pathotypes of Leptosphaeria maculans, the causal agent of blackleg of crucifers, using RAPDs. Guthrie et al. (1992) reported that some isolates of Colletotrichum graminicola have characteristic RAPD signatures based on geographical location. Van Der Vlugt-Bergmans et al. (1993) studied the segregation of molecular markers in the progeny between Italian strains known to contain the mating type alleles, MAT-1 and MAT-2, and eight Dutch strains of Botrytis cinera Pers.: Fr.. Analysis of the progeny by RAPDs revealed that both mating type alleles were present among three of the eight Dutch strains.

1.5 OBJECTIVES

Very little is known about the mycoflora of oilseed rape in Great Britain and its potential for the production of mycotoxins. Therefore, a survey was initiated to determine the principal fungal contaminant in oilseed rape at harvest, during storage and after conversion into meal. This was determined to be Alternaria spp. (see chapter 2) and subsequently, research primarily focused on the detection and isolation of mycotoxins produced by the genus i.e. ALT, ATX-I, AOH, TeA and AME. Specific research objectives included the following:

(i) development of an isocratic HPLC system for the detection and quantification of ALT, AOH, AME, TeA and ATX-I utilising solvent optimisation techniques
(ii) appraisal and confirmation of the biological activity of metabolites produced by *Alternaria* spp.

(iii) growth of *Alternaria* spp. and determination of their toxigenicity

(iv) isolation and characterisation of any new, toxic metabolites produced by *Alternaria* spp.

(v) examination of the genetic variation and its relationship to toxigenicity of *Alternaria* spp. using RAPDs generated by PCR.
CHAPTER 2.

APPRAISAL OF THE MYCOFLORA IN OILSEED RAPE

The ability of moulds to contaminate oilseed rape, together with their evidently important mycotoxigenic capacity was reviewed in chapter one. For example, Mills and Abramson (1986) demonstrated *Penicillium* spp. and *A. versicolor* as common contaminants in stored rapeseed in Canada, whereas Visconti et al. (1992) isolated *A. alternata* from oilseed rape grown in Italy. A number of these isolates produced mycotoxins in culture. Therefore, a survey was initiated in Great Britain to determine the predominant moulds colonising oilseed rape at harvest, during storage and after conversion into meal.

2.1 MATERIALS AND METHODS

2.1.1 Source of samples

2.1.1.1 At harvest

Within 2 months of the oilseed rape harvest (August-September, 1990), 55 samples of 00 cvs. of rapeseed were collected from lorries unloading seed at either Archer Daniels Midland (ADM) Erith Oil Works, London or Bibby Edible Oils Company Limited (BEOCO), Liverpool. Seed (ca. 1 kg) was acquired by suction through a 2-3 metre aluminium probe powered by a commercial vacuum cleaner. To ensure that representative samples were taken, the probe was inserted into different areas of the lorry load for roughly 1 minute.

Detailed histories of samples, including location of growth and cultivar type, were acquired from the lorry drivers or rapeseed merchants (e.g. United Oilseeds Marketing Limited, Devizes) who managed the seed. Most samples were traced back to locations in Oxfordshire, Berkshire, Cambridgeshire, Surrey, Norfolk, Essex and Kent. Samples taken from lorries which did not freight rapeseed from a single source (e.g. one silo) or directly convey seed to the crushing mills were disregarded.
2.1.1.2 During storage

Ten samples of rapeseed (ca. 1 kg) were collected at 2 months storage (September, 1990), then again at 5, 7 and 10 months from Camgrain Silo (holding capacity; 2000 tonnes), Wittleford, Cambridgeshire. Seed samples were collected either by implanting a 3m hollow rod directly into the stored seed or during collection by the lorries. The seed contained in Camgrain silo included the cvs. Lictor, Cobra, Libravo, Ariana, Score, Capricorn and Susanna.

2.1.1.3 After conversion into meal

In January, 1991, 20 samples of meal (ca. 500 g) were collected from Erith Oil Works generally within 3 days of oil extraction.

Upon receipt, rapeseed and meal samples were conveyed to UCL in clean, dry, plastic bags. Those samples not examined immediately were stored at 7°C.

2.1.2 Determination of moulds

2.1.2.1 Isolation

For rapeseed collected at harvest, 100 intact, visibly-mould free seeds of each sample were selected randomly for examination. Samples taken during storage were examined as 10 replicates, 50 seeds per replicate, for each specified storage period (i.e. 2, 5, 7 and 10 months).

Seeds were sterilised in 0.4% sodium hypochlorite (NaOCl) for 2 minutes to eliminate superficial microorganisms. Preliminary experiments showed lower concentrations of NaOCl allowed the rapid development of spreading mucoraceous moulds (e.g. Rhizopus spp.) which inhibited the slower growing fungi, such as Alternaria spp. The seeds were rinsed thoroughly in sterile water to remove residues of the sterilant.

After drying with sterile tissue, seeds were transferred aseptically to dichloran rose bengal chloramphenicol (DRBC) agar in 9cm diameter petri
dishes, 10 seeds per dish. Medium containing DRBC is reported to restrict spreading of mould colonies without affecting spore germination unduly (King et al., 1979). Plates were incubated for 4-5 days at 25°C on a light bench illuminated by four fluorescent tubes (12 hours photoperiod; 65/80W; Thorn, UK) and one near-ultra violet (UV) light (TLD 36W; Philips, UK).

Moulds isolated from meal samples were enumerated by sprinkling meal (0.2 g/plate) on DCRB medium and incubating under the same incubation regime used for plating seed. Samples which developed colonies that were not separated from each other were disregarded and repeated using a serial dilution method. Meal (0.1 g/plate) was suspended in 0.9ml sterile water and serial 10 fold dilutions were prepared. Aliquots (0.1ml) of the dilutions were spread on to DCRB plates.

2.1.2.2 Identification

Fungi were identified according to the nomenclature of Simmons (1967), Ellis (1971), Barnett and Hunter (1972), Pitt and Hocking (1985) and a laboratory manual edited by Dr John David, International Mycological Institute (IMI), Egham, UK. Moulds which were not categorised in the keys (e.g. P. hordei) were sent to IMI for further identification.

Identification of moulds was accomplished using a low-magnification stereo-microscope on the infected seed coat and surrounding DRBC medium or by inspecting a slide preparation made in 1% acid-fuscin using a high-magnification (X100-1000) light microscope. Moulds were then subcultured onto three types of media; malt extract agar (MEA), Czapek yeast agar (CYA) and 25% glycerol nitrate agar (G25N). Cultures on CYA and G25N were incubated at 25°C and MEA at 5°C, 25°C and 37°C for 7 days in the dark. Fungal species were recognized by macroscopic inspection of the colony (colour, diameter, mycelial texture) and by light microscopy (Pitt and Hocking, 1985).
Other media (e.g. CYA containing 20% sucrose - CY20S - after 14 days at 25°C) were required for confirmation of certain fungal species (e.g. Eurotium spp.). Moulds which were not identified at species level because of poor spore production were transferred to dichloran chloramphenicol peptone agar (DCPA) and rapeseed agar (RA) for 7 days at 25°C under fluorescent and near-UV light. The medium DCPA is reported to be particularly effective for the identification of Alternaria spp. because DCPA promotes spore production (Pitt and Hocking, 1985).

Chemical constituents of the media (DRBC, CYA, G25N, MEA, CY20S, DCPA and RA) used for isolation and identification of moulds are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Media</th>
<th>Chemical components (per L of water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czapek yeast agar (CYA)</td>
<td>Czapek concentrate (i.e. NaNO₃ 30 g; KCl 5 g; MgSO₄·7H₂O 5 g; FeSO₄·7H₂O 0.1 g in 100 ml water) 10 ml; K₂HPO₄ 1.0 g; yeast extract 5 g; sucrose 30 g; agar 15 g.</td>
</tr>
<tr>
<td>Czapek yeast agar containing 20% sucrose (CY20S)</td>
<td>Czapek concentrate 10 ml; K₂HPO₄ 1.0 g; yeast extract 5 g; sucrose 200 g; agar 15 g.</td>
</tr>
<tr>
<td>Dichloran rose bengal chloramphenicol agar (DRBC)</td>
<td>glucose 10 g; peptone 5 g; KH₂PO₄ 1.0 g; MgSO₄·7H₂O 0.5 g; rose bengal 50 mg; chloramphenicol 0.1 g; agar 15 g.</td>
</tr>
<tr>
<td>Dichloran chloramphenicol peptone agar (DCPA)</td>
<td>peptone 15 g; KH₂PO₄ 1.0 g; MgSO₄·7H₂O 0.5 g; chloramphenicol 0.1 g; dichloran 2 mg; agar 15 g.</td>
</tr>
<tr>
<td>Glycerol nitrate agar (G25N)</td>
<td>K₂HPO₄ 0.75 g; Czapek concentrate 7.5 ml; yeast extract 3.7 g; glycerol (A grade) 250 g; agar 12 g.</td>
</tr>
<tr>
<td>Malt extract agar (MEA)</td>
<td>malt extract 20 g; peptone 1.0 g; glucose 20 g; agar 20 g</td>
</tr>
<tr>
<td>Rapeseed agar (RA)</td>
<td>rapeseed (lightly crushed) 20 g; agar 15 g.</td>
</tr>
</tbody>
</table>

Table 2.1 Chemical components of fungal media
2.1.2.3 Preservation

Single spores from some isolated moulds (e.g. *A. alternata*) were grown on media (e.g. DCPA). They were then flooded with a sterile solution of aqueous glycerol (10%) and agitated with a glass rod previously flamed in ethanol. The suspensions containing spores were adjusted to approximately $1 \times 10^3$/ml using a haemocytometer and transferred to sterile, plastic vials (1ml in Nunc Cryotubes [2ml], Inter-Med, Kamstrup, Denmark). Storage was at -70°C.

2.2 RESULTS

2.2.1 At harvest

Mycological analysis showed an average of 17.84% seed was colonised by moulds. Nineteen genera were identified; *Alternaria* spp. (10.82%) predominated, followed by *Penicillium* spp. (2.11%), *Eurotium* spp. (0.93%), *Cladosporium* spp. (0.84%) and *Aspergillus* spp. (0.82%) (Fig. 2.1).

Although there was considerable variation (1-52%) in the occurrence of moulds between sample points in the country, *Alternaria* spp. were detected in 54 of 55 (98.2%) samples and were the dominant (i.e. ≥50%) moulds in 76.4% samples. A similar amount of variation occurred in samples (8) taken near ADM Oil Works in Kent (11-32%), although *Alternaria* spp. were detected in all eight samples and were the principal (≥50%) species in seven (Fig. 2.2).

Five species of *Alternaria* were identified from 595 isolates of the genus, including *A. alternata* (86.2% of *Alternaria* isolates), *A. infectoria* (8.2%), *A. tenuissima* (4.0%), *A. brassicicola* (1.1%) and *A. brassicae* (0.5%) (Fig. 2.3A).

The percentage seed containing *Penicillium* spp. (2.1%) was much lower than *Alternaria* spp. (10.8%). Species of *Penicillium* identified in decreasing order of incidence included *P. hordei*, *P. citrinum*, *P. puberulum*, *P. chrysogenum*, *P. corylophilum*, and *P. waksmanii* (Fig. 2.3B). Species of *Penicillium* occurred in 67.2% samples and were the predominant (≥50%) moulds in 9.9% samples - notably lower values than those of *Alternaria* spp.
Fig. 2.1 Incidence of moulds in rapeseed collected at harvest in the UK
Fig. 2.2  Incidence of moulds in rapeseed collected at harvest in Kent
Fig. 2.3 Proportions of (A) *Alternaria* spp. (average incidence in rapeseed; 10.82%), (B) *Penicillium* spp. (2.1%) and (C) *Aspergillus* spp. (0.8%) in rapeseed collected at harvest in the UK.
A very low (0.8%) proportion of the potentially mycotoxigenic genus *Aspergillus* was found in rapeseed. Of the 12.7% of samples which were contaminated by *Aspergillus* spp. it was the dominant genus (≥ 50%) in only one (1.8%). The predominant species detected was *A. candidus* but 2 isolates of *A. parasiticus*, which is a potential aflatoxin producer, were identified in a sample collected from a property near Radstock, Avon (Fig. 2.3C).

### 2.2.2 During storage

Postharvest incidence of moulds after storage for 2, 5, 7 and 10 months was 7.0 ± 2.63*, 12.25 ± 3.82, 12.5 ± 3.09 and 9.25 ± 3.01% respectively, and lower than the incidence at harvest (17.8%) (Fig. 2.4). Statistical analysis showed there was no significant reduction in mould levels with longer storage. At 2, 5, 7, and 10 months storage, the incidence of *Alternaria* spp. (4.8, 8.3, 8.2 and 7.8% respectively) was greater than *Penicillium* spp. (1.5, 1.2, 2.4 and 0.9% respectively) and *Aspergillus* spp. (0, 0.7, 0.5 and 0.3% respectively). The incidence of the dominant species of *Alternaria* identified for all storage periods were:

- *A. alternata* (85.0%)
- *A. infectoria* (7.1%)
- *A. tenuissima* (4.4%)
- *A. brassicae* (3.5%)

### 2.2.3 After conversion into meal

From the 20 samples of meal, an average of 65 mould colonies per gram of meal developed on DRBC agar. Colonies detected in decreasing order of incidence included the moulds:

* represents standard deviation (sd)
Fig. 2.4 Postharvest incidence of moulds in rapeseed collected from Camgrain Silo, Cambridge
*Penicillium* spp. (approximately 41 colonies/g; 61.7% of the total mould incidence)

*Monilinia* spp. (13; 19.7%)

*Aureobasidium* spp. (5; 7.0%)

*Rhizomucor* spp. (3; 4.9%)

*Aspergillus candidus* (2; 2.8%)

*Mucor* spp. (1; 2.1%)

*Alternaria alternata* (1; 1.8%)

*Penicillium* spp. occurred in 18 of 20 samples (90%), *Monilinia* spp. in 50% of samples, *Rhizomucor* spp. 30%, *A. alternata* 15%, *Mucor* spp. 15%, *Aureobasidium* spp. 15% and *Aspergillus candidus* 5%. Species of *Penicillium* identified included *P. hordei, P. citrinum, P. puberulum, P. restrictum* and *P. oxalicum*.

### 2.3 DISCUSSION

Although the rapeseed collected at harvest showed an average mould content of 17.8%, there was considerable variation in mould incidence (1-52%) amongst samples in the UK. This was also the case for samples collected near ADM Oil Works in Kent, where mould occurrence ranged between 11 to 32%.

The high variability of moulds was probably attributable to factors such as climate, soil type, cultivar and agronomic practices (e.g. fungicide application). For these factors to be assessed accurately, increased sampling would be required.

However, *Alternaria* spp., principally *A. alternata*, were established as the predominant moulds contaminating rapeseed at harvest in the UK. *Penicillium* spp., which were the next most common moulds on rapeseed (2.1%), occurred at roughly a fifth of the incidence of *Alternaria* spp.

Other surveys show *Alternaria* spp. to be common contaminants in cereals, including wheat, barley, rye, oat, maize and rice (Bruce et al., 1984; Logrieco et al., 1990; Sanchis et al., 1993), and in oilseed rape grown in Italy.
(Visconti et al., 1992). Surveys undertaken in England established A. brassicae and brassicicola as major pathogens of oilseed rape (Evans and Cox, 1982; Humpherson-Jones, 1985), but regrettably did not report on other Alternaria spp.

Pitt and Hocking (1985) stated that some mycotoxigenic species of Aspergillus (e.g. A. flavus) have a particular attraction for oilseeds (e.g. groundnuts) as substrates, although the reason is not understood. However, the incidence of Aspergillus found on rapeseed in this study was low (0.8%), possibly because of low temperatures encountered during winter. In general, Aspergillus spp. is tolerant of, or thrives in, elevated temperatures; e.g. the temperature range for growth of A. flavus is minimum near 12°C, maximum near 48°C and optimal between 25-42°C (Pitt and Hocking, 1985).

Postharvest incidence of moulds (7.0, 12.25, 12.5 and 9.25% at 2, 5, 7 and 10 months respectively) was lower than that at harvest (17.8%). This suggests that viability of mycelium and spores decreases after harvest but surprisingly, in this study, mould incidence was generally constant during storage (note sds of sample means in Fig. 2.4).

However, it was later discovered that Satisfar (active ingredient: etrimfos), a postharvest insecticide, was applied to the seed prior to storage. Bioassay studies demonstrated a Satisfar level greater than 10 μg (P<0.05) inhibits the growth of A. alternata (Appendix 1). Therefore, application of Satisfar to the seed may have killed moulds during storage. In addition, Satisfar may have inhibited fungal growth during the isolation procedures, particularly in samples taken at 2 months storage.

As expected, Alternaria spp. were clearly the dominant fungi for all storage periods. In mould infected seeds at 2, 5, 7, and 10 months storage, the proportion of Alternaria spp. was approximately 61.4, 67.8, 65.6 and 84.3% respectively. This indicates that the spores or mycelium of Alternaria spp. easily
survive storage for 10 months (the period for which industrial rapeseed is often stored for prior to oil extraction).

The mycoflora detected on meal samples showed considerable differences from that found in seed. *Penicillium* spp. occurred in all samples whereas *A. alternata* was present in only 15% of samples. Evidently, oil extraction and subsequent events caused postharvest changes in the mycoflora. Moulds such as *Penicillium* spp. were probably introduced after oil extraction since their conidia are prolific, small (approximately 2-10 μm diameter) and thus readily airborne. *Penicillium* spp. has been reported as a common contaminant of a number of food commodities, including cereals and groundnuts (Pitt and Hocking, 1985), and rapeseed (Mills and Sinha, 1980; White and Jayas, 1989).

Despite the fact that *Penicillium* spp. was the most commonly occurring mould in meal, intact conidia of *Alternaria* spp. were often detected within colonies of other moulds, especially spreading mucoraceous fungi (e.g. *Rhizomucor* spp.). Production of metabolites and competition for nutrients and air by spreading fungi may have suppressed the germination of conidia of *Alternaria* spp.. Other types of media which inhibit spreading moulds - e.g. 7.5% sodium chloride in potato dextrose agar (PDA) (Mislivec and Bruce; 1988) - may be more effective than DRBC in allowing colony formation of *Alternaria*.

Further contamination of meal by *Alternaria* spp. was believed to occur during transit to the compound feed companies. Frequently, lorries which unloaded rapeseed for crushing at the oilworks then immediately conveyed meal to the compound feed companies. Obviously, it was most likely that *Alternaria* spp. (the major moulds found in seed) were introduced to the meal during transport, but this could not be verified as it was not possible to take samples at the final destinations (i.e. the compound feed companies).

*Fusarium* spp. were not detected in any samples making contamination of rapeseed or meal by *Fusarium* toxins (e.g. T2-toxin and zearalenone) unlikely.
Alternaria spp. were established as the principal mycotoxigenic moulds colonising rapeseed at harvest and during storage, and A. alternata was present in the meal. The species were detected in seed after surface disinfection, indicating penetration, growth and possible toxin production. It is possible therefore for that any Alternaria toxins contaminating the seed may then be transferred to the meal or oil. Such transfer has been shown for damaged olives, although the proportion was low (about 4% from the original olive sample) (Visconti et al., 1986)
CHAPTER 3

HPLC SOLVENT OPTIMISATION OF *ALTERNARIA* TOXINS

Various solvent systems for HPLC analysis of *Alternaria* toxins have been devised (see Table 1.3) but none using a reverse phase isocratic solvent system incorporating TeA with ALT, ATX-I, AOH and AME. One approach for separating these metabolites is to optimise the solvent system based on the principle of the solvent selectivity triangle, as described in section 1.3. The primary strategy for optimising the separation of *Alternaria* metabolites was to select an isoeluotropic plane which gave acceptable RTs of the toxins and to modify the composition of the four solvents on the plane in order to achieve the best overall selectivity. Philips Solvent Optimisation PU6100 Software was employed to assist with this approach and to monitor the RTs of the components in the varying solvent mixtures.

3.1 MATERIALS AND METHODS

3.1.1 Mycotoxins

Standards of AME and the copper salt of TeA were available commercially (Sigma Chemical Co. Ltd., Poole, UK), and ALT, ATX-I and AOH were a generous gift from Mr M. Solfrizzo (ITMPV, Bari, Italy).

Free TeA was regenerated by passing a Cu-TeA solution (ca. 1 mg in 5 ml dichloromethane) through a column of cation exchange resin (Dowex 50W-X8; 18-52 mesh), previously washed with dichloromethane. Addition of excess dichloromethane (10 ml) eluted the free acid (Scott and Kanhere; 1980), which was then concentrated under vacuum using a rotary evaporator (Rotavapor R110, Buchi Laboratoriums-Technik AG, Flawil, Switzerland). Tenuazonic acid remained in the residue as a brown oily gum.
Single (approximately 150 µg/ml in MeOH) and mixed toxin solutions (roughly 30 µg/ml of each metabolite) were prepared immediately prior to HPLC solvent optimisation, as TeA on standing for long periods loses its optical activity and crystallises; this is attributed to the formation of iso-TeA (Stickings, 1959). The concentrations of the compounds were determined using a spectrophotometer (Philips PU8740, PYE UNICAM Ltd., Cambridge, UK) connected to a Philips printer/plotter.

3.1.2 Chromatography

A Philips HPLC system was used, incorporating a PU4100 quaternary pump module equipped with a Rheodyne 7125 injection valve, and a PU4021 diode array detector interfaced to a P3202 personal computer containing PU6003 diode array software. Chromatography was performed on a 150X4.6 mm I.D. reverse phase column (Jones Chromatography, Hengoed, UK) with Spherisorb octyl (C8) (5 µm) packing. A Jones Chromatography guard column (20X4.6 mm I.D.) of identical packing was used to protect the analytical column.

The solvents used were MeOH, ACN, THF (HPLC grade from Fisons Ltd., Loughborough, U.K.) and aqueous zinc sulphate solution (ZnSO₄•7H₂O; 300 mg/L) since incorporation of a divalent metal complexing agent such as Zn²⁺ to the mobile phase is reported to improve the peak efficiency of TeA (Stack et al., 1985).

All chromatography was carried out at room temperature (ca. 20°C) with a flow rate of 1000 µl/min. The system was equilibrated with each solvent mixture for 30-40 min. before sample injection (5.0 µl). To ensure bubbles did not develop in the system, solvents were continuously degassed with dry helium gas.

3.1.3 Solvent optimisation

Philips solvent optimisation software PU6100 was applied to the separation of Alternaria toxins. The overall process involves identifying the
isoeluotropic solvent composition plane, peak finding, retention modelling and optimisation, as summarised in Fig. 3.1.

3.2 RESULTS

3.2.1 Identification of the isoeluotropic plane

Two MeOH/zinc sulphate solution gradients (5 and 15 min.; 0-100%) were run on the HPLC in order to establish accurately the first and last peaks within an acceptable $k'$ range (i.e. 1-10) (Fig. 3.2). The data from these gradients were compared. In the 15 min. gradient, the RTs of the first (TeA) and last (AME) peaks were 13.22 and 16.82 min. respectively. At $k'=5$, the isocratic MeOH/zinc sulphate composition was predicted as 85.8% MeOH with the last peak eluting at 22.20 min. In contrast, the isocratic MeOH/zinc sulphate composition predicted from the five minute gradient was 95.5% MeOH (first peak ALT, RT = 7.93 min.; last peak TeA, RT = 10.30 min.), which eluted the last peak (for the same $k'$ value) at 13.5 min..

The isocratic solvent mixture predicted from the five minute gradient was selected as the total analysis time of Alternaria metabolites was shorter (13.5 min.) than the analysis time estimated from the 15 min. gradient (22.2 min). To identify the isoeluotropic plane, the other two corner eluents - ACN/zinc sulphate and THF/zinc sulphate - were calculated using designated transfer rules (Schoenmakers et al., 1981) (Fig 3.3A).

Upgrading of the corner points of the isoeluotropic plane estimated was necessary as Alternaria metabolites differed in their behaviour according to the solvent mixture selected. The MeOH/zinc sulphate corner required two HPLC runs, THF seven runs and ACN seven runs until the RTs of the last peaks were close ($\pm 0.13$ min.) to the predicted RT of 13.5 min.
Fig. 3.1 Flow diagram of PU6003 solvent optimisation

1. **IDENTIFY ISOELEOTROPIC PLANE**
   - Run ZnSO₄ solution/MeOH gradient; Revise MeOH, ACN, THF corners if required and predict isocratic solvents within a suitable (1-10 k' range

2. **COMPILE 10 CHROMASCANS**

3. **SELECT A REFERENCE CHROMASCAN**
   - Number desired peaks; process and store reference spectra

4. **PROCESS 10 CHROMASCANS**
   - Find and classify peaks using multiple wavelengths detection and deconvolution techniques

5. **MODEL AND PREDICT**
   - Model the retention behaviour of peaks across the isoeleotropic plane;
     - Model the response surface to show the quality of resolution of peaks;
     - Predict optimum separation of peaks
Fig. 3.2  HPLC gradient runs (MeOH/zinc sulphate solution; 0-100%) of Alternaria toxins. Chromatograms A and B correspond to 5 and 15 min. gradients respectively. Peak 1 = TeA; 2 = ALT; 3 = ATX-I; 4 = AOH; 5 = AME
### Isoeluotropic Solvent Composition Plane

#### (A)

<table>
<thead>
<tr>
<th>MeOH</th>
<th>ACN</th>
<th>THF</th>
</tr>
</thead>
<tbody>
<tr>
<td>95.5%</td>
<td>0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>63.0%</td>
<td>0.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>31.5%</td>
<td>31.5%</td>
<td>31.5%</td>
</tr>
<tr>
<td>55.2%</td>
<td>27.6%</td>
<td>0.0%</td>
</tr>
<tr>
<td>0.0%</td>
<td>20.5%</td>
<td>41.0%</td>
</tr>
</tbody>
</table>

#### (B)

<table>
<thead>
<tr>
<th>MeOH</th>
<th>ACN</th>
<th>THF</th>
</tr>
</thead>
<tbody>
<tr>
<td>91.5%</td>
<td>0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>60.4%</td>
<td>0.0%</td>
<td>13.4%</td>
</tr>
<tr>
<td>30.2%</td>
<td>30.2%</td>
<td>30.2%</td>
</tr>
<tr>
<td>28.0%</td>
<td>14.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>0.0%</td>
<td>13.4%</td>
<td>26.9%</td>
</tr>
</tbody>
</table>

---

**Fig. 3.3**  
(A) The isoeluotropic plane calculated by the software based on the MeOH/zinc sulphate solution 5 min. gradient.  
(B) The final isoeluotropic plane updated in the light experimental data
Once the corner solvent mixtures were finally established, the remaining seven solvent points lying on the plane were run on the HPLC (Fig 3.3B). Retention times for the five metabolites in all 10 mobile phases are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Chromascan number</th>
<th>Retention time (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT</td>
</tr>
<tr>
<td>1</td>
<td>1.98</td>
</tr>
<tr>
<td>2</td>
<td>2.27</td>
</tr>
<tr>
<td>3</td>
<td>2.20</td>
</tr>
<tr>
<td>4</td>
<td>2.90</td>
</tr>
<tr>
<td>5</td>
<td>2.72</td>
</tr>
<tr>
<td>6</td>
<td>2.78</td>
</tr>
<tr>
<td>7</td>
<td>3.15</td>
</tr>
<tr>
<td>8</td>
<td>3.15</td>
</tr>
<tr>
<td>9</td>
<td>3.38</td>
</tr>
<tr>
<td>10</td>
<td>3.55</td>
</tr>
</tbody>
</table>

Table 3.1 Retention times of the five *Alternaria* toxins in the 10 chromascans
3.2.2 Assignment of chromascans

Peak finding was acquired from the second derivative of each chromatogram using a maxplot function* >A240 nm. Chromascan 4 was used as the reference chromascan as all five metabolites were resolved adequately to produce true spectra (Fig. 3.4). Peaks in the other nine chromascans were matched with the reference chromascan using a least squares fit and labelled accordingly. To ensure this procedure was carried out correctly, peak matching was confirmed manually.

3.2.3 Optimisation

Retention surfaces of the five metabolites were constructed from the data of the 10 chromascans (Fig. 3.5). These were uniform, indicating that the assignments were correct. Finally, a response curve was created which showed the quality of resolution across the isoeuotropic plane, the peaks corresponding to solvent compositions which gave optimum separations (Fig. 3.6A). This was best represented as a contour plot where the darkest areas signify the solvent compositions for optimum separation of compounds. For the Alternaria toxins examined here, two such areas were displayed (zones 1 and 2) (Fig. 3.6B).

Simulated chromatograms show solvent compositions predicted in zone 1 (e.g. 58.2% aqueous zinc sulphate, 1.2% MeOH, 10.1% ACN and 30.5% THF) yielded longer RTs of ALT, TeA, ATX-I, AOH and AME (3.41, 4.94, 6.24, 7.68 and 11.45 min. respectively) than the solvent combinations selected in zone 2 (e.g. for 41.7% aqueous ZnSO₄, 30.3% MeOH, 18.1% ACN and 9.9% THF, the predicted RTs of ALT, ATX-I, AOH, TeA and AME were 2.76, 3.45, 4.07, 5.13 and 7.31 min. respectively (Fig. 3.7). However, solvent assignments assigned in zone 1 caused tailing of TeA and subsequent coelution with ATX-I and AOH. The symmetry of TeA could be measured by assigning chromatograms (at A280 nm) which represented 10, 25 and 50% of absorbance of the peak (100% absorbance) (Fig. 3.8). For the solvent composition selected in zone 1 above

* maxplot describes the absorbance of the diode of highest absorbance at each individual time unit
Fig. 3.4 UV Spectra of *Alternaria* metabolites. Spectrum 1 = ALT; 2 = ATX-I; 3 = AOH; 4 = TeA; 5 = AME. Y axis = absorbance (AU), X axis = $\lambda$ (nm)
Fig. 3.5 The retention surfaces for ALT (A), ATX-I (B), AOH (C), TeA (D) and AME (E) showing the variation in retention time with solvent composition.
Retention $[k']$ for ATX-1 from opt2a

$40.7 \% \text{THF} \quad 0.0 \% \text{ACN} \\
0.0 \% \text{MeOH} \quad 59.3 \% \text{Water}$

Range of function = 2.9E-0001 to 4.3E+0000

B (Fig. 3.5)
Retention(I') for ADH from opt2a

40.7 % THF, 0.0 % ACN
0.0 % MeOH, 59.3 % Water

Range of function = 3.0E-0001 to 4.4E+0000

C (Fig. 3.5)
Retention $k'$ for TEA from opt2a

$40.7\%$ THF $0.0\%$ ACN
$0.0\%$ MeOH $59.3\%$ Water

Range of function = $1.9E+0000$ to $7.8E+0000$

D (Fig. 3.5)
Retention [R'] for AME from opt2a

0.0 % THF  0.0 % ACN
0.0 % MeOH  99.3 % Water

Range of function = 5.7E-0001 to 7.7E+0000

E (Fig. 3.5)
Fig. 3.6  Response surface (A) and contour plot (B) showing the effect of solvent composition on the quality of resolution. The peaks in (A) and zones 1 and 2 in the triangle (B) indicate optimum solvent compositions.
Fig. 3.7  Predicted retention times of ALT (stick 1), ATX-1 (2), AOH (3), TeA (4) and AME (5). Chromatograms 3 and 6 (Y axis) represent optimum solvent compositions predicted in zones 1 and 2 respectively.
Fig. 3.8 The effect of MeOH as a mobile phase on the resolution of TeA. Chromatograms are displayed as three sets of sticks (1 and 7; 2 and 6; 3 and 5) which represent 10\%, 25\% and 50\% absorbance (A280 nm) of the TeA peak (stick 4) respectively.
(i.e. 58.2% aqueous zinc sulphate, 1.2% MeOH, 10.1% ACN and 30.5% THF), the width of TeA, for example at ±25% absorbance of the peak, was approximately 4.65 min.; in zone 2, it was 0.98 min.. Evidently, as the proportion of MeOH in the solvent mixture increases, so too does the resolution of TeA.

Several solvent mixtures assigned in zone 2 were run on the HPLC. The solvent system which best separated *Alternaria* metabolites was 41.7% ZnSO₄ solution, 30.3% MeOH, 18.1% ACN and 9.9% THF. The RTs of ALT, ATX-I, AOH, TeA and AME were 2.76, 3.52, 4.13, 5.12 and 7.08 min. respectively, which closely corresponded to those RTs predicted by the software (Fig. 3.9).

3.3 DISCUSSION

Use of solvent optimisation in separating compounds by HPLC is limited owing to the difficult problem in monitoring overlapping peaks with changing solvent mixtures. However, Cox (1984) determined the optimum solvent system for separating anticonvulsant drugs and Chen *et al.* (1991) optimised the separation of the plant phytotoxins solanopyrones A, B, and C, by applying Philips PU6100 computer software.

In this study, solvent optimisation of *Alternaria* metabolites by HPLC using the PU6100 software was successful in achieving baseline separation of all five metabolites. This method of separation for *Alternaria* toxins has the major advantage over other known HPLC systems that all metabolites are quantified on a single isocratic run thus reducing the analysis time of samples (RT of last peak; 7.08 min.). Other workers have generally quantified TeA separately from the dibenzopyrone and perylenequinone compounds (Heisler *et al.*, 1980; Stack and Prival, 1986; Palmisano *et al.*, 1989), although Ozcelik *et al.* (1990) developed an isocratic system (see Table 1.3) which separated ALT, AOH, AME, together with TeA on a normal phase (silica) column. However, ATX-I was not included in the solvent system and, as mentioned previously in section 1.2.6.2, normal phase chromatography has several disadvantages compared with reverse phase.
Fig 3.9  HPLC chromascan (A) and chromatogram (at λ245 nm) (B) of ALT (peak 1; RT = 2.76 min.), ATX-I (2; 3.52 min.), AOH (3; 4.13 min.), TeA (4; 5.12 min.) and AME (5; 7.08 min.)
chromatography - more expensive solvents are used, the mobile phase takes longer to equilibrate and the mode is less reproducible (Lindsay, 1992).

The initial phase of the optimisation procedure was to select a solvent isoeluotropic plane. While it is feasible to accumulate data to model retention throughout the complete factor space of the tetrahedron, this is normally of limited use because the corner region containing solvent mixtures which are high in water often lead to prolonged analysis times, while solvent mixtures low in water cause peaks to elute too near the solvent front (Wright, 1990). If only an isoeluotropic plane is considered, the number of variables is restricted to two as any point on the plane can be generated by mixing appropriate proportions of the three corner eluents, in this case MeOH/aqueous zinc sulphate, ACN/zinc sulphate and THF/zinc sulphate.

In this study, the isoeluotropic plane predicted from the five minute zinc sulphate solution/MeOH gradient (RT of last peak; 13.50 min.) was selected for the optimisation procedure. This was because the RT of the last peak (22.20 min.) estimated from the longer gradient (15 min.) was too prolonged for practical quantification of Alternaria metabolites.

A possible reason why the isoeluotropic plane identified from the 15 min. gradient differed from the plane predicted from the shorter (5 min.) gradient was because the metal complexation capacity of TeA to Zn$^{2+}$ was dependant on the concentration of aqueous ZnSO$_4$ in the mobile phase. This can be shown by the fact that for the 15 min. gradient, TeA was the first peak (13.22 min.) to elute, whereas during the five minute gradient it was the last peak (10.30 min.). Lebrun et al. (1989) noted in their studies of TeA that the RT of TeA can be slightly reduced ($k' = 4.5$ to 3.3) on addition of the metal complexant EDTA (10 mM) to the mobile phase (55% MeOH in phosphate buffer; pH 6.0, containing 5 mM hexadecyltrimethylammonium bromide). Similar results were seen here, where an increase in the concentration of zinc sulphate solution from 300 to 600 mg
lessened the RT of TeA by approximately 40 sec., during analysis at 41.7% ZnSO₄ solution, 30.3% MeOH, 18.1% ACN and 9.9% THF.

Once the isoeluotropic plane was established, the 10 solvent mixtures lying on the plane were run and the movement of all five peaks was carefully monitored using sophisticated chemometric techniques. Although peak recognition by the PU6100 computer software was generally reliable, it was sometimes necessary to manually correct peak mis-matches between AOH and AME as their spectra were virtually identical. In addition, if a peak totally coeluted with another peak or was flattened to the point where recognition was not possible, the run was repeated using single standards, with, if necessary, a higher injection volume (e.g. 10 µl).

An interesting feature found during the monitoring of Alternaria metabolites was that TeA behaved in a contrary manner to the other toxins with varying proportions of MeOH. As the proportion of MeOH increased (and the percentage of the more polar solvent, aqueous zinc sulphate decreased), the RTs of TeA became longer, suggesting that TeA was subjected to normal phase chromatography on this column. This phenomenon was also reported by Heisler et al. (1980) who found that with a C-18 column and a MeOH-water mobile phase, the $k'$ value for TeA increased with decreasing proportions of water.

Once all Alternaria peaks were assigned, a contour plot was generated from the data (Fig. 3.7). Here the two light areas corresponded to solvent compositions giving the best separation of Alternaria metabolites. In general, simulated chromatograms showed the solvent compositions predicted in zone 1 yielded longer retention times of ALT, TeA, ATX-I, AOH and AME than the solvent combinations selected in zone 2 (see Fig. 3.8 for solvent compositions and RTs). Longer retention times can often produce better resolution of compounds but in the case of Alternaria toxins, there was prolonged tailing of TeA.

It was found that the extent of TeA tailing was dependent on the proportion of MeOH in the solvent phase. Solvent combinations in zone 2, which contained
higher amounts of MeOH (28-37%) than zone 1 (0-2%), reduced the tailing of TeA. A possible explanation for this may be due to the fact that solvent mixtures containing little or no MeOH may reduce the metal chelating properties of TeA with Zn\(^{2+}\). Perhaps aqueous ZnSO\(_4\) solution precipitated in certain solvent mixtures (e.g. with low or no MeOH), thus reducing the concentration of the zinc cation in solution.

Tailing of acidic compounds is common in HPLC as they may partly dissociate into ionic molecules. Addition of a metal complexing reagent such as Zn\(^{2+}\) to the mobile phase has been recommended by workers to improve peak sharpness of TeA (Stack and Prival, 1986; Visconti et al., 1986), although Lebrun et al. (1989) found no reduction of tailing of TeA on addition of a metal complexing reagent (EDTA) or the acidification of the mobile phase (different water-MeOH mixtures) with phosphoric acid (pH 2.0) to suppress the ionic form of TeA. They did however confirm that addition of EDTA to the mobile phase improved the efficiency of the TeA peak during ion-pair chromatography.

In addition to the tailing of TeA, the sharpness of the AME peak also changed with varying solvent mixtures; as ACN increased, the sharpness of the AME peak decreased. Since AME has no ionic properties, the shape of its peak was probably dependent its solubility in ACN. In general, AME is more soluble in MeOH than in ACN (pers. comm., Mr M. Solfrizzo).

It was thought that tailing of the TeA and AME peaks may be reduced by optimising with a solvent different from ACN. However, there was no suitable solvent available which was compatible with water, THF and MeOH, as well as having distinct selectivity properties, as described by Synder (1978). For example, the solvent system used by Heisler et al., 1980 for separating ALT, AOH and AME (65 acetone:35 water) was not acceptable for quantifying TeA as this solvent system absorbed strongly at \(A278\) nm.
In summary, a reverse phase isocratic HPLC system has been developed which separates five important *Alternaria* mycotoxins. Using this system it was possible to quantify *Alternaria* toxins produced on rice, rapeseed and rapeseed meal, as discussed in the next chapter.
Chapter one reported that natural contamination of Alternaria toxins (e.g. TeA) occurs in various oilseed crops, including sunflower (Logrieco et al., 1988) and olives (Visconti et al., 1986). Also, Alternaria spp. isolated from oilseed rape grown in foreign countries (e.g. Italy) produced mycotoxins when cultured on natural substrates (e.g. rice) (Visconti et al., 1992). However, little is known about mycotoxigenic Alternaria spp. which colonise oilseed rape in the UK. For this reason, the ability of Alternaria spp. to produce mycotoxins on rice, rapeseed and rapeseed meal were analysed under laboratory conditions. Although toxin production in the laboratory is not directly related to that occurring under natural systems, these observations are applicable to the problem of ALT, ATX-I, AOH, TeA and AME production in nature.

In addition, the biological activity of Alternaria metabolites are appraised in this chapter in order to learn (i) the toxicity of metabolites and (ii) whether bioassays can be utilised as means of a detection of Alternaria metabolites in crude extracts.

4.1 MATERIALS AND METHODS

4.1.1 Biological activity of Alternaria toxins

Several types of bioassays were examined, including measurement of the germination rate of rapeseed, extent of chlorosis in detached rapeseed leaves, viability of cells isolated from oilseed rape leaves and motility of Artemia salina (brine shrimp).

4.1.1.1 Rapeseed germination assay

Seed from oilseed rape (cv. Bravo) were placed in wells (3 per well) of a 24 well microtest plate containing 20 μl water and 5 μl of methanolic toxin
solution. After incubation for 60 hours at 20°C in the dark, the rate of germination was scored on a scale from 0 to 3, as follows:

- 0 = no germination
- 1 = radicle ≤ 3 mm
- 2 = radicle > 3 mm
- 3 = radicle > 3 mm with cotyledons present

Results were compared with controls i.e. seeds in water containing 20% MeOH solution. Five replicates (of 3 seeds) per test were used.

### 4.1.1.2 Oilseed rape leaf assay

Toxins dissolved in MeOH (0.25, 0.5, 1, 2 mM) were applied as drops (5 μl) on oilseed rape leaves and incubated at 20°C in petri dishes containing filter paper moistened with water. Controls consisted of MeOH only.

### 4.1.1.3 Oilseed rape leaf cell assay

Oilseed rape leaf cells were isolated using modifications of the protocol described by Shoet and Strange (1989). From plants (cv. Cobra) grown for three weeks in John Innes No. 2 soil (William Sinclair Holdings Group, Lincoln, UK) at approximately 20°C in a greenhouse, first or second true expanded leaves were excised and then cut into 5 mm² segments. These portions were vacuum infiltrated (5-10 sec.) in the following enzyme digestion solution; 0.1% Macrerozyme R10 (Yakult Honsha Co. Ltd., Tokoyo, Japan), 0.01% Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokoyo, Japan) and 0.005% bovine serum albumin (Sigma Chemical Co., St. Louis, USA), dissolved in a holding buffer comprising glucose (10%), citric acid monohydrate (1.05%), CaCl₂·2H₂O (0.0735%), KH₂PO₄·7H₂O (0.0136%), MgSO₄·7H₂O (0.0247%) and NaOH (0.062%), and adjusted to pH 6.1. After gentle swirling on a flask shaker (MkV orbital shaker, LH Fermentation) for 15-20 min., clumps of cells were separated by filtration through four layers of muslin. Cells were washed three times by alternate centrifugation at 100 g for 5 min. and resuspension in holding buffer, and finally suspended in holding buffer (ca. 1.0 x 10⁵ cells/ml). Fluorescein
diacetate (FDA) dissolved in acetone (0.5 mg/ml) was added to the cell suspension (0.001 mg/ml).

Methanolic solutions of toxin were diluted in nine volumes of holding buffer and then serially diluted two-fold in holding buffer contained in the wells of a microtest plate (50 µl/well). Cells (50 µl) were pipetted into the first well, allowed to settle for 2 min. and then viewed under an inverted fluorescent microscope (Olympus, model IMT). The centre of the well, which had been previously marked with a small dot underside, was aligned to the centre of a graticule (i.e. the 5 mm measurement) fixed within the microscope eye piece. Cells that fluoresced yellow-green (50-60% of cells) within the field of view at X200 magnification (i.e. ca. 0.785 mm²) were counted as live (generally 50-120 cells) and the procedure was repeated for the remaining wells. To prevent the disturbance of cells in wells, the microtest plate was handled with care. Plates were incubated at room temperature (ca. 20°C) and covered with aluminium foil to reduce evaporation of solutions. After 3.5 hours, each field of view per well was realigned using the dot and graticule, and cells which continued to fluoresce were recounted. Results were presented as percent relative viability, after correcting for viability in the controls i.e. cells in holding buffer with 5% MeOH or less. Three replicates per treatment were performed.

4.1.1.4 Brine shrimp assay

Decapsulated cysts of brine shrimp (New Technology Products, East Peckham, UK) were permitted to hatch at 25°C for 36-48 hours in the outer sector of a triple-sector plastic receptacle (Hykro, Udine, Italy) containing brine shrimp medium (3.5% NaCl in water), which was aerated vigorously. The sectors were partially obstructed by a series of screens which allowed active nauplii (but not inactive ones) to pass from the hatching sector to the centre of the apparatus. Brine shrimp were attracted to the centre with light, making use of the positive phototaxis feature of the swimming larvae.
In order to measure the motility of brine shrimp, an apparatus was constructed similar to that of the optical motility counter devised by Schmidt and Schmidt (1986) (i.e. Motimat 700 or 716, HWS, Mainze, Germany). Motility was represented by the sum of interruptions caused by the larvae moving through a light beam recorded with an optical sensor.

Serial two-fold dilutions of toxin standards were made in MeOH and evaporated to dryness in wells of 24 well microtest plates (Flow Laboratories, Irvine, UK). Brine shrimp (ca. 20) in 100 µl of brine shrimp medium were added to the wells. After 20 hours incubation at 25°C in the dark, motility was estimated by placing the wells under the light beam for 2 min. All samples were compared to controls (i.e. brine shrimp media) so that relative motility could be calculated. Three replicates per treatment were performed.

As a preliminary investigation, brine shrimp were also challenged with extracts of *Alternaria* cultures (see sections 4.2.2.2 and 4.2.2.3 for growth and extraction procedures of fungal cultures). The equivalent amount of original culture assayed corresponded to 1 and 0.1 g/ml. Extracts of uncultured rice, rapeseed and rapeseed meal were used as controls.

4.1.2 Determination of mycotoxigenic *Alternaria*

4.1.2.1 Fungal isolates

Twenty-three isolates of *Alternaria* spp. (*A. alternata*, 13 isolates; *A. infectoria*, 4; *A. tenuissima*, 2; *A. brassicicola*, 1; *A. brassicae*, 1; unidentified species of *Alternaria* *, 2) were obtained from seeds of oilseed rape collected from various areas in the UK (Table 4.1).

* these isolates were not identified to species level as their conidial production was too poor for reliable determination (confirmation by IMI)
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>A. infectoria</em></td>
<td>Charing, Kent</td>
</tr>
<tr>
<td>B</td>
<td><em>A. alternata</em></td>
<td>Tenbury Wells, Shropshire</td>
</tr>
<tr>
<td>C</td>
<td><em>A. alternata</em></td>
<td>Farringdon, Oxfordshire</td>
</tr>
<tr>
<td>D</td>
<td><em>Alternaria</em> spp.</td>
<td>Shrewton, Wiltshire</td>
</tr>
<tr>
<td>E</td>
<td><em>A. alternata</em></td>
<td>Gloucester, Gloucestershire</td>
</tr>
<tr>
<td>F</td>
<td><em>A. brassicicola</em></td>
<td>Winchester, Hampshire</td>
</tr>
<tr>
<td>G</td>
<td><em>A. alternata</em></td>
<td>Writtleford, Cambridgeshire</td>
</tr>
<tr>
<td>H</td>
<td><em>A. alternata</em></td>
<td>Shrewton, Wiltshire</td>
</tr>
<tr>
<td>I</td>
<td><em>A. alternata</em></td>
<td>Stomanet, Suffolk</td>
</tr>
<tr>
<td>J</td>
<td><em>A. alternata</em></td>
<td>Writtleford, Cambridgeshire</td>
</tr>
<tr>
<td>K</td>
<td><em>A. infectoria</em></td>
<td>Redhill, Surrey</td>
</tr>
<tr>
<td>L</td>
<td><em>A. alternata</em></td>
<td>West Wickham, Cambridgeshire</td>
</tr>
<tr>
<td>M</td>
<td><em>A. alternata</em></td>
<td>Alton Pancreas, Hampshire</td>
</tr>
<tr>
<td>N</td>
<td><em>A. brassicae</em></td>
<td>Writtleford, Cambridgeshire</td>
</tr>
<tr>
<td>O</td>
<td><em>Alternaria</em> spp.</td>
<td>Bircher, Hereford and Worcestershire</td>
</tr>
<tr>
<td>P</td>
<td><em>A. alternata</em></td>
<td>Taughton, Somerset</td>
</tr>
<tr>
<td>Q</td>
<td><em>A. alternata</em></td>
<td>Maidenhead, Berkshire</td>
</tr>
<tr>
<td>R</td>
<td><em>A. infectoria</em></td>
<td>Maidstone, Kent</td>
</tr>
<tr>
<td>S</td>
<td><em>A. alternata</em></td>
<td>Pershore, Hereford and Worcestershire</td>
</tr>
<tr>
<td>T</td>
<td><em>A. alternata</em></td>
<td>Epping, Essex</td>
</tr>
<tr>
<td>U</td>
<td><em>A. tenuissima</em></td>
<td>Wye, Kent</td>
</tr>
<tr>
<td>V</td>
<td><em>A. infectoria</em></td>
<td>Tunbridge Wells, Kent</td>
</tr>
<tr>
<td>W</td>
<td><em>A. tenuissima</em></td>
<td>Tenterden, Kent</td>
</tr>
</tbody>
</table>

Table 4.1 Isolates of *Alternaria* spp. used in this study
4.1.2.2 Mycotoxin production

Strains of *Alternaria* were cultured on rice as well as on rapeseed and its meal in order to facilitate the isolation of toxins. The following media were distributed in 250 ml conical flasks and autoclaved at 121°C for 20 min.: 

(i) 20 g rice (Sainsbury’s American long grain; Sainsbury’s Ltd., Camden, UK) + 30 ml distilled water (total MC = 60%)

(ii) 20 g rapeseed (OO cultivars, collected from Camgrain Silo, Cambridge) + 20 ml distilled water (MC = 55%)*

(iii) 20 g meal (collected from ADM Erith Oil Works, London) + 20 ml distilled water (MC = 53%)*

The media were inoculated with spore suspensions of the fungi (20 μl; ca. 1X10^3 spores/ml) previously prepared in 10% glycerol (see section 2.1.2.3) and incubated at 25°C** in the dark.

4.1.2.3 Extraction of the cultures

After incubation for three weeks, cultures were frozen (-70°C), lyophilised (Birchover Instruments Ltd., Freeze Dryer 7.5) and then extracted by homogenising (Sorvall Omni-mixer, Ivan Sorall Inc., Newtown, USA) in MeOH (100 ml). Solids from methanolic extracts were removed by centrifugation (Gallenkamp Labspin at 5 000 g for 10 min. Samples (25 ml) taken from each culture supernatant were evaporated to dryness under vacuum at 30°C. Residues were reconstituted in 1.0 ml MeOH to give extracts that contained the equivalent of 5 g culture substrate/ml and stored at -70°C.

* the MC of ‘stock’ rapeseed and meal (ca. 9 and 6% respectively) was measured by heating 5 g of each substrate at 100°C for 3 hours, cooling to room temperature in a desiccator containing silica gel (ca. 30 min) and comparing the weight of these substrates with their original weights (implemented by quality control technicians, ADM Erith Oilworks)

** Preliminary studies demonstrated that the optimum growth of *Alternaria* on rapeseed agar occurred at approximately 25°C (Appendix 2)
4.1.2.4 Analysis by TLC

Culture extracts were chromatographed (10 μl bands) on silica gel thin layer plates (0.2 mm thickness) with fluorescence indicator (Kieselgel 60 F₃₅₄, 20 X 20 mm, Merck, Lutterworth, England), that had been activated at 100°C for two hours. Toluene-ethyl acetate-90% formic acid (6:3:1) was used as the solvent system. Toxins were visualised under long (λ365 nm) and short-wave (λ254 nm) UV light (Chromato-vue cabinet model CC-20, Ultra-violet Products Inc., San Gabriel) and by spraying with ethanolic ferric chloride (1%).

Isolation of the metabolites for subsequent HPLC analysis was accomplished by chromatographing the extracts on TLC plates with the TEF system, scraping bands containing the toxin from the plate and eluting them from the silica gel with MeOH (0.5-1.0 ml). Elutates were dried under vacuum and made up to 20 μl (corresponding to 2.5 g/ml).

4.1.2.5 Analysis by HPLC

HPLC analysis was used for confirmation of TLC results and quantification of ALT, ATX-I, AOH, TeA and AME. The diode array HPLC system and mobile phase used for analysis of mycotoxins are described in chapter three. Mycotoxins (5 or 10 μl injections) were recognised by their retention times (see section 3.2.3) and UV spectra (see Fig. 3.4). ALT was quantified by processing chromatograms from the chromascans at λ240 nm; ATX-I, AOH and AME at λ260 nm and TeA at λ280 nm. The absorbance of the toxin peaks was determined and quantified by comparing with standard calibration curves. In some samples, it was necessary to dilute with MeOH (e.g. rice extract of isolate T) so that the height of the toxin peaks were within the range of the standard calibration curve.
4.1.3 The effect of temperature, moisture content and incubation period on the production of TeA

4.1.3.1 Fungal cultures, mycotoxin production and extraction

Three studies were performed:

(I) Flasks (conical, 250 ml) containing rapeseed (20 g) were divided into four treatment units of three replicates, with each treatment unit containing different amounts of distilled water (0, 5, 10 and 20 ml)*. The flasks were autoclaved at 121°C for 20 min. and inoculated with spore suspensions of isolate U (20 μl; ca. 1X10³ spores/ml). The four MC treatments of rapeseed were incubated at five temperatures, 10, 15, 20, 25 and 30°C, for 3 weeks. In order to determine whether MCs changed over the course of the experiment, flasks were weighed immediately before and after autoclaving, and at the termination of the experiment (i.e. three weeks). A similar study was run in parallel with experiment I but rapeseed was substituted by rapeseed meal**.

(II) Three MC treatment units of both rapeseed and meal (for 0, 2.5 and 5 ml of added distilled water, the MC of rapeseed was 9, 19 and 27% respectively, and meal, 6, 16 and 25% respectively) were inoculated with isolate U and rapeseed (9% MC) with isolate T, as described in experiment I. Flasks were incubated at 10, 15 and 20°C as replicates of three.

Extraction of mycotoxins from cultures was carried out as detailed in section 4.1.2.3.

* the MCs of rapeseed containing 0, 5, 10 and 20 ml distilled water were 9, 27, 39 and 55% respectively

** the MCs of meal containing 0, 5, 10 and 20 ml distilled water were 6, 25, 37 and 53% respectively
4.1.3.2 Analysis of mycotoxins

An HPLC solvent system was developed using the isoeluotropic solvent composition plane described in chapter 3, which allowed direct analysis of TeA from crude extracts, or after suitable dilution with MeOH. Quantification was accomplished by integrating the peak area and comparison with a standard calibration curve, using Philips PU6100 Integration Software. Where TeA was not detected, samples were concentrated using TLC (refer to section 4.1.2.4) and then re-examined by HPLC.

4.1.4 Statistical analysis

Analyses of variance (ANOVA) and significance testing of mean values (least significance difference - LSD) were calculated using the computer programme STATGRAPHICS (STSC Inc., Rockville, USA). Simple statistical computations of the mean, range and sd were performed on a hand held pocket calculator (Casio fx-85v).

4.2 RESULTS

4.2.1 Biological activity of Alternaria toxins

4.2.1.1 Rapeseed germination assay

Detection of TeA and AME by measuring the germination rate of rapeseed was only suitable for TeA, which reduced radicle elongation at 100 µg/ml (0.507 mM). AME had no effect on rapeseed germination at concentrations less than or equal to 100 µg/ml (0.388 mM) (Fig. 4.1).

4.2.1.2 Rapeseed leaf assay

On application of Alternaria toxins to rapeseed leaves, only TeA (2 mM) induced chlorotic lesions (Fig. 4.2).

4.2.1.3 Oilseed rape leaf cell assay

Control solutions holding 5% MeOH or less sustained live cells for up to 3.5 hours with 97.8% viability. With samples containing 0.1 and 0.2 mM of toxin, TeA (LD$_{50}$; approximately 0.165 mM) was more toxic to rapeseed cells
Fig. 4.1 The effect of TeA and AME on rapeseed germination after 60 hours at 20°C
Fig. 4.2 Toxicity of TeA to a detached leaf of oilseed rape. A = 2 mM (5 μl drop); B = 1 mM; C = 0.5 mM; D = 0.25 mM
than the other *Alternaria* toxins. Estimation of LD$_{50}$ values of ALT, ATX-I, AOH, and AME were not feasible as the highest concentrations used (0.2, 0.1, 0.2 and 0.1 mM respectively) killed less than 50% of cells. The ATX-I and AME toxins could not be tested at concentrations greater than 0.1 mM because the availability of ATX-I was limited and the solubility of AME in MeOH was poor (roughly 2.5 mM) (Fig. 4.3A and B).

### 4.2.1.4 Brine shrimp assay

Toxicity of *Alternaria* metabolites as measured by the decreased motility of *Artemia salina* is shown in Fig. 4.4. In general, TeA appears to be the most toxic metabolite, although ATX-I was not analysed. On addition of rice, rapeseed and rapeseed meal culture extracts (from isolates E and F), motility of brine shrimp was strikingly reduced, particularly at concentrations of 1 g substrate/ml extract (Table 4.2). However, as the control extracts (*i.e.* from uninoculated rice, rapeseed or rapeseed meal) also reduced brine shrimp motility (except the rice control extract corresponding to 0.1 g/ml), alternative factors contributed to the decrease in brine shrimp motility. This is further supported from the fact that culture extracts of isolates E and F were later determined by HPLC analysis to contain no toxins (see section 4.2.2.2).
Fig. 4.3  (A) Leaf cells of oilseed rape stained with fluorescein diacetate. Live cells fluoresce green under UV light (magnification X200) (B) Toxicity of Alternaria metabolites to isolated leaf cells of oilseed rape
Fig. 4.4  Toxicity of *Alternaria* metabolites to motility of brine shrimp
### MOTILITY (%)###

<table>
<thead>
<tr>
<th>SUBSTRATE</th>
<th>CONCENTRATION (g/ml)</th>
<th>CONTROL</th>
<th>A. alternata (isolate E)</th>
<th>A. brassicicola (isolate F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RICE</td>
<td>0.1</td>
<td>93</td>
<td>74</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>62</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RAPESEED</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RAPESEED MEAL</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.2 Toxicity of crude *Alternaria* extracts on the motility of brine shrimp

4.2.2 Determination of mycotoxigenic *Alternaria*

4.2.2.1 TLC of mycotoxins

The Rf values of the ALT, ATX-I, AOH, TeA and AME on TLC plates were 0.27, 0.44, 0.56, 0.47 and 0.68 respectively, which generally corresponded to those values reported by Visconti et al. (1986). Under long wave UV light, AME and AOH were visualised by their blue fluorescence; ALT and ATX-I fluoresced yellow-green and brown-orange, respectively. On exposure to short wave UV radiation, fluorescence spots were observed for ALT (light blue), AOH (blue) and AME (blue), and quenching spots for ATX-I and TeA (Figs. 4.5A and B). Spraying plates with 1% ethanolic ferric chloride caused TeA to appear as a red spot.
Fig. 4.5 TLC of culture extracts of *Alternaria* spp. under (A) long wave (λ365 nm) UV light and (B) short wave (λ254 nm) UV light. Lane 1 = TeA standard (a); 2 = ALT (b), ATX-I (c), AOH (d) and AME (e) standards; 3 = rice extract of isolate N; 4 = meal extract of N; 5 = rice extract, U; 6 = meal extract, U; 7 = rice extract, T; 8 = rice extract, O
4.2.2.2 HPLC of mycotoxins

Standard calibration curves had a linear relationship with a concentration range (0.25-4.0X10^3 µmoles) of toxin reference standards. The correlation coefficients (r) for ALT, ATX-I, AOH, TeA and AME were 0.998, 0.998, 0.999, 0.998 and 0.997 respectively (Fig. 4.6).

Of the 23 isolates of *Alternaria* spp. grown on rice at 60% MC and 25°C for three weeks, the majority produced ATX-I (7 isolates) and AOH (7), followed by AME (5), TeA (4) and ALT (4) (Table 4.3). Thirteen isolates produced at least one of these toxins while none produced all toxins. Two isolates (T and U) produced four mycotoxins (ALT, AOH, TeA and AME), as did isolate W (ALT, ATX-I, AOH and AME). Isolates that yielded three toxins were N (ALT, AOH and AME), V (TeA, AOH and AME) and O (ATX-I, AOH and AME).

The highest producers of mycotoxins on rice were isolate U (1.7400 µmoles ALT/g), isolate O (0.1320 µmoles ATX-I/g), V (2.2875 µmoles AOH/g), T (32.070 µmoles TeA/g) and T (7.3887 µmoles AME/g).

Five isolates produced toxins on oilseed substrates (Table 4.4). Meal was generally a better substrate for growth of *Alternaria* spp. and subsequent production of toxins than rapeseed (e.g. production of TeA by isolate U on meal and rapeseed was 0.0594 and 0.0255 µmoles/g respectively). Average TeA production of those five isolates (0.3791 and 0.2132 µmoles/g meal and rapeseed, respectively) was higher than AME (0.0342; 0.0192), ALT (0.0125; 0.0038) and AOH (0.0122; 0.0065). There was no production of ATX-I on oilseed rape substrates.
Fig. 4.6  Standard curves of peak height of ALT (\(\lambda_{240}\) nm; \(r=0.999\)), ATX-I (\(\lambda_{260}\) nm; \(r=0.998\)), AOH (\(\lambda_{260}\) nm; \(r=0.999\)), TeA (\(\lambda_{280}\) nm; 0.999) and AME (\(\lambda_{260}\) nm; \(r=0.997\)), as determined by HPLC analysis.
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mycotoxin content (µmoles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT</td>
</tr>
<tr>
<td><strong>A. alternata</strong></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
</tr>
<tr>
<td>H</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>-</td>
</tr>
<tr>
<td>M</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>-</td>
</tr>
<tr>
<td>Q</td>
<td>-</td>
</tr>
<tr>
<td>S</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>0.1400</td>
</tr>
<tr>
<td><strong>A. brassicaceae</strong></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>0.2375</td>
</tr>
<tr>
<td>N*</td>
<td>0.0575</td>
</tr>
<tr>
<td><strong>A. brassicicola</strong></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>-</td>
</tr>
<tr>
<td><strong>A. infectoria</strong></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td><strong>A. tenuissima</strong></td>
<td></td>
</tr>
<tr>
<td>U</td>
<td>1.7400</td>
</tr>
<tr>
<td>W</td>
<td>0.1470</td>
</tr>
<tr>
<td><strong>Alternaria spp.</strong></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>O</td>
<td>-</td>
</tr>
</tbody>
</table>

* when grown at 60% MC; 25°C; 2 weeks

** these isolates were not identified to species level as their conidium production was too poor for reliable determination (confirmation by IMI)

- not detected

Table 4.3 Production of toxins by *Alternaria* spp. grown on rice (60% MC) for 3 weeks at 25°C
<table>
<thead>
<tr>
<th>Isolate of Alternaria spp.</th>
<th>Mycotoxin content (μmoles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ALT</td>
</tr>
<tr>
<td>N</td>
<td>0.0510</td>
</tr>
<tr>
<td>N*</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>-</td>
</tr>
<tr>
<td>U</td>
<td>0.0113</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
</tr>
<tr>
<td>W</td>
<td>-</td>
</tr>
</tbody>
</table>

**Meal**

<table>
<thead>
<tr>
<th></th>
<th>ALT</th>
<th>ATX-I</th>
<th>TeA</th>
<th>AOH</th>
<th>AME</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.0114</td>
<td>-</td>
<td>-</td>
<td>0.0102</td>
<td>0.0150</td>
</tr>
<tr>
<td>N*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T</td>
<td>-</td>
<td>-</td>
<td>1.0250</td>
<td>-</td>
<td>0.0465</td>
</tr>
<tr>
<td>U</td>
<td>0.0074</td>
<td>-</td>
<td>0.0255</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>-</td>
<td>0.0156</td>
<td>0.0224</td>
<td>0.0133</td>
</tr>
<tr>
<td>W</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.0210</td>
</tr>
</tbody>
</table>

**Rapeseed**

* when grown at 53-55% moisture; 25°C; 2 weeks

- not detected

Table 4.4  Production of toxins by Alternaria spp. grown on rapeseed (55% MC) and meal (53% MC) for 3 weeks at 25°C
4.2.3 The effect of temperature, moisture content and incubation period on the production of TeA

4.2.3.1 Development of an HPLC solvent system for quantifying TeA

The best solvent system which gave suitable separation of TeA (at λ280nm) from oilseed rape components was 50.3% MeOH, 18.3% THF and 31.4% aqueous zinc sulphate solution (300 mg/L). The RT of TeA was 5.71 min. (Fig. 4.7).

4.2.3.2 Variability in moisture content of rapeseed and meal

Autoclaving had minimal effect (generally within a 3% change in weight) on the MC of oilseed substrates. After incubation for three weeks, MC loss was relatively small, with most water evaporating from the 30°C; 20 ml water treatments. On average, the weight lost from those meal and rapeseed cultures was 10.9 and 13.0% of respectively. Loss of water was not measured for cultures incubated for four months.

4.2.3.3 Production of TeA

Temperature and MC markedly affected the ability of *Alternaria* spp. to grow and produce TeA on rapeseed and meal. When isolate U was incubated for three weeks, optimum conditions for production of TeA occurred at about 25-30°C; 53-55% MC. As temperatures and moisture contents decreased, so too did TeA production. For example, TeA production on rapeseed at 10°C; 53-55% MC (0.0047 μmoles/g) was roughly a fifth of that produced under optimum conditions (0.0271 μmoles/g). Although increasing temperatures generally promoted production of TeA, this was highly dependent on MC. At 6% MC in meal and 9% MC in rapeseed, there was no production of TeA at 10, 15, 20, 25 or 30°C. At the next higher MCs (25 and 27% on meal and rapeseed respectively), TeA was only detected at 30°C on meal (0.0044 μmoles/g) and between 20-30°C on rapeseed (0.0025-0.0041 μmoles/g) (Fig. 4.8A and B).

Although further incubation (four months) of isolate U increased production of TeA at 20°C; 25-27% MC on rapeseed (0.0157 μmoles/g) and
Fig. 4.7 HPLC chromatogram (λ280 nm) of a crude culture extract of *A. tenuissima* (U) grown on rapeseed. The mobile phase was 50.3% MeOH, 18.3% THF and 31.4% aqueous zinc sulphate solution (300 mg/L); RT of TeA = 5.71 min.
Fig. 4.8 The effect of temperature and moisture content on TeA production by *A. tenuissima* (U) grown on (A) rapeseed and (B) meal for three weeks.
meal (0.0239 \( \mu \)moles/g), there was no TeA formation on either of these substrates under drier conditions (6-9% MC) at 10, 15 or 20°C. Isolate T, which was found to be a superior TeA producer to isolate U (see Tables 4.3 and 4.4), was also unable to produce TeA at moisture levels of 9%, when grown on rapeseed at 10, 15, 20°C for 16 weeks (Table 4.5).

One other interesting feature observed here was that meal was generally preferable to rapeseed for growth of \textit{Alternaria} spp. and production of TeA. For example, at 25°C; 53-55% MC; 3 weeks growth, levels of TeA on meal and rapeseed were 0.0494 and 0.0271 \( \mu \)moles/g respectively.

<table>
<thead>
<tr>
<th>TeA content (( \mu )moles/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10°C</strong></td>
</tr>
<tr>
<td>Rapeseed (isolate U)</td>
</tr>
<tr>
<td>Rapeseed meal (isolate U)</td>
</tr>
<tr>
<td>Rapeseed (isolate T)</td>
</tr>
</tbody>
</table>

Table 4.5  Production of TeA by \textit{Alternaria} spp. on rapeseed and rapeseed meal after four months incubation
4.3 DISCUSSION

Biossay results indicated TeA was the most toxic of the compounds produced by Alternaria spp. This confirms earlier work by Meronuck et al. (1972) and Harvan and Pero (1976). Although ATX-I is considered to be quite important (Schade and King; 1984), the toxin is normally produced in small amounts both in culture and nature (Visconti et al., 1986; Logrieco et al., 1990). Owing to the small quantity of the metabolite available for this study, it was not always appraised in bioassay tests.

Detection of Alternaria toxins (AME and TeA) by the rapeseed root assay was only feasible for TeA at 100 µg/ml (0.508 mM), which inhibited root growth by approximately 50%. Visconti et al. (1992) also found that TeA inhibited the root growth of oilseed rape but at lower concentrations e.g. 50% inhibition at 10 µg/ml.

The toxicity of Alternaria metabolites was determined by challenging detached leaves of oilseed rape with toxin solutions. Detached leaves of Brassica species and potato have been used previously to detect phytotoxins produced by A. brassicae (Bains and Tewari, 1987). In this study, TeA was the only Alternaria metabolite that induced leaf chlorosis.

Using cells from oilseed rape leaves to measure toxicity of Alternaria metabolites, TeA was found to be the more toxic compound. This method appeared to be more sensitive in detecting Alternaria toxins, particularly TeA (LD₅₀; ca. 0.165 mM) than the detached leaf and root germination assay. Advantages of employing cells in bioassays in comparison to seeds, detached leaves, plant cuttings or whole plants are that only relatively small quantities of test solutions are needed, results can be acquired within a matter of hours and difficulties associated with toxin penetration are resolved (Earle et al., 1978).

The brine shrimp assay demonstrated that TeA but not other Alternaria toxins (i.e. ALT, AOH and AME) significantly reduced motility of brine shrimp.
Similarly, Visconti et al. (1992) found that TeA produced from Alternaria spp. killed brine shrimp larvae after 36 hours at 27°C (e.g. 56% larva mortality occurred at 20 μg/ml of TeA). However, it is unlikely that the brine shrimp assay could be used for the detection Alternaria toxins in rapeseed and rapeseed meal cultures because (i) the controls generally reduced motility of brine shrimp at the same rate as culture extracts and (ii) the culture extracts tested (i.e. isolates E and F) contained no known toxins, suggesting that reduction of motility could be caused by the presence of compounds other than mycotoxins already identified. Similar results were found by Zajkowski et al. (1991), who found there was no correlation between toxicity of wheat culture extracts to the motility of brine shrimp and quantity of mycotoxins (e.g. 8 330 μg/ml TeA) in culture. Long-chained fatty acids, which may be present in the substrates, are toxic to brine shrimp (Curtis et al., 1974). Although the brine shrimp assay is easy to perform, relatively inexpensive and quite sensitive to mycotoxins (e.g. LD₅₀ of sterigmatocystin, 0.54 μg/ml; Harwig and Scott, 1971), it may be unsuitable for evaluation of mycotoxins present in animal feedstuffs owing to the high incidence of interfering compounds (Watson and Lindsay, 1982; Buckle et al., 1990).

For estimating the mycotoxigenic capability of isolates of Alternaria spp, researchers normally use unsupplemented milled rice under optimum moisture (ca. 50%) and temperature (20-25°C) conditions (Mortimer et al., 1988; Logrieco et al., 1990; Bruce et al. 1984; Visconti et al., 1992). This is because Alternaria spp. generally produced greater quantities of mycotoxins on rice than on other solid substrates, including wheat, rye, oats, maize, barley, olives and sorghum (Burroughs et al. 1976; Visconti et al., 1986, 1987; Mortimer et al., 1988; Logrieco et al., 1990; Kostecki et al., 1991), and liquid media (e.g. 2% yeast extract-4% sucrose broth - YES) (Young et al., 1980; Maas et al., 1981; Wei and Swartz 1985). On rapeseed, Visconti et al. (1992) observed that production of TeA was about quarter that produced on rice; only traces of ATX-I was found and no AOH, AME and ATX-II could be detected in rapeseed cultures.
Similarly, the results presented here show that production of mycotoxins by *Alternaria* spp. was greater on rice than oilseed rape substrates. However, the proportion of *Alternaria* isolates which produced mycotoxins on rice (13 from 23 isolates produced at least one mycotoxin) was lower than that stated in other studies (Bruce et al., 1984; Visconti et al., 1986; Logrieco et al., 1990). For example, Visconti et al. (1992) found that all strains of *Alternaria* (11) isolated from oilseed rape grown in Italy produced at least one mycotoxin (e.g. ALT) on rice.

With regard to mycotoxin levels found in rapeseed and meal cultures, meal was generally better for production of toxins by *Alternaria* spp. than rapeseed (e.g. production of TeA by isolate U on meal and rapeseed was 0.0594 and 0.0255 μmoles/g respectively) (see Table 4.4). Perhaps the surface area per volume ratio of crushed meal was greater than that of rapeseed which allowed faster growth of the mould and subsequent TeA production.

The literature generally acknowledges that rice cultures of *Alternaria* produce higher levels of TeA than ALT, ATX-I, AOH and AME (Logrieco et al., 1990; Visconti et al., 1986; 1992). Bruce et al. (1984) observed that the average yield from 18 cultures which produced TeA was 2.2 mg per gram (11.17 μmoles/g) of rice and the average yields from 12 cultures which produced AOH and AME were 0.5 mg (1.94 μmoles) and 0.4 mg (1.47 μmoles) per gram of rice, respectively. Comparable results were noticed in this study, where TeA producing isolates of *Alternaria* (P, T, U and V) produced higher mean levels of TeA (9.4165 μmoles/g) than those isolates which produced AME (2.4800 μmoles/g), AOH (0.6605), ALT (0.5661) and ATX-I (0.0354) grown on rice for three weeks (Table 4.3). This was also the case for production of mycotoxins on rapeseed and meal, where average production of TeA from isolates which produced mycotoxins on rapeseed and meal (N, T, U, V and W) was greater than average levels of AME, AOH, ALT and ATX-I (see Table 4.4 for exact quantities).
It is apparent then that TeA is more toxic and produced in greater quantities than the other Alternaria metabolites, under favourable growing conditions for Alternaria i.e. 25°C; 53-60% MC. Therefore, TeA would be potentially more hazardous to the UK oilseed rape industry than any other Alternaria mycotoxin. However, data presented in this study also showed that production of TeA on rapeseed and rapeseed meal was highly dependent on MC and temperature. Optimum production of TeA occurred about 25-30°C; 53-55% MC but as conditions cooled and became drier, growth of Alternaria spp. and production of TeA was reduced.

Previous publications report that high moisture conditions i.e. a water activity above 0.84 (King and Schade, 1984) or MC between 28-34% (Stinson, 1985) favour growth of Alternaria spp.. Young et al. (1980) observed that production of TeA by A. tenuissima on cottonseed at 20°C was higher at 37.46% MC (52.27 ± 0.96 µg/g) than cottonseed at lower MCs (e.g. at 22.31 and 11.71% water, production of TeA was 24.14 ± 2.02 and 0 µg/g respectively). Young et al. (1980) also found that the optimum temperature for TeA formation in cottonseed was 20°C, with maximum quantity of Tea (0.105 mg/g seed) being attained after 24 days.

Mills and Sinha (1980) estimated a guideline for maximum periods (ca. five months) of safe storage for rapeseed in Canada from spoilage by moulds (e.g. Penicillium spp.) at different temperature and moisture levels. Generally speaking, rapeseed stored at 20°C or below with a MC less than 9% was safe from microbial spoilage. At 10°C, MC was permitted at 12%, whereas between 25 and 40°C, MC was 7-8%. This guideline is consistent with results presented here, where there was no production of TeA on meal and rapeseed by Alternaria at 10-30°C and approximately 9% moisture when incubated for three or 16 weeks.

In summary, if TeA is to be actively produced in rapeseed and meal, it would most probably occur if MC was greater than approximately 9% and at
temperatures which are optimum for *Alternaria* growth (25-30°C). However, production of TeA is unlikely to be a problem during industrial storage of rapeseed and meal because:

(i) the ambient temperatures to which industrial rapeseed and meal are exposed are low, particularly during autumn and winter (0-15°C)

(ii) maximum moisture content of industrial rapeseed (prior to crushing) and meal is ≤9%, as enforced by EEC regulations.
Chapter four demonstrated that *Alternaria* spp. isolated from oilseed rape grew more rapidly and produced greater quantities of toxins on rice than rapeseed and meal. However, the growth of one isolate of *A. alternata* (isolate B) on rice was slower than on other substrates (*i.e.* rapeseed, meal and MEA) and appeared to die after 2-3 weeks (Fig. 5.1). Although it is not uncommon for some isolates of *Alternaria* spp. to exhibit retarded growth and produce few spores, this is often owing to poor maintenance or frequent sub-culturing of spores or mycelium (Dr John David, pers. comm., IMI, Egham, UK). However, all the isolates studied here were only subcultured three times and spores of single-spored cultures were then stored at -70°C in 10% glycerol. Contamination by bacteria and subsequent production of toxic metabolites may also inhibit the development of fungal cultures but this was not the case in this study since no bacteria were detected in rice cultures of isolate B.* Therefore, it was thought that an inhibitory compound was produced by the fungus itself on rice but not on rapeseed, meal or MEA.

Another interesting feature of isolate B was that when a spore suspension of the mould was sprayed on leaves of oilseed rape plants, slight chlorotic lesions developed around the germinating spores (see 5.1.10 and 5.2.8). This suggested isolate B was a pathogen of oilseed rape and not a true saprophyte as are most isolates of *A. alternata*. Since opportunistic (*i.e.* virulent) forms

---

* methods used for detecting possible contamination of bacteria included light microscopy and streaking a small portion of rice culture on nutrient agar (Oxoid Ltd., Basingstoke, UK)
Fig. 5.1  Growth of *Alternaria* spp. (isolates B and G) on rice (60% moisture content) at 25°C for three weeks
of *A. alternata* normally yield phytotoxins (e.g. AF toxin I by a pathotype of *A. alternata* that infects strawberries causes necrosis of leaves of Morioka-16 strawberry and Nijisseiki pears) (Scheffer, 1992), it is possible a metabolite was produced here which aided in the infection process.

This chapter is concerned with the isolation of a compound, designated compound B, which inhibited the growth of spores produced by *A. alternata* and examination of its properties and possible role in pathogenicity of oilseed rape.

### 5.1 MATERIALS AND METHODS

#### 5.1.1 Media and growth conditions of *A. alternata*

Cultures of isolates B and G* were grown on rice according to the procedure described in 4.1.2.2.

#### 5.1.2 Extraction

Cultures (3 weeks old) were frozen (−70°C), lyophilised and homogenised in MeOH (1 g/5 ml). After filtering (Whatman filter paper, No. 1), the solids were re-homogenised with chloroform (ca. 1 g/2 ml). The MeOH and chloroform culture extracts were evaporated independently under reduced pressure and reconstituted to the equivalent of approximately 20 g rice per ml of MeOH and chloroform, respectively.

#### 5.1.3 Detection

Crude extracts of isolate B and G were applied as spots (< 5 mm diameter) to TLC plates (0.2 mm, Kieselgel 60 F$_{254}$, Merck, Lutterworth, UK) that had been activated at 100°C for two hours. Initially, a simple spot test was employed to detect the presence of any toxic metabolites. In order to separate these metabolites, plates were developed in one of the following solvent systems:

* these isolates did not produce ALT, ATX-I, AOH, TeA and AME (see chapter 4)
(1) toluene:acetone (12:7)
(2) toluene:ethyl acetate:acetone (7:2:1)
(3) chloroform:acetone (88:12)
(4) MeOH:chloroform (1:9)
(5) MeOH:ACN (1:9); (1:3); (2:3); (1:1); (4:3)

After drying, plates were sprayed with a spore suspension of A. alternata Cladosporium (ca. 1 X 10^4 spores/ml) or Cucumerinum (ca. 1 X 10^5 spores/ml) in double strength Czapek Dox liquid medium (Oxoid Ltd.). Incubation of plates was at high humidity in the dark at 25°C for 48 hours. A region which showed antifungal activity indicated the presence of a toxic metabolite.

5.1.4 Purification

Solid phase cartridges (360 mg; C18 plus, Millipore [UK] Ltd, Watford, UK) were employed for 'cleaning-up' the crude (MeOH) extract of isolate B. Cartridges were conditioned with MeOH (5 ml) followed by 5 ml of citrate buffer (0.1 M; pH 3.0):MeOH (1:1 v/v). Before the column dried, citrate buffer:MeOH culture extract (1:1 v/v) was passed slowly through the cartridge (10-15 ml) and washed with 10 ml of citrate buffer/MeOH (1:1 v/v). Addition of 0.5 ml MeOH shifted the desired component to the lower region of the cartridge, which was eluted with further MeOH (1 ml); this fraction was collected for further purification. The cartridge was washed with MeOH (ca. 10 ml) and then reconditioned using the procedure described previously. Cartridges were re-used three times only.

Fractions collected from solid phase clean-up were pooled, concentrated under vacuum and further refined using preparative TLC. A long, narrow band (160X5 mm) was applied to a TLC plate (0.5 mm Kieselgel 60 F_{254}) and developed using the solvent system n-butanol:acetic acid:water (4:1:1) (BAW). After drying, the band containing compound B (Rf=0.18) was isolated by scraping the appropriate region of silica from the plate. The silica was mixed with MeOH (ca. 0.1 g silica/1 ml MeOH), centrifuged at 12 900 g (Micro-
centaur, MSE, Loughborough, UK) for 3 min. and the resulting supernatant was transferred to a clean pear-shaped flask (25 ml). This procedure was repeated with further MeOH (ca. 0.1 g silica/1 ml MeOH) and the resulting extracts were combined, concentrated under vacuum and stored in MeOH at -20°C.

5.1.5 Purification studies using centrifugally accelerated TLC

Isolation of compound B was investigated using the 'Chromatotron' (Model 7924T, Harrison Research, Palo Alto, USA), which is a preparative, centrifugally accelerated, radial thin layer chromatograph (Fig. 5.2). The system consists of a thin layer of sorbent on a glass rotor which is rotated at constant speed (750 rpm). Solutions of samples are introduced by a polyester wick at the inner edge of the rotating sorbent layer. Elution with a particular solvent system results in concentric bands of separated compounds which exit the rim of the rotor together with the solvents; these are collected as fractions.

The sorbent layer in this study was prepared on a clean glass rotor as follows: distilled water (130 ml), chilled at 4°C, was added to 65 g of silica gel 60 PF254 containing calcium sulphate as the binder, and shaken vigorously for 30 sec.. While the rotor was slowly rotated by hand, the mixture was poured in a continuous stream in overlapping circles near the central metal shaft. The rotor was carefully jolted to liquify the mixture and improve the uniformity of the sorbent by the release of air bubbles. After drying at room temperature (ca. 20°C; ca. 12 hours) under a plastic container to prevent cracking, the plate was dried in a oven at 65°C for 5 hours. The sorbent layer was shaved to 2 mm in depth using a special metal scraping tool.
Fig. 5.2 Blueprint of the 'chromatotron' (Model 7924T, Harrison Research, Palo Alto, USA)
Before the sample solutions (i.e. from the solid phase sample) were introduced (ca. 1 ml), the sorbent layer was equilibrated with the solvent system, which included one of the following:

(1) BAW; rate of delivery was approximately 2 ml/min.
(2) MeOH:ACN; equilibration at 4:3, then step gradients at 4:3 (ca. 50 ml), 6:3 (ca. 50 ml), 8:3 (ca. 50 ml) and 10:3 (ca. 50 ml); rate of delivery was approximately 6 ml/min.

Fractions were collected as 1.0-1.5 ml aliquots.

5.1.6 Analysis by TLC

Analytical TLC plates (0.2 mm, Kieselgel 60 F$_{254}$, Merck) were developed in either BAW or MeOH:ACN (4:3). After drying, plates were examined under short and long wavelength UV light and then sprayed with one of the following reagents:

(1) Bromocresol green*; 0.04 g bromocresol green was dissolved in 100 ml ethanol and 0.1 M sodium hydroxide added until a blue colour just appeared

(2) Dragendorff's reagent*; equal volumes of solution A (0.85 g basic bismuth nitrate dissolved in a mixture of 10 ml acetic acid and 40 ml water) and solution B (8 g potassium iodide in 20 ml water) were mixed as a stock solution. The spray reagent consisted of 1 ml of stock solution together with 2 ml acetic acid and 10 ml water. After treatment, the plate was sprayed with 0.1 M sulphuric acid to increase the sensitivity of detection.

(3) Anisaldehyde-sulphuric acid**; 0.18% anisaldehyde solution dissolved in a mixture of 7 ml MeOH, 1 ml glacial acetic acid and 0.5 ml concentrated sulphuric acid, and the plate was heated at 100°C for approximately 5 min.
(4) Folin-Ciocalteu's reagent; purchased from BDH Laboratory Supplies, Merke Ltd., UK.

(5) Isatin-sulphuric acid*; 1 g isatin was dissolved in 100 ml concentrated sulphuric acid and the plate was heated at 100°C for approximately 5 min.

(6) Ninhydrin*; 0.2 g ninhydrin was dissolved in 100 ml ethanol and the plate was heated at 100°C for approximately 5 min.

(7) Ferric chloride*; 5% solution of ferric chloride in 0.5 M hydrochloric acid

(8) Vanillin-sulphuric acid*; 1 g vanillin was dissolved in 100 ml concentrated sulphuric acid and the plate was heated at 100°C for approximately 5 min.

5.1.7 Spectrophotometry

The absorption spectrum of compound B in MeOH was measured using a Philips UV-VIS spectrophotometer, scanning between λ200 and 450 nm.

5.1.8 Analysis by HPLC

Chromatography was performed on a C8 column (150X4.6 mm I.D.), protected by a guard column of similar packing (20X4.6 mm I.D.) using the Philips HPLC system previously described in section 3.1.2. The mobile phase consisted of a 20 min. gradient commencing with water (100%) and ending with ACN (100%), with a flow rate of 1 ml/min.

* Krebs et al., 1969
** M. Solfrizzo, pers. comm.
5.1.9 Analysis by mass spectrometry

Compound B was analysed by electron impact mass spectrometry (ionising voltage; 10 eV) with a direct insertion probe (School of Pharmacy, University of London, London, UK).

5.1.10 Oilseed rape leaf assays

Oilseed rape plants (cv. Bravo) were grown according to the procedures stated in section 4.1.1.4. After 3-4 weeks growth, spore suspensions of isolates B, G, T and U (ca. 1X10^4 spores/ml water) were sprayed on leaves and incubated at approximately 20°C in plastic bags to promote high humidity. Leaves were then examined for symptoms of disease (e.g. chlorotic lesions).

In order to examine the toxicity of compound B, MeOH containing compound B was applied as drops to detached rapeseed leaves. The leaves were placed on moist filter paper in petri dishes and incubated at 25°C for up to four days. Results were compared with controls i.e. drops of 100% MeOH.

5.2 RESULTS

5.2.1 Detection by antifungal activity

Preliminary spot studies of the MeOH culture extract of rice cultures on TLC plates showed that a compound (or several compounds) produced by isolate B but not (or present in lesser concentration) by isolate G inhibited *A. alternata* (isolate B) (Fig. 5.3). The chloroform extract (i.e. culture solids remaining after MeOH extraction and extracted by chloroform) exhibited no zones of inhibition for either isolates indicating that MeOH is an efficient solvent for extracting the toxic metabolite/s.

Development of TLC plates with MeOH:ACN (4:3) produced two zones (Rfs = 0.11 and 0.70) from isolate B but none with isolate G which inhibited spore germination of *A. alternata* (Fig. 5.4). As the lower spot was discrete and inhibited the growth of spores more effectively than the higher one, work focussed on the lower zone, which was designated compound B. Although a
Fig. 5.3  Bioautographic assay (*A. alternata*; isolate B) of MeOH rice culture extracts (isolates B and G). Spots have been diluted with MeOH (X2) from left to right
Fig. 5.4 Bioautographic assay (*A. alternata*; isolate B) of MeOH rice culture extracts (isolates B and G). Spots (for each isolate) were diluted (X5) with MeOH from left to right. The TLC plate was developed with the solvent system MeOH:ACN (4:3).
number of other TLC solvent systems were examined (see 5.1.3), none moved compound B from the baseline (e.g. the Rf value of compound B using toluene:acetone [12:7] was zero). Compound B also inhibited C. cucumerinum, although the zone of inhibition was less intense than for A. alternata.

Owing to the fact that detection of compound B by bioautography assay was slow (i.e. 48 hours, in order to allow for optimum growth of fungal spores), a more rapid method of detection was developed by spraying TLC plates with anisaldehyde-sulphuric acid solution; compound B appeared as a pink/purple spot (see Table 5.1). This in turn permitted the use of an acidic solvent system BAW* - that gave superior resolution and separation of compounds to that produced by MeOH:ACN (4:3). The Rf value of compound B using this solvent system was 0.18 (see Fig. 5.5).

5.2.2 Purification

Solid phase clean-up of the MeOH extract eliminated most interfering compounds (e.g. amino acids) but further purification by preparative TLC was necessary to completely isolate compound B. Preparative TLC plates were developed with BAW. The progressive purification of compound B from initial MeOH extraction to preparative TLC is displayed in Fig. 5.5.

5.2.3 Purification studies using centrifugally accelerated TLC

Large, pure preparations of compound B were not practical using the chromatotron owing to prolonged tailing of the compound. To illustrate, compound B was collected in 110 fractions (ca. 110 ml) and 75 fractions (ca. 75 ml) using solvent systems (1) and (2) respectively (see 5.1.5 for description of solvent systems). Although some fractions contained pure compound B (e.g. fractions 88-107; solvent system 2), these were of low intensity (when analysed

* this solvent system was not feasible for bioautographic detection of compound B as acetic acid killed the fungal spores
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Result</th>
<th>Diagnostic colour observed</th>
<th>Compound or classes of compounds which are detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromocresol green</td>
<td>+</td>
<td>light green</td>
<td>organic acids</td>
</tr>
<tr>
<td>Dragendorff’s reagent</td>
<td>+</td>
<td>bright orange</td>
<td>alkaloids and other nitrogen-containing compounds</td>
</tr>
<tr>
<td>Anisaldehyde-sulphuric acid</td>
<td>+</td>
<td>pink/purple</td>
<td>sugars, steroids, terpenes, etc</td>
</tr>
<tr>
<td>Folin-ciocalteu’s reagent</td>
<td>-</td>
<td>-</td>
<td>phenols</td>
</tr>
<tr>
<td>Isatin-sulphuric acid</td>
<td>+</td>
<td>light brown</td>
<td>thiophene derivatives</td>
</tr>
<tr>
<td>Ninhydrin</td>
<td>-</td>
<td>-</td>
<td>amino acids, amines and aminosugars</td>
</tr>
<tr>
<td>Ferric chloride</td>
<td>-</td>
<td>-</td>
<td>phenols and hydroxamic acids</td>
</tr>
<tr>
<td>Vanillin-sulphuric acid</td>
<td>+</td>
<td>purple</td>
<td>higher alcohols, phenols, steroids and essential oils</td>
</tr>
</tbody>
</table>

Table 5.1 Reagents sprayed on TLC plates for detection and characterisation of compound B
Fig. 5.5 Thin layer chromatography of the various purification stages of compound B. 'A' corresponds to plates sprayed with ninhydrin and then heated to 100°C for approximately 5 min.; 'B' corresponds to plates sprayed with anisaldehyde-concentrated sulphuric acid and then heated at 100°C for approximately 5 min. Arrow signifies compound B (pink/purple spot). Lane 1 = crude extract; lane 2 = 50% citrate buffer/crude extract, post-solid phase; lane 3 = 50% citrate buffer/MeOH wash; lane 4 = 0.5 ml MeOH wash; lane 5 = 1 ml MeOH aliquot eluting compound B; lane 6 = 2nd 1 ml MeOH aliquot (eluting very small amounts of compound B); lane 7 = 3rd 1 ml MeOH aliquot (no compound B present); lane 8 = pure compound B, post-preparatory TLC. Remarks: Solid phase effectively removed most contaminating compounds such as amino acids from the crude extract; the resolution of metabolites was considerably enhanced using the solvent system n-butanol:acetic acid:water (4:1:1).
by TLC and subsequently sprayed with anisaldehyde-sulphuric acid solution, and thus of relatively low concentration.

5.2.4. Analysis by TLC

Results of the various reagents sprayed on TLC plates for detection and characterisation of compound B are listed in Table 5.1 and Fig. 5.6. In general, data indicated that compound B is weakly acidic and contains nitrogen and thiophene derivatives.

Although compound B had no fluorescence under long wave UV radiation, detection on TLC plates ($F_{254}$) with heavy loading was possible since it quenched short wave UV radiation.

5.2.5 Spectrophotometry

The UV spectrum of compound B in MeOH showed no characteristic spectral features; the absorption maximum was about $\lambda_{200-210}$ nm (Fig. 5.7).

5.2.6 Analysis by HPLC

Gradient analysis (ACN/H$_2$O, 0-100%; 20 min. on a C8 column) of the MeOH solution containing pure compound B eluted the metabolite at 17.83 min.; no contaminating compounds were detected (Fig. 5.8).

5.2.7 Analysis by mass spectrometry

The parent ion of compound B was approximately $m/z$ 350 (relative abundance; 52%). Other major fragments included $m/z$ 294 (34%), 280 (100%), 263 (48%), 256 (57.5%), 228 (54.5%), 185 (57%) and 137 (82%). The fragmentation peak at 149 (52.5%) was most likely to be caused by plasticisers (i.e. phthalates), as reported by McLafferty and Turecek (1993) (Fig. 5.9).
Fig. 5.6 Detection of compound B using various spray reagents and UV radiation. Lane 1 = Bromocresol green (TLC solvent system; MeOH:ACN [4:3]); 2 = Anisaldehyde (BAW); 3 = Vanillin (BAW); 4 = Dragendorff's reagent (BAW); 5 = Isatin (BAW); 6 = short wave UV light (4:3)
Fig. 5.7 Absorption spectrum of compound B in MeOH ('1' represents maximum at λ203.2 nm)
Fig. 5.8  High performance liquid chromatogram (λ207 nm) of (A) a pure preparation containing compound B (RT = 17.83 min.) and (B) blank run. Stationary phase, C8 column; mobile phase, gradient run 0-100% (ACN/water), 20 min.
Fig. 5.9  Electron impact mass spectrum of compound B
5.2.8 Oilseed rape leaf assays

A spore suspension of isolate B (ca. $1 \times 10^4$ spores/ml) sprayed on oilseed rape plants (cv. Bravo) caused chlorotic lesions around germinating spores on 'older' (ca. 3-4 weeks) leaves (but not the 'younger'; ≤ 3 weeks) after 4-5 days (Fig. 5.10). Spores of other isolates, including some of those which are capable in producing high quantities of TeA (isolates T and U), caused no symptoms of disease.

Application of compound B to rapeseed leaves induced no disease symptoms indicating that the compound is unlikely to play a significant role in pathogenesis.

5.3 DISCUSSION

Compound B produced by isolate B of A. alternata was self-inhibitory and perhaps explained the death of the fungus grown on rice. Similarly, Marumo et al., (1984) observed that production of stemphol by Pleospora herbarum (Pers.:Fr) Rabenh. grown on potato agar with 2% sucrose at 25°C for seven days acted as a self-inhibitor. At the highest concentration examined, (400 µg/ml), growth was retarded 63.2% in relation to that of the control.

As well as compound B being self-inhibitory, it also inhibited C. cucumerinum. Production of a metabolite that is toxic to susceptible individuals of like or related species has been reported in a number of organisms, particularly yeasts. One metabolite isolated from a strain of Aspergillus giganteus was identified as a small polypeptide of approximately 6 kD that was toxic to related forms (Chet, 1993). Beed (1993) also observed that ascochitine, which is produced by Ascochyta fabae Speg. and believed to be important in ascochyta blight of the faba bean (Vicia faba L.), inhibited C. cucumerinum. In general, secretion of an agent toxic to other strains of the same species or other genera may be viewed as a competitive advantage. However, this is probably not the case with isolate B as compound B has an additional self-inhibitory effect on the growth of the fungus, as previously discussed.
Fig. 5.10  Formation of chlorotic lesions on leaves of oilseed rape sprayed with spores produced by isolate B (ca. $1 \times 10^4$ spores/ml water)
Purification of a small amount of compound B was accomplished in two steps, initially with solid phase and then preparative TLC, although centrifugal TLC (cTLC) procedures were attempted. Several workers have used cTLC for preparative separation of compounds, including alkaloids (e.g. rhoeadine) extracted from *Papaver rhoeas* flowers (Ferrari and Verotta, 1988) and polar substances (e.g. quadranguloside) from methanolic extracts of leaves of *Passiflora quadrangularis* L. (Orsini and Verotta, 1985). However, the major problem with cTLC in this study was that compound B tailed excessively during the solvent run and consequently coeluted with other compounds. Two different solvent systems were examined (i.e. BAW and a four step gradient run of MeOH:ACN, starting at 4:3 and ending at 10:3; see 5.1.5) which both gave excellent resolution of compounds on analytical TLC (0.2 mm) and BAW on preparatory TLC (0.5 mm). However, it seemed that with the thicker layer of silica used for cTLC (2 mm), the resolving powers of chromatography diminished dramatically. Perhaps a 0.5 mm layer of silica, similar to that used in normal preparative TLC (see above), would have been better suited for purification of compound B, although 2 mm silica layers have been previously demonstrated to successfully separate compounds by cTLC, such as diastereoisomeric 2-aryl propionic acid derivatives (Maitre et al., 1986).

It was also suspected that BAW was too viscous for use as a solvent system for cTLC, as the solvent mixture tended to skate over the surface and in effect cause a forwards slope within the silica layer. Although a reduced rate of solvent flow (ca. 2 ml/min.) was tested to prevent band sloping, some skimming of the solvent mixture was still observed during the run. In the light of these difficulties, cTLC of compound B was discontinued and normal preparative TLC was employed for purification of the metabolite.

A number of chemical tests were applied in order to characterise the properties of compound B. Spray reagents on TLC plates are important for identifying certain compounds produced by *Alternaria* spp., including ethanolic ferric chloride which detects TeA as a orange/red spot (Schade and King, 1984)
and diazotized sulphanilic acid which colours ALT yellow, AOH cherry-red and AME wine-coloured (Pero et al., 1971). In this study Dragendorff’s reagent which revealed nitrogen in the molecule, clearly distinguished compound B from other metabolites. Various other Alternaria toxins are known to possess nitrogen atoms (e.g. TeA and phytotoxins such as the AAL-toxins) but these have different UV and mass spectrometry properties and different molecular weights from those shown by compound B.

The parent ion in the mass spectrum of compound B (ca. at m/z 350) was close to the parent ions of the altertoxins (i.e. ATX-I, II and III; at m/z 352.095, 350.08 and 348.06, respectively). However, the fragmentation patterns of the altertoxins (e.g. major fragments of ATX-II; m/z 332, 321, 305 and 291) (Stack et al., 1986) differed from the mass spectrum of compound B.

HPLC analysis demonstrated that compound B was retained by a C8 column (RT = 17.83 min.) using a gradient solvent system (0-100%; ACN/water). However, as the metabolite was primarily detected in the low UV range (λ200-210 nm), derivatisation is recommended for further HPLC analyses. By analogy, derivatisation of AAL-toxins with maleic anhydride allowed detection of the metabolites by HPLC at the higher wavelength of λ_{max}=250 nm (Stack et al. 1986).

Pathogenicity studies indicated isolate B was virulent to ‘older’ leaves (ca. four weeks old) but not ‘younger’ leaves (less than three weeks) of oilseed rape. In order to discuss this pathogenic association, some framework of fungal/plant relationships is required. The simplest fungus/plant relationship is saprophytic growth on plant material i.e. the mould never invades normal living cells. However, some saprophytes may have a more complex relationship with plants as opportunistic pathogens. These types are low in virulence and usually require old or stressed host tissue for colonisation and disease expression. Opportunistic pathogens may adapt a step further, whereby they become specialised and confined to a single host, or to a group of related plant species.
Host-selectivity and virulence of some moulds, such as A. alternata, are known to depend on production and release of host-specific toxins. Some examples of host specific toxins produced by A. alternata include AL-A and B toxins (host; tomato), AF-I, II and III (strawberry), ACTG and ACT-I and II toxins (tangerine) and AK toxin (Japanese pear) (Scheffer, 1992). Since no disease symptoms were observed with application of compound B to leaves of oilseed rape in this study, the metabolite probably had inconsequential or no role in pathogenicity.
CHAPTER 6.

USE OF RANDOM AMPLIFIED POLYMORPHIC DNA FOR ASSESSMENT OF GENETIC VARIABILITY IN *ALTERNARIA* SPP.

The principles of RAPD analysis and its usefulness in identifying fungi, as well as grouping isolates in relation to various attributes such as host species, pathogenic variability, geographic origin and mating type, were outlined in chapter 1. The objectives of this chapter are to investigate the use of RAPD analysis as means of evaluating the extent of intraspecific variability of *Alternaria* spp. and, if feasible, to distinguish between toxin and non-toxin producing isolates.

6.1 MATERIALS AND METHODS

6.1.1 Growth conditions

Liquid cultures of fungal isolates were grown in malt extract medium containing 20 g malt extract, 20 g glucose and 1 g peptone in one litre. Conical flasks containing 50 ml of liquid medium were inoculated with spore suspension (50 μl; 1X10^3 spores/ml) and incubated at 25°C in the dark. After 6-8 days, the mycelium was harvested by pouring off the liquid medium and blotting on filter paper (Whatman filter paper, No. 1), frozen at -70°C for several hours and lyophilised.

6.1.2 Preparation of Genomic DNA

Lyophilised mycelium was ground in liquid nitrogen and genomic DNA was extracted using the procedure of Raeder and Broda (1985). Approximately 50 mg of ground mycelium was placed in a 1.5 ml micro-centrifuge tube and homogenised with 500 μl of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). The homogenate was mixed with 350 μl of phenol (BDH Analar, molten at 45°C and equilibrated with one volume of
extraction buffer), after which 150 µl of chloroform was added. After centrifugation at 12 900 g (Micro-centaur, MSE, Loughborough, UK) for one hour in the cold (ca. 4°C), the upper aqueous phase was transferred to another tube containing 25 µl RNAse solution (Sigma RNAse A No. 4875, 70 Kunitz Units/mg, 20 mg/ml in 10 mM Tris HCl pH 7.5, 15 mM NaCl and boiled for 10 min.) and incubated for 10 min. at 37°C. Chloroform (ca. one volume) was added and centrifuged (12 900 g) for 10 min. The supernatant containing the DNA was removed and DNA precipitated by the addition of ice-cold isopropanol (250 µl). The DNA was pelleted by brief centrifugation (ca. 1 min.) at 12 900 g. DNA pellets were rinsed in 70% ethanol, dried under vacuum and re-suspended in 100 µl of 10 mM Tris HCl, 0.1 mM EDTA pH 8.0 solution (TE). Storage of DNA was at -20°C.

6.1.3 Oligonucleotide primers

Oligonucleotide decamer primers (60-70% GC content) were purchased from Operon Technologies Inc., Alameda, USA. The sequences of the primers tested are shown in Table 6.1.
<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP1</td>
<td>GTGTCTCAGG</td>
</tr>
<tr>
<td>OP2</td>
<td>TCGGCACGCA</td>
</tr>
<tr>
<td>OP4</td>
<td>GTGTCTCAGG</td>
</tr>
<tr>
<td>OP6</td>
<td>GTGGGCTGAC</td>
</tr>
<tr>
<td>OP7</td>
<td>GTCCATGCCA</td>
</tr>
<tr>
<td>OP8</td>
<td>ACATCGCCCA</td>
</tr>
<tr>
<td>OP9</td>
<td>GTGGTCCGCA</td>
</tr>
<tr>
<td>OP10</td>
<td>TCCCCGCTAC</td>
</tr>
<tr>
<td>OX1</td>
<td>CTGGGCACGA</td>
</tr>
<tr>
<td>OX3</td>
<td>TGGCGCAGTG</td>
</tr>
<tr>
<td>OX7</td>
<td>GAGCGAGGCT</td>
</tr>
<tr>
<td>OX9</td>
<td>GGGAGGCTCA</td>
</tr>
<tr>
<td>OX11</td>
<td>GGTCTGGTTG</td>
</tr>
<tr>
<td>OX13</td>
<td>ACGGGAGGCAA</td>
</tr>
<tr>
<td>OX15</td>
<td>CAGACAAGCC</td>
</tr>
<tr>
<td>OX17</td>
<td>GACACGGACC</td>
</tr>
<tr>
<td>OX19</td>
<td>TGGCAAGGCA</td>
</tr>
<tr>
<td>OX20</td>
<td>CCCAGCTAGA</td>
</tr>
</tbody>
</table>

Table 6.1 Sequences of oligonucleotide primers
6.1.4 Amplification of DNA

Standard amplification reaction mixtures consisted of 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris HCl pH 9.0 (at 25°C), 1% Triton X-100, two units of Taq DNA polymerase (Promega Corp., Madison, USA), 100 μM of each 2'-deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP; Pharmacia LKB Biotechnology, Uppsala, Sweden), 400 ng primer and approximately 10 ng of genomic DNA in a final volume of 50 μl, overlaid with two drops of mineral oil. Amplification by PCR was performed in a thermal cycler (Model HBTR1, Hybaid Ltd., Teddington, UK) with the following temperature profile:

<table>
<thead>
<tr>
<th>Time</th>
<th>Temperature</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>94°C</td>
<td>initial</td>
</tr>
<tr>
<td>1 min</td>
<td>94°C</td>
<td>1 cycle denaturation</td>
</tr>
<tr>
<td>1 min</td>
<td>38°C</td>
<td>1°C/15 sec.</td>
</tr>
<tr>
<td>2 min</td>
<td>72°C</td>
<td>45 cycles ramping</td>
</tr>
</tbody>
</table>

Reproducibility of amplification patterns was confirmed for each primer tested by incorporating a control DNA (+ve control) from a previously amplified Alternaria isolate. Inclusion of a -ve control, which consisted of all reaction components except genomic DNA, ensured detection of extraneous DNA. Samples were stored at -20°C until analysed.

6.1.5 Electrophoresis

Amplified DNA samples (9 μl) were mixed with 3 μl of loading buffer (0.25% bromophenol blue and 60% sucrose in TE buffer) and resolved by electrophoresis (100 V for up to two hours) in 1.5% agarose gels in a Tris borate EDTA (TBE) buffer system (0.089 M Tris-Hcl, 0.089 M boric acid and 0.002 M EDTA, pH 8.0), using a mini gel apparatus (Model HB-B2, Hybaid Ltd.) connected to a Gibco BRL power pack (Model 400h, Life Technologies Ltd., Paisley, UK). Gels were stained with ethidium bromide (0.5 mg/ml) for 15 min. and washed briefly in distilled water. DNA bands were visualised on a UV
(λ302 nm) transilluminator and photographed using polaroid positive/negative film (Polaroid film type 665). The migration distances and intensities of PCR products were estimated by scanning the negatives with a gel densitometer (LKB 2222-020 Ultrascan XL, Bromma, Sweden) programmed with Gelscan XL (Vers. 2.0) software. A one kb DNA ladder (Gibco BRL) was used as the molecular standard (0.5 μl marker + 3 μl loading buffer + 8.5 μl TE) to assess the mobility of DNA fragments*. The negative scanned images were then magnified by roughly four times and the printouts from these images were used for scoring.

6.1.6 Data analysis

For each primer, intensities of DNA fragments were recorded as a proportion of the fragment of maximum intensity, which was taken as one absorbance unit (AU). Bands were scored as present (i.e. as the intensity value of the DNA fragment) or absent (0) and a matrix of continuous quantitative data was assembled using this procedure.

Cluster analysis was performed on the data using the CLUSTAN 3.2 statistical package (Wishart, 1987). Similarity between isolates were evaluated using squared euclidean distance, which determines a centre point for each group and calculates the sum of squares of distances from the centre to each group member (Pankhurst, 1991). Data matrices and genetic distance (GD) dendrograms were generated according to the genetic profile of each isolate. Several types of clustering methods were applied to the data, including agglomerative clustering - single linkage (SL) and unweighted pair group arithmetic average method (UPGMA) - and Ward’s method of minimum variance spherical clustering. In addition, a principal components analysis (PCA) was tested with some primers to verify dendrogram grouping of isolates. For an explanation of the clustering methods and PCA used here, refer to section 6.3.3.

---

* the mobility of marker DNA (0.153-4.07 kb) was inversely correlated to the loge of molecular size (r = 0.996 ± 0.002)
6.2 RESULTS

6.2.1 Preparation of genomic DNA

Electrophoresis (100 V for 2.5 hours) of genomic DNA on an agarose gel (1%) showed a minimal amount of DNA shearing for all 23 isolates, indicating the extraction method was effective (Fig. 6.1).

6.2.2 Development of a reliable PCR method

Preliminary investigations (ca. 40 PCR experiments) were carried out in order to develop a method which produced clear, reproducible amplification products. Amplification of *Alternaria* DNA was affected by the following factors:

(i) *Taq* polymerase; generation of DNA products was possible using 'Promega' *Taq* DNA polymerase enzyme and its associated reaction buffer, but not with other enzymes (e.g. 'Ampli'taq; Perkin Elmer Cetus, USA)

(ii) template DNA concentration; in initial RAPD reactions, no amplification products were observed and this was believed to have been caused by insufficient DNA. For those PCR experiments, spectrophotometric measurements (λ260 nm) were used for estimating concentrations of *Alternaria* DNA and perhaps these were not accurate owing to interfering compounds (e.g. phenolics), although some spectra produced were characteristic of relatively pure DNA (e.g. isolate K; λ280/260 nm ratio of AU = 0.66) (Fig. 6.2). To investigate this, the concentration of genomic DNA (isolate O) was assessed by fluorometric quantification with a known concentration of marker DNA (Lambda Hind III, Sigma Co. Ltd.) of similar size - ca. 23 kb - stained with ethidium bromide under UV light using a gel scanning densitometer (Fig. 6.3). From this procedure, it was found that the DNA concentration was approximately 100 times less than that estimated by spectrophotometry. Using the fluorometric DNA quantification method and the PCR protocol described in section 6.1.4, approximately 10 ng of template DNA generated bright PCR fragments. Greater amounts of genomic
Fig. 6.1  Agarose gel of genomic DNA of *Alternaria* spp.. Letters signify isolate codes; Marker DNA is represented as `-` (Lambda Hind III; lower band = 23.13 kb)
Fig. 6.2  Spectrum of genomic DNA of *Alternaria* spp. (isolate K). The $A_{280}/260$ nm ratio of absorbance is 0.66
Fig. 6.3  Fluorometric quantification of genomic DNA of *Alternaria* spp. (isolate O). (A) Lanes 1, 3, 5, 7, 10, 12, 14 and 16 contain 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 ng marker DNA (Lambda Hind III) respectively. Lanes 2, 4, 6, 8, 11, 13 and 15 contain genomic DNA (X2 dilution from left to right); (B) standard calibration curve of the intensity of marker DNA (23.13 kb fragment) verses concentration.
DNA (ca. 20, 40 and 80 ng) appeared to have little effect on the quality of amplification products (Fig. 6.4).

(iii) cycling parameters; initial exposure to 94°C for 1 min. (i.e. 'hot start') separated any DNA pairing, therefore eliminating any improper primer binding that may have occurred during preparation of the reaction mixture and preventing amplification of contaminating sequences (Foster et al., 1993). Also, a rate of changing temperature (ramping rate) at 1°C per 15 seconds from 38°C to 72°C was optimal for effective generation of DNA fragments. Faster ramping rates decreased the number of bands (e.g. at 1°C/5 sec., there was no generation of target DNA) whereas slower rates (e.g. 1°C/20 sec.) resulted in smearing of bands and gave non-reproducible results (Fig. 6.5).

(iv) magnesium concentration; Mg²⁺ was necessary for the production of arbitrary primed DNA fragments. A concentration of 1.5 mM was found to produce bright bands (Fig. 6.6).

6.2.3 Variability of the Alternaria using random primers

Genomic DNA extracted from the 23 Alternaria isolates selected for toxin production (see Table 5.1) were amplified at random with 18 decamer primers (see Table 6.1), giving a total of 414 reactions. All of these reactions yielded PCR products, with the exception of nine*. Positive control treatments (i.e. isolate O and primer OP2) generally produced invariant RAPD fragments for each primer examined. For the -ve controls, no products were amplified indicating samples were free from foreign DNA.

* isolate U; primer OP9
  isolate C; primer OX9
  isolate E; primer OX9
  isolate C; primer OX19
  isolate M; primer OX19
  isolate C; primer OX20
  isolate O; primer OX20
  isolate Q; primer OX20
  isolate V; primer OX20
Fig. 6.4 The effect of different concentrations of *Alternaria* DNA (isolate O) on PCR. Lanes 1, 2, 4 and 5 contain ca. 10, 20, 40 and 80 ng template DNA respectively. Marker DNA (1 kb ladder) is represented in lane 3.
Fig. 6.5 The effect of different ramping rates on PCR banding patterns of *Alternaria* spp. (isolate O). Lane 1 corresponds to no ramping (i.e. fastest obtainable transition in temperature), lane 2 (1°C/5 sec.), 4 (1°C/10 sec.), 5 (1°C/15 sec.) and 6 (1°C/20 sec.). Marker DNA (1 kb ladder) is represented in Lane 3.
The effect of different concentration of Mg$^{2+}$ on the PCR of *Alternaria* spp. (isolate O). Lanes 1 corresponds to no Mg$^{2+}$ in the reaction mixture, lane 2 (0.5 mM), lane 3 (1.0 mM), lane 5 (1.5 mM), lane 6 (2.0 mM) and lane 7 (2.5 mM). Marker DNA (1 kb ladder) is represented in lane 4.
None of the primers selected generated molecular patterns that were identical in any isolate assayed. Bands that were not clear (i.e. smeared or of very low intensity) were not included in the analyses. Summing all the isolates tested, a total of 2314 amplification products were obtained and ranged from 0.19 to 4.06 Kb. Numbers of bands amplified per isolate varied from 0 and 14, giving an average of around six RAPD products per isolate per primer.

The effect of changing the primer on DNA bands of individual isolates was profound, as both the number and size of DNA fragments altered with each primer. For example, the mean number and range of band size for primer OP6 (ca. 10 and 0.23-2.82 kb respectively) was markedly greater than that for primer OX9 (ca. 3 and 0.22-3.13 kb respectively).

The primary clustering statistic used for the data was Ward’s method although SL and UPGMA were also examined. In general, it was found SL and UPGMA methods could not differentiate groups of similarity as proficiently as Ward’s method, and SL tended towards ‘chaining’ i.e. where single isolates divided off one at a time and the corresponding dendrogram appeared as a staircase. Analysis by PCA (see section 6.3.3 for an explanation of the statistic) was often indicative of groupings produced cluster analysis but the accumulative percentage variation of the first two transformed variables (eigenvectors) was relatively low (ca. 25%) and therefore 2D plots represented only about a quarter of the character set. Although the accumulative percentage variation of the first three transformed variables was slightly higher (ca. 35%), 3-D plots were not possible with the graphics programme (Harvard Graphics 2.0) available for the study.

6.2.4 Differentiation of species

Examination of data showed there was considerable variation among isolates of *Alternaria* spp., with most DNA fragments being polymorphic. However, PCR of isolates with primer OX17 each generated a common DNA product (0.75 kb), indicating some degree of genetic homogeneity among the 23
isolates (Fig. 6.7).

Although primers differed in their capacity to differentiate A. alternata, A. infectoria, A. tenuissima, A. brassicae and A. brassicicola, no single primer effectively distinguished all five species. No loci were conserved in isolates of A. alternata or/and A. infectoria which differed from other Alternaria spp. but several unique loci were generated by A. tenuissima (e.g. primer OP1; 0.69 kb), A. brassicicola (primer OP1; 0.29 kb) and A. brassicae (e.g. primer OP1; 0.77 kb) (Table 6.2). In addition, the total number of characteristic DNA fragments yielded by A. brassicicola and A. brassicae isolates (30 and 36 respectively) were substantially higher than those generated by the other isolates of Alternaria spp. (e.g. isolate A; 11) (Table 6.3). These results indicate that A. tenuissima, A. brassicicola and A. brassicae differ considerably in the organisation of their DNA from A. alternata and A. infectoria and this is also expressed in differences in their morphology (Fig. 6.8A-F).

Similarly, no cluster analysis for a given primer effectively segregated the five species of Alternaria. However, clustering data from primer OX15 (using Ward’s method) effectively separated A. tenuissima (isolates U and W; GD = 0.196, maximum identity is represented by 0), primer OP8 isolated A. brassicae (isolate N; GD = 0.119) and primer OX1, less clearly, branched A. brassicicola (isolate F; GD = 0.130) (and again A. tenuissima; GD = 0.188) from other Alternaria spp. The dendrograms and corresponding PCR patterns for primers OX15, OP8 and OX1 are shown in Figs. 6.9, 6.10 and 6.11 respectively.

With regard to clustering of A. alternata and A. infectoria, there was no apparent grouping with any of the primers used, and the levels of GD between isolates were high. For example, GD was 0.031-0.142, 0.006-0.143 and 0.014-0.181 for primers OX15, OP8 and OX1 respectively.

The Alternaria isolates which were not identified to a species level because of poor conidium production (i.e. isolates D and U) showed no special
Fig. 6.7  RAPD amplifications of *Alternaria* spp. with primer OP17. Letters below lanes signify isolates; > signifies monomorphic DNA fragment (0.75 kb); + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control (no template DNA)
<table>
<thead>
<tr>
<th>PRIMER</th>
<th>A. brassicae (isolate N)</th>
<th>A. brassicicola (isolate F)</th>
<th>A. tenuissima (isolates U and W)</th>
<th>HT producers (isolates T, U, V and W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP1</td>
<td>0.77</td>
<td>0.29</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>OP2</td>
<td>0.82, 0.38</td>
<td>0.88</td>
<td>0.93, 0.29</td>
<td></td>
</tr>
<tr>
<td>OP4</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP6</td>
<td>0.99</td>
<td>2.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP7</td>
<td>1.24</td>
<td></td>
<td>1.75, 0.45</td>
<td></td>
</tr>
<tr>
<td>OP8</td>
<td>0.25, 0.32</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP9</td>
<td>0.38, 0.28</td>
<td>0.54, 1.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP10</td>
<td>0.98, 2.95</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX1</td>
<td>0.63</td>
<td>3.14, 1.04</td>
<td></td>
<td>1.53</td>
</tr>
<tr>
<td>OX3</td>
<td>2.00</td>
<td>1.42, 0.57</td>
<td>0.71</td>
<td>1.72</td>
</tr>
<tr>
<td>OX7</td>
<td>1.24</td>
<td>1.77, 0.38</td>
<td>0.50</td>
<td>1.05</td>
</tr>
<tr>
<td>OX9</td>
<td>0.55</td>
<td>3.13, 1.62</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>OX11</td>
<td>0.20, 0.38</td>
<td>0.40, 0.94</td>
<td></td>
<td>1.29</td>
</tr>
<tr>
<td>OX13</td>
<td>0.44, 0.59</td>
<td>0.45, 0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX15</td>
<td>0.33</td>
<td>0.40, 0.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX17</td>
<td>2.09, 2.52</td>
<td>2.96, 1.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX19</td>
<td>1.3</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OX20</td>
<td>1.48, 3.32</td>
<td>1.14</td>
<td></td>
<td>1.68</td>
</tr>
</tbody>
</table>

Table 6.2 Polymorphic loci for A. brassicae (isolate N), A. brassicicola (F), A. tenuissima (U and W) and HT producing isolates of Alternaria (T, U, V and W)
<table>
<thead>
<tr>
<th>ISOLATE</th>
<th>TOTAL NUMBER OF UNIQUE DNA FRAGMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
</tr>
<tr>
<td>D</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>15</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
</tr>
<tr>
<td>G</td>
<td>12</td>
</tr>
<tr>
<td>H</td>
<td>7</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>J</td>
<td>13</td>
</tr>
<tr>
<td>K</td>
<td>8</td>
</tr>
<tr>
<td>L</td>
<td>7</td>
</tr>
<tr>
<td>M</td>
<td>5</td>
</tr>
<tr>
<td>N</td>
<td>36</td>
</tr>
<tr>
<td>O</td>
<td>9</td>
</tr>
<tr>
<td>P</td>
<td>8</td>
</tr>
<tr>
<td>Q</td>
<td>9</td>
</tr>
<tr>
<td>R</td>
<td>8</td>
</tr>
<tr>
<td>S</td>
<td>10</td>
</tr>
<tr>
<td>T</td>
<td>16</td>
</tr>
<tr>
<td>U</td>
<td>8</td>
</tr>
<tr>
<td>V</td>
<td>13</td>
</tr>
<tr>
<td>W</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 6.3 The total number of DNA fragments for all primers used (18) which are characteristic for individual isolates
Fig. 6.8 Conidia of *Alternaria* spp. isolated from oilseed rape (A) *A. alternata* (magnification X1000) (B) *A. alternata* (hypertrophic i.e. swollen and distorted; see text) (X1000); (C) *A. infectioria* (X1000); (D) *A. brassicae* (X400); (E) *A. brassicicola* (X1000); (F) *A. tenuissima* (X1000)
Fig. 6.8 (cont’d)
Fig. 6.9 (A) RAPD amplifications of *Alternaria* spp. with primer OX15. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.10 (A) RAPD amplifications of *Alternaria* spp. with primer OP8. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.11 (A) RAPD amplifications of *Alternaria* spp. with primer OX1. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
molecular characteristics and were generally segregated with *A. alternata* and *A. infectoria* for all primers tested.

It is interesting to note that isolate B, which was shown in chapter 5 to produce a toxic nitrogen-containing metabolite, produced an average number of polymorphic DNA fragments (14; see Table 6.3), although cluster analysis of data obtained with primer OP9 using Ward's method separated the mould (GD = 0.116) from the other *Alternaria* isolates (Fig. 6.12).

### 6.2.5 Differentiation of toxin and non-toxin producing isolates

While RAPD studies could not completely separate mycotoxigenic moulds from non-mycotoxigenic moulds, they could to some degree recognise isolates of *A. alternata* (T), *A. infectoria* (V) and *A. tenuissima* (U and W) which were capable of 'high' toxin (HT) production. The molecular profile of isolate N (*A. brassicaceae*), which was also an HT producing isolate, appeared not to be closely associated with the other HT producers. It is possible that other traits affiliated with isolate N, such as being a pathogen of oilseed rape, influenced the pattern of its RAPDs.

Monomorphic DNA fragments associated with HT producing isolates but also polymorphic to non/low toxin (NLT) producers are listed in Table 6.2. Note that within HT producing isolates there are additional DNA fragments which distinguish the *A. tenuissima* (isolates U and W) from *A. alternata* (T) and *A. infectoria* (V). As reported in the previous section, PCR products may relate to morphological characteristics of *A. tenuissima*. There is also some possibility that polymorphic DNA distinguishes HT producers of *A. tenuissima* from NLT individuals of the same species, but this was not examined here.
Fig. 6.12 (A) RAPD amplifications of Alternaria spp. with primer OP9. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Subsequent cluster analyses of DNA amplification products showed that there was often (i) distinct segregation of isolates T and V from NLT producing isolates and (ii) U-W were more closely associated to T-V than to NLT producers. Cluster dendrograms (Ward's method) and related RAPD profiles of four primers (OP2, OP4, OP7 and OX13) which segregated HT from NLT producers are shown in Fig.s 6.13, 6.14, 6.15 and 6.16 respectively. In each case, HT and NLT producers divided into two broad clusters (for primers OP2, OP4, OP7 and OX13, the GD values were 0.215, 0.263, 0.259 and 0.209 respectively). Within the HT producing cluster, T and V divided from U and W (GD = 0.109, 0.102, 0.106 and 0.118 respectively), substantiating that the relationship of U-W was closer to T-V than to NLT producers. The GDs between T and V (0.048, 0.047, 0.057 and 0.039 respectively), and U and W (0.028, 0.033, 0.012 and 0.020 respectively) were minimal.

Segregation of T-V and U-W from NLT producers with primers OP2, OP4, OP7 and OX13 was supported using PCA analyses on the data (Fig. 6.17A-D). Although the first and second principal factors contributed a small proportion of the total variation between Alternaria isolates (22.02, 31.12, 24.01 and 21.88% respectively), 2D plotting of eigenvectors revealed specific spatial patterns among isolates T-V and U-W from NLT producers.

6.3 DISCUSSION
6.3.1 Development of a reliable PCR method
Random oligodeoxynucleotide primers have been demonstrated here to amplify effectively DNA from Alternaria spp. by PCR. However, certain elements were critical for the reproducibility of the RAPD protocol and should be addressed. For example, Taq polymerase acquired from Promega Ltd. produced banding patterns whereas other commercially-available enzymes (e.g. Amplitaq) did not. Although the reasons for this were not understood, recent reports have stated that the source of Taq polymerase (e.g. type of bacterium) may be an important factor in determining RAPD profiles (Williams et al., 1990; Fekete, 1992).
Fig. 6.13 (A) RAPD amplifications of Alternaria spp. with primer OP2. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.14 (A) RAPD amplifications of *Alternaria* spp. with primer OP4. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward’s method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.15 (A) RAPD amplifications of Alternaria spp. with primer OP7. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward's method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.16 (A) RAPD amplifications of *Alternaria* spp. with primer OX13. Letters below lanes signify isolates; + = +ve control (primer OP2-genomic DNA from isolate O); - = -ve control. (B) Cluster dendrogram (Ward's method) based on the RAPD products in (A); y-axis = Genetic Distance (GD); x-axis = isolate
Fig. 6.17 First and second principal components of RAPD data: (A) OP2 (proportion of total variation; 22.02%); (B) OP4 (31.12%); (C) OP7 (24.01%); (D) OX13 (21.88%)
Fig. 6.17 (cont’d)
The concentration of template DNA evidently has a marked influence on amplification products. Excess genomic DNA may result in smearing of bands whereas too little often gives irreproducible results (Welsh and McCelland, 1990; Williams et al., 1990). In this study initial PCR experiments yielded no amplification products despite the apparently adequate concentrations of template DNA as calculated from UV spectrophotometry. The genomic DNA was then estimated by measuring the intensity of fluorescence of DNA fragments stained with ethidium bromide and viewed under UV light. Using this technique, generation of RAPDs was both attainable and reproducible, although increasing concentrations of template DNA (10-80 ng) appeared to have little effect on RAPD patterns. It seems that quantification of template DNA by UV spectrophotometry determined inadequate concentrations of DNA, possibly owing to interfering compounds such as low molecular weight proteins or phenolics originally present in the mycelium. Subsequently, RAPD products seldom developed.

Another important factor which determined banding profiles was the rate of ramping. Slowly increasing the annealing temperature to that of extension resulted in increased numbers of amplified fragments. A number of studies have used ramping to produce RAPDs from fungi (Guthrie et al., 1992; Ouellet and Seifert, 1993). As it is generally accepted that random primers sometimes anneal incorrectly and that production of DNA fragments may occasionally arise from mis-priming (Rafalski et al., 1991), perhaps ramping favours mismatching. However, the slow ramping rate used in this study (1°C per 15 seconds) facilitated the production of RAPD bands and improved reproducibility of the analyses.

It is advantageous to optimise the concentration of magnesium in the reaction mixture in order to obtain more pronounced bands. Magnesium ion concentration may influence the following; primer-template annealing, strand uncoupling temperatures of both template and PCR product, product specificity, development of primer artifacts and enzyme activity and fidelity (Saiki, 1989;
Innis et al., 1990). The work presented here showed that a concentration of 1.5 Mm MgCl$_2$ was suitable for production of well defined bands.

Standardisation of conditions by using a previously amplified template DNA/primer combination for each set of reactions is crucial if the results are to be meaningful. In addition, the ability of PCR to generate large numbers of copies of a sequence from minute quantities of DNA requires that precautions must be taken to avoid introduction of foreign DNA (e.g. from dust) and the resulting confusion of the data obtained. Therefore, a control without genomic DNA was included with each primer selection.

The method of selecting RAPD bands to score presents a problem. Even with careful standardisation of techniques, certain bands may amplify poorly owing to factors other than the genetic diversity of the organism being tested, such as inconsistencies in template sequence copy number and altering degrees of mismatch between primer and binding site. Several researchers have approached these problems in various ways. Guthrie et al. (1992), in discriminating isolates of Colletotrichum graminicola, measured the intensity of bands by an image processing system, and selected those for analysis above an arbitrarily-designated intensity. Goodwin and Annis (1991) in their RAPD studies of Leptosphaeria maculans allocated double weight to bright bands and single weight to weak ones. In a further extension of this principle, Demeke et al. (1992) who studied the taxonomy of Brassica spp., graded the intensity of RAPD fragments from 0 (no band) to six (very bright).

In this study, quantitative analysis of DNA segments (using intensity values calculated by a densitometer) was more informative than data analysed in a qualitative binary manner (i.e. 0 = band absent; 1 = band present). Bands that smeared or were difficult to perceive owing to low intensity were excluded from analyses.
6.3.2 Characterisation of groups by polymorphic DNA

The major aim of the research was to differentiate species of *Alternaria* and to identify HT producing isolates. One method of discriminating these specific groups was to determine the polymorphic DNA. The other analytical approach was cluster analysis of the intricate RAPD patterns and display any significant molecular variation of the species by dendrograms (see section 6.3.3).

Since a number of polymorphic DNA distinguished isolates of *A. tenuissima*, *A. brassicae* and *A. brassicicola*, and HT producers of *Alternaria*, the development of a species-specific or HT-specific probes seems plausible. Probes created from RAPD products may be used as diagnostic tools in blot techniques, or on gels of RFLPs. Another strategy for developing a DNA probe is to sequence the polymorphic DNA segment in question and construct a specific primer which is homologous to, or part of, this sequence (typically 18-30 nucleotides in length).

Several studies have demonstrated the use of polymorphic DNA for developing species-specific probes; examples include *F. solani* f.sp. *cucurbitae* (Crowhurst et al., 1991), *F. graminearum* Schwabe (Ouellet and Seifert, 1993) and *Mycosphaerella fijiensis* Morelet and *M. musicola* Leach ex Mulder (Johanson, 1993). Goodwin and Annis (1991) in their studies of *Leptosphaeria maculans* isolated from oilseed rape were able to determine so-called 'aggressive' strains from 'non-aggressive' strains. Development of a specific probe to toxin producing *Alternaria* spp. could be a valuable tool in detecting spores or mycelium of mycotoxigenic *Alternaria* spp. present in rapeseed or meal.

Although the development of molecular probes specific to *A. tenuissima*, *A. brassicae*, *A. brassicicola* and HT producers of *Alternaria* seems possible, synthesis of probes specific to isolates of *A. alternata* and *A. infectoria* is doubtful as no polymorphic DNA sequences were conserved within (or between) either of these species. This suggests that isolates of these genera are genetically...
6.3.3 Characterisation of groups by cluster analysis

Cluster statistics is a technique by which more data are included in the determination of individuals i.e. the incorporation of DNA fragments which are conserved, and those which are not conserved between individuals. Various types of clustering methods are available. In this study, Ward's method was generally superior to the agglomerative clustering methods (i.e. SL and UPGMA analyses) for separating different species and HT producing isolates of Alternaria, although some primers (e.g. OP7) produced similar results with the UPGMA and SL analyses (Fig. 6.18A-B).

Ward's method of clustering is based upon the minimisation of sum of squares and uses squared euclidean distance as the measure of distance between individuals (Pankhurst, 1991). The method examines the variation present and forms groups by segregating individuals in such a manner that, when a new individual is added to an existing group, a minimum amount of variation is added to that group (i.e. uses the principle of parsimony). As the clusters constructed are thus relatively heterogeneous, any one level may vary in different characters. In addition, Ward's method assesses all individuals in a group when adding a new individual and thereby has several advantages over most other methods (except maybe UPGMA; see below paragraph) which tend to consider only two individuals at a time. One advantage in relation to the RAPD data presented here is that Ward's method did not emphasise solitary bands but considered all the bands for a given primer. As a result, individuals which varied in a specific band were still placed together.

A different type of clustering method is the agglomerative clustering technique. This is where clusters are arrived at by starting with individual objects and joining them into successive larger groups until there is only one group remaining, which is the group of all objects. Single linkage (or nearest neighbour) estimates similarity between two groups by taking the highest
Fig. 6.18 Cluster dendrograms based on the RAPD products of primer OP7. (A) UPGMA (unweighted pair group arithmetic average method); (B) SL (Single linkage). The y-axis = Genetic Distance (GD); x-axis = isolate
similarity (or lowest dissimilarity) between the two most similar objects. This statistic is generally better at dealing with very different individuals since it takes into consideration the total distance of all members of a group from the new individual being examined. However, it is inefficient at identifying larger groups, inclines to exaggerate similarity between groups and has a tendency to link similar objects (i.e. chaining). In comparison, UPGMA is a method where the average of all the similarities between pairs of objects, one from each group, are computed. With most cases in this study, the results from SL and UPGMA were unsuitable and therefore Ward’s method was employed for all analyses.

Cluster analysis (Ward’s method) suggested that with certain primers, different species of Alternaria form distinct groups. Although the interpretation of the data for A. brassicaceae and A. brassicicola was limited (since there was only one isolate of each), there was considerable variability between isolates of A. alternata and also A. infectoria. This was confirmed by the fact that (i) the GD between isolates (for a given primer) was relatively wide compared to isolates belonging to another species (e.g. A. tenuissima) and (ii) batching of isolates was not uniform with each primer used. These results are consistent with those obtained by Petrunak and Christ (1992), whose isozyme studies of A. alternata isolated from potato showed high molecular diversity between individuals. Furthermore, RFLP analyses detected relatively high levels of variation in nuclear rDNA of Japanese pear pathotype populations of A. alternata (Tanabe et al., 1990; Adachi et al., 1993).

The high level of genetic variability in A. alternata and A. infectoria can be illustrated by their morphology. Identification of ‘small-spored’ Alternaria such as A. infectoria and A. alternata is not definite (Simmons, 1967; Lucas, 1971; Simmons 1981; 1986; Chekowski and Visconti, 1992). Alternaria alternata is an ubiquitous species and has no specific definition. Consequently, virtually every small-spored Alternaria is generally identified as this single species (De Hogg and Gueho, 1985; Nishimura and Kohmoto, 1983; Simmons, 1990; 1992; 1993A). On closer inspection however, there appear to be discrete
morphological disparities. For example, Simmons (1993B) demonstrated that six common sporulation patterns were present among isolates (several hundred were studied) of *A. alternata* from Japanese pear. In the present study, there were considerable morphological differences in the conidia of *A. alternata* and *A. infectoria*, including those which became hypertrophic (i.e. swollen and distorted). Cells of other species of *Alternaria*, such as *A. chlamydospora*, become hypertrophic, so that the conidia appear to be chlamydospores. This is not the case here as these hypertrophied conidia freely produce secondary conidiophores, as reported by Simmons (1981).

Petrunak and Christ (1992) suggested one possible reason for the high level of genetic variability of *A. alternata* isolated from potato may be natural mutation. *A. alternata* is proficient in producing large numbers of spores in a short period of time, and together with natural mutation rates, this could lead to a relatively high level of diversity. It has also been reported that with some asexual populations of fungi, exchange of genetic material is unlikely (Burdon and Roelfs, 1985). Although the sexual stage of *A. alternata* is not known, the telemorph of *A. infectoria* has been described as *Lewia infectoria* (Fuckel) Barr and Simmons (Simmons, 1986).

Another explanation as to why *A. alternata* and *A. infectoria* have high genetic variability is that both species usually exist as common saprophytes. Perhaps the saprophytic nature of the moulds may have forced them to adapt to a more divergent range of substrates, which may have then led to individuals becoming more genetically variable.

With the *Alternaria* spp. surveyed in this study, clustering of RAPD patterns appears to be successful in segregating mycotoxigenic (HT) individuals from non-mycotoxigenic (NLT) individuals. As mycotoxigenic isolates of *Alternaria* are morphologically indistinguishable from non-mycotoxigenic individuals, identification of toxin-producing *Alternaria* can be time consuming i.e. culturing of isolates on media, chemical extraction of possible toxins and
detection of these metabolites using methods such as TLC and HPLC. Therefore, development of a rapid PCR method which generates unique RAPD patterns for mycotoxinogenic *Alternaria* spp. is significant.

One criticism of clustering procedures is they set out by presuming clusters exist. Analysis by ordination (PCA) avoids this assumption as it is possible to transform variables so that, in many cases, much of the total variation is integrated in the first few dimensions. The statistic first presumes that the data points of the individuals in question can be depicted as variables in hyperspace, with each data point plotted along a separate axis. If the hyperspace is rotated in such a manner in order to minimise the scatter of data points around each axis, the set of angles of rotation can then be used to compute an eigenvector loading for each of the variables on each axis, or component as it is now recognised. The eigenvectors come out in decending order of size difference until all the variation is accounted for.

Under ideal situations, most of the total variation is contained in the first two or three variables. Although the ordination patterns shown here confirmed separation of HT producing isolates of *Alternaria*, only about 25% of the total variability was condensed in the first two factors, and therefore 2D plots of isolates must be interpreted with care. This is because closer relationships may not always be represented by shorter distances and more distant relationships by greater distances. In addition, actual distances between points on the plots may also be unreliable.

The major limitation in this study was that the number of isolates appraised was too small, and therefore some of the results obtained may be misinterpreted. For example, the HT producing isolates T, U, V and W were isolated from regions relatively close together (*i.e.* Essex and Kent). As RFLP patterns of *Alternaria* spp. have been reported to vary with geographical location (Adachi et al., 1993), perhaps locality as well as toxin production influenced the molecular profiles of these isolates. In order to give a more comprehensive
picture of distinguishing mycotoxigenic *Alternaria* spp. from NLC producers, and also differentiating different species of *Alternaria*, more isolates from areas further afield, and additional representative isolates from those already surveyed should be considered.
CONCLUSIONS

*Alternaria* spp. were the predominant moulds contaminating rapeseed samples in Great Britain. Although no samples were collected and analysed for the presence of mycotoxins (most importantly TeA), it is improbable that *Alternaria* metabolites are or will be a threat to the oilseed rape industry in the UK. This is owing to the stringent governmental controls of rapeseed and rapeseed meal quality (e.g. ≤9% MC), and low ambient storage temperatures (generally 0-15°C). However, in countries which have hot, humid climates and where storage facilities for rapeseed or meal are crude (e.g. in Pakistan), *Alternaria* toxins may be a problem. Therefore, transfer of new methodology determined in this study (e.g. HPLC solvent optimisation of *Alternaria* toxins) to less developed countries may be of importance to their oilseed rape industries.

A nitrogen-containing metabolite produced by an isolate of *A. alternata* (isolate B) was a self inhibitor of spore germination and hyphal extension. Further work requires the development of a method for isolating large (mg) quantities of the compound, perhaps using cTLC, identification by NMR analysis and bioassay tests (e.g. other fungi and brine shrimp) in order to determine the range of biological activity.

Perhaps the most interesting feature of the study was that DNA polymorphisms generated by the PCR and subsequent cluster dendrograms were able to distinguish some species of *Alternaria* (e.g. *A. tenuissima*) and high toxin producing *Alternaria* isolates. However, further RAPD investigations are recommended using additional *Alternaria* isolates as the number studied here (23 isolates) was small. Future work may then involve synthesis of a primer which is complementary to one of these polymorphisms which could be used to identify potential mycotoxigenic isolates of *Alternaria* spp..
Appendix 1 The effect of Satisfar on the growth of *A. alternata* on rapeseed agar.
Appendix 2 The effect of temperature on the growth of A. tenuissima on rapeseed agar
BIBLIOGRAPHY


Curtis R.F., Coxon D.T. and Levett G. Toxicity of fatty acids in assays for mycotoxins using the brine shrimp (*Artemia salina*). *Food and Cosmetic Toxicology* 12, 233-235.


Evans J. and Cox T. (1982) *Alternaria* is now the major disease of oilseed rape crop. *Arable Farming* April, 78-84.


Pero R.W and Harvan D. (1973) Simultaneous detection of metabolites from several toxigenic fungi. *Journal of Chromatography* 80, 335-336.


208


