Factors affecting mycotoxin production by *Fusarium* species

By

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Abstract

Studies were undertaken to investigate the factors affecting mycotoxin production in *Fusarium* phytopathogens. The conclusions of this work are divided into the following categories.

Substantive findings:
Contamination of cereal grains with *Fusarium* mycotoxins is a global and continuing issue and is likely to remain so until a systematic elucidation of controlling factors has been accomplished.

An important observation at the commencement of the research programme examined the validity of using direct TLC analysis of agar culture plugs to establish mycotoxin profiles in *Fusarium* species.

The results in this thesis indicate that this method only detected a limited number of mycotoxins and this was not consistent between surfaces of the plugs tested or between experiments in the preliminary series.

A noteworthy observation was that HT-2 toxin production from T-2 toxin was not time-related as claimed by other workers. There may be a real species differences in this respect between *F. sporotrichioides* and *F. poae*.

Mixtures of carbendazim and propiconazole or carbendazim plus maneb or carbendazim plus maneb plus tridemorph all enhanced T-2 toxin formation.

The fungicide-induced enhancement of mycotoxin production has now been extended, for the first time, to HT-2 toxin and NEO.

A substantive finding, not previously noted, is that difenoconazole failed to stimulate T-2 toxin formation at 25°C but was capable of transforming it to HT-2 toxin and then stimulating the production of the latter product.

Difenoconazole appears to be a fungicide in a class of its own in that DAS and NEO production are consistently higher than for a large majority of other fungicides tested.

NEO production was not substantially affected by fungal exposure to Bavistin, whereas carbendazim acted in a stimulatory manner. It is suggested that this discrepancy is due to fungicide form, an effect not previously reported.
A novel finding has been a consistent indication of reduced efficacy of pesticides for the control of ZEN in the 25-11°C regime compared to the constant incubation at 25°C.

The effects of incubation temperature on T-2 toxin production is dependent upon fungicide type whereas in the case of HT-2 toxin, DAS and NEO, there is a clear effect of temperature. Contrasting effects were observed in fungicide efficacy for the control of HT-2 toxin in the two temperature regimes. At 25°C, stimulation occurred at the 100 µg/ml dose but in the 25-11°C regime, HT-2 toxin production was almost eliminated at the same concentration.

It is suggested that the complex interactions involving fungicides, temperature and duration of exposure to these factors may be critical in the timing of fungicide applications in the field.

A new classification for fungicide efficacy is proposed, based on the capacity to completely control FHB/grain infection/fungal growth and mycotoxin production on grain or in culture. Three classes have been identified. Most fungicides appear in the Class III group, incorporating fungicides that are either ineffective or capable of stimulating trichothecene production.

Although maneb has been identified as a Class I fungicide from evidence in the literature, its high efficacy is not conferred to mixtures incorporating this fungicide.

A novel finding in the present study with *F. culmorum* was the demonstration of 3-ADON enhancement induced by the herbicide combination of bromoxynil, ioxynil and mecoprop added as Swipe.

A radical re-design of pesticides is proposed to incorporate efficacy for regulation of mycotoxin biosynthesis.

Secondary findings:
A rule-of-thumb based on colour differences of *Fusarium* colonies is proposed. The present results suggest that the striking differences in pigmentation might be associated with the pattern of mycotoxin production. Thus yellow is possibly indicative of the presence of T-2 toxin, HT-2 toxin, DON, 15-ADON and 3-ADON while pink/red coloration might suggest the production of ZEN. This rule-of-thumb, if sufficiently developed, might find application in field assessments of toxigenic potential in cases of cereal diseases such as FHB.

The lack of efficacy of propiconazole to regulate trichothecene production has been extended to instances where it is combined with other fungicides.

Confirmatory findings:
Present results with *F. sporotrichioides* confirm for only the second time in recent years that this phytopathogen is a consistent producer of ZEN.

Biosynthesis of ZEN is clearly temperature-sensitive but there may be species differences regarding this effect.

The enhancement effect of fungicides on T-2 toxin production has now been extended to carbendazim.

New hypothesis:
The current studies on gene expression indicate future potential for elucidating the biochemical mechanisms underlying trichothecene biosynthesis as affected by factors such as incubation temperature and fungicide applications. Such an approach might lead to more effective pesticides.

Declaration

This thesis is my own composition, the results presented are of investigations conducted by myself, work other than my own is clearly indicated by reference to relevant workers or their publications and it has not been presented in any previous application for a higher degree.

Cristina - Mihaiela Müller - Placinta

"For nothing is impossible with God"

Luke 1:37

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List of Abbreviations

A	Adenine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pares
C	Cytosine
cm	centimetre
cm^2	square centimetre
CM	chloroform : methanol
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.$1^{3,7}$]decan}-4-yl)phenyl phosphate
d	day
DAS	diacetoxyscirpenol
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DON	deoxynivalenol
3ADON	3 acetyldeoxynivalenol
15ADON	15 acetyldeoxynivalenol
EDTA	ehylenediaminetetraacetic acid
FHB	fusarium head blight
FUS	fusarenon X
g	gram
G	Guanine
GYEP	glucose yeast extract peptone
h	hour
HT-2	HT-2 toxin
kb	kilo bases
l	Litre
LB	Luria-Bertani medium
LD_{50}	Lethal dose
LSD	Least significant difference
MES	2-[N-morpholino]ethanesulfonic acid
min	minute
mg	milligram
ml	millilitre
M	molar
mM	millimolar
MOPS	3-[N-morpholino] propanesulfonic acid
NBT	nitroblue tetrazolium
NEO	neosolaniol

ng	nanogram
NIV	nivalenol
nm	nanometre
O.D.	optical density
PCR	polymerase chain reaction
PDA	potato dextrose agar
Rf	relative mobility
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	rotations per minute
s	second
SDS	sodium dodecyl sulfate
SDW	sterile distilled water
SEM	standard error of the mean
SSC	saline sodium citrate
T	Thymine
T-2	T-2 toxin
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEF	toluene : ethyl acetate: formic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)amino methane
tRNA	transfer RNA
U	Uracil
UV	ultra violet
V	volt
ZEN	zearalenone
µg	microgram
µl	microlitre
µM	micromolar
°C	degree Celsius

Chapter 1

Introduction

1 Introduction

The secondary metabolism of fungi results in the production of a diverse array of bioactive metabolites of critical significance to plant, animal and human health. Three major groups of secondary metabolites may be recognised. Species of *Cochliobolus*, and *Alternaria* elaborate compounds which instigate specific plant diseases including, respectively, victoria blight of oats and Alternaria stem canker of tomato. These metabolites are designated 'host-specific toxins' (HSTs). Other species of *Alternaria* and certain members of the *Fusarium* genus synthesise 'non-specific toxins' which have been implicated as virulence factors in plant disease. The term 'mycotoxins' refers to a third group of bioactive substances which induce specific syndromes and other adverse effects in animals and humans. A wide variety of fungi are capable of producing mycotoxins, but particular emphasis is currently being accorded to *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* species. These fungi may act as plant pathogens or as spoilage organisms and, consequently, mycotoxin contamination of raw materials can arise in the field or during storage of harvested commodities. The *Aspergillus* genus contains species responsible for the production of two major groups of mycotoxins, namely the aflatoxins and ochratoxins. Certain *Penicillium* species also synthesise ochratoxins as well as other mycotoxins such as citrinin and patulin. The *Fusarium* phytopathogens elaborate trichothecenes, zearalenone, fumonisins, fusaric acid, moniliformin, fusaproliferin and beauvericin as the major mycotoxins, but production is often species-related. The *Alternaria* mycotoxins include alternariol, alternariol methyl ether, altenuene, tenuazonic acid, tentoxin and altertoxin I. Although HSTs, non-specific toxins and mycotoxins may be considered as distinct groups, there are at least two common features. Thus the fumonisins are structural analogues of an HST produced by *Alternaria alternata*, designated AAL-toxin while fusaric acid can act as both a non-specific toxin in plants and as a mycotoxin. In addition, a number of other mycotoxins have been implicated as virulence factors in plant disease. Many mycotoxins are associated with acute toxicity, with relatively low LD_{50} values under experimental conditions and high mortality in field incidents such as turkey X

disease, attributed to aflatoxin contamination of groundnuts. However, the syndromes induced by chronic exposure are of potentially greater economic concern in animal production. Thus, the ochratoxins cause porcine nephropathy while specific *Fusarium* mycotoxins induce a greater diversity of syndromes including feed refusal and emesis, oral and other gastrointestinal lesions, infertility, porcine pulmonary oedema and equine leukoencephalomalacia. In humans chronic exposure to aflatoxins and fumonisins have been associated with the incidence of cancer in Africa and China. In the Balkans ochratoxins have been implicated in endemic nephropathy, a condition analogous to the porcine syndrome cited above.

Mycotoxin exposure in animals and humans continues despite awareness of health risks and imposition of regulations and directives on permissible levels in primary foods such as cereal grains and in animal feeds. Much work has been conducted on factors affecting aflatoxin production in *Aspergillus* species and on the molecular basis underlying synthesis of this group of compounds. However, the study of *Fusarium* mycotoxins in all these facets is still in its infancy. Thus, the occurrence and roles of fumonisins, fusaproliferin and beauvericin have only recently been elucidated. The experiments in the present thesis were designed to examine the extent to which physical and biotic factors interact to determine mycotoxin production in three *Fusarium* phytopathogens subjected to different types and levels of pesticides. Molecular biology techniques have also been employed in an attempt to elucidate how fungicides may influence specific steps in the pathways of trichothecene biosynthesis. This thesis also contains a review of contamination of cereal grains with *Fusarium* mycotoxins and of the toxicological issues arising from acute and chronic exposure in animals. Thus an attempt is made to present as complete a perspective of *Fusarium* mycotoxins as current evidence allows.

Chapter 2

Literature Review

2 Literature review

2.1 Background

In global terms the *Fusarium* genera constitute a significant group of fungi colonising cereal plants and grains. *F. culmorum*, *F. graminearum*, *F. poae* and *F. sporotrichioides* are prominent species in temperate zones, while in warm countries *F. moniliforme* and *F. proliferatum* are also important in crop ecology. Several *Fusarium* species associated with crop diseases such as fusarium head (ear) blight also have the propensity to synthesise mycotoxins and contamination of grain is, therefore, to be expected. Since fungicides are used to control *Fusarium* diseases of crop plants, the effects on mycotoxin production need to be considered. Although much work has been conducted on the identification and quantification of the *Fusarium* mycotoxins, more work is required to determine the wide variety of factors which affect the production of these compounds. This is all the more relevant in the current context of reduced pesticide inputs in Europe, and global climate change, all of which may affect mycotoxin production from *Fusarium* species. Furthermore, no commercial techniques are available for removal or reduction of *Fusarium* mycotoxins in contaminated grain. The well recognised development of resistance to fungicides in *Fusarium* species and the co-existence of these organisms with other fungal species in the field indicate the need for further work on the factors which affect the production of mycotoxins from *Fusarium* species. Although it is widely acknowledged that these factors are likely to exert their effects through interactions, few studies have been conducted to elucidate the extent and complexity of such interactions. In addition, the efficacy of fungicides in controlling mycotoxin production has been assessed in only a limited number of investigations, with indeterminate outcomes.

The strategy adopted in the following review involves a critical examination of published evidence with the ultimate objective of developing a rationale for the work presented in this thesis. Pursuance of this approach has necessitated a re-assessment

of published data to maximise impact, to improve clarity, to establish underlying concepts, to identify anomalies and to provide alternative interpretation of the evidence. In consequence, all tables and figures in this review are original, although based on published data.

2.2 Production of major Fusarium mycotoxins

The principal mycotoxins produced by *Fusarium* species include the trichothecenes, zearalenone, fumonisins, moniliformin, wortmannin, fusarochromanones, fusaric acid, fusarin C, beauvericin and fusaproliferin.

The trichothecenes are a diverse group comprising:

Type A - for example, T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), neosolaniol (NEO),

Type B- for example, fusarenon-X, nivalenol, deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON),

Type C - verrucarin and roridin.

The pathways for the biosynthesis of trichothecenes are now well established (Desjardins *et al.*, 1993) and will be discussed later in this review. The essence of these pathways is the ordered series of cyclizations, oxygenations and acetylations. Furthermore, it is known that T-2 toxin is metabolized to HT-2 toxin (Kotsonis and Ellison, 1975) and that 3-ADON is the precursor of DON (Yoshizawa and Morooka, 1975).

The production of the important trichothecenes and zearalenone (ZEN) by different *Fusarium* species is summarized in Table 2.1, derived from work over the past 20 years. Compilation of Table 2.1 was considered necessary to resolve conflicting issues in the literature concerning the production of type A and type B trichothecenes. For example, the production of DON and 3-ADON by *F. sporotrichioides* was claimed in one study but discounted in another (Table 2.1).

Table 2.1 Production of mycotoxins [a,b] by different *Fusarium* species (compiled from original publications)

Fusarium species	T-2	HT-2	DON	3-ADON	15-ADON	NIV	NEO	DAS	ZEN	Refs[c]
F. sporotrichioides	+									1
	+	+					+			2
	+		-		-			+		3
	+	+								4
	+	+	+		+	+	+	+		5
	+	+					+	+		6
	+							+		7
	+	+					+		+	8
	+	+					+	+		9
F. culmorum						+				10
			+	+					+	11
			+		+					4
									+	2
			+						+	5
			+						+	6
			+	+						12
			+						+	13

Table 2.1. continued

Fusarium species	T-2	HT-2	DON	3-ADON	15-ADON	NIV	NEO	DAS	ZEN	Refs
F. graminearum			+							14
			+		+					15
									+	16
			+						-	17
			+	+	+				+	3
			+							18
			+		+	-				4
									+	19
									+	2
									+	20
		+	+		+					5
			+		+				+	21
			+			+			+	6
			+							22
			+						+	13
			+	+					+	23
F. roseum									+	2
			+							24
			+	+					+	3
									+	2
									+	25

Table 2.1 continued

Fusarium species	T-2	HT-2	DON	3-ADON	15-ADON	NIV	NEO	DAS	ZEN	Refs
F. equiseti									+	26
									+	2
			-		-				+	21
									+	6
F. tricinctum	+									27
	+									16
	+						+	+		28
F. oxysporum									+	2
F. poae	+	+				+				4
		+	+		+					5
						+				29
			-	-		+	-	+	-	30
	+	+					+			8

[a] DON=deoxynivalenol; 3-ADON=3-acetyldeoxynivalenol; 15-ADON=15-acetyldeoxynivalenol; NIV=nivalenol; NEO=neosolaniol; DAS=diacetoxyscirpenol; ZEN=zearalenone

[b] + = confirmed presence; - = confirmed absence; empty cell = no data.

[c] 1. Proctor *et al.* (1995); 2. Richardson *et al.* (1985); 3. Miller *et al.* (1983); 4. Wong *et al.* (1995); 5. Abramson *et al.* (1993); 6. Thrane (1986); 7. Moss and Frank (1985); 8. Szathmary *et al.* (1976); 9. Marasas *et al.* (1987); 10. Gareis & Ceynowa (1994); 11. Snijders & Perkowski (1990); 12. Greenhalgh *et al.* (1986); 13. O'Neill *et al.* (1993); 14. Scott *et al.*(1984); 15. El-Bahrawy *et al.* (1985); 16. Halasz *et al.* (1989); 17. Miller *et al.* (1985); 18. Milus & Parsons (1994); 19. Berisford & Ayres (1976); 20. Cuero *et al.* (1988); 21. Bosch *et al.* (1992); 22. Boyacioglu *et al.* (1992); 23. Greenhalgh *et al.* (1983); 24. Yoshizawa and Morooka (1975); 25. Draughon & Churchville (1985); 26. Paster *et al.* (1991); 27. Paster *et al.* (1986); 28. Roinestad *et al.* (1993); 29. Pettersson (1991); 30. Sugiura *et al.* (1993)

Virtually all *Fusarium* species produce a mixture of mycotoxins. The major toxins of *F. sporotrichioides* are T-2, HT-2, DAS, NEO, whereas in *F. culmorum* and *F. graminearum* the major toxicants are DON and ZEN, and these fungi would therefore appear to be more toxigenic than other *Fusarium* species. For example in *F. roseum* and *F. equiseti* ZEN appears to predominate. There is a clear indication of absence of DON and 15-ADON in *F. sporotrichioides* (Miller *et al.*, 1983). However, Abramson *et al.* (1993) provide evidence of the presence of DON in *F. sporotrichioides*. This may be due to differences in culture conditions and substrate nature. Similar comments may apply to the discrepancies in NIV and ZEN production by *F. graminearum*.

F. poae produces mostly HT-2, NIV and T-2. With reference to DON and NEO some authors (Sugiura *et al.*, 1993) maintain that these mycotoxins are not produced by *F. poae*, others claim that DON (Abramson *et al.*, 1993) and NEO (Szathmary *et al.*, 1976) are produced by this fungal species. There is evidence that ZEN and 3-ADON are not produced by *F. poae*.

Over the past 20 years ZEN was found to be produced by *F. sporotrichioides* on only one occasion (Szathmary *et al.*, 1976). Since ZEN is now an established oestrogen its production by different *Fusarium* species is worthy of further research and forms the basis of several experiments in the current investigations.

Co-production of different trichothecenes with ZEN by particular *Fusarium* phytopathogens is the dominant issue in Table 2.1. Co-production also occurs in other important *Fusarium* fungi. Thus, *F. moniliforme* produces at least three mycotoxins, namely the fumonisins, moniliformin and fusarin C. The fumonisins (FB_1, FB_2 and FB_3) are regarded as unique in that they are also structural analogues of the host-specific AAL-toxins synthesized by *Alternaria alternata*, the causative agent of stem canker of tomato. In addition, however, *F. moniliforme* and *F. proliferatum* have recently been linked with the natural co-contamination of maize with FB_1, and two relatively novel mycotoxins, fusaproliferin and beauvericin (Placinta *et al.*, 1999). Moniliformin is also synthesized by *F. oxysporum* which, in addition, is a recognized source of the mycotoxins wortmannin and fusaric acid. The diversity of *Fusarium* mycotoxins is further illustrated by the production of

fusarochromanones (TDP-1, TDP-2 and TDP-6) by *F. equiseti* which also synthesizes several trichothecenes as well as ZEN (Placinta *et al.*, 1999).

The reason why fungi such as *Fusarium* produce this array of secondary metabolites has been the subject of much speculation and may, indeed not be resolved in the foreseeable future. It is possible that synthesis of mycotoxins may confer competitive advantage over other organisms. Production of mycotoxins may be initiated by stress factors such as diminishing substrate or unfavourable environmental conditions. It has also been suggested that trichothecenes may act as instruments in plant pathogenesis by *Fusarium* species (Desjardins *et al.*, 1993). In addition, there are claims, at least for ZEN, that it may act as a hormone governing the development of sexual stages in *Fusarium* fungi (Wolf *et al.*, 1972). The universal production of ZEN by *Fusaria* may be a reflection of this function (Table 2.1). The effects of biotic and environmental stress on mycotoxin production form part of the present studies. However, the investigations were not designed to specifically address the reasons why *Fusaria* synthesize these deleterious compounds.

2.3 Worldwide contamination of cereal grains with *Fusarium* mycotoxins

Since toxigenic species of *Fusarium* are commonly associated with cereal plants as pathogens, contamination of grain is virtually inevitable. It follows that this contamination may be transferred to cereal-based animal feeds. The occurrence of *Fusarium* mycotoxins in cereals and animal feed has been summarised by Scott (1989) and Yoshizawa (1991), but considerable data has since been published on these and the more novel mycotoxins such as fumonisins. A clear need for an up-dated review was perceived and a paper has now been published (Placinta *et al.*, 1999).

2.3.1 Trichothecenes

Table 2.2 has been extracted from the review of Placinta *et al.* (1999), with important modifications to include data for the type A trichothecenes. It is instructive to consider the values in Table 2.2 in relation to the observed incidence of co-contamination, where this information is available. Thus, in the German study (Muller and Schwadorf, 1993), 79 of the 84 wheat samples analysed contained between two and six *Fusarium* mycotoxins, with 20% of samples co-contaminated with DON and ZEN. The most frequent combination included DON, 3-ADON and ZEN. In the Lublin region of south-eastern Poland, type A trichothecene contamination of barley grain was linked with the natural incidence fusarium head blight in which the predominating organism was *F. sporotrichioides* (Perkowski *et al.*, 1997). Of 24 barley grain samples, 12 were positive for T-2 toxin, with the values shown in Table 2.2. In 21% of these samples, co-contamination with HT-2 toxin occurred (Table 2.2) and two samples also contained T-2 tetraol.

In Bulgaria, *F. graminearum* is a major pathogen of wheat and consequently grain can be expected to be contaminated with type B trichothecenes. Vrabcheva *et al.* (1996) recorded relatively low levels of DON (Table 2.2), but the incidence of contamination was 67% and was accompanied by 3-ADON and 15-ADON.

Oat grains produced in Norway by commercial growers were found to be more heavily contaminated with DON than barley or wheat kernels (Table 2.2) and this has been attributed to edaphic and agronomic factors and to different infection pathways by *Fusarium* pathogens (see Placinta *et al.*, 1999). In addition to NIV (Table 2.2), other contaminants included 3-ADON and fusarenon-X. For example 56% of certain oat samples contained detectable quantities of 3-ADON at 0.03 mg/kg or more.

Table 2.2 Global distribution of deoxynivalenol (DON), nivalenol (NIV), other trichothecenes and zearalenone (ZEN) in cereal grains and animal feed (mg/kg)[a]

Country	Cereal/ feed type	DON	NIV	Other trichothecenes	ZEN
Germany	Wheat	0.004-20.5	0.003-0.032	T-2 toxin, max 0.250; HT-2 toxin, max 0.020	0.001-8.04
Poland	Wheat Barley	2-40	0.01	T-2 toxin, max 2.4; HT-2 toxin, max 0.37; T-2 tetraol, max 0.21	0.01-2
Poland	Maize kernels Maize cobs: axial stems	4-320 9-927		15-ADON, 3-86 15-ADON, 6-606	
Bulgaria	Wheat	up to 1.8		3-ADON, 0.1 15-ADON, 0.1	up to 0.12
Finland	Feeds and grains Oats	0.007-0.3 1.3-2.6		3-ADON, 0.01-0.6	0.022-0.095
Norway	Wheat Barley Oats	0.45-4.3 2.2-13.33 7.2-62.05	max 0.054 max 0.77 max 0.67	3-ADON, 0.03	
Netherlands	Wheat Barley Oats Rye	0.020-0.231 0.004-0.152 0.056-0.147 0.008-0.384	0.007-0.203 0.030-0.145 0.017-0.039 0.010-0.034		0.002-0.174 0.004-0.009 0.016-0.029 0.011
South Africa	Maize	up to 1.83	up to 0.37		
South Africa	Cereals/ animal feed			T-2 toxin, DAS (values not given)	0.05-8.0
India	*Paspalum palidosum* straw Mixed concentrate				0.422 0.843
Philippines	Maize		0.018-0.102		0.059-0.505
Thailand	Maize				0.923
Korea	Barley Maize	0.005-0.361 mean 0.145	0.040-2.038 mean 0.168	Fusarenon-X, 0.015-0.072	
Vietnam	Maize powder	1.53-6.51	0.78-1.95		
China	Maize	0.49-3.10	0.6		

Table 2.2 continued

Country	Cereal/ feed type	DON	NIV	Other trichothecenes	ZEN
Japan	Wheat Barley	0.03-1.28	0.04-1.22		0.002-0.025 0.010-0.658
Japan	Wheat Barley	0.029-11.7 61-71	0.01-4.4 14-26	 3-ADON, max 18.7	0.053-0.51 11-15
New Zealand	Maize	max 3.4-8.5	max 4.4-7.0		max 2.7-10.5
USA	Wheat	up to 9.3			
USA	Wheat (winter), 1991 Wheat (spring), 1991 Wheat, 1993 Barley, 1993	<0.1-4.9 <0.1-0.9 <0.5-18 <0.5-26			
Canada	Wheat and barley	up to 0.5		T-2 toxin, 0.16-0.31; HT-2 toxin, 0.12-0.44; DAS, 0.11	up to 0.3
Canada	Wheat (hard) Wheat (soft, winter) Wheat (soft, spring) Maize	0.01-10.5 0.01-5.67 0.01-1.51 0.02-4.09		HT-2 toxin, 0.06-0.59 (in Durum wheat)	
Canada	Animal feeds	0.013-0.2	0.065-0.311		
Argentina	Wheat	0.10-9.25			
Brazil	Wheat	0.47-0.59	0.16-0.40	T-2 toxin, 0.04-0.80; DAS, 0.60	0.04-0.21

[a] Adapted from Placinta *et al.* (1999)

Furthermore, a study of cereals in the Netherlands revealed the co-occurrence of DON, NIV and ZEN (Table 2.2). Of the 29 samples tested, 90% and 79% were positive for DON and NIV respectively, with 76% containing both mycotoxins as co-contaminants.

In Natal, South Africa, 417 samples comprising of maize, compound animal feeds, oilseeds and forage were examined for mycotoxins, of which 19% were contaminated with DON, NIV, DAS and an unknown trichothecene, but results were not quantified (Dutton and Kinsey, 1995). Subsequently Dutton and Kinsey (1996) found that 10% of animal feeds and cereals in Kwazulu Natal were contaminated with DON, NIV, DAS and T-2 toxin, but quantitative results were not presented. Earlier however, Rheeder *et al.* (1995) concluded that contamination of commercial maize with trichothecenes (Table 2.2) constituted little risk to animal health in South Africa.

In Korea, both barley and maize harvested in 1992 were co-contaminated with DON and NIV (Table 2.2), with a higher incidence for NIV in barley (93%) than in maize (53%). However for DON, incidence of contamination was higher in maize (93%) than in barley (67%). In addition fusarenon-X occurred in 33% of maize samples.

The issue of co-contamination was taken a step further with a report by Wang *et al.* (1995) who suggested that for the first time in North Vietnam some maize samples destined for animal feeds were found with DON, NIV (Table 2.2), fumonisins and AFB_1.

Two sets of data from Japan showed strikingly different values for trichothecene contamination of cereal grains (Table 2.2). The first group, based on the results of Sugiura *et al.* (1993) indicated relatively low levels of DON and NIV in wheat. However, a more recent set of data (Yoshizawa, 1997) indicated considerably higher values for these trichothecenes in both wheat and barley. In addition, an appreciable number of barley samples were found with 3-ADON. In highly contaminated grains, a positive correlation occurred between levels of DON and its acetyl derivatives. DON levels were always higher than those of 3-ADON and 15-ADON, with ratios ranging from 2.9 to 155. Furthermore, regional differences were observed in that DON was the major contaminant in grain from northern districts of Japan, whereas in central districts NIV was the predominant trichothecene. These differences were correlated with chemotype variants of *Fusarium* species.

In New Zealand, all samples tested from the 1992 harvest were positive for DON/NIV, this incidence declining to 97% in the 1994 harvest. Maximum levels of

these trichothecenes and ZEN are shown in Table 2.2. In 1992, 24% of samples had high levels of DON + NIV (in excess of 2 mg/kg) which corresponded with adverse weather conditions and the consequent delay in harvest. In 1994, however, 60% of samples had less than 0.4 mg/kg and only 5% were found with DON + NIV levels in excess of 2 mg/kg.

Trichothecene contamination of grain and animal feeds in the United States of America and Canada is extensive and has been adequately reviewed by Scott (1989). The impetus for monitoring has continued particularly with respect to DON in wheat and barley (Table 2.2). In addition, regional variations in DON contamination of wheat have been observed (Fernandez *et al.*, 1994). Highest levels in the 1991 harvest in the United States were seen in Missouri, North Dakota and Tennessee. Even higher levels of contamination of cereals with DON were seen in the 1993 harvest (Table 2.2), with 86% of samples from Minnesota and up to 78% of samples from North and South Dakota containing levels in excess of 2 mg/kg.

In a study of trichothecene contamination of grain in Atlantic Canada (Table 2.2), DON was detected in 53% to 62% of samples tested. Of 55 samples, five contained T-2 toxin, two were contaminated with HT-2 toxin and DAS occurred in two samples (Stratton *et al.*, 1993). A comprehensive review of trichothecene levels in Canadian grain is now available (Scott, 1997) indicating higher values for DON in cereal grains (Table 2.2) than those reported by Stratton *et al.* (1993). In Ontario, DON incidence was consistently higher for maize than for soft wheat over a 15-year period (Scott, 1997). Of particular note is the lower levels of DON in soft spring wheats over this period (Table 2.2). It was concluded that DON was a frequent contaminant of Canadian grains. However, NIV and T-2 toxin were also detected in these cereals.

Analysis of animal feeds in 94 cases of suspected mycotoxicosis of farm animals in western Canada, over the period 1982-1994, indicated relatively low levels of DON, NIV (Table 2.2) and several other trichothecenes (Abramson *et al.*, 1997). However, in four of these cases feed refusal in pigs was linked with levels of DON ranging from 0.013 to 0.095 mg/kg feed.

Argentinian sources of maize have been monitored for DON (Scott, 1989) but a recent analysis of 60 wheat samples indicated high incidence of contamination (over 93%) with the levels shown in Table 2.2. In Brazil, 13 out of 20 grain samples from experimental plots in wheat-producing areas of the State of Sao Paulo were free of mycotoxin contamination. Relatively low levels of DON and NIV were found in positive samples (Table 2.2). In addition, T-2 toxin and DAS occurred in three of these samples. However, the authors cautioned that their findings may not reflect the potential for contamination in other wheat-growing regions of Brazil.

2.3.2 Zearalenone

The predominant feature of ZEN distribution in cereal grains and animal feed is its co-occurrence with other *Fusarium* mycotoxins, including trichothecenes (Table 2.2). This observation is consistent with the confirmed production of ZEN by virtually all toxigenic and plant pathogenic species of *Fusarium* (Table 2.1). The highest values for ZEN in Table 2.2 (11 and 15 mg/kg) relate to two barley samples from the Fukuoka region of Japan (Yoshizawa, 1997). Co-contamination of maize with ZEN, NIV, fumonisins and aflatoxins is an emerging issue in South East Asia (Table 2.2) and elsewhere in the humid tropics. Consequently, although only four sets of data in Table 2.2 indicate serious ZEN contamination of grain and feeds, synergistic effects on animal health may arise at lower levels if ZEN occurs in combination with other mycotoxins or oestrogenic compounds.

2.3.3 Fumonisins

Widespread contamination of maize and animal feed with fumonisins has recently been reported (Table 2.3). High incidence rates and co-contamination have been major features of recent reports. In most instances the predominant fumonisin was FB_1. Highest values for FB_1 were recorded for maize samples in China, where AFB_1

co-occurred in 85% of samples, and in Thailand. Multiple contamination of maize with fumonisins, DON, NIV and AFB_1 was also observed in Vietnam (see Placinta *et al.*, 1999). For FB_2, highest values in maize were found in samples from Argentina. In Philippines, Thailand and Indonesia, FB_1 and FB_2 occurred in over 50% of maize samples and, furthermore, these mycotoxins co-occurred with aflatoxins in 48% of samples.

Table 2.3 Worldwide contamination of maize and animal feeds with fumonisins B_1, B_2 and B_3 (FB_1, FB_2, FB_3;μg/kg) [a]

Country	FB_1	FB_2	FB_3	Total
	MAIZE			
Benin	nd [b]-2630	nd-680		nd-3310
Botswana	35-255	nd-75	nd-30	35-305
Mozambique	240-295	75-110	25-50	340-395
South Africa	60-70	nd	nd	60-70
South Africa	max 2000			
Malawi Zambia	nd-115 20-1420	nd-30 nd-290	nd	nd-135 20-1710
Zimbabwe	55-1910	nd-620	nd-205	55-2735
Tanzania	nd-160	nd-60	nd	nd-225
Honduras	68-6555			
Argentina	85-8791	nd-11300	nd-3537	85-16760
Uruguay	nd-3688			
Costa Rica	1700-4780			
Italy	10-2330	nd-520		10-2850
Portugal	90-3370	nd-1080		90-4450
USA	nd-350			
Vietnam	268-1516	155-401	101-268	524-2185
China	160-25970	160-6770	110-4130	430-36870
Philippines	57-1820	58-1210		
Thailand	63-18800	50-1400		
Indonesia	226-1780	231-556		
	ANIMAL FEED			
South Africa	4000-11000			
Uruguay	256-6342			
India	20-260			

[a] Adapted from Placinta *et al.* (1999); [b] nd= not detectable

In addition these fumonisins co-occurred with NIV and ZEN. Incidence rates of 82 to 100% were recorded for samples from Italy, Portugal, Zambia and Benin. In Honduras, FB_1 was detected in all 24 samples maize tested. Wide within-country and between-country variations in levels of contamination have emerged (Table 2.3). The differences in South African sources of maize and animal feed are particularly worthy of note. Of additional concern is the recent demonstration of co-contamination of pre-harvest maize ears in Italy with FB_1, fusaproliferin and beauvericin, at 300, 500 and 520 mg/kg respectively (Ritieni *et al.*, 1997). The levels of FB_1 are well in excess of those shown in Table 2.3. In Argentina, fumonisins were detected during ear development and their occurrence was closely correlated with the natural infection with *F. moniliforme* and *F. proliferatum*. In Costa Rica, significant regional differences were observed in contamination of maize with FB_1 (see Placinta *et al.*, 1999).

2.3.4 Co-contamination: a conspectus

It is clear that co-production of *Fusarium* mycotoxins, as exemplified in studies with pure cultures (Table 2.1), is reflected in the practical issue of co-contamination of cereal grains with the same compounds (Tables 2.2 and 2.3). An analysis of the data in these tables led Placinta *et al.* (1999) to conclude that, although sample size has been small in a number of surveys, there is unequivocal evidence of continuing global co-contamination of grains with several trichothecenes, ZEN and fumonisins. Of particular concern is the co-occurrence of several *Fusarium* mycotoxins with AFB_1. Introduction of legislation for the control of certain trichothecenes and fumonisins in cereals and animal feed is long overdue (Placinta *et al.*, 1999). However, any efforts to define tolerance limits and to formulate regulatory guidelines are likely to be undermined by multiple contamination of these commodities with different *Fusarium* mycotoxins and AFB_1.

2.4 Factors affecting the production of *Fusarium* mycotoxins

A wide range of factors might be expected to affect the production of *Fusarium* mycotoxins. Based on extensive work with *Aspergillus* species, three major categories of factors have been identified: physical, biotic and chemical and it is instructive to assesss the impact of these elements on mycotoxin production in *Fusarium* species. However, the literature relating to physical factors is somewhat sparse. Moreover, it is likely that any effects will be the result of complex multifactorial interactions. In assessing these effects, evidence from both laboratory and field studies will be reviewed and further areas of research will be identified. It is instructive to first recognise underlying features which may impinge on the various factors to be considered.

2.4.1 Underlying features

It is axiomatic that mycotoxin production is a direct function of fungal growth as determined under *in vitro* conditions, or of fungal infection as might occur under field conditions. With laboratory cultures it is possible to demonstrate parallel time-course patterns for both fungal growth and mycotoxin production. This principle is illustrated by the data of D'Mello *et al.* (1998) in their investigations on 3-ADON formation in *F. culmorum*. Data for the control strain have been extracted from D'Mello *et al.* (1998) and plotted in Figure 2.1. Both radial growth and 3-ADON production followed corresponding trends throughout the 57 days period of investigation. Thus any factors affecting fungal growth would also be expected to influence mycotoxin biosynthesis. While these relationships hold for a variety of conditions, there are factors which influence mycotoxin production more than mycelial growth. Thus it is possible to distinguish between direct and growth-determined inhibitions of mycotoxin production and to identify factors which elicit these contrasting effects. In addition it is possible to identify factors which enhance

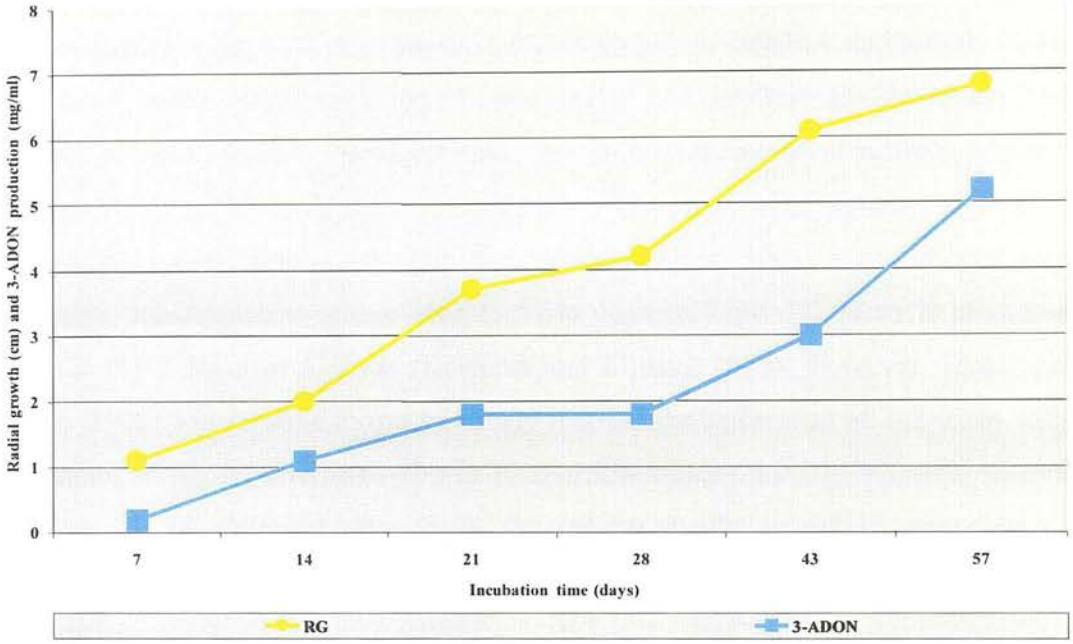

Figure 2.1 Radial growth (RG, cm) and 3-ADON production (mg/ml culture extracts) in *F. culmorum* in relation to incubation time (days). Plotted from data of D'Mello *et al.* (1998).

production followed corresponding trends throughout the 57 days period of investigation. Thus any factors affecting fungal growth would also be expected to influence mycotoxin biosynthesis. While these relationships hold for a variety of conditions, there are factors which influence mycotoxin production more than mycelial growth. Thus it is possible to distinguish between direct and growth-determined inhibitions of mycotoxin production and to identify factors which elicit these contrasting effects. In addition it is possible to identify factors which enhance mycotoxin production without necessarily affecting growth. Another aspect often observed, although not represented in Figure 2.1, is the decline in mycotoxin levels after fungal growth has maximized. Moss and Frank (1985) reported such an effect for T-2 toxin production by *F. sporotrichioides*. Time-induced reductions of FB_2 production have also been shown for one isolate of *F. proliferatum* but this effect was associated with an increase in FB_1 biosynthesis over the same period (Marin *et*

al., 1995). In the absence of comments or direct studies, it is relevant to speculate on the mechanisms involved. It is possible that depletion of substrate induces mycelial death and results in the secretion of lytic enzymes which initiate and sustain the mycotoxin decay process. There may also be a shift in the partition between primary and secondary metabolism as survival processes take precedence under conditions of declining nutrient supply. Alternatively, or in addition, time-course effects may represent transformation of one mycotoxin to another. Thus T-2 toxin is produced prior to HT-2 toxin in *F. poae* (Kotsonis and Ellison, 1975). However, Moss and Frank (1985) were unable to quantitatively link the disappearance of T-2 toxin with formation of HT-2 toxin and NEO in *F. sporotrichioides*. Finally, physical factors such as pH or chemical changes in the growth media caused by secretion of extracellular enzymes by the fungus may induce degradation or transformation of mycotoxins.

2.4.2 Physical factors

For the purposes of this review physical factors include temperature, humidity/ water activity, time, pH, exposure to light and gamma irradiation, use of modified/controlled atmospheres and agitation levels in liquid cultures. It is not clear how pH affects *Fusarium* mycotoxin production, although Greenhalgh *et al.* (1983) stated that the optimum range for DON formation by *F. graminearum* is 5.2-6.5. In particular weather conditions, ambient temperature, humidity and duration of exposure to these elements will exert predominant effects on *Fusarium* diseases of plants and on mycotoxin production in harvested commodities. Field observations have led to a number of conclusions concerning the development of fusarium head blight of cereal crops. Thus Parry *et al.* (1995) indicated that warm dry soil conditions during the initial phase of the growing season facilitated the development of *Fusarium* foot rot and the supply of inoculum, while high rainfall at anthesis aided the dispersal of the inoculum to ears. Rain impact is thought to play an important role in dispersal of *Fusarium* inoculum. It was concluded that protracted periods of warm

humid weather conditions promoted the development of fusarium head blight which in turn would result in mycotoxin contamination of grain (Parry *et al.*, 1995). These authors further suggested that when the conditions cited above prevail in a particular season, it might be possible to predict the incidence of fusarium head blight and consequent mycotoxin contamination of grain. It is salutary to note that the higher than average levels of DON in the 1991 wheat harvests from Missouri, North Dakota and Tennessee were tentatively attributed to increased rainfall (Fernandez *et al.*, 1994).

Despite the foregoing, the primary evidence of the effects of physical factors emanates from laboratory studies. Such investigations permit substantially more control over individual factors and also allow an assessment of the impact of different types of interactions. Nevertheless, there is still a measure of controversy associated with the findings.

2.4.2.1 Trichothecenes

Data on the effects of physical factors on trichothecene synthesis are somewhat sparse and there is no recent work (post-1985) on the effects of temperature or humidity/water activity. The limited evidence suggests that trichothecene synthesis on grain substrates is possible under a variety of physical conditions. Greenhalgh *et al.* (1983) observed that DON production on rice inoculated with *F. graminearum* occurred at initial moisture contents ranging from 30 to 48% at temperatures of 19.5, 25 and 28°C but the levels varied with duration of exposure to these two factors. Maximum production occurred at after 24 days of incubation at 28°C in rice with an initial moisture content of 40%. O'Neill *et al.* (1993) showed that DON production from *F. culmorum* and *F. graminearum* is possible at 25°C in a process dependent upon time and substrate type. In both species there were instances with certain substrates when peak concentrations of DON were followed by a rapid fall in production of the mycotoxin. A similar time-course pattern was seen with acetyl-DON, although the identity of the isomer was not disclosed. Similar temperatures

(23-25°C) are required for NIV formation from *F. poae* cultured on rice, but exposure to near UV light can enhance mycotoxin production (Pettersson, 1991). Furthermore, at low doses γ-irradiation can almost double T-2 toxin production in *F. tricinctum* cultured on rice (Halasz *et al.*, 1989). However, as might be expected, moisture content of grain exerts marked effects on T-2 toxin and HT-2 toxin synthesis by *F. sporotrichioides* (Richardson *et al.*, 1985). In a rice substrate progressive increases in synthesis of both mycotoxins were observed as moisture levels were raised from 33 to 60%. At the latter moisture level, HT-2 toxin production was also enhanced in soybean meal. Predictably, the results of Richardson *et al.* (1985) also demonstrated that particle size may be of importance in that soybean meal elicited higher yields of T-2 toxin, HT-2 toxin and T-2 tetraol than cracked or whole soybeans. In liquid cultures, agitation rate may be critical in maximising mycotoxin production. Moss and Frank (1985) showed that T-2 toxin formation in cultures of *F. sporotrichioides* was linearly enhanced by increasing agitation rates from 140 to 180 revolutions/min, but production was stimulated further and in a linear manner by inclusion of a fungicide in the culture mixture. The effect of agitation rate was markedly greater in the presence of fungicide than in its absence. These observations on the importance of aeration are consistent with role of oxygenation reactions in the biosynthesis of T-2 toxin (McCormick *et al.*, 1996), and with inhibition of production of the mycotoxin on exposure of *F. sporotrichioides* to atmospheres enriched with CO_2 (Paster *et al.*,1986). In an atmosphere of 50% CO_2 and 20% O_2, growth of *F. tricinctum* was reduced to 81% of that achieved in controls exposed to air, but T-2 toxin production was reduced to 19% of control values.

2.4.2.2 Zearalenone

There is some disagreement about the effects of temperature on ZEN production with Merino *et al.* (1993) maintaining that a thermic shock in the middle of the incubation period was required for mycotoxin formation in *F. graminearum* cultured on maize kernels. Synthesis of ZEN was expressed following incubation at 28°C for 10 days

followed by 20 days at 11°C, whereas a constant 28°C yielded no mycotoxin. They suggested that production of abundant mycelium at 28°C followed by the reduction in temperature were important elements in ZEN synthesis, citing other papers based on the production of penicillin by *Penicillim chrysogenum* to support their arguments. The observations of Merino *et al.* (1993) were consistent with those of Chelkowski *et al.* (1984) who showed that a higher temperature of 20°C during the first week of incubation also favoured ZEN production in isolates of *F. culmorum*. In direct contrast, Milano and Lopez (1991), in studies with *F. graminearum* and *F. oxysporum*, observed that incubation at 25°C for 4 weeks enhanced ZEN formation whereas reducing the temperature to 11°C during the last 2 weeks of incubation totally or partially inhibited its production. These results were consistent with those of Cuero and Smith (1987) who concluded that low temperatures were not essential for ZEN synthesis in *F. graminearum*. They were able to maintain relatively high production of ZEN at a constant 11°C or 25°C by manipulating water activity and by the use of an appropriate substrate (maize grain instead of rice). The temperature-water activity interaction is shown in Figure 2.2. It is clear that if water activity is set at 0.90, ZEN production is maintained at 25°C but eliminated at 11°C.

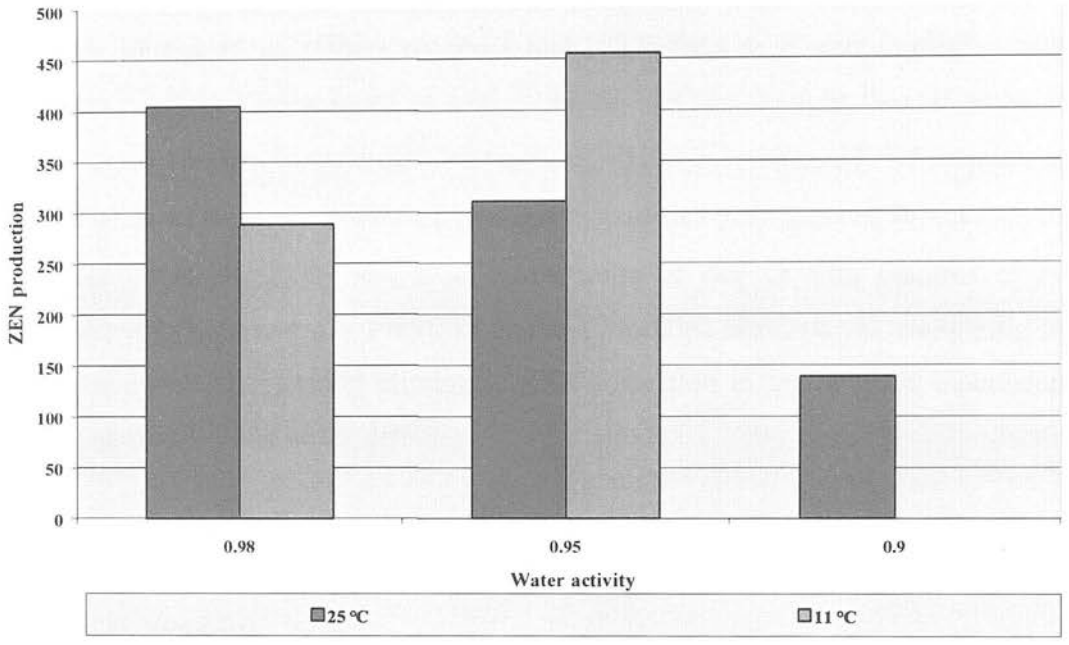

Figure 2.2 The effects of temperature and water activity on ZEN production (ng/g) in *F. graminearum* cultured on cracked maize grain. Plotted from data in Cuero *et al.* (1987).

O'Neill *et al.* (1993) also demonstrated ZEN production at constant 25°C from *F. graminearum* and *F. culmorum* cultured on a variety of grain substrates but levels were dependent upon incubation time, substrate and *Fusarium* species. Maximum synthesis of ZEN occurred at 28 days on maize inoculated with *F. graminearum* and at 21 days with *F.culmorum* cultured on rice. Incubation time was important in another respect. At 42 days ZEN production markedly declined in the *F. graminearum* maize culture but was maintained in the *F. culmorum* rice culture. Time-related reductions were also noted with DON production in maize and in rice inoculated with *F. culmorum*, but the mechanisms for such an effect were not discussed (O'Neill *et al.*, 1993). The substrate differences observed by O'Neill *et al.* (1993), were attributed to a physical effect in that ZEN (and DON) formation was lower in grains with husks (eg barley) than in those without husks (eg wheat or

maize). Another physical dimension in ZEN production is the effect of exposure to light. Di Menna *et al.* (1991) observed that production in *F. crookwellense* was greater at ambient temperatures varying from 16-29°C in daylight than at ambient temperatures varying from 18-23°C in darkness. Like T-2 toxin, ZEN formation is also sensitive to exposure to γ-irradiation and to modified atmospheres. Production of ZEN can be increased by low level γ-irradiation of rice or corn cultures of *F. graminearum* (Halasz *et al.*, 1989). In contrast modified atmospheres containing in excess of 20% CO_2 virtually eliminated ZEN formation in maize grain inoculated with *F. equiseti* (Paster *et al.*, 1991).

2.4.2.3 Fumonisins

The effects of temperature and water activity over different periods of time and their interactions on fumonisin production have been investigated in two experiments. Cahagnier *et al.* (1995) showed that the production of FB_1 by *F. moniliforme* cultured on maize grain was determined primarily by water activity. Fumonisin B_1 production declined threefold after water activity was reduced from 1 to 0.95, whereas fungal biomass remained unaffected. When water activity was reduced from 1 to 0.90, there was a 20-fold fall in fungal biomass but FB_1 synthesis was reduced 300-fold. Incubation time was also important. After 8 days of culture, 500 μg FB_1/g were produced at a water activity of 1 whereas 30 days were required to yield the same quantity of mycotoxin at a water activity of 0.95. These results are consistent with the role of *F. moniliforme* as a plant pathogen with the capacity to synthesise fumonisins in the standing crop where moisture content will be maximal. Temperature conditions were not defined by Cahagnier *et al.* (1995), but in the study of Marin *et al.* (1995) temperature, time and *Fusarium* isolates were investigated in a factorial approach using maize grain as substrate. Relatively high production of FB_1 occurred following inoculation with *F. moniliforme* on grain maintained at a water activity of 0.956 or 0.968 (Marin *et al.*, 1995). Under these conditions, values were maximised 6 weeks of incubation at 30°C, with markedly lower levels of FB_1 in

cultures maintained at 25°C or in those sampled at 3 weeks. In contrast, two isolates of *F. proliferatum* yielded substantially lower levels of FB_1 at both temperatures and times. Overall, irrespective of species, isolate type or temperature, FB_1 production was markedly reduced at water activities of 0.944 and 0.925. FB_2 production was also determined and found at much lower concentrations than FB_1, but the ratio of FB_1:FB_2 was higher in one isolate of *F. proliferatum* than in the other isolate or that found in *F. moniliforme*. Although a water activity of 1 was not tested there is some measure of consistency between these results and those of Cahagnier *et al.* (1995) in that the two fumonisin producers are essentially pathogens of standing crops requiring humid and warm conditions for infection and mycotoxin formation. There is some lack of agreement with regard to the effects of temperature on fumonisin synthesis. Marin *et al.* (1995) observed greater production of FB_1 at 30°C for water activities of 0.968 and 0.956 at 6 weeks of incubation. However, Le Bars *et al.* (1994) showed that maximal production in a maize culture of *F. moniliforme* occurred at 20°C following 5 weeks of incubation. Their results are of additional interest since they provide a rare example of the effects of a wide range of temperatures on mycotoxin production from a *Fusarium* species. It is unfortunate that the data were not presented to maximum effect to indicate optimum temperature and, in addition, moisture content of the incubation mixture was not given. However, using values estimated from a figure in Le Bars *et al.* (1994), it is possible to ascertain the optimum temperature for maximum yield of FB_1. Values estimated at 5 weeks of incubation are plotted in Figure 2.3.

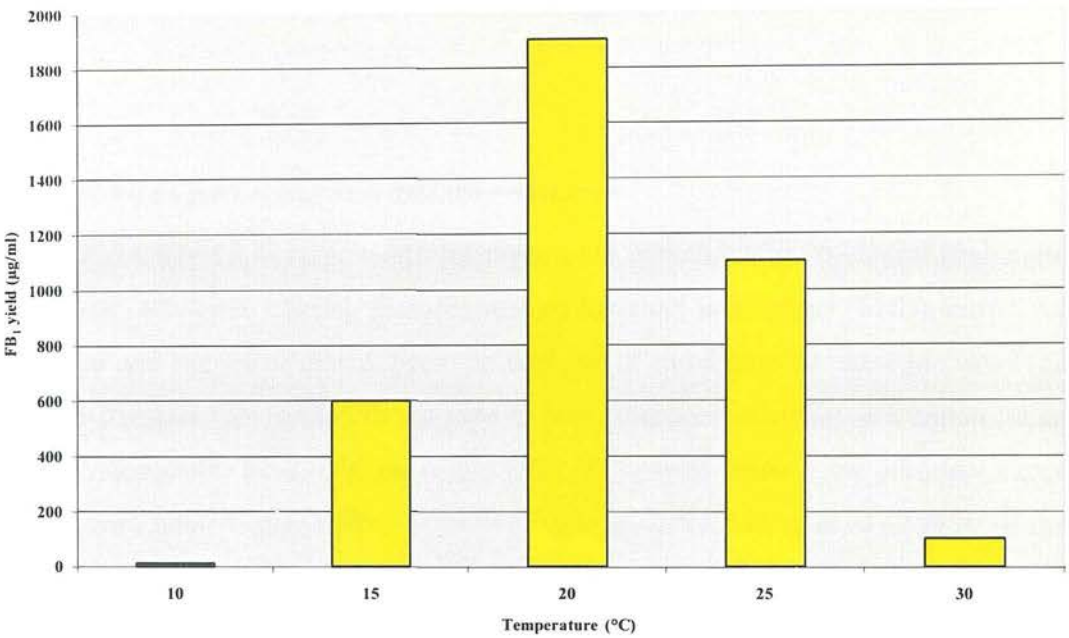

Figure 2.3 The effects of incubation temperatures on fumonisin B_1 production in a maize culture of *F. moniliforme*. Values at 5 weeks of incubation were estimated from a figure in Le Bars *et al.* (1994).

Despite the discrepancies just outlined, the physical conditions, as optimised in laboratory cultures, are reasonably consistent with the ecological niche of *F. moniliforme* and the natural occurrence of fumonisins in the tropics and other high-temperature regions (Table 2.3).

2.4.3 Biotic factors

Biotic factors considered in this chapter include the effects of toxigenic *Fusarium* fungi as plant pathogens; the effects of disease resistance in economic plants; and the

effects of fungal interactions. In evaluating these factors the implications for mycotoxin contamination of harvested commodities is given particular emphasis.

2.4.3.1 Plant pathogens and disease resistance

Many *Fusarium* fungi responsible for mycotoxin contamination of cereal grains are also plant pathogens, causing diseases such as fusarium head blight (FHB), crown rot of wheat and ear rot of maize. From an analysis of the dominant pathogen involved in FHB it is possible to discern the type of trichothecene occurring as a contaminant in harvested grain. Thus, in Canada and USA, *F. graminearum* is the principal cause of fusarium head blight (FHB; Milus and Parsons, 1994; Wong *et al.*, 1995). In the Netherlands, on the other hand, *F. culmorum* is the pathogenic fungus causing FHB (Snijders and Perkowski, 1990). In both cases DON is the major trichothecene found in grain (Table 2.2). Cereal genotype has an effect on the incidence of FHB and this in turn affects DON contamination of the heads (Figure 2.4). For example, wheat genotypes resistant to head blight caused by *F. culmorum* were found to produce heads with low levels of DON (1.2-2.3 mg/kg grain). On the other hand, wheat genotypes susceptible to the disease, produced grain with higher levels of DON (12.1-14.7 mg/kg).

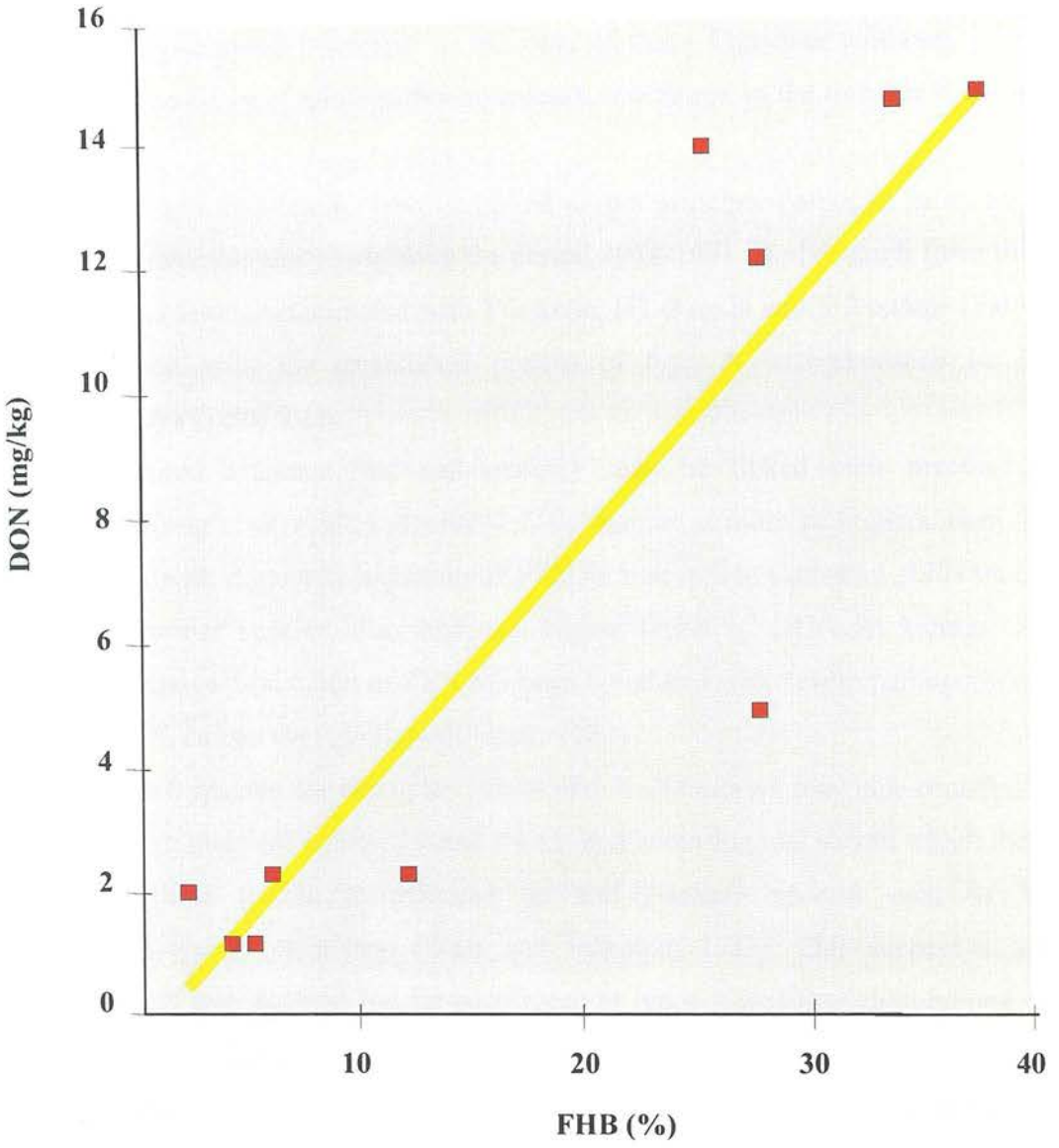

Figure 2.4 Incidence (%) of fusarium head blight (FHB) and deoxynivalenol (DON) contamination (mg/kg) of wheat grains (plotted from data of Snijders and Perkowski, 1990).

The regression equation is:

y=-0.40+0.40x (R=0.80)

Similar results have been recently shown by Wong *et al.* (1995). Chinese cultivars of wheat are more resistant to FHB than the Canadian cultivars, and levels of DON higher in the susceptible varieties. In the case of these Canadian cultivars, HT-2 production was observed whereas this mycotoxin was absent in the resistant Chinese cultivars.

In Poland, *F. sporotrichioides* was identified as the principal pathogen in an area where FHB was observed yearly over the period 1988-1991. Barley grain from this area was found to be contaminated with T-2 toxin, HT-2 toxin and T-2 tetraol (Table 2.2), consistent with the established pattern of type A trichothecenes in *F. sporotrichioides* (Table 2.1).

There is limited evidence that pathogenicity may be linked with mycotoxin production. Wong *et al.* (1995) identified *F. culmorum* as more pathogenic than *F. graminearum* with respect to induction of FHB in susceptible Canadian cultivars of wheat The former species also produced higher levels of DON in kernels. In addition, excessive production of ZEN has been correlated with severe pathogenicity in isolates of *F. culmorum* (Chelkowski *et al.*, 1984).

Other *Fusarium* species, for example *F. poae* and *F. avenaceum* may also contribute to FHB through incipient colonisation of the ear and enclosing leaf sheath which then predisposes these tissues to infection by end-of-season species such as *F. graminearum* and *F. culmorum* (Sturz and Johnston, 1983). This succession of *Fusarium* fungi may account for the occurrence of types A and B trichothecenes in harvested grain (Table 2.2).

Toxigenic species of *Fusarium* have also been implicated in other cereal diseases. Thus crown rot of wheat is attributed to *F. graminearum* (Wildermuth and McNamara, 1994) while ear rot of maize is associated with both *F. graminearum* and *F. moniliforme* (Schaafsma *et al.*, 1993). The implications for mycotoxin contamination have yet to be elucidated. However, it is established that aspergillus ear rot of maize is linked with aflatoxin contamination of kernels and the potential clearly exists for mycotoxin production from infection of maize crops with *F. graminearum* and *F. moniliforme*.

The incidence of plant disease by toxigenic species of *Fusarium* is not always associated with mycotoxin contamination of products. For example, although *F. oxysporum* can cause wilt diseases of tomato (Gapillout *et al.*, 1996), there is no evidence of contamination of fruit with fusaric acid or with any of the other mycotoxins characteristic of this phytopathogen.

2.4.3.2 Fungal interactions

Analysis of grain reveals a complex mycoflora, implying the potential for fungal interactions both in the standing crop and during storage of grain (D'Mello *et al.*, 1993). Studies of the interactions between fungi may lead to improved understanding of the ecology of these organisms and to biological methods of mycotoxin control. Paster *et al.* (1992) showed that *Aspergillus niger* is capable of producing metabolites inhibiting growth of *A. flavus* and aflatoxin formation. Cotty (1994) demonstrated that field application of an atoxigenic strain of *A. flavus* to soils planted with cotton resulted in progressive displacement of toxigenic strains of the fungus and reduced aflatoxin contamination of cottonseed. On the other hand, Fabbri *et al.* (1984) concluded that higher aflatoxin production may be expected when *Fusarium* species co-exist with aflatoxigenic strains of *Aspergillus*. This conclusion was based on an experiment showing substantial increases in aflatoxin production following growth of *A. parasiticus* in a medium containing T-2 toxin. In general, however, the effects of mutualistic or antagonistic interactions on mycotoxin production in *Fusarium* species have not been assessed in any systematic manner. The study of Cuero *et al.* (1988) represents an exception in that ZEN production was determined in maize kernels following co-inoculation with *F. graminearum* and *A. flavus*. Under these conditions, formation of ZEN was reduced to negligible levels at incubation temperatures of 16°C in the presence of *A. flavus* but remained largely unaffected at 25°C. In pure culture, ZEN production was not influenced by incubation temperature. Much still needs to be elucidated on the question of fungal interactions, not least on their effects in trichothecene biosynthesis.

2.4.4 Chemical factors

A variety of chemical factors are known to affect the growth of fungi and their capacity to produce mycotoxins, both in the field and during storage of harvested grain. In the case of *Fusarium* species and their mycotoxins, there are notable effects of fungicides, insecticides, other inhibitors and substrate type and composition. Although herbicides are used extensively in crop production, effects of any such residues on mycotoxin synthesis by *Fusarium* pathogens remain totally unexplored.

2.4.4.1 Fungicides

These chemicals are widely used in the prevention of disease in crop plants, acting by inhibiting RNA synthesis (Phenyl amides), sterol biosynthesis (triazoles and morpholines), tubulin synthesis (benzimidazoles) and DNA synthesis (dicarboximides). However, the efficacy of fungicides in controlling mycotoxin production has come under scrutiny for two important reasons. Firstly, despite regular applications of fungicides worldwide, *Fusarium* mycotoxin contamination of cereal grains continues to be a problem, even in developed countries (Tables 2.2 and 2.3). Secondly, fungicide resistance is easily generated particularly in instances where the mode of action of the fungicide is under simple gene control. Although the development of fungicide resistance in *Fusarium* phytopathogens has long been recognised (Sozzi and Gessler, 1980), the implications for mycotoxin control are only now emerging, forming the basis of a separate series of studies (D'Mello *et al.*, 1997 and 1998). However, it is clear that attempts to explore the impact of fungicide resistance has been hampered by a lack of understanding of the effects of fungicides in normal, non-resistant strains of toxigenic *Fusarium* phytopathogens and by inconsistent evidence in the literature.

On purely theoretical grounds it is logical to expect that fungicides should control both diseases such as FHB and the accompanying mycotoxin contamination. This expectation is not supported by the evidence derived from field and laboratory studies (Tables 2.4 and 2.5). The data reviewed here was adapted by D'Mello *et al.*

Table 2.4 Fungicide efficacy: field evidence

Fungicides	Methods/Conditions	Efficacy	Reference
Propiconazole	Foliar application (250g ai[a]/ha) at two growth stages of wheat	FHB[b] reduced from 94 to 55%; DON[c] levels in wheat kernels not affected	Martin and Johnston (1982)
Propiconazole	Oats grown in field with history of DON contamination; no details of application	NIV[d] content of grain reduced from 3.4 to 1.2 mg/kg; DON not found	Pettersson (1991)
Propiconazole, thiabendazole, triadimefon	Ears of wheat crop sprayed with *F. graminearum* inoculum 2d after anthesis; fungicides applied in separate treatments prior to, during and after inoculation; rates (ai/ha): propiconazole 120g; thiabendazole 360g; triadimefon at 60g	Propiconazole reduced kernel infection by 39-55% and DON levels by 34-78%. Thiabendazole had no effect on infection level, but DON contamination reduced by up to 83%. Triadimefon reduced infection by up to 61% and DON levels by 65-79%	Boyacioglu *et al.* (1992)
Propiconazole, thiabendazole, tebuconazole, flusilazole	Wheat inoculated with *F. graminearum* at 3 stages from beginning of flowering; fungicides rates (ai/ha): 140g, 280g, 140g and 140g, respectively, as individual treatments	FHB incidence 83-84% (control, 87%); DON levels 12.9-19.0 mg/kg grain (control, 12.0 mg/kg)	Milus and Parsons (1994)
Tebuconazole	Natural *Fusarium* infection of wheat; rate, 250 g ai/l	DON levels in grain reduced by 73%	Suty *et al.* (1996)
Tebuconazole with triadimenol (Matador)	Ears of wheat crop inoculated with *F. culmorum*; rate: 1l/ha	FHB reduced ; 16-fold increase in NIV content of grain from fungicide treatment	Gareis and Ceynowa (1994)
Thiophanate-methyl (carbendazim presursor)	Efficacy investigated over 3 seasons (1982, 1983, 1987) on incidence of FHB in barley and wheat	Overall reduction of disease and grain mycotoxin contamination; seasonal/cultivar variations; DON and NIV in one cultivar of barley and wheat not affected in 1983	Ueda and Yoshizawa (1988)
Maneb (protectant dithiocarbamate)	Maize crop inoculated with *F. graminearum* 20d after silk development	ZEN[e] contamination of grain eliminated	Draughon and Churchville (1985)

[a] active ingredient
[b] fusarium head blight
[c] deoxynivalenol
[d] nivalenol
[e] zearalenone

Table 2.5 Fungicide efficacy: *in vitro* studies with pure cultures of *Fusarium* species

Fungicides	Methods/Conditions	Efficacy	Reference
Thiabendazole, tebuconazole, prochloraz, tridemorph, fenpropimorph	*F. graminearum* cultured in nutrient broth at 21° C and 16h/8h light/dark cycle; static culture assumed; fungicides dissolved in acetone or methanol	Morpholines had no effect on 3-ADON[a] production; at 1 µg/ml thiabendazole and tebuconazole each inhibited 3-ADON formation; at 0.1 µg/ml tebuconazole caused 4-fold increase in 3-ADON synthesis; at 0.005 µg/ml prochloraz reduced 3-ADON production	Matthies and Buchenauer (1996)
Thiabendazole	*F. tricinctum* cultured on maize at 10° C; fungicide added as an aqueous suspension at 100 µg/ml	Growth and T-2 toxin production totally suppressed	Gabal (1987)
Tridemorph	*F. sporotrichioides* cultured in defined medium under shake-flask conditions at 25°C; fungicide added at 6 and 36 µg/ml; aqueous addition of fungicide	Growth enhanced at 6 µg/ml but T-2 toxin and DAS[b] production inhibited; at 36 µg/ml, growth reduced but T-2 toxin formation stimulated	Moss and Frank (1985)
Difenoconazole	*F. culmorum* cultured on PDA[c] at 25° C in dark; fungicide added as Plover dissolved in ethanol	At 0.1 µg/ml, difenoconazole markedly increased 3-ADON production without affecting radial growth; at 1 µg/ml growth reduced by 9% but 3-ADON synthesis totally suppressed	D'Mello *et al.* (1997b)
Dicloran, iprodione, vinclozolin	Fungicides added separately at levels of up to 500 µg/ml potato-dextrose broth; static culture of *F. graminearum* at 28° C; fungicides dissolved in ethanol	Dose-related reductions of growth and production of DAS and ZEN[d]; vinclozolin less effective for ZEN suppression.	Hasan (1993)

[a] 3-acetyl deoxynivalenol
[b] diacetoxyscirpenol
[c] potato dextrose agar
[d] zearalenone

(1997a; 1998) to address key issues on the comparative efficacy of fungicides. In a few investigations, fungicide concentrations tested under laboratory conditions exceeded the maximum solubility levels achievable in aqueous media. The interpretation of these results is, therefore, not straightforward.

Propiconazole is widely recommended for control of a variety of cereal diseases such as powdery mildew, septoria, rusts and eyespot and its efficacy for FHB and mycotoxin control has been the subject of a series of field trials (Table 2.4). The consensus is that propiconazole is largely ineffective as a means of controlling FHB, kernel infection or mycotoxin contamination. Results are, however, highly variable, with Pettersson (1991) and Boyacioglu *et al.* (1992) respectively showing considerable reductions, but not elimination, in grain levels of NIV and DON. At the other end of the spectrum, data of Martin and Johnston (1982) and Milus and Parsons (1994) demonstrated, at best, no effect of propiconazole and at worst consistent increases in grain concentrations of DON. It should be noted that Milus and Parsons (1994) tested a wider range of fungicides than that indicated in Table 2.4, some at two dose levels. These included triadimefon with mancozeb, benomyl and fenbuconazole. With all fungicides and doses tested, DON levels in grain were arithmetically higher than in the non-sprayed check. The increases, although not significant ($P>0.05$), form a consistent pattern which will become relevant later in this section.

The discrepancies in the observations cited above are difficult to resolve in field studies since the incidence of FHB and contamination with DON/NIV would be affected by a wide range of factors including environmental conditions, inoculum and fungicide levels, timing of and application techniques used in fungicide treatments. In respect of timing of fungicide applications the results of Boyacioglu *et al.* (1992) indicated that propiconazole was least effective in reducing DON contamination of grain when delivered at the pre-inoculation stage. Efficacy maximised when propiconazole was applied 2 days after inoculation; at this stage DON levels were reduced by 78% (Table 2.4). In contrast, thiabendazole reduced DON production by 83% when applied prior to inoculation and by 16% when sprayed 2 days after inoculation. Timing of application and dose levels may account for the discrepancies in the effects of thiabendazole on DON contamination of wheat kernels (Table 2.4).

Tebuconazole is specifically recommended for use against FHB and other diseases of cereals (UK Pesticide Guide, 1999) but its efficacy with regard to DON control is

highly variable (Table 2.4). In natural *Fusarium* infection of wheat Suty *et al.* (1996) observed a 73% reduction in grain levels of DON following application of tebuconazole whereas in an experimental inoculation of wheat with *F. graminearum*, no such effect was observed (Milus and Parsons, 1994). More importantly, tebuconazole in combination with triadimenol induced a 16-fold increase in NIV contamination of wheat kernels (Gareis and Ceynowa, 1994; Table 2.4).

The carbendazim precursor fungicide, thiophanate-methyl, is not specifically recommended for cereals but its efficacy for FHB and mycotoxin control in barley and wheat cultivars has been examined over three seasons (Ueda and Yoshizawa, 1988; Table 2.4). Overall, the results indicated three groups of efficacy of mycotoxin control depending upon season and cultivar. In 1982 and 1987 the fungicide was highly effective for all cultivars of barley and wheat with marked reductions in DON and NIV contamination of harvested grain. In a few instances levels were reduced to below detectable limits. However, in 1983 using a selection of the same cultivars thiophanate-methyl was only moderately effective for two cultivars of barley. Also in 1983, a third category of results showed that the fungicide was ineffective for control of NIV and DON in an additional cultivar of barley and in one cultivar of wheat. Indeed, DON contamination was higher in the third cultivar of barley following application of thiophanate-methyl. It should be noted that the above classification of efficacy has been derived in the absence of statistical analyses (Ueda and Yoshizawa, 1988). It should also be pointed out that reduced efficacy of thiophanate-methyl in that study was associated with considerably higher levels of *Fusarium* infection of grain in 1983 relative to levels in 1982 and 1987.

Maneb is recommended as a protectant before the establishment of diseases such as rusts and septoria in cereal crops (UK Pesticide Guide, 1999). In a field trial (Table 2.4), weekly applications of maneb completely prevented contamination of maize grain with ZEN. However, the effectiveness of maneb for control of other *Fusarium* mycotoxins (Tables 2.1 and 2.2) was not determined despite the use of *F. graminearum* as inoculum (Draughon and Churchville, 1985). Nevertheless, this is the only example of complete fungicide efficacy among the field trials listed in Table 2.4.

It might be anticipated that under controlled laboratory conditions, with pure cultures, more consistent effects of fungicides would be demonstrated. In one particular respect this expectation appears to be justified. Thus, three of the five studies listed in Table 2.5 show marked increases in mycotoxin production following imposition of fungicide treatments. Matthies and Buchenauer (1996) and D'Mello *et al.* (1997b) reported stimulation of 3-ADON production in *Fusarium* isolates treated with tebuconazole and difenoconazole ($P<0.05$) respectively at 0.1 μg/ml (Table 2.5). In the third study, T-2 toxin synthesis in *F. sporotrichioides* was enhanced ($P<0.01$) on addition of tridemorph to the culture medium (Moss and Frank, 1985). Another morpholine, fenpropimorph, which stimulated synthesis of two aflatoxins in *A. parasiticus* (Badii and Moss, 1988) also failed to control 3-ADON formation in *F. graminearum* (Table 2.5; Matthies and Buchenauer, 1996). Nevertheless, the strength of the laboratory evidence concerning mycotoxin enhancement is somewhat diminished by the absence of statistical analyses in the work of Matthies and Buchenauer (1996). However, when considered in the light of field evidence (Table 2.4; Milus and Parsons, 1994; Gareis and Ceynowa, 1994; Ueda and Yoshizawa, 1988), there appears to be a consistent effect of fungicide-induced stimulation in trichothecene biosynthesis. Further investigation of factors affecting this enhancement seems to be justified. A mechanism for the stimulatory effects of tridemorph has been postulated to centre on its inhibition of sterol biosynthesis and greater availability of the shared precursor, mevalonate, for T-2 toxin formation (Moss and Frank, 1985). This mechanism might also explain the relative inefficacy of propiconazole and tebuconazole in the control of trichothecene biosynthesis (Table 2.4).

Further indications of consistent laboratory evidence relate to the effects of thiabendazole (Table 2.5). Both 3-ADON and T-2 toxin were suppressed following exposure of two different *Fusarium* species to the fungicide but in the case of 3-ADON, inhibition was achieved with just 29% retardation of mycelial growth (Matthies and Buchenauer, 1996). The latter observation is consistent with the lack of effect of thiabendazole on infection level under field conditions (Table 2.4; Boyacioglu *et al.*, 1992). However, in terms of overall efficacy of thiabendazole for

mycotoxin control, the discrepancy between the field and laboratory evidence emerges as the overriding feature (Tables 2.4 and 2.5).

Laboratory studies with pure cultures allow the use of graded levels of fungicides thus enabling an assessment of optimum dose. The effect of sub-lethal doses of fungicides on enhancement of trichothecene biosynthesis has been discussed above. Depending upon fungicide type, such doses may also completely inhibit formation of *Fusarium* mycotoxins, while growth continues, albeit at substantially reduced rates. Thiabendazole, difenoconazole, dicloran, iprodione and vinclozolin are examples of fungicides with this type of activity (Table 2.5). The discrepancy relating to the efficacy of tridemorph may be explained in terms of dose rates assuming that Matthies and Buchenauer (1996) used maximum levels not exceeding 1 μg/ml media. It is clear from the results of Moss and Frank (1985) that much higher doses are needed to exert an inhibitory effect on trichothecene production (Table 2.5). Although dicloran, iprodione and vinclozolin are not intended for control of *Fusarium* diseases of plants, marked dose-related effects on mycelial growth were reported as well as differential effects on mycotoxin biosynthesis (Table 2.5; Hasan, 1993). At the highest dose used (500 μg/ml), growth of *F. graminearum* continued with each of the fungicides, but DAS production ceased at much lower concentrations (250 μg/ml) with iprodione and with vinclozolin whereas for dicloran, 500 μg/ml were required for complete inhibition. In contrast, at 250 μg/ml, dicloran was more effective in preventing ZEN formation while vinclozolin failed to completely inhibit ZEN production despite dose-related reductions in biosynthesis. The relationship between fungicide type and dose is depicted in Figures 2.5 and 2.6 using dicloran and vinclozolin, respectively, to highlight the differential aspects on DAS and ZEN biosynthesis. It is clear that with dicloran, DAS production is a function of growth whereas ZEN inhibition was affected directly and to a greater extent than growth. However, growth-dependent inhibition of both DAS and ZEN was observed following exposure of *F. graminearum* to vinclozolin. A point of caution is appropriate in this interpretation in that *F. graminearum* is not known to be a DAS producer (Table 2.1) and the exact identity of the trichothecene may therefore be in doubt.

It is relevant here to compare fungicide dose effects in field trials (Table 2.4). It will be recalled that Milus and Parsons (1994) reported only minor effects on FHB following application of propiconazole to wheat whereas Martin and Johnston (1982) had observed substantial amelioration of the disease, albeit wihout any benefits in terms of DON contamination of grain. This discrepancy has been attributed to two applications of the fungicide amounting to a total dose of 500 g ai/ha in the latter study, four times the permitted rate in the United States of America (Milus and Parsons, 1994).

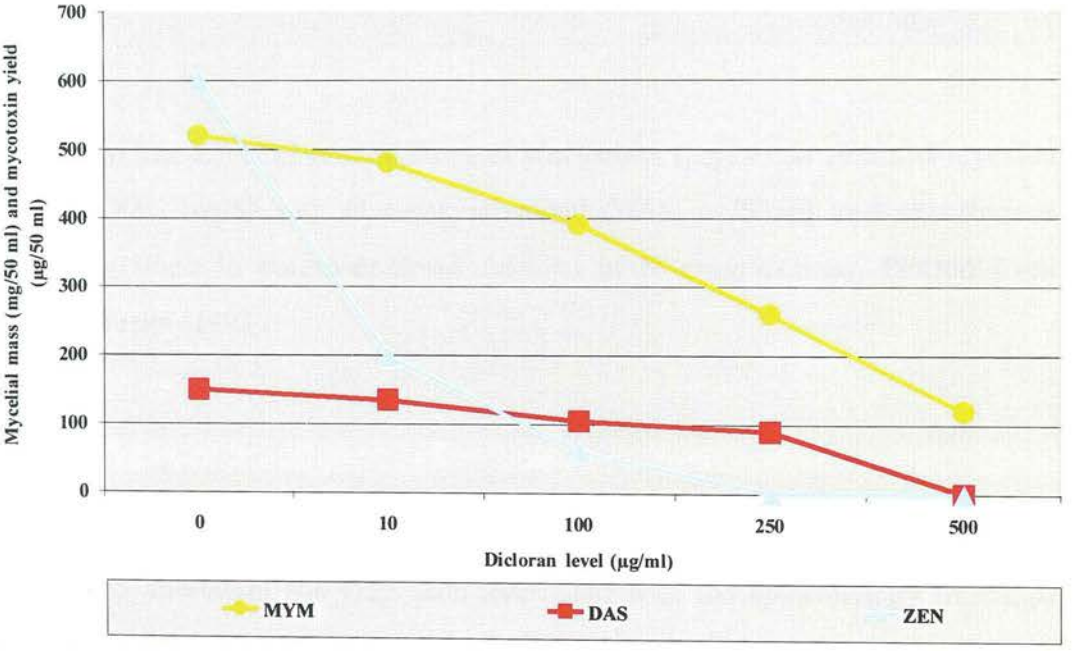

Figure 2.5 The effect of graded doses of dicloran (µg/ml) on yields of mycelial mass (MYM, mg/50 ml), diacetoxyscirpenol (DAS, µg/50ml) and zearalenone (ZEN, µg/50ml) in potato-dextrose cultures of *F. graminearum*. Plotted from data of Hasan (1993).

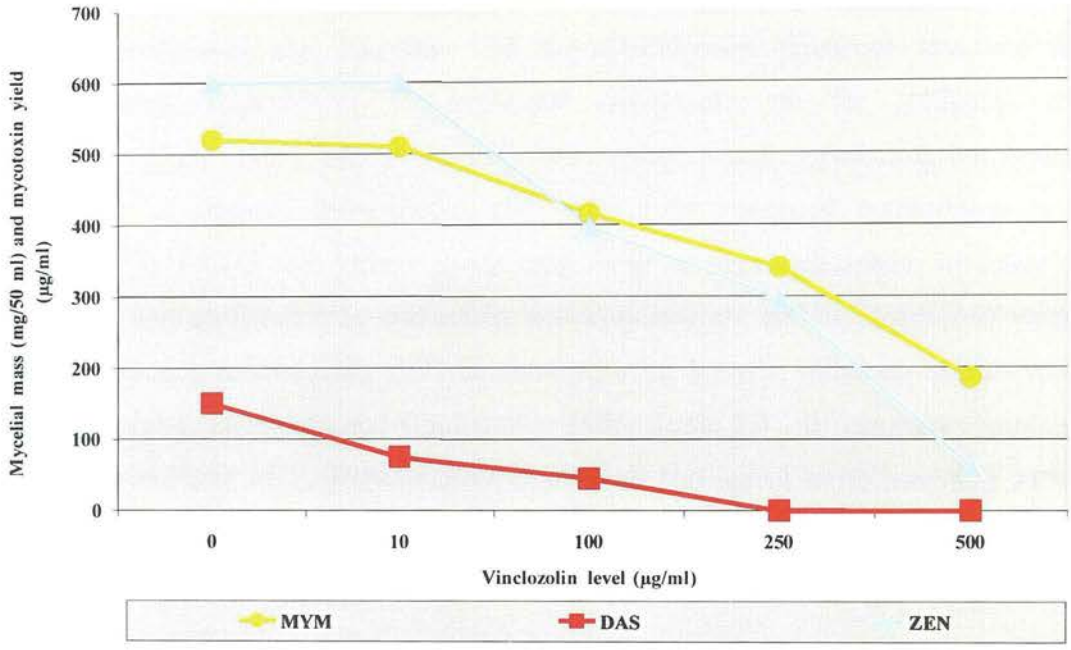

Figure 2.6 The effect of graded doses of vinclozolin (µg/ml) on yields of mycelial mass (MYM, mg/50 ml), diacetoxyscirpenol (DAS, µg/50ml) and zearalenone (ZEN, µg/50ml) in potato-dextrose cultures of *F. graminearum*. Plotted from data of Hasan (1993).

2.4.4.2 Insecticides

Insect pest infestation has long been associated with the epidemiology of fungal diseases of crop plants (Windels *et al.*, 1976). Insects may act as vectors or the physical damage they cause may facilitate entry of fungi into standing crops and harvested grain (Farrar and Davies, 1991). Adequate control of pests through effective insecticide applications should, therefore, result in reduced fungal infection of crops and mycotoxin contamination of harvested commodities. Dual-function insecticides would confer environmental benefits by reducing the overall need for and usage of pesticides. Additionally, there may be a biochemical basis for direct and potentially effective mycotoxin control by certain insecticides. For example,

organophosphate insecticides inhibiting acetylcholine esterase activity in insects and other animals may also interfere with the esterification processes involved in trichothecene biosynthesis. Experimental validation of the efficacy of organophosphate insecticides in trichothecene control is still outstanding but is the subject of a separate investigation (D'Mello, 1999, personal communication). However, in a field trial with a maize crop, both an organophosphate insecticide (fonofos) and an insecticide containing anticholinesterase activity (carbaryl) were highly effective for reducing ZEN contamination of kernels, although maneb was more effective (Draughon and Churchville, 1985; Table 2.4). In laboratory studies with pure cultures of *F. graminearum*, fonofos at 100 μg/ml broth inhibited ZEN production by 90% while mycelial mass was reduced by only 56%. At the same level of addition, carbaryl inhibited ZEN production by 94% and mycelial growth by 71%. In contrast, at 10 μg/ml, naled totally inhibited mycelial growth and, therefore, ZEN production (Draughon and Churchville, 1985), fully confirming earlier observations for ZEN control with this insecticide (Berisford and Ayres, 1976). In the latter study, however, mycelial inhibition was only 25% of control values at the 10 μg/ml addition of naled. Thus, as with fungicides, it is possible to demonstrate direct and growth-dependent effects of insecticides on ZEN production by *F. graminearum*.

2.4.4.3 Other inhibitors

A wide range of other chemicals have been tested or used to prevent fungal growth and mycotoxin production in stored products. Propionic acid and sorbic acid are two examples of such inhibitors. However, at sub-inhibitory levels sorbic acid has been shown to stimulate T-2 toxin synthesis in cultures of *F. acuminatum* (Gareis *et al*, 1984). Another compound of interest is sodium bicarbonate, which inhibits formation of aflatoxin by *Aspergillus parasiticus*. Recent studies suggest that biosynthesis of trichothecenes in cultures of *Fusarium tricinctum* is also inhibited by sodium bicarbonate. In particular, acetyl T-2 toxin and NEO synthesis were totally

suppressed, while there was a 7 to 51 fold decrease in the production of DAS and T-2 toxin (Roinestad *et al.*, 1993).

2.4.5 Substrates

It will be apparent from the review thus far that *Fusarium* species are essentially cereal pathogens and, in consequence, trichothecene, ZEN and fumonisin contamination is confined to cereal grains (Tables 2.2 and 2.3). The question arises as to why grain from leguminous plants, for example, is rarely associated with these mycotoxins. It is possible that endogenous protease inhibitors and secondary compounds in legumes confer resistance to *Fusarium* infection. It is also apparent from Table 2.3 that fumonisin contamination is confined to maize whereas trichothecene contamination occurs across virtually all major cereal types (Table 2.2). Furthermore, Greenhalgh *et al.* (1983) observed that all three isolates of *F. graminearum* tested produced DON and ZEN on maize kernels, whereas on rice only two of the isolates produced significant amounts of these mycotoxins. Quantitative differences are also seen. Thus O'Neill *et al.* (1993) showed that *F. culmorum* produced more DON on rice and maize substrates than on barley and wheat grains. Furthermore, measurable quantities were produced within 14 days of incubation on rice and maize, but much later on wheat (28 days) and barley (42 days) grains. By day 42 of incubation, ZEN production by *F. culmorum* was also higher on maize and on rice substrate than on barley and wheat grains. Similar effects were observed with *F. graminearum* cultured on these four grain substrates. The differences observed due to grain type have been attributed to the physical effects of husks present on barley and oats (O'Neill *et al.*, 1993), but other factors such as starch type and availability may exert some influence on mycotoxin yields. It is worth noting that Cuero and Smith (1987) recorded substantial production of ZEN in cracked maize inoculated with *F. graminearum* (Figure 2.2) whereas under the same conditions, rice was totally unsuitable for this purpose. The importance of substrate was further highlighted by Thrane (1986) who showed that ali 33 cultures of *F. culmorum* tested

produced ZEN when the substrate employed was yeast extract sucrose agar whereas only 5 out of 7 cultures produced ZEN when the substrate used was rice meal agar. It is thus clear that substrate type and composition are important factors determining production of *Fusarium* mycotoxins under field and laboratory conditions. However, the chemical basis of the differences outlined above requires elucidation.

2.5 Control strategies

Although worldwide regulations exist to control aflatoxins in animal feed, similar legislation is not available for any of the *Fusarium* mycotoxins, including the carcinogenic fumonisins (Placinta *et al.*, 1999). However, in the USA advisory directives exist for concentrations of DON in grains and by-products. A value of 10 mg/kg has been set for such feeds offered to cattle and chickens. For pigs the advisory level is set at 5 mg/kg (see Placinta *et al.*, 1999). In wheat products such as flour and bran, destined for human consumption, DON levels should not exceed 1 mg/kg.

Once contamination has occurred in grains, a number of options are available for limiting adverse effects in livestock. Thus, DON levels have been reported to be reduced by as much as 75% during milling and other forms of physical treatment. Density segregation enables separation of mouldy grain and this technique may also result in reduced levels of trichothecenes and ZEN in the residual batch. Chemical treatments involving calcium hydroxide monomethylamine, sodium bisulphite or ammonia have also been examined. However, although effective to different extents, these physical and chemical methods have yet to be applied in commercial situations (Placinta *et al.*, 1999). Dilution of contaminated grain with other feed components is another option, providing that monitoring is performed before grains are incorporated into compound feeds. All too often analyses are conducted after deleterious effects on livestock performance have been observed.

In view of the foregoing, it is axiomatic that preventive rather than remedial measures are likely to be more effective for minimising risk. The efficacy of

fungicides to control *Fusarium* mycotoxins is at best questionable (Table 2.4) and the search for alternative methods is now under way. Selection of cultivars of cereal plants resistant to *Fusarium* pathogens is currently viewed as a viable and sustainable option for reducing mycotoxin contamination of grain. In experimental studies, exploitation of genetic resistance to FHB in wheat has been successfully used to restrict kernel contamination with DON. Similarly, selection of Chinese genotypes of wheat which are naturally resistant to this disease has also resulted in lower levels of DON in grain, compared with values for susceptible Canadian cultivars (Wong *et al.*, 1995). Potential benefits of this approach have been quantified but are most visibly demonstrated in Figure 2.4, plotted from tabulated values in Snijders and Perkowski (1990). Wheat genotypes with increased susceptibility to FHB yielded grain with higher DON levels than resistant cultivars.

2.6 Toxicology

As with other bioactive compounds, *Fusarium* mycotoxins are attributed with both acute and chronic features of toxicity. It is generally accepted that among the trichothecenes, type A members are more toxic than those in the type B category. Thus Leeson et al. (1995) listed LD_{50} values, determined with young chickens, of 2 to 5.9 mg/kg body weight for DAS and 3.6 and 5.25 mg/kg body weight for T-2 toxin. In contrast, a single LD_{50} value of 140 mg/kg body weight was cited for DON. Similarly, acute tests indicate that ZEN is of relatively low toxicity, with an LD_{50} value of 2-10 g/kg body weight as determined with mice (Flannigan, 1991). However, with several *Fusarium* mycotoxins, particularly DON, ZEN and fumonisins, chronic exposure results in well-defined effects. The major features summarized in Table 2.6 should be considered in the context of the disorders and syndromes described by D'Mello *et al.* (1999). From their analysis it emerged that the syndrome involving feed refusal, emesis and anorexia in pigs, although originally associated with DON, should now be extended to other trichothecenes, particularly NIV and T-2 toxin (Table 2.6). The syndrome characterized by precipitation of oral

and other gastro-intestinal lesions in poultry has been elaborately linked with DAS, but other feed trichothecenes namely NIV and T-2 toxin are also implicated. Furthermore, T-2 toxin can cause dermatitis of the snout and associated structures in the pig (Table 2.6). Comprehensive evidence exists for the role of ZEN in a syndrome recognized by profound reproductive dysfunction in pigs and ruminant animals. The role of ZEN as a mammalian endocrine disruptor needs to be considered in relation to human exposure and reduced fertility in men. Fumonisins have been linked with three specific conditions. In South Africa, enhanced risk of oesophageal cancer in human populations has been statistically correlated with consumption of maize contaminated with *F. moniliforme* and fumonisins. In farm animals, porcine pulmonary oedema and equine leukoencephalomalacia are two syndromes definitively linked with dietary exposure to fumonisins (Table 2.6). Other conditions including ovine ill-thrift, acute mortality syndrome in poultry and in duodenitis/proximal jejunitis of horses were outlined by D'Mello *et al.* (1999) but further work is required to confirm the link with specific *Fusarium* mycotoxins.

As emphasised earlier in this review, co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a recurring issue. Combinations of *Fusarium* mycotoxins generally result in additive effects, but synergistic interactions have been reported between DON and fusaric acid; DON and FB_1; and DAS and the aflatoxins. Limited evidence of potentiation between FB_1 and DON or T-2 toxin requires confirmation (D'Mello *et al.* 1999). Additive and synergistic effects between these mycotoxins and unidentified metabolites may account for the greater toxicity of *Fusarium*-contaminated diets. Transmission of DON into eggs and ZEN into edible porcine organs has been demonstrated experimentally. However, lactational transfer of FB_1 appears not to occur, at least in cows and sows. D'Mello *et al.* (1999) concluded that animal health and productivity may be severely compromised by consumption of DON, T-2 toxin, DAS, ZEN and fumonisins and by interactions among these mycotoxins. Importantly, safety of some edible offal may also be at risk.

Table 2.6 Adverse effects of major *Fusarium* mycotoxins[a]

Mycotoxin	Syndromes/disorders	Species	Major/additional features
Deoxynivalenol	Emesis, feed refusal and depressed appetite; increased corrugation of gastric mucosa	Pigs	Low threshold range (2-5 mg/kg feed), with higher doses inducing marked reductions in feed intake; partial dose-dependent recovery possible
Nivalenol	Reduced feed intake	Pigs	Also increased time to consume feed
Nivalenol	Depressed feed intake and growth; gizzard erosions	Broiler chickens	Also reduced liver weights
T-2 toxin	Reduced feed intake; dermatitis of snout and nose	Pigs	Effects related to dose
T-2 toxin	Depressed weight gains; oral lesions	Broiler chickens, turkey poults, ducklings	Also reduced weights of thymus, spleen and bursa of Fabricii in ducklings
Diacetoxyscirpenol	Reduced feed intake, bodyweights and feed efficiency; oral lesions	Broiler chickens, turkey poults	Oral lesions in broiler chickens dependent upon dose
Zearalenone	Anoestrus; reduced conception rates; stillbirths; enlargement of uterus	Sows	Decreased production of luteinising hormone and progesterone; congenital lesions of the external genitalia in piglets suckling sows fed contaminated feed
Zearalenone	Depressed testes weight and spermatogenesis; feminisation and suppression of libido	Boars	Reduced serum levels of testosterone
Zearalenone	Infertility; depressed conception rates	Cows	Depressed milk production
Zearalenone	Increased number of barren ewes; fewer ewes with twins	Ewes	Pastures in New Zealand implicated in this form of infertility
Fumonisins	Oesophageal cancer	Humans	Based on epidemiological evidence from South Africa
Fumonisins	Pulmonary oedema	Pigs	Pulmonary hypertension; reduced and erratic feed intake and growth patterns; sphingolipid pertubations; dose-dependent effects; death
Fumonisins	Leukoencephalomalacia	Equines	Reduced appetite; oedema of lungs and brain; death

[a] Adapted from D'Mello *et al.* (1999)

2.7 Dynamics of hyphal growth

Apart from some green algae, the fungi are almost unique in the living world in utilizing a microscopic cylinder, the hypha, as their basic constructional unit. Hyphae have walls and may be partitioned along their length into cells by incomplete, or more rarely complete, transverse septa, or may lack transverse septa. The hyphae are capable of branching at a regular distance, characteristic of the species, behind the apex. Hyphae respond to a variety of environmental stimuli, by directed growth and by differentiation. It is suggested that hyphae respond to volatile chemicals, soluble chemicals, to the physical nature of surfaces, to temperature gradients, light and applied electrical fields. The cellular and molecular basis of hyphal growth are not clearly understood. When cells are pressurized, turgor is the primary force that drives cellular expansion. It was proved that turgor pressure is only one characteristic of a growing fungal cell, rather than the ultimate driving and controlling variable that determines the rate and pattern of growth.

The process of apical growth of hyphae requires the controlled expansion of the apical wall, which must be transformed subsequently into a wall that resists turgor pressure and maintains the tubular shape of the hypha. The term "steady state" refers to the presence of a steady state amount of plastic wall material at the apex (Wessels, 1993). The wall at the apex must be plastic to allow its extension by insertion of new material. Thus it must be progressively rigidified as it is transformed to become the lateral wall of the hypha (Gooday, 1995). It is clear that two processes involved in this rigidification are the covalent cross-linking of wall materials, especially chitin and β-glucans, and the hydrogen-bonding of adjacent polysaccharide chains, especially chitin, to give microfibrils. There is good evidence that cytoskeletal elements such as actin and microtubules are also important in the maintenance of polarity by transporting vesicles to the apex.

Chitin, one of the main wall components, plays a key role in hyphal growth and differentiation. It is synthesized by the enzyme chitin synthase. Uncoordinated increases in chitin deposition result from treatment with a range of antifungal compounds, interfering with chitin microfibril assembly and azole derivatives inhibiting ergosterol biosynthesis (Gooday, 1995). Branching of hyphae occurs

where a localized area of the rigidified wall of the hypha becomes plastic under the action of chitinases and β-1,3 glucanases.

Fluorescence microscopy constitutes a valuable aid in understanding the structures of higher fungi. Calcofluor binds to β-(1,4)-linked glucans such as cellulose, chitosan and chitin (Sbrana *et al.*, 1995) and therefore can be used for studying the structure of the hyphal wall and the effects of pesticides on hyphal development.

2.8 Trichothecenes gene expression studies

2.8.1 Economic importance of fungi and biotechnology

The economic importance of the fungi is a reflection of their diverse, and often unique, metabolic capabilities. The interaction of fungi with mankind is both beneficial and harmful and is deeply rooted in the history of human society and agriculture. Over the centuries, man has sought to manipulate the growth of fungi to his advantage. The ability to secrete enzymes into the environment makes filamentous fungi attractive to exploiters of modern biotechnology. Industrially, the filamentous fungi are best known as sources of antibiotics, organic acids and enzymes. For example, *Trichoderma* species are used commercially as the producers of a range of hydrolitic enzymes, which are secreted into the growth medium. In laboratory, they are model systems for basic research. The approach and techniques of molecular biology enable us to ask and answer fundamental questions about many aspects of fungal biology, and open the way to the directed manipulation of fungal metabolism.

The detrimental economic effects of fungi as agents of plant diseases are of even greater importance than the beneficial role of fungi in biotechnology. Most phytopathogenic fungi are not amenable to study by the classical methods of genetics and biochemistry and, as a result, the basic mechanism of fungal pathogen-plant host interactions is poorly understood. However, the approach and techniques of

molecular genetics bypass many of these difficulties and are transforming knowledge of all aspects of the biology of these fungi. Clearly there is a long way to go before we understand the molecular basis of fungal pathogenicity, but sound foundations are being laid.

Resistance is a major factor limiting the effectiveness of all classes of pesticides applied to control pests and fungal diseases. Where resistance develops soon after the introduction of a new pesticide, development costs may not be recovered, and the target site may cease to be a resource available to both growers and the agrochemical sensitivity, and strategies implemented to combat spread should resistance emerge. A wide range of epidemiological, biochemical and genetic information about resistance must be combined if strategies are to be effective, and knowledge of mode of action and mechanism of resistance will often be crucial. Pesticides generally inhibit structural or enzymatic proteins, and to understand the mechanism of resistance, traditional biochemical methods of protein separation and enzyme assays are increasingly augmented with a battery of powerful recombinant DNA techniques.

Nucleic acids are of vital importance because of their central position in plant cell manipulation. The importance of a precise knowledge of the structure and function of genes and the end product of gene expression, proteins, is vital to the application in crop protection of the wide range of techniques categorised under the term of genetic engineering.

2.8.2 Genome organization in filamentous fungi

In addition to their economic importance, filamentous fungi have interesting biological properties such as a complex life cycle, cell differentiation, highly regulated metabolic pathways and efficient secretion of proteins which makes them attractive as a model for basic biological research of eukaryotic organisms.

Fungi are eukaryotic in organization and possess more complex genomes than bacteria (Figure 2.7).

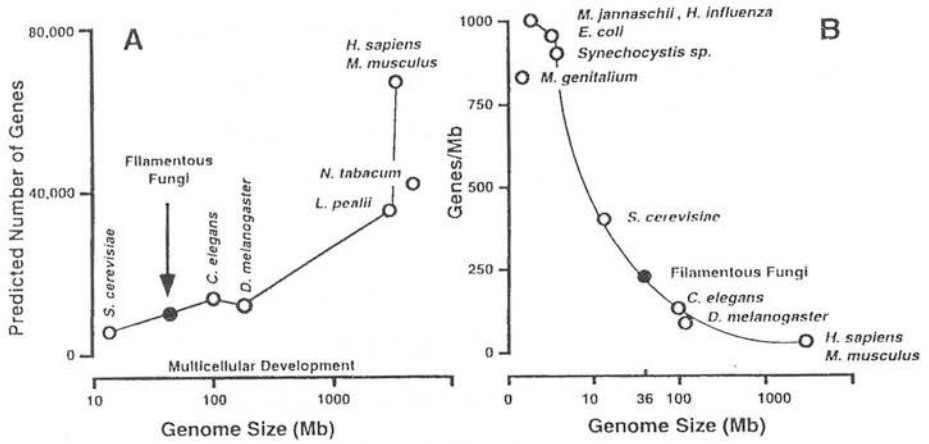

Figure 2.7 Correlation of genome size and number of genes in eukaryotes (A) and prediction of gene density based on evolutionary relationships (B), from Kupfer *et al.* (1997)

The organization of genes in eukaryotes differs from that in prokaryotes in several major respects (Hawkins, 1996). At gross level, eukaryotic genes are distributed on a number of chromosomes rather than one. This has important consequences for regulation, as regulated genes are often located on different chromosomes. The concept of operon does not seem to apply in eukaryotes and polycistronic mRNA is rare or non-existent. Furthermore, eukaryotic genes are often interspersed between an excess of non-coding DNA sequences. An important difference is that, eukaryotic protein-coding genes are often split and the coding "exons" are interspersed with (generally) non-coding "introns". A consequence of this is the enormous size of some eukaryotic genes. An insight into the molecular arrangement of filamentous fungal genes has been made possible by recent development of efficient DNA-mediated fungal transformation systems coupled with standard recombinant DNA technology. Most of the molecular studies have concentrated on the ascomycetous fungi *Neurospora crassa* and *Aspergillus nidulans* for which there has been extensive information provided by classical genetic analyses, now well complemented by molecular approaches.

Organization of the operon type is still controversial in fungi. The indications so far are that there may be as many, or more, regulatory genes in fungi as there are genes with protein structure-determining functions. The study of genetic regulation is at present in an immature phase of its development in which new discoveries tend to complicate the picture rather than making it easier to understand.

In general, therefore, regulation in eukaryotic cells looks as though it will prove to be somewhat more complex than the operon model. These observations make it clear that the regulation of gene action in eukaryotes is not yet understood.

The study of the regulation of functionally and co-ordinately controlled structural genes has been a major research for fungal geneticists over the last decades.

2.8.3 Gene clusters in filamentous fungi

Clusters of functionally related genes are a general feature of prokaryotic gene organization but are much less prevalent in eukaryotes. The discovery that genes for certain types of metabolic pathways are clustered in filamentous fungi is relatively recent. Fungal gene clusters can be broadly defined as the close linkage of two or more genes that participate in a common metabolic or developmental pathway. Fungi possess numerous pathways for what can be described as "dispensable" metabolic functions, and research over the last 5 years has shown that the genes for these dispensable pathways are often organized in gene clusters. The term dispensable metabolic pathways is used here to describe pathways that either are not required for growth or are only required for growth under a limited range of conditions. Dispensable metabolic pathways are typically expressed under suboptimal growth conditions and most likely function to enhance fungal survival in response to nutrient deprivation or competing organisms. Two types of dispensable pathways are the catabolic pathways for the utilization of low molecular weight nutrients such as proline and quinate (nutrient utilization pathway) and biosynthetic pathways for low molecular weight compounds, which include antibiotics and mycotoxins (natural product pathways). Natural product pathways result in the production of structurally

diverse metabolites also referred to as secondary metabolites. It is not uncommon for fungi to produce a number of different natural products and a single natural product can consist of as many as 25 different genes and occupy up to 60 kb of DNA. One proposed function for some natural products is that they contribute to some aspect of organism ecology. Evidence supporting this function has recently come from a number of studies implicating natural products in specific fungal-plant interactions. Currently, studies of natural product pathway regulation are not as advanced as those for nutrient utilization. Positive acting pathway-specific regulators are known for the trichothecene and aflatoxin/sterigmatocystin pathways. Progress in the characterization of dispensable pathways is now sufficient to allow meaningful generalization concerning gene structure and gene organization within pathway gene clusters.

2.8.4 Regulation of transcription in eukaryotes

Gene transcription is accomplished by the transfer of genetic information from DNA to RNA molecules and then from RNA to protein molecules. RNA molecules are synthesized by using the base sequence of one strand of DNA as a template in a polymerization reaction that is catalyzed by enzymes called DNA-dependent RNA polymerases or simply RNA polymerases. Transcriptional regulation in eukaryotes can occur at a variety of levels. As with prokaryotes, proteins can interact with DNA sequences to either stimulate or repress activity of a particular gene; but quite unlike prokaryotes, changes may also occur via differential usage of promoter, termination and splice sequences in various tissues to produce different mRNAs from the same gene. The synthesis of a product within a cell, using the information encoded by the nucleotide sequence, is dependent on a related series of events. The gene is transcribed into a messenger RNA (mRNA), processed, transported into the cytoplasm and by the process of translation the information in the mRNA is decoded into a polypeptide chain.

In general, therefore, regulation in eukaryotic cells looks as though it will prove to be somewhat more complex than the operon model. Regulation in eukaryotes is likely to be complicated because of increased cell size and complexity and it seems probable that different organisms could have evolved different regulatory mechanisms. These observations make it clear that the regulation of gene action in eukaryotes is not yet understood.

2.8.5 Trichothecene biosynthetic pathway

Many species of *Fusarium* produce trichothecenes that have been implicated in mycotoxicoses of humans and animals. Genetic manipulation should be a useful technique for studying the regulation of toxin production and for determining the relationship of toxin production to plant pathogenicity. However, although genetic systems have been developed in some *Fusarium* species, the association of such a genetic system with trichothecene toxin production has been elusive. Genetically characterized isolates of the heterothallic species *Nectria haematococca* (*F. solani*), *Gibberella fujikuroi* (*F. moniliforme*), *G. baccata* (*F. lateritium*) and *G. tricincta* (*F. tricinctum*) do not include any documented trichothecene producers. Conversely, many trichothecene producing species, such as *F. sporotrichioides* and *F. poae*, produce no known teleomorph. In other species, such as *G. zeae* (*F. graminearum*), all documented toxin producing strains are homothallic.

The results obtained by Desjardins *et al.* (1993) suggest that DAS production (biosynthesis) is controlled by multiple unlinked loci. These findings are consistent with the fact that DAS biosynthesis involves a complex series of reactions. The data are also consistent with results obtained in the genetic analysis of a variety of toxins produced in other fungi via complex biosynthetic pathways.

Little is known about the regulation of enzymes involved in fungal secondary metabolism. This is partly due to difficulties in isolating and studying many of these enzymes. Recently, as part of the investigations on trichothecene biosynthesis, Hohn and Beremand, (1989b) have described the isolation of trichodiene synthase (TS)

from *F. sporotrichioides* NRRL 3299. TS catalyses the isomerization - cyclization of farnesyl pyrophosphate (FPP), resulting in formation of the bicyclic olefin, trichodiene. This reaction is the first unique step in trichothecene biosynthesis and a potential site for pathway regulation (Fig. 2.8).

The results obtained for trichothecene production and growth are similar to those previously reported for *F. sporotrichioides*. They clearly demonstrate that the initiation of trichothecene biosynthesis occurs with a high concentration of glucose remaining in the culture medium. This is in contrast to the results reported for *F. graminearum* grown in GYEP medium which indicated that the initiation of trichothecene biosynthesis was dependent on the reduction of glucose in the medium to low levels (Miller *et al.*, 1983).

The lag between the appearance of TS and the initial detection of trichothecenes could be the result of TS expression occurring prior to that of other pathway enzymes. It may also be due to the fact that TS catalyses the first step in the pathway and that the trichothecenes analysed are pathway end products (Fig. 2.8).

The regulation of trichodiene synthase (TS) and its relationship to trichothecene biosynthesis was investigated in *F. sporotrichioides* NRRL 3299 and *Gibberella pulicaris* R - 6380 (Hohn and Beremand, 1989b). Cultures were analysed for the presence of TS activity, trichothecenes, and immunodetectable TS polypeptide over a time period of 144 h. Enzyme activity increased from barely detectable to maximum levels over a period of 3 h for *F. sporotrichioides*, while in *G. pulicaris*, a steady increase was observed over 144 h. Increases in TS activity of 50 - fold for *F. sporotrichioides* and 10 - fold for *G. pulicaris* preceded by several hours the detection of trichothecenes. Immunoblot analysis employing polyclonal serum specific for the enzyme from *F. sporotrichioides* showed that increases in the levels of TS polypeptide correspond to the observed changes in enzyme activity for both organisms. These data indicate that the regulation of TS activity is accomplished through increases in its cellular concentration and that TS may serve as a useful indicator of trichothecene biosynthetic activity.

In summary, *F. culmorum*, *F. sporotrichioides*, and *G. pulicaris* share most of the initial scheme of oxygenations and cyclizations in trichothecene biosynthesis.

Trichodiene synthase is the first unique enzyme in the trichothecene pathway and catalyzes the cyclization of *trans*, *trans*-farnesyl PP_i to trichodiene.

Although specific nutritional factors involved in the induction of trichothecene biosynthesis have not been identified, media containing a high ratio of carbon to nitrogen have been found to give the highest yields of trichothecenes.

2.8.6 Trichothecene genes

The functional categories of genes found in dispensable metabolic pathway gene cluster include genes encoding enzymes, transcription factors, and transporters.

Analysis of the trichothecene pathway gene cluster in *F. sporotrichioides* has so far resulted in the identification of nine genes within a 25 kb region (Keller and Hohn, 1997). *Tri8*, *Tri7*, *Tri3*, *Tri4*, *Tri5*, *Tri6*, *Tri11* and *Tri12* genes are all contiguous members of a gene cluster. The function of eight pathway gene products have been determined and include six biosynthetic enzymes, a transcription factor, and a transport protein. Of the six known biosynthetic enzymes, two are cytochrome P450s that function either in the addition of a hydroxyl group at C-15, (*Tri11*), or in the oxygenation of trichodiene (*Tri4*) to yield a product of unknown structure. The remaining enzyme encoding genes in the cluster include trichodiene synthase, the branch point step in the pathway catalyzing the cyclization of farnesyldiphosphate, and three acetyltransferases that are involved in the acetylation of the trichothecene hydroxyl groups. The cluster gene, *Tri6* encodes a transcription factor and is required for pathway gene expression. Finally, a gene (*Tri12*) encoding an apparent transport protein has been identified by Alexander *et al.* (1997).

Proctor *et al.* (1995) have shown that several genes required for biosynthesis of the trichothecene mycotoxin T-2 by *F. sporotrichioides* are closely linked.

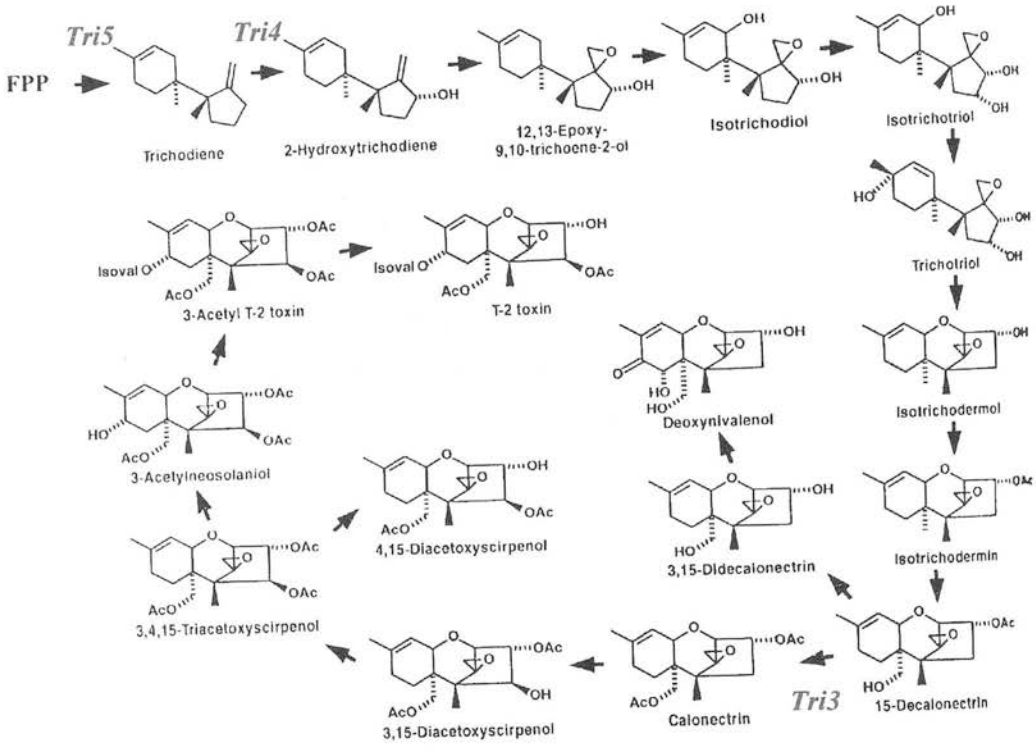

Figure 2.8 Trichothecene biosynthetic pathway in *Fusarium* species, from Desjardins (1993)

Tri5

The *Tri5* gene encodes trichodiene synthase, the first unique enzyme in the trichothecene biosynthetic pathway, present as a single copy in *F. sporotrichioides* and *G. pulicaris* (Hohn *et al.*, 1989a). In *G. pulicaris*, the level of *Tri5* gene mRNA increased 47-fold in the early stationary phase. These results indicate that transcriptional controls play an important role in the regulation of *Tri5* expression and that genes involved in trichothecene biosynthesis in *G. pulicaris* may be linked to *Tri5*.

Tri4

Hohn *et al.* (1995) determined the sequence of *Tri4* and locations of 3 introns were identified. Analysis of *Tri4* mRNA levels revealed that transcription reached maximum levels coincidentally with the onset of trichothecene biosynthesis and then declined 20-fold over the next 8 h. Disruption of *Tri4* resulted in loss of production of both trichothecenes and apotrichodiol and the accumulation of the unoxigenated pathway intermediate trichodiene. Transformants lacking a functional *Tri4* gene were able to convert isotrichotriol, an early pathway intermediate, to T-2 toxin suggesting that most pathway enzymes are present in *Tri4*- mutants. These data suggest that the enzyme encoded by *Tri4* catalyses the first oxygenation step in trichothecene pathway and participates in apotrichodiol biosynthesis.

Tri3

McCormick *et al.* (1996) isolated an acetyltransferase gene (*Tri3*) from *F. sporotrichioides*, closely linked to three other trichothecene biosynthetic pathway genes. Comparison of the *Tri3* sequence with its cDNA revealed the presence of 4 introns. Regulation of *Tri3* transcription in liquid cultures appeared identical to that of other trichothecene pathway genes. Disruption of the *Tri3* gene resulted in the accumulation of deacetylated calonectrins rather than T-2 toxin. It was concluded that *Tri3* encodes an acetyltransferase that converts 15-decalonectrin to calonectrin.

Tri6

Further characterization of this gene cluster revealed a gene (*Tri6*) that specifies a 217-amino-acid protein with regions similar to Cys_2His_2 zinc finger proteins (Proctor *et al.*, 1995). Temporal expression of *Tri6* is similar to that of trichothecene biosynthetic pathway genes. Analysis of *Tri6* transcripts indicated that transcription is initiated in two regions and that within each region there may be at least four initiation sites. Disruption of *Tri6* resulted in a mutant that did not produce trichothecene but that did accumulate low levels of the trichothecene precursor

trichodiene. The *Tri6* mutant was unable to convert 6 trichothecene biosynthetic intermediates to T-2 toxin, and transcription of 2 biosynthetic genes (*Tri4* and *Tri5*) was greatly reduced in the mutant relative to the wild type. It is suggested that *Tri6* encodes a protein involved in the transcriptional regulation of trichothecene biosynthetic genes in *F. sporotrichioides*.

Chapter 3

Rationale

3 Rationale

It is clear from the literature review that factors influencing the production of mycotoxins by *Fusarium* species are poorly understood. In particular, factors such as temperature, fungal interaction and the effect of pesticide form and combinations on mycotoxin production are not fully elucidated. In addition, the effect of interactions between these factors on the production of individual mycotoxins is not known. The objective of this research is to clarify these issues.

3.1 Choice of *Fusarium* species

Three species of *Fusarium* were selected for study on the basis of their global importance as cereal phytopathogens. Additionally, these species typify diversity in terms of type A and type B trichothecene production. In particular *F. sporotrichioides* is capable of synthesizing up to four trichothecenes (Table 2.1) and the need to confirm ZEN production appeared to be an important objective in order to establish the universality of ZEN formation. The importance of this objective resides in the proposal by D'Mello and Macdonald (1998) that ZEN be considered as an environmental oestrogen of relevance to the decline in fertility in men. This diversity of mycotoxin production made *F. sporotrichioides* an ideal candidate organism for examining the effects of a range of factors on trichothecene and ZEN formation.

3.2 Mycotoxin profiles

There is currently considerable interest in using mycotoxin profiles in chemotaxonomy of *Fusarium* species (Ichinoe *et al.*, 1983). To facilitate this approach Thrane (1986) developed a thin layer chromatography (TLC) screening procedure based on direct analysis of *Fusarium* agar culture plugs. These plugs were

placed directly on TLC plates and run with developing solvent systems. A preliminary experiment was designed to explore further the potential of direct plug analysis in comparison with analysis of entire colonies.

3.3 Choice of media

O'Neill *et al*, (1993) was unable to explain variation in mycotoxin yields caused by different grain substrates, therefore homogeneity of substrate was considered to be a desirable feature in the current studies.

Yeast extract sucrose agar (YES) is recommended as a useful medium for mycotoxin production in all three genera (*Aspergillus*, *Fusarium* and *Penicillium*) and potato sucrose agar (PSA) is recommended for detection of different mycotoxins in *Fusarium* species (Filtenborg and Frisvad, 1980). PDA is also a common substrate for culturing *Fusarium* fungi. A preliminary experiment was planned in order to compare YES with PDA media with the intention of choosing the appropriate one for the present study.

Glucose yeast extract peptone (GYEP), a liquid media, was chosen for molecular biology studies, due to a greater yield in mycelial production, essential for RNA extraction and multiple Northern blots.

3.4 Choice of fungicide

Carbendazim was selected as the major fungicide in the present series for three reasons. It is recommended as a fungicide with curative and protectant activity for a variety of cereal diseases such as eyespot and rhynchosporium. Secondly, according to Hollomon *et al.* (1996) carbendazim is effective for controlling FHB and a *Fusarium* disease of rice ('bakanae' disease) but development of carbendazim resistance is a practical problem. Thirdly, carbendazim is a regularly used as a component of mixtures of fungicides as a means of counteracting fungicide

resistance in cereal pathogens. Also, a comparison between the active ingredient carbendazim (98% purity) and the proprietary brand Bavistin was envisaged.

3.5 Fungicide combinations

Fungicide mixtures are routinely recommended as a means of overcoming problems associated with fungicide resistance in *Fusarium* phytopathogens. It is a matter of considerable concern that only one field trial has been conducted to assess the efficacy of this practice for mycotoxin control (Gareis and Ceynowa, 1994). The issue is compounded by enhanced NIV contamination of grain following application of a fungicide mixture to a wheat crop (Table 2.4). Furthermore, *in vitro* determinations of efficacy of mixtures are lacking altogether (Table 2.5). Consequently, a study was undertaken to examine the effectiveness of carbendazim, propiconazole, maneb and tridemorph in combinations similar to those employed in proprietary mixtures for cereals. Additionally, it will be recalled that maneb and tridemorph represent fungicides of differing efficacy with respect to mycotoxin regulation (Tables 2.4 and 2.5).

3.6 Fungicide efficacy

Since fungicides are used to control FHB and other diseases of cereal crops, possible effects on *Fusarium* mycotoxin production provide important criteria for the assessment of efficacy. Evaluation of field evidence in Table 2.4 indicated overall lack of efficacy of fungicides to adequately control trichothecene contamination of cereal grains. Furthermore, only two trials have been conducted with the aim of examining effects on two or more *Fusarium* mycotoxins (Table 2.4). It will also be recalled that, with one exception (Milus and Parsons, 1994), only single doses of fungicides have been tested in field trials, while two of the five *in vitro* studies summarized in Table 2.5 were conducted with merely one or two dose levels.

Although maneb is effective for control of ZEN in maize, its efficacy against the trichothecenes has not been established. Furthermore, the efficacy of maneb and other fungicides in mixtures is not sufficiently documented. Finally, lack of statistical analysis and absence of peer review in certain published experiments has made appraisal of the effects of fungicides more speculative. There is consequently a need for further clarification of issues such as mycotoxin enhancement and other facets of inefficacy using a multi-factorial approach where appropriate.

3.7 Herbicide efficacy

Herbicides are used to combat weeds present in cereal crops. There is no evidence of work on the effect of herbicides on mycotoxin production. Therefore, in one experiment in the present study, an attempt was made to study the effect of different levels of herbicide bromoxynil+ioxynil+mecoprop, added as Swipe, on mycotoxin production by *Fusarium culmorum*, kept under two temperature regimes, namely 25°C and 25-11°C.

3.8 Temperature

It is not clear from either field or laboratory trials how trichothecene production is affected by environmental or incubation temperatures. Greenhalgh *et al.* (1983) suggested that higher incubation temperatures (28°C) favoured the production of DON in *F. graminearum*, but it is not known whether this general rule applies to all trichothecenes. The lowest temperature tested by Greenhalgh *et al.* (1983) was 19.5°C and there is clearly a need for further investigation using lower temperatures with different *Fusarium* species. Ratios of FB_1:FB_2 in *F. moniliforme* are affected by temperature (Marin *et al.*, 1995) and similar sensitivity may be displayed by the conversion of T-2 toxin to HT-2 toxin.

As stated earlier (section 2.4.2.2), there is considerable disagreement about the effects of temperature on ZEN formation, with some studies indicating the need for thermic shock while others showing maximal production at constant temperatures. A concerted series of experiments with different *Fusarium* species and two distinct temperature regimes were therefore planned. The choice of temperature regime was selected as follows: since 25°C represented an approximate mean of the higher temperature used in published work, it was the chosen temperature for the upper limit. The lower temperature of 11°C was selected to enable a study of the effect of a temperature shock since it is known that ZEN is sensitive to temperature.

3.9 Fungal interactions

The effects of mutualistic or antagonistic interactions on mycotoxin production in *Fusarium* species have not been assessed in any systematic manner. Therefore, the effects of fungal competition on growth and mycotoxin production were explored.

3.9.1 Effects on mycotoxin transformations

The metabolism of T-2 toxin to HT-2 toxin and NEO and that of 3-ADON to DON has been commented upon in the review (section 2.2). It is not known, however, to what extent these transformations are affected by external factors such as fungicide applications. In addition temperature may interact with fungicides to alter relative proportions of these mycotoxins. Ratios of FB_1:FB_2 in *F. moniliforme* are sensitive to incubation temperatures (Marin *et al.*, 1995) while ratios of AFB_1:AFG_1 in *A. parasiticus* were affected by addition of fenpropimorph to the incubation medium (Badii and Moss, 1988). On the basis of such evidence, a number of experiments were designed to examine trichothecene metabolism in reponse to incubation time, temperature and fungicide additions.

3.9.2 Fungal morphology

One of the aims of the present studies was to further clarify the relationship between fungal growth and mycotoxin production and to determine the effects of fungicides and temperature on this relationship.

According to Hewitt (1998), the synthesis of the pigment, melanin, by fungi is important for pathogenicity. The thesis being tested in one of the present experiments is whether pigmentation in *Fusarium* species is also necessary for toxigenicity.

Moss and Frank (1985) noted that enhanced T-2 toxin production in *F. sporotrichioides* treated with tridemorph was consistently accompanied by changes in hyphal morphology. Fluorescence microscopy was employed in one of the current experiments to investigate other changes in morphology in response to incubation temperature and fungicide application.

3.9.3 Fluorescence microscopy

Yellow and pink/red pigmentation was observed in *F. sporotrichioides* and *F. culmorum* grown on PDA with or without the addition of carbendazim, under two temperature regimes, 25°C and 25-11°C respectively. These differences in colour development were consistent throughout this study suggesting a modification in the morphology of *F. sporotrichioides* and *F.culmorum*. Therefore, a step further in finding more about hyphal development was considered appropriate. For this reason, in experiment 7, where carbendazim was used alone or combined with other fungicides (propiconazole, maneb and maneb+tridemorph), mycelium was stained using calcofluor white in order to localize β-1, 4 glucans, mainly chitin, in the cell wall.

3.9.4 Time-course studies

Time is an important factor in determining mycotoxin production as discussed in the literature review (see Figure 2.1). Thus, in the current studies, duration of exposure to fungicides and temperature was an integral part of the design of experiments.

3.9.5 The experiments in outline: a multi-factorial approach

It will be clear from the foregoing rationale that there is a need for a multi-factorial approach incorporating elements such as temperature, fungal species and graded doses of different pesticides, ideally in time course studies. Such a strategy would facilitate identification of important interactions. The translation of this rationale into an experimental programme is depicted in Table 3.1.

Table 3.1: The experimental programme

Exp. No.	Fungal species	Pesticide	Temperature regime (°C)	Time points	Page no.
1	*Fusarium sporotrichioides*	Carbendazim (added as Bavistin)	25	1	103
2	*Fusarium sporotrichioides*	Carbendazim (added as Bavistin)	25 - 11	1	103
3	*Fusarium sporotrichioides*	Carbendazim (added as Bavistin)	25 25 - 11	3	104
4	*Fusarium sporotrichioides*	Difenoconazole (added as Plover)	25 25 - 11	1	104
5	*Fusarium sporotrichioides*	Bavistin/ Carbendazim 98% a.i.	25	3	104
6	*Fusarium sporotrichioides/ Aspergillus ochraceus*	Bavistin/ Carbendazim 98% a.i.	25	3	105
7	*Fusarium sporotrichioides*	Carbendazim Carbendazim+ Maneb Carbendazim+ Propiconazole Carbendazim+ Maneb+ Tridemorph	25 25-11	3	105
8	*Fusarium sporotrichioides*	Difenoconazole (added as Plover)	25	2	106
9	*Fusarium sporotrichioides*	None	25	9	106
10	*Fusarium sporotrichioides*	Carbendazim	25	11	107
11	*Fusarium culmorum*	Carbendazim (added as Bavistin)	25 25 - 11	1	107
12	*Fusarium culmorum*	Carbendazim (added as Bavistin)	25 25 - 11	1	108
13	*Fusarium culmorum*	Bromoxynil+ ioxynil+mecoprop (added as Swipe)	25 25 - 11	1	108
14	*Fusarium graminearum*	Difenoconazole (added as Plover)	25 25 - 11	1	109

3.9.6 Justification of experimental methods

3.9.6.1 *In vitro* studies of fungicide efficacy

An *in vitro* approach to assess fungicide efficacy for mycotoxin control was necessitated by the need to conduct multifactorial experiments. *In vitro* methodology confers several advantages over field trials. A comparison ot the two procedures is presented in Table 3.2. In summary, the main attributes of *in vitro* assessments are

Table 3.2 Comparison of *in vitro* methods with field trials for assessment of fungicide efficacy for control of *Fusarium* mycotoxins

In vitro methods	Field trials
Allows wider range of fungicides to be tested	Limited range of fungicides can be assessed in any one trial
Wider dose levels can be tested	Generally, only single levels have been tested
A greater number of combinations of fungicides can be tested	A resticted number of combinations have been tested
Greater control over environmental conditions such as temperature and humidity	No control over environmental conditions
Greater control over infection from other fungal pathogens	No control over other plant diseases
Greater variety of factors can be imposed within a study	Limited range of factors tested in any one trial
Possibility of separating effects of fungicides on fungal growth and on mycotoxin production	Also possible to separate effects on infection and on mycotoxin production, but evidence to date is inconclusive
Greater replication possible	Limited replication
Rapid assays possible	Essentially long-term, over a full season
Cost-effective	Relatively high financial costs
Careful interpretation of findings required	Existing evidence on fungicide efficacy is inconclusive

that a wider variety of fungicides can be tested at different dose levels and combinations. Concurrently, the effects of temperature and time intervals can also be determined. Greater replication can be achieved to allow safe conclusions to be drawn. Several assays can be conducted within a given period of time in a cost-effective way. In field trials background infection may interact with the inoculated phytopathogen, whereas in *in vitro* studies secondary infection can be virtually eliminated. However, careful interpretation of findings is necessary when extrapolating to practical conditions, but it is important to note that field trials conducted so far have yielded inconclusive results (Table 2.4).

3.9.6.2 TLC and densitometry versus HPLC

Thin layer chromatography (TLC) in conjunction with densitometry was chosen as the main technique for mycotoxin analysis.

Advantages of using TLC over HPLC are:

- User friendly
- Less expensive to run
- Less time consuming
- Possibility of multiple samples application at one time
- As effective as HPLC in accurate quantification when used in conjunction with the densitometry
- TLC is also used to confirm or refute components as being mycotoxins. However, an UV cabinet is essential firstly to determine the presence of mycotoxins and secondly to confirm the information obtained from densitometer readings.

3.9.6.3 Trichothecene gene expression studies

In order to fully understand the influence of pesticides on toxin production at the genetic level, a molecular biological approach was adopted. Previous experiments suggest that pesticides could have an effect on gene expression, therefore this hypothesis was tested. Experiments employed in this test were aimed at quantification of signal density and/or presence/absence of signal on a Northern blot. In order to study gene expression, "calibration" of the system with respect to a time course for mycotoxin production in a liquid medium suitable for mycelium production for RNA extraction was investigated. The genes studied were: β-tubulin (a house - keeping gene) *Tri 4*, *Tri 5* and *Tri 6*.

Why did we study mRNA? mRNA was studied because of the interest in the effect of fungicides on gene expression, in regulation of trichothecene biosynthesis in *Fusarium sporotrichioides*. All cells and tissue functions are ultimately ruled by gene expression and a commonly studied parameter of gene expression is the relative abundance of cellular mRNA transcripts. Such an approach was envisaged by the fact that the abundance of a particular RNA species may represent a significant biochemical event in the cell cycle such as the initiation of toxin production.

The rationale behind the time - course experiment was to establish whether the commencement of mycotoxin production in liquid media was correlated to an induction of trichothecene gene expression as witnessed through alterations in mRNA transcript levels.

Molecular experimentation was completed, utilizing the trichothecene genes (*Tri4* and *Tri5*) known to be expressed at the beginning of the trichothecene biosynthetic pathway. In addition, a gene (*Tri6*) regulating this pathway was investigated.

Chapter 4

Materials and Methods

4 Materials and methods

4.1 Experimental design

A total of 14 experiments were conducted with three different toxigenic species of *Fusarium*. Mycotoxin production was determined in all experiments and in addition in three of these experiments, aspects of gene expression were investigated.

Cultures were exposed to graded levels of different fungicides or herbicide and to two temperature regimes and incubated for variable periods of time. Fungicide and herbicide exposure was accomplished by growing cultures on different media. Liquid media was used when cultures were grown for molecular biology investigations. In all experiments fungicide or herbicide was dissolved in ethanol prior to addition to sterilized media. Ethanol without fungicide was added to control media. In order to assess the effects of ethanol addition, an experiment (Experiment 2) was conducted with two sets of controls, one with ethanol and another without ethanol.

4.1.1 Fusarium sporotrichioides

Ten experiments were conducted with *F. sporotrichioides* (IMI 309349) of which 7 (Exp. 1-7) involved the use of solid media while three (Exp. 8-10) employed liquid media under shake culture conditions. A preliminary experiment was conducted using yeast extract sucrose media with or without trace elements and different pH levels.

F. sporotrichioides was used in order to prepare 5-day-old cultures on potato dextrose agar (PDA) (Exp. 1-7) and V8 juice agar (Exp. 8-10) in Petri dishes (~9.0 cm diameter). Peripheral plugs were removed using a flamed cork borer and a dissecting needle from the margin of the 5-day-old colonies and placed centrally onto PDA containing fungicides at different levels.

4.1.1.1 Experiment 1 (Preliminary experiment) : The effects of medium, pH, temperature and carbendazim on fungal growth and mycotoxin production

Experiment 1A : The effects of medium type, pH and temperature at different stages of growth

F. sporotrichioides grown on PDA at 25°C was used as inoculum for yeast extract sucrose media with or without trace elements and pH adjusted to 3.0, 5.4, 6.4, 7.0. Two replicates per treatment were used. The Petri dishes were incubated at 25, 18 and 13°C and in order to examine mycotoxin production; agar plug technique in conjunction with thin layer chromatography (TLC) was involved.

Experiment 1B : The effects of carbendazim and temperature

YES media containing carbendazim (added as Bavistin) at concentrations of 0.0, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml and a constant temperature of 25°C were used. Colonies were measured on the 2^{nd}, 4^{th} and 7^{th} day after inoculation and mycotoxin analysis performed using agar plug technique in combination with TLC.

Experiment 1C : The effects of medium type and temperature shock at different stages of growth

A comparison between YES and PDA media was carried out. Colonies of *F. sporotrichioides* grown on YES and PDA were exposed to 25°C for 5days followed by a transfer of all replicates to 11°C until the completion of experiment. Analysis of mycotoxin production was performed at six time points.

4.1.1.2 Experiment 2 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

The fungicide was added as Bavistin (a.i. 50%) diluted with ethanol, with carbendazim concentrations of 0.0, 0.1, 1.0, 10.0, 100.0 μg/ml PDA and a set of controls without the addition of ethanol was used. The plates were incubated at 25°C

for five days then transferred to 11°C until the completion of experiment. Four replicates per treatment were used. The extraction of the media and colony was performed on the 28th day.

4.1.1.3 Experiment 3 : The effects of carbendazim (added as Bavistin), temperature regime and time on pigmentation, fungal growth and mycotoxin production

The concentration of carbendazim, added as Bavistin (a.i. 50%), was of 0.0, 1.0, 2.5, 5.0, 7.5, and 10.0 μg/ml PDA. The plates were incubated for 4-5 days at 25°C then half of the replicates, selected at random, were transferred at 11°C. Four replicates per treatment were used. Extractions were performed at 6, 14 and 26 days of growth.

4.1.1.4 Experiment 4 : The effects of difenoconazole (added as Plover) and temperature regime on fungal growth and mycotoxin production

Petri dishes containing PDA with different concentrations of difenoconazole (added as Plover) 0.0, 0.1, 1.0, 10.0, 100.0 μg/ml were used. The plates were incubated at 25°C for 5 days then half of the replicates transferred to 11°C until the completion of the experiment. Four replicates per treatment were used. The extraction of the media and colony was performed on the 28th day.

4.1.1.5 Experiment 5 : The effects of carbendazim (added as Bavistin and/or pure substance) and time on fungal growth and mycotoxin production

Preliminary experiments showed that concentrations of 5.0 and 10.0 μg/ml carbendazim were lethal. Consequently, Petri dishes containing PDA with carbendazim (98% a.i.) and Bavistin (50% a.i.) at 0.0, 0.1 and 1.0 μg/ml were used.

Plates were incubated at 25°C for the entire period. Two replicates per treatment were used. Extractions were performed on the 7, 14 and 29th day from inoculation.

4.1.1.6 Experiment 6 : The effects of carbendazim (added as Bavistin and/or pure substance), fungal interaction and time on fungal growth and mycotoxin production

F. sporotrichioides was used in combination with *Aspergillus ochraceus* in order to study the interaction between these fungi in terms of growth and mycotoxin production. The same fungicide type and concentration as above were used. There was a set of controls inoculated with *Aspergillus* only, another set with *Fusarium* only and a set with *Aspergillus* inoculated at three centimetre distance from *Fusarium* on the ~9.0 cm Petri dish. Apart from the control set there were identical sets inoculated on 0.1, 1.0 μg/ml Bavistin (50% a.i.) and carbendazim (98%). Two replicates per treatment were used. All the plates were incubated at 25°C for the entire length of the experiment and extractions performed on the 7, 15 and 28th day.

4.1.1.7 Experiment 7 : The effects of fungicide combinations, temperature and time on pigmentation, growth and mycotoxin production

Plugs from 5-day-old colonies of *F. sporotrichioides* were used to inoculate PDA containing various mixtures of fungicides in order to study the effect of the fungicide combinations on growth and mycotoxin production. The fungicides used were carbendazim, maneb, propiconazole, and tridemorph, in the following combinations; carbendazim+maneb (50:320 g/l), carbendazim+propiconazole (20:25% w/w), carbendazim+maneb+tridemorph (40:320:90 g/l). A set of controls with ethanol was used and additionally a set of "controls" with carbendazim for comparison with mixture of fungicides was used. The levels of fungicide combination used were: 0.0, 0.1 and 1.0 μg/ml. There were two temperature regimes used: 25°C and 25-11°C.

After 5 days incubation at 25°C half of the replicates, at random, were transferred to 11°C for the remaining of the experiment. The colonies were measured and described on the 2nd and 4th day and extractions performed on the 7, 14 and 25th day. Two replicates per treatment were used.

Due to a very distinct coloration between the colonies kept at 25°C (yellowish) and the ones kept at 11°C (pink) for the main part of the experiment, fluorescence microscopy was performed in order to observe any differences at the cellular level.

4.1.1.8 Experiment 8 : The effects of difenoconazole (added as Plover) and time on mycotoxin production in liquid culture

A spore suspension with a concentration of $5x10^4$/ml was used to inoculate glucose (5.0%): yeast extract (0.1%): peptone (0.1%) (GYEP) media (125 ml/flask), containing difenoconazole (added as Plover) at concentrations of: 0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml. The flasks were placed on a shaker (180 rpm) at 25°C and extractions performed at 24 and 48 hours.

4.1.1.9 Experiment 9 : The effect of time on toxin production in liquid culture

In this experiment, a spore suspension with a concentration of $5x10^3$/ml media (GYEP 10:1:1 g/l, pH 6.5, 100 ml media/250 ml flask) was used. Flasks placed on shaker (rpm=180) were kept at 25°C. Extractions were performed at: 0, 6, 12, 24, 48, 72, 120, 168 and 264 hours. The filtered mycelium was flash freezed in liquid nitrogen and stored at -80°C until all samples were filtered, then freeze dried overnight followed by storage at -80°C. The filtrates were used for mycotoxin analysis and the mycelium for RNA extraction, dot and Northern blots analysis.

4.1.1.10 Experiment 10 : The effects of carbendazim and time on gene expression and mycotoxin production

The same concentration of spores as in experiment 9 was used to inoculate GYEP media containing carbendazim at 0.0, 1.0, 2.0 and 3.0 µg/ml. The flasks were placed on a shaker at 25°C and extractions performed at: 0, 16, 20, 22, 24, 30, 36, 48, 72, 120 and 264 hours. Mycelia obtained from the concentration: 0.0 and 1.0 µg/ml were used for RNA extraction and the filtrate obtained from the concentrations: 0.0, 1.0, 2.0 and 3.0 µg/ml, were checked for mycotoxin production. Two replicates were used per treatment.

4.1.2 Fusarium culmorum

Three experiments were conducted with *F. culmorum* cultured on solid media (PDA) with Bavistin (Exp. 11 and 12) and with herbicide Swipe (Exp. 13).
Preparation of peripheral plugs and inoculation of media were performed according to protocols described for Experiments 1-7.

4.1.2.1 Experiment 11 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

The fungicide was added as Bavistin, (a.i. 50%) diluted in ethanol, with carbendazim concentration of 0.0, 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml of media. The plates were incubated at 25°C for four days then half of the replicates (at random) were transferred to the 11°C incubator for the remaining 25 days until extraction was performed. Two replicates were used per treatment.

4.1.2.2 Experiment 12 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

The same method as above for preparation of inoculum was used. The fungicide used was carbendazim at 0.0, 0.1, 1.0 and 10.0 μg/ml (added as Bavistin). The plates were incubated at 25°C for 5 days, then half of the replicates, at random, were transferred to 11°C. The extraction was performed on the 27th day. Four replicates were used per treatment.

4.1.2.3 Experiment 13 : The effects of bromoxynil + ioxynil + mecoprop (added as Swipe) and temperature regime on mycotoxin production

This experiment was conducted in the same fashion as experiment five with a few exceptions: the pesticide used was bromoxynil + ioxynil + mecoprop, added as Swipe 560 EC incorporated in PDA at 0.1, 1.0, 10 and 100 μg/ml media. The extraction was performed after 33 days of incubation. Four replicates were used per treatment.

4.1.3 Fusarium graminearum

One experiment has been conducted using *F. graminearum* cultured on solid media (PDA) containing Plover. Preparation of peripheral plugs and inoculation of media were performed in the same manner as for previous experiments with *F. sporotrichioides*.

4.1.3.1 Experiment 14 : The effects of difenoconazole (added as Plover) and temperature regime on fungal growth and mycotoxin production

A four-day-old culture was prepared and PDA plates containing difenoconazole at 0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml media were inoculated. All the plates were incubated at 25°C for five days followed by a transfer of half the plates to 11°C. The extraction took place at 32 days from inoculation. Four replicates were used per treatment.

4.2 Fungal isolates

4.2.1 Preparation

The following fungal species in form of freeze-dried cultures were purchased from the International Mycological Institute (IMI) and used in the current studies:

- *Aspergillus ochraceus* IMI 132429
- *Fusarium culmorum* IMI 309344 (as used by O'Neill *et al.*, 1993)
- *Fusarium graminearum* IMI 263189
- *Fusarium sporotrichioides* IMI 309349

The ampoule containing the freeze-dried culture was marked with a file near the middle of the cotton wool plug. Using gloves and tissue paper, the ampoule was snapped open inside the laminar flow cabinet. The opening of the ampoule was flamed and the plug removed. Ringer solution was added gradually in order to resuspend the contents and the plug was replaced. The vial was rolled in hand and then left in the rack for 5 minutes in order to reconstitute the organism. Using a sterile Pasteur pipette the content of the vial was dispensed in shape of drops onto Petri dishes containing PDA. The method used for preparation of fungal isolates is a modified version of the IMI method for opening and reviving freeze-dried cultures.

4.2.2 Maintenance of fungal isolates

Stock cultures were maintained on PDA in Universal vials (10 ml PDA slants) and Petri dishes (20 ml PDA). All fungal isolates were inoculated on a regular basis on slopes and Petri dishes. A plug constituted the inoculum. To avoid dryness the Petri dishes were sealed with parafilm. A set of isolates was kept at room temperature and a stock of isolates in the cold room (4°C).

4.2.3 Inoculum form / appearance

4.2.3.1 Plug - used for inoculations onto solid media

Using the flamed cork borer (5.0 mm diameter), plugs were cut from the periphery of the fungal colony and with an ethanol-flamed dissecting needle the plugs were transported and placed in the centre of the agar plates (Filtenborg & Frisvad, 1980; Filtenborg *et al.*, 1983; Frisvad & Filtenborg, 1990). Inoculation was performed in a microbiological class II safety cabinet.

4.2.3.2 Spore suspension - used for inoculations into liquid media

Sterile distilled water was dispensed using a sterile pipette onto the media with colony, then using a flamed glass spreader, the mycelia was detached from the media with circular movements. Using a graded sterile pipette the suspension was transferred into sterile 50 ml centrifuge tube (Sorvall®) and then inverted gently. Another centrifuge tube containing water and having the same weight as the first was used for balance in the centrifuge. The tubes were spun at 10000 rpm for 10 minutes. The supernatant was removed and the pellet resuspended in a known volume of sterile distilled water. A count of spores was performed using a haemocytometer (Improved Neubauer depth 0.1 mm, 1/400 mm^2 Weber) and the inoculum measured

and adjusted to give a count of $5x10^3$/ml or $5x10^4$ /ml media. The spore count was performed according to Weber Scientific International Ltd. method, as follows:
The haemocytometer and cover slip were cleaned and dried. The cover slip was rubbed against the haemocytometer using the thumbs and index fingers until the Newton's rings appeared. Using a sterile Pasteur pipette, a small volume of spore suspension (agitated previously in order to ensure an even distribution of spores for an accurate reading) was released onto the two grids of the haemocytometer (one each side of the slide). Under low power of the microscope (x10) the main square (A) of the grid was located. This square is made up of 25 smaller squares (B) which each contain 16 very small squares (C). The whole large square (A) contains 400 of these very small squares (C). The volume of each very small square is 1/4000000 ml. The number of spores present in 5 out of 25 smaller squares (B) was counted and the following calculation was then used to determine the number of spores per ml.

- the total number of spores in 5 small squares (B) was calculated =X
- the number of spores in the whole grid was calculated as = 5xX (as there are 25 small squares (B) in total)
- the number of spores in each very small square (C) was calculated as = (5xX)/400 (as there are 400 of these in the whole grid)
- the volume of each very small square (C) was calculated as =1/4000000 ml, therefore the number of spores per ml = (5xX)/400 x 4000000

Two readings were performed for each spore suspension and the average taken. When finished, the haemocytometer and cover slip were rinsed in distilled water and dried with a lens tissue. Inoculation took place in the microbiological safety cabinet.

4.3 Media

Media was prepared as shown below and dispensed using one of the following manners: sterile pipettes, peristaltic pump (Watson-Marlow 505 DU/RL) or a plate pourer (Pourmatic™ Model MP-320).

4.3.1 Potato dextrose agar (PDA)

Potato dextrose agar (Oxoid CM 139) was prepared according to the manufacturer's instruction (3.9 g PDA/litre distilled water). PDA was autoclaved in Duran bottles for 15 minutes at 121°C. When the media cooled down to 55°C, 20 ml aliquots were dispensed into each plastic Petri dish. In the case of preparation of several litres, a water bath set at 55°C was kept close by.

4.3.2 Yeast extract sucrose (YES)

To a litre of water, the following were added:

Yeast extract	20.0 g
$MgSO_4$ $5H_2O$	0.5 g
Sucrose	150.0 g
Agar	20.0 g

Trace metal solution	1.0 ml consisting of:
$ZnSO_4$ $7H_2O$	1.0 g
$CuSO_4$ $5H_2O$	0.5 g
Water	100 ml

The pH of YES media was adjusted from 6.4 to 3.0, 5.4, and 7.0 using NaOH or HCl.

4.3.3 V8 juice agar

For every litre of media, the following components were mixed: V8 juice (Savacentre) 200 ml, agar (Agar technical - Oxoid) 20.0 g, $CaCO_3$ (AnalaR - BDH chemicals) 3.0 g and water 800 ml. The pH was checked and adjusted to 5.5. Media was autoclaved in Duran bottles for 15 minutes at 121°C, then poured into plastic Petri dishes.

4.3.4 Glucose yeast extract peptone (GYEP)

There were two recipes for glucose yeast extract:

1) glucose 5.0%, yeast extract 0.1% and peptone 0.1% where the glucose was autoclaved separately and added aseptically to the media, 125 ml/250 ml flask.

2) to every litre of distilled water, 10.0 g glucose, 1.0 g yeast extract, 1.0 g peptone were added. The pH of the media was checked and adjusted to 6.5 (100 ml/250 ml flask) (Ueno, *et al.*, 1975)

4.3.5 Luria - Bertani (LB medium)

To 950 ml of deionized water, the followings were added: bacto-tryptone 10.0 g, bacto-yeast extract 5.0 g, and NaCl 10.0 g. The mixture was shaken until the solutes dissolved. The pH was adjusted to 7.0 with 5N NaOH (~0.2 ml). The volume of the solution was adjusted to 1 litre with deionized water. The media was then sterilised by autoclaving for 15 minutes at 121°C on liquid cycle.

Prior to working with this media, ampicillin (Sigma) was added to it. The concentration of the ampicillin stock solution was 50 mg/ml H_2O, it was sterilized by filtration and kept at -20°C, and the concentration of the working solution was 60 µg/ml.

4.3.6 SOC medium

Composition: 2.0% Tryptone
0.5% Yeast Extract
10.0 mM NaCl
2.5 mM KCl
10.0 mM $MgCl_2$-$6H_2O$
20.0 mM glucose

For one litre of media, 20 g Tryptone, 5 g Yeast Extract and 0.5 g NaCl were dissolved in 950 ml deionized water. A 250 mM KCl solution was prepared by dissolving 1.86 g of KCl in 100 ml of deionized water and 10 ml of this stock solution added to previous solution. The pH was adjusted to 7.0 with 5 M NaOH, then the volume brought up to 980 ml with deionized water. Both solutions were autoclaved. Meanwhile, a 2 M solution of glucose was prepared by weighing out 36 g glucose and dissolving it in a final volume of 100 ml deionized water and filter sterilized. The autoclaved solutions were allowed to cool down to about 55°C, then 10 ml of the filter sterilized 2 M glucose solution and 10 ml 1 M $MgCl_2$ were added.

4.3.7 Addition of pesticides

To the sterile PDA medium various pesticides were added (Table 4.1). Carbendazim and difenoconazole were added to GYEP media in accordance with Table 4.1.
Pesticides were prepared in ethanol due to their greater solubility in this solvent (Table 4.2).

Table 4.1 A summary of pesticide addition to different types of media used in the present study

Pesticide type	Media	Level of pesticide (µg/ml)	Experiment no.	Page no.
Carbendazim (added as Bavistin)	YES	0.1, 0.5, 1.0, 5.0, 10.0	1	103
	PDA	0.1, 1.0, 10.0, 100.0	2	103
		1.0, 2.5, 5.0, 7.5, 10.0	3, 11	104,107
		0.1, 1.0	5, 6	104, 105
		0.1, 1.0, 10.0, 100.0	12	108
Carbendazim (98% purity)	PDA	0.1, 1.0	5, 6, 7	104,105
	GYEP	1.0, 2.0, 3.0	10	107
Carbendazim+Maneb 50:320 g/l Carbendazim+ Propiconazole 20:25% w/w Carbendazim+Maneb +Tridemorph 40:320:90 g/l	PDA	0.1, 1.0	7	105
Difenoconazole	PDA	0.1, 1.0, 10.0, 100.0	4, 14	104,109
	GYEP	0.1, 1.0, 10.0, 100.0	8	
Bromoxynil+ioxynil+ mecoprop 56:56:448 g/l	PDA	0.1, 1.0, 10, 100	13	108

Table 4.2 Details of solubility in water versus ethanol for the pesticides used in this study

Pesticide	Solubility				
	Water			Ethanol	
	Temperature (°C)	pH	Value (mg/l)	Temperature (°C)	Value (g/l)
Carbendazim	24 24 24	4.0 7.0 8.0	29 8 7	24	0.3
Difenoconazole	25		15	25	330
Maneb			Insoluble		
Propiconazole	20		100		Completely miscible
Tridemorph	20	7.0	1.1		Miscible
Bromoxynil	20		130	25	70
Ioxynil	20		50	25	20
Mecoprop	25		734	20	>1000

In the case of pesticide addition, the required concentrations were prepared while media was autoclaving, then added to it prior to pouring onto the Petri dishes.

4.4 Growth determination

4.4.1 Solid state media

Colonies grown on solid state media were measured using a ruler. Two perpendicular diameters were measured and the mean value recorded for each colony.

4.4.2 Liquid state media

Mycelial growth was determined in this case by measuring the dry weight. Recovered mycelia, after centrifugation (Sorvall® RC 5C) in sterile centrifuge buckets (Sorvall® Instruments Du Pont, 250 ml) followed by vacuum filtration on filter paper (Whatman®, Qualitative Circles 55 mm diameter, 6, Vacuum pump Whatman, Type N 726 FT.18), was transferred to a pre-weighed 5 ml tube (Kartell, Merk), snap frozen in liquid nitrogen, freeze dried (Freeze-drier Edwards Freeze Drier, Super Modulyo) and then sample + vial weighed (balance Sartorius GMBH Gottingen, Type 1872) to obtain mycelial weight.

4.5 Mycotoxin investigations /studies

Mycotoxin studies comprised of: extraction of samples, preparation of mycotoxin standards and mycotoxin analysis. All extracts obtained during this study were subjected to qualitative analysis followed by quantitative analysis.

4.5.1 Extraction procedure

Chloroform and acetonitrile were tested for extraction procedures and due to better results, chloroform was the chosen solvent.

4.5.1.1 Solid state media

Using a scalpel the contents of each plate was cut in small cubes and introduced into 54 ml test tubes (Quickfit Bibby) with the help of clean forceps. Ten ml of chloroform were added to each tube, tops fitted and then shaken using a vortex mixer. The tubes were left in the metal rack on the bench for half an hour. The extract was then filtered through a phase separator paper (Phase Separators 1PS,

Silicone Treated Filter Paper, Circles 110 mm diameter, Whatman) in order to remove any residual water, into a round bottom flask (Quickfit® Corning Ltd 100 ml) and another 10 ml chloroform added to the test tubes, repeating the previous steps. The cumulated extract was dried using a rotary evaporator (Vacuum Rotary Evaporator, Type 349/2) then the dried extract was washed with 3 x 0.5 ml chloroform into 2.0 ml glass vials (Vial with metal foil cap, Wheaton) using Pasteur pipettes (Volac Disposable). At this stage the extract was dried using nitrogen (Nitrogen M.30 - NG), on a heating module (Pierce, Reacti - Therm III™), then all the samples were transferred in the freezer (-20°C).

4.5.1.2 Liquid state media

After filtration, the content of the conical flasks was transferred into 250 ml separating funnels (Quickfit, Rotaflo) and 50 ml chloroform added to each of them. Stoppers were fitted and the whole unit shaken by hand, then the tops being slowly released because of the pressure formed inside the funnel. The separating funnels were then placed into the stands and left still until a separation between layers was observed. The lower layer was filtered through a phase separator paper and collected in round bottom flasks and another 50 ml of chloroform added to the separating funnels. The process was repeated two more times. The cumulated extract was dried using the rotary evaporator and the dried extract washed into the 2.0 ml glass vials using 3x 0.5 ml chloroform. At this stage the extract was dried using nitrogen and then all the samples were stored in the freezer (-20°C) until quantification was required.

4.5.2 Mycotoxin standards

The following standards were obtained from SIGMA and the details of physical and chemical characteristics are presented in Table 4.3. R_f values were determined in two

solvent systems: chloroform with methanol (CM) and toluene with ethyl acetate and formic acid (TEF). The lower limits of detection (LLOD) for the main mycotoxins investigated in this study, are presented in Table 4.3.

Table 4.3 Compiled data on chemical and physical aspects of mycotoxin standards employed in the current research project

Mycotoxin standard	Empirical formula	Lower limits of detection (mg/ml extract)	R_f		Colour under UV light		
			CM[a]	TEF[b]	No deriv.	After derivatization	
						$AlCl_3$	H_2SO_4
T-2	$C_{24}H_{34}O_9$	0.05	0.83	0.53			Blue
T-2 triol	$C_{20}H_{30}O_7$			0.32			
Neosolaniol	$C_{19}H_{26}O_8$	0.03	0.52	0.33			Blue
HT-2 toxin	$C_{22}H_{32}O_8$	0.003	0.40	0.38			Blue
Diacetoxyscirpenol	$C_{19}H_{26}O_7$	0.04	0.80	0.49			Rusty pink
Nivalenol	$C_{15}H_{20}O_7$			0.34			
Deoxynivalenol	$C_{15}H_{20}O_6$	0.02		0.36			Blue
3-acetyldeoxynivalenol	$C_{17}H_{22}O_7$	0.02		0.51			Blue
15-acetyldeoxynivalenol	$C_{17}H_{22}O_7$			0.48			Blue
Zearalenone	$C_{18}H_{22}O_5$	0.005		0.75	Blue		
Zearalanone	$C_{18}H_{24}O_5$			0.68	Blue		
α-zearalenol	$C_{18}H_{24}O_5$			0.46	Blue		
α-zearalanol	$C_{18}H_{26}O_5$			0.60	Blue		
β-zearalenol	$C_{18}H_{24}O_5$			0.45	Blue		
β-zearalanol	$C_{18}H_{26}O_5$			0.62	Blue		
Ochratoxin	A $C_{20}H_{18}ClNO_6$ B $C_{20}H_{19}NO_6$			0.78	Blue		
Sterigmatocystin	$C_{18}H_{12}O_6$			0.78	Brick	Yellow	
FusarenonX	$C_{17}H_{22}O_8$					Blue	

[a]CM = chloroform: methanol

[b]TEF = toluene: ethyl acetate: formic acid

4.5.2.1 Standard preparation

The metal cans/plastic containers with standards obtained from Sigma were opened in the fume cabinet, and with care, the vial containing the mycotoxin standard brought out. The metal centre part of the top was detached and a sterile needle (Microlance® 3Becton-Dickinson), used as an escape valve, inserted into the rubber top avoiding contact with the standard. Using a sterile syringe (Plastipak® Becton-Dickinson®) fitted to another needle, a volume of chloroform (1 or 5 ml) adjusted to the amount of standard present in the vial, 1 or 5 mg respectively, was introduced into the vial to give a concentration of 1 mg/ml. The needles were removed, the top covered with parafilm and the vial vortexed. The content of the vial was divided into 0.2 ml aliquots, one kept for preparation of the standard mixture (working solution) and the others dried under nitrogen (kept as stock at -20°C). The concentrations of mycotoxin standards used were 0.5 mg/ml for the trichothecenes group but for zearalenone a dilution of 0.05 mg/ml was used due to its greater fluorescence under the UV light.

4.5.2.2 Standard curves

Standard curves were produced in order to verify the linearity of the standards, so the results obtained would be comparable. Linearity was observed and an example is presented in Appendices (section 8.2, Figure 8.1).

4.5.3 Mycotoxin analysis

The analysis of mycotoxins employed two aspects, namely the qualitative analysis and the quantitative analysis. All extracts obtained during this study were subjected to both aspects.

4.5.3.1 Qualitative analysis

Qualitative analysis involved screening of culture extracts by thin-layer chromatography (TLC) for the presence and identification of mycotoxins.

4.5.3.2 Quantitative analysis

Quantitative analysis entailed subjection of TLC plates to quantification by visual and densitometric methods.

1) Visual method

Quantification using this method involved visual comparison of samples with standards spotted in graded concentrations. The visual comparison was performed under UV light.

This method was used in a quantitative manner only in experiment 2. However, this method was also used in conjunction with densitometry for preliminary and verification purposes.

2) Densitometric method

A second method used for quantification of mycotoxin production involved the use of a densitometer. Quantification of mycotoxins was achieved with Camag Densitometer CD60.

4.5.4 Thin layer chromatography (TLC)

TLC is the technique of separation and identification of chemical substances by a moving solvent on a thin layer of suitable adsorbent.

The furthest point reached by the advancing solvent on the paper is termed the solvent front. This can be used as reference point for describing the relative distance of travel of different substances in a chromatogram. The symbol used to designate this relative distance of travel is R_f, and is defined by the following equation:

$$R_f = x/y$$

Where : x = distance compound has moved from origin

y = distance of solvent front from origin

As the denominator is always larger than the numerator, the R_f would be a decimal. For convenience it can be expressed as a percentage by multiplying by 100. The R_f is a useful figure because it is a constant when all conditions are exactly reproduced. The R_f of a given compound will generally be different for different solvents. Some of the factors which affect R_f values are the type of plate and the temperature at which chromatography is run.

4.5.4.1 One - way TLC

In this procedure, standards and agar plugs and/or sample extracts were applied onto silica gel plates: aluminium (1.05553 DC - Alufolien, Kieselgel 60, Merck) or glass (1.05721, DC - Platten 20x20, Kieselgel 60, Merck). An example of one way TLC is presented in Appendices (section 8.2, Plate 8.1A).

1) Agar plug

This technique involved the use of agar plugs combined with TLC. Agar plugs, obtained in the same manner as for inoculation purposes (see 4.2.3.1) were used as follows; the agar side of the plug was pressed gently, directly onto the TLC plate, then, a drop of chloroform/methanol (2:1) was applied to the mycelial side of the plug and subsequently pressed lightly onto the TLC plate. A standard mixture

together with the spots produced, situated at equidistant intervals, were allowed to dry and the plate developed in a glass tank (see 4.5.4.4 -1) containing a developing solvent which ascended the TLC plate by a form of capillary action. The migrating distance was set to 10 cm. This technique was used in the preliminary experiment (see experiment 1).

2) Sample extract

Standards as well as samples resuspended in chloroform, were loaded equidistantly onto the plate, which when dried was placed into a tank/chamber to develop (see 4.5.4.4). The migrating distance was also set to 10 cm. This method was considered more accurate, and therefore implemented in experiments 2-14.

4.5.4.2 Two - dimensional TLC

Two - dimensional TLC was employed for two purposes. Firstly, this procedure provided greater confidence in the identity of a particular mycotoxin and secondly, improved separation was possible in instances when fungal pigments and other substances interfered with chromatography.

Using a 20x20 cm plate, the sample of interest was spotted (Figure 4.1) in the left hand corner at 2.0 cm distance from both sides of the TLC plate, and the standard or mixture of standards was spotted on the right hand side of the plate and symmetrically at a 90 degrees angle on the other side (see also Appendices, section 8.2, Plate 8.1B).

The plates were developed first in chloroform: methanol (93:7), removed from the tank and allowed to dry then turned through an angle of 90 degrees and placed into the second solvent TEF (5: 4: 1). When the solvent reached the 10 cm distance for development, the plate was removed from the tank allowing it to dry.

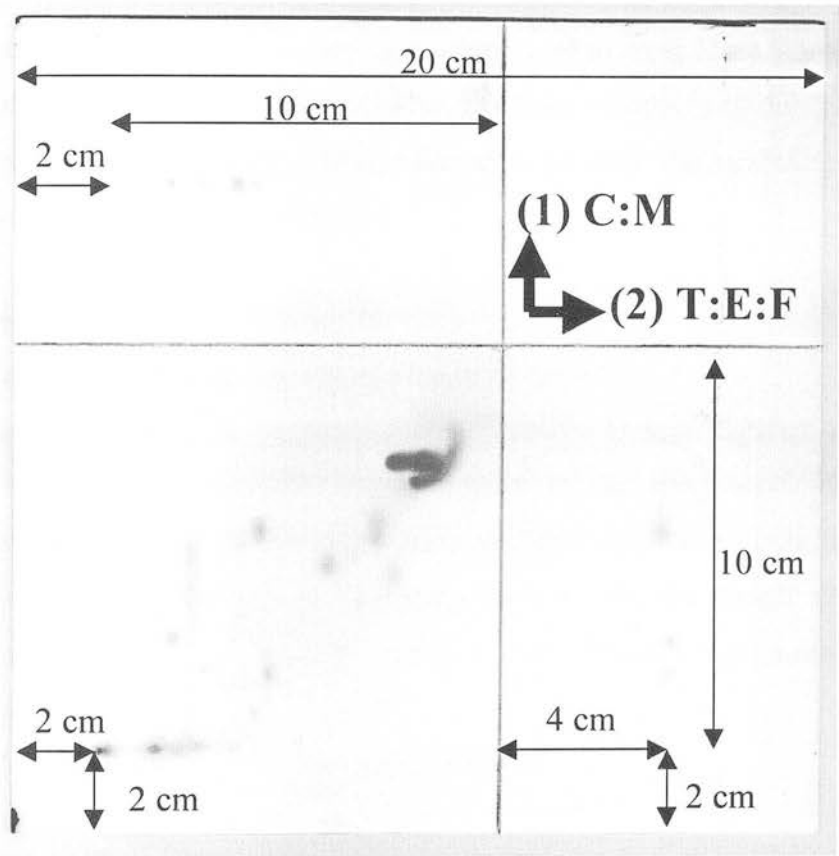

Plate 4.1 An example of two dimensional TLC plate for analysis of *Fusarium* mycotoxins

4.5.4.3 Spotting technique

Samples were resuspended in 100 µl chloroform and when required, dilutions performed accordingly. Standards (5.0 µl) were spotted on the same plate in order to screen a number of extracts for presence of mycotoxins.

Two methods of spotting were used:

1) Manual

This method was performed using a Gillson pipette with tips, and a constant volume of 5.0 µl/spot. To apply the extract solution as a spot, 5.0 µl were taken and applied

to the thin layer in a drop not larger than 2-3 mm diameter taking care that the pipette tip did not damage the coated surface. Being necessary to apply more than one drop, the spot was allowed to dry each time before applying another, until the 5.0 µl were completed. The manual spotting took place in a portable fume cabinet (Astecair 3000, Astec Environmental Systems Ltd.).

2) Automatic

The automatic spotting took place also in the fume cabinet.

The advantage of using the automatic spotter (Camlab, Desaga, Sarstedt - Gruppe, AS 30) is the defined position, the uniformity of the spot (band) useful for quantification. The tremendous disadvantages are that it needs 25 µl from the 100 µl sample to load a 5.0 µl spot and the syringe used to load the sample clogs even though it is washed in between samples. The following parameters were programmed:

- plate length: size of the used TLC plate (20x20)
- positions: number of lanes per plate
- length: length of a lane. If the length is 0= spot
- distance: distance from the middle of the first lane to the middle of the second lane
- start: middle of the first lane
- volume: application quantity per lane
- time: application time for one microlitre sample
- cycles: number of application steps per application
- break: time between two application steps
- edge clearance: distance between edge of the TLC plate to the beginning of the first lane (minimum edge clearance 5.0 mm given from the software)

After the parameters had been keyed in, a correlation of the input was carried out. This meant that to each line, a sample (or standard) was assigned. For example:

Lane	Vial	Factor
1	1	x1
2	2	x1
3	3	x1
4	1	x2
5	2	x2
6	3	x2

An input of a factor "n" means that the volume will be applied "n" times.

4.5.4.4 Development

Two methods of developing TLC plates were used:

1) In a glass tank

When using this method, the development of TLC plates took place in a sealed glass tank (Panglas® Shandon Southern, T.L.C. Chromatank®), lined with chromatographic paper (Whatman 1) in order to obtain a saturated atmosphere with the developing solvent. The spotted plates were placed coated surfaces facing each other in the tank, after the solvent mixture had been poured into it, the lid was placed on the tank and the run commenced. When the solvent reached the 10 cm mark, the plates were removed from the tank and dried standing at an angle in the fume cupboard with the fan on or in the Camag TLC Spray Cabinet II.

2) In an automatic developing chamber

When using this method, an automatic developing chamber (Camlab, Desaga Sarstedt - Gruppe) was employed. The advantages of using the developing chamber are: development of TLC plates automatically without supervision, generation of reproducible results, low solvent consumption, protection of plates from the atmosphere and from light and removal of solvent vapours by an integrated fan after

development has been accomplished. Because it operates with a sensor to recognise the solvent front in order to report the exact development time, only translucent plates (glass) have to be used. However only one plate can be placed for development at any particular time.

The following parameters were programmed:

- preconditioning time or equilibration: the dry plate was conditioned in the vapour without contact with mobile phase.
- maximum time: a safety parameter, serving to terminate chromatography if the front height was not reached because of too little mobile phase.
- height of front: maximum 18 cm, the sensor recognising the front as a result of the differing reflection properties of dry and wet areas of the TLC plates.
- drying time.
- signal tone: provided an acoustic warning of the completion of the chromatography.

4.5.4.5 Solvents

Solvents were measured in graded cylinders, poured into a conical flask, mixed thoroughly and then poured into the chromatographic tank / developing chamber.

The following combinations of solvents were used:

- toluene: ethyl acetate: formic acid, 5:4:1 (TEF)
- chloroform: acetone: 2 - propanol, 85:15:20 (CAP)
- toluene: acetone: methanol, 5:3:2 (TAM)
- chloroform: methanol, 93:7 (CM)

From the various combinations tested, the solvent systems yielding optimum separation were: TEF for one way TLC and for the two way TLC, solvent one was chosen to be CM and solvent 2, TEF.

4.5.4.6 Detection

The visualisation of TLC plates was done under white light and UV light (short and long wavelength). The colourless compounds were treated with a suitable reagent, which made them visible by reacting with them to produce coloured spots. In order to visualise the trichothecenes group under the UV light; two ways of derivatization were used:

1) By spraying

Spraying TLC plates with aluminium chloride ($AlCl_3$) and sulphuric acid (H_2SO_4) using spray guns (Shandon Scientific Company Ltd. or Merck Type:GW 1).

- $AlCl_3$ solution as spray reagent. 20 g $AlCl_36H_2O$ were dissolved in 100 ml alcohol -H_2O (1+1)
- H_2SO_4 solution as spray reagent

The cloud of droplets was directed from the jet towards the plate 25-40 cm away and the bottle moved to cause the spray to traverse the plate. Spraying was carried out evenly and lightly and over spraying was avoided.

Although spraying allowed the visualisation of mycotoxins present, the aerosol distributed the spray reagent unevenly over the surface of the plate. This led to difficulties (inaccurate/inconsistent) in densitometric measurements. Also higher concentrations of reagent were required and this was environmentally less acceptable. The aerosol creates fine particles in the atmosphere and so becomes a health hazard. Therefore, when the purchase of an immersion device was possible, the following method was used:

2) By dipping

Dipping was used together with Camag, Chromatogram Immersion Device III. TLC plates were immersed in aluminium chloride and sulphuric acid (8%) dipping solution. Dipping ensured uniform and reproducible reagent transfer, improved detection limits and increased reproducibility. The dipping time and distance were

also set in order to standardise the method. By using the dipping technique, it was possible to lower the H_2SO_4 concentration to 8% with no loss in spot resolution. Using this method, an even film was created over the entire TLC plate surface, which made it more desirable for quantification purposes and also reduced the amount of chemicals in the atmosphere.

- $AlCl_3$ solution as dipping reagent. 1.5 g $AlCl_36H_2O$ were added to 15 ml H_2O and 85 ml alcohol. On a steam bath, these ingredients were mixed until dissolved.
- H_2SO_4 as dipping solution. 80 ml H_2SO_4 in 920 ml distilled water was chosen as the proportion that gives better results.

After spraying or dipping, the plates were heated in the oven (Mino/30/Clad) at 110-120°C for 5-10 minutes (when spraying) and 15-20 minutes (when dipping). The reason the time was increased is that silica gel plates that had been dipped absorbed more liquid and they took longer to dry.

The advantage of using aluminium plates is that they are easy to cut in sections and used for development of techniques.

The advantages of using glass plates are: they do not curl up at the corners while in the oven compare to the aluminium ones, they are better secured in the densitometer compare to the aluminium ones which have to be fixed with blue tack on a glass plate.

The R_f values and colours obtained after the visualisation procedures were compared with the standards.

4.5.5 Densitometry

Quantification of mycotoxins was achieved with Camag Densitometer CD60. The CD60 is a PC-controlled device for absorption and fluorescence measurements in remission or transmission geometry. The diagram of the optical path of commercially available densitometers for absorption scanning, is shown on page 1185, AOAC

methods. The standard software (program version 4.0) includes the control of the instrument and the evaluation and documentation of the measured data and results adapted to good laboratory practice (GLP).

Most of the menu points in the main menu consist of several submenus.

The program includes:

1 Manual control

2 Processing of methods

3 Measurement of chromatograms

4 Measurement of spectra

5 Assisting programs

6 User programs

7 Configuration

8 Ending of the program

In order to perform the quantification of the existent mycotoxins, a method has to be created. A start method was automatically loaded and parameters set as required by editing it ("Editing of Methods"). Once a method was created, it was saved and loaded when required.

Using the submenu "Manual Control", one had to select, in the area of input, between the different parameters, as follows:

- Stage X, Y.

  Where X = position that corresponds to a measuring lane (in mm)

  Y = position that corresponds to the start point of measurement on the selected lane (in mm)

  In the display area the actual signal is displayed numerical.

- Monochromator: Lambda.

  This step enabled the setting of the wavelength (increase or reduce lambda)

- Select type of signal
  - Remission/ transmission
  - Extinction/ fluorescence
  - Signal positive/ signal negative
  - Tungsten-deuterium lamp/ mercury arc lamp

- Set scaling. At this stage, the length of the measuring lane and the scaling of the graphic window for manual measurement of chromatograms or spectra were determined. The actual position of the stage in Y-direction (the Y-position) was used as the start point of the measurement and as origin of the abscissa in the graphic display when taking a chromatogram. The abscissa in this case was the Y-position, while in the case of measuring spectra the abscissa was the wavelength. With "Y-value from start point", the distance to be evaluated was set, starting at the actual Y-position. The value Y-interval added to the actual Y-position of the stage gave the end values of the abscissa in the graphic display when measuring chromatograms.

Parameter of the signal processing. At this stage, the following parameters can be controlled:

- Resolution of measurement.
- Number of measurements per point = the number of measurements taken in one single position and thus the speed of measurement. By increasing the number of measured data per point the signal/noise ration is improved. The standard setting for measurements is "8".
- Smoothing factor. The smoothing factor determines the degree of smoothing of the chromatograms and spectra immediately after the measurement. This factor was set to "0" for the reason that when smoothing data, peaks may be reduced in height or small peaks can be suppressed.
- Automatic zero compensation. The automatic zero compensation determines whether the value at the start of the measurement is used as point of reference-chromatograms start automatically at the origin - or not.
- Background correction. The background correction is only possible if meander shaped measurements are used.
- Slit height/ slit width. At this stage, the slit height and slit width of the scanning light beam were entered.
- Define meander

- Chromatogram. Using this menu item, measurements and analyses of the chromatograms were done, using the following functions: Measurement: with this command a chromatogram was measured and when the end value of the abscissa was reached, the measurement ended automatically. Window: by entering numbers within this function, new diagram-limits were set. Zoom: with this function, a part of the graphics was cut out to display it in full-size.
- Spectrum. Using this menu, we measured "manually" the spectrum of interest. The wavelength axis reaches from 200-700 nm. This function is used in order to confirm whether a component is the same as a standard. Under the diagram, the following points appear: measurement, window, zoom, new plot, printing, etc. these functions being identical with the ones available for chromatograms.
- Chromatogram/ spectrum save

The wavelength method was chosen from the software options and used to find the optimum wavelength for the standard components (see Appendices, section 8.2, Figure 8.2). Then, the external standard method was used in order to locate the R_fs of standard components on the lane. This information was programmed into the method together with standard concentration and used to quantify sample components relative to standard.

The lane containing the standards was scanned first in order to find the optimum wavelength for the standard mixture (274 nm was chosen). The prepared TLC plates were fixed onto the mobile track and using the software (program version 4.0) provided, the parameters were keyed in, then the scanning procedure begun. The lanes were scanned one by one and the respective chromatogram was visible on the monitor. One has to select whether the peak area or the peak height is used for the calibration. If slim peaks are present calibration to the height is more favourable while with broad peaks calibration to the area is more suitable.

When all the lanes from the plate had been scanned, a printout of peaks list and results was obtained and carefully studied. When needed, several scans were performed on the same plate. Apart from comparing sample chromatograms with

standard ones, a spectral analysis was also performed in order to enable the identification of substances.

Table 4.4 Details of necessary equipment for mycotoxin quantification by densitometry

Apparatus	Specifications	Components
Densitometer	Densitometer CD60, 230 V Desaga GmbH Maaβstraβe 26-28 69123 Heidelberg	lamps: deuterium lamp: 200-339nm tungsten halogen lamp: 340-700nm mercury arc lamp: fluorescence excitation photo multiplier: -remission -transmission -reference
Computer	IBM PC AT or compatible mathematic coprocessor harddisk at least 40 MB 1 drive 1.2 MB or 1.4 MB Free working buffer (RAM) 520 KB VGA - board and monitor 640x480 mouse driver (Microsoft compatible)	DELL - Pentium
Printer	HP or Epson compatible printer	Hewlett Packard, Deskjet 660C

4.5.6 Decontamination procedure

At the end of determinations, all glassware and plasticware in contact with mycotoxins or extracts were immersed in sodium hypochlorite solution (1%) for 15-20 min, then immersed in disinfectant (Virkon solution 1%) for 2 h and finally washed by standard procedures.

4.5.7 Disposal of waste material

The microbial waste was rendered safe either by autoclaving at 121°C for 15 minutes or by incineration.

The Pasteur pipettes, syringes, needles used with mycotoxin standards and extracts were immersed in hypochlorite (1%) for at least 20 minutes, then transferred in cinbins (sharp container, Labco Ltd.) for disposal.

4.6 Fluorescence microscopy

Each colony was tested at three sites. For each site a slide (76x26x1, 0-1,2 mm thick) was prepared using an aqueous solution of 0.01% Calcofluor white (aqM2R) and the entire area examined under a Leitz Ortholux II epi-illumination fluorescence microscope (Leitz Wetzlar Germany Type 307-148.002 514 687 D69027), fitted with a mercury vapour UV light source, using a x40 objective and a system filter 1 G (UV light, BP 350-460 nm, RKP 510 nm, LP 520 nm), k 460. Pictures were taken for the representative areas.

4.7 Trichothecene gene expression studies

4.7.1 General rules/precautions

The difficulties associated with the isolation of full length, intrinsically labile RNA are further compounded by the ubiquity of ribonuclease (RNase) activity. RNases are a family of enzymes that degrade RNA molecules through both endonucleolytic and exonucleolytic activity. It is therefore a prerequisite to ensure that both equipment and reagents are "cleaned" of nucleases from the beginning of the experiment. In order to achieve this, several steps were taken. Protective clothing (labcoat) and disposable gloves were worn at all times. The glassware was treated in the oven at 180°C overnight. The plastic ware was treated by autoclaving twice at 120°C for 15 minutes. All the buffers were treated with diethyl pyrocarbonate (DEPC) unless otherwise stated. DEPC is a chemical that binds to RNases and destroys them. Solvents and chemicals were kept aside for RNA use only. The working surface was kept clean at all times. RNAaseZAP (Ambion® Inc.) was used for decontaminating electrophoresis equipment, quartz cuvettes and equipment used for Northern blots. So, for most RNA-minded molecular biologists, saying that a reagent or apparatus is sterile is more than that, it is RNase-free.

4.7.2 Buffers and reagents

See Appendices, section 8.3.

4.7.3 Isolation of mycelia from liquid media

There were two methods used for isolation of mycelia from liquid media:

4.7.3.1 Collected on filter paper following freeze-drying

Mycelia were filtered under vacuum, through a 9.0 cm diameter C test filter paper (Whatman®, grade 181). Then, mycelia and paper were transferred into a sterile flat base reagent tube (Sarstedt), covered with muslin, fixed with autoclavable paper, wrapped in aluminium foil, dipped into liquid nitrogen and placed into the freezer at -70°C until required for freeze drying. Mycelia was then removed from the filter paper by scraping, using clean spatulas, and then transferred into 5.0 ml tubes (Kartell).

4.7.3.2 Collected wet immediately after filtration

Mycelia was filtered under vacuum through 3 filter papers 5.5 cm diameter, scraped from the filter paper and transferred into a 5.0 ml tube (Kartell), dipped into liquid nitrogen, then placed into the -70°C freezer until required for freeze drying.

The second method would be recommended due to better recovery of mycelia from the filter paper.

4.7.4 Isolation of RNA from fungal mass

The isolation of high quality RNA from whole cell samples is the first critical step in the evaluation of a model system.

Guanidine was chosen for extraction of RNA because lysis buffers that contain guanidinium yield the highest quality samples (Logemann *et al.*, 1987). This is due to the extreme chaotropic nature of these chemicals; they are among the most effective protein denaturants. The efficiency of protein denaturation (including disruption of RNases) may be enhanced by the inclusion of β-mercaptoethanol; this reducing agent acts to break intramolecular disulphide bonds.

Fifty ml of 8 M guanidine were added to a 50 ml Greiner centrifuge tube. The pH of guanidine was checked and adjusted to 7.00 and the solution was filter sterilised. Then, in a fume hood 175 µl of β-mercaptoethanol were added to the sterilised guanidine. The tubes with dried mycelia were placed on ice and 3 glass beads (5-8 mm diam.) were introduced into each tube. The samples were ball milled (Glen Creston 8000 Mixer Mill) until a fine powder was obtained, then 2.0 ml of guanidine were added to each tube followed by a short milling. In the fume hood the content of each tube was transferred to a 15 ml Greiner tube and the remainder of the sample was washed with more guanidine up to 7.00 ml in total, in order to retrieve all the sample. An equal amount (7.00 ml) of phenol: chloroform (5:1, Sigma) was added to each tube. The tubes were well shaken by hand and left on ice for 30 minutes. After that, the tubes were centrifuged at 4°C, 3000 rpm for 20 minutes. Two sets of 15 ml tubes were prepared and labelled; one (P: C) and the other (C). After centrifugation, the upper layer was transferred to the tubes labelled (P: C) using sterile pipettes and an equal amount of phenol: chloroform (1:1) was added. The tubes were centrifuged for another 20 minutes, the transparent upper layer being transferred into the second set of tubes (C) and an equal amount of chloroform added. The tubes were centrifuged for another 20 minutes under the same conditions and the transparent layer transferred to oak ridge centrifuge tubes (Nalgene®). This time ethanol (0.7 of the sample volume) and acetic acid (0.2 of the sample volume) were added. The closed oak ridge tubes were gently inverted and rolled by hand, then left at –20°C overnight for the RNA to precipitate. The following day, the tubes were centrifuged at 4°C, 10000 rpm for 15 minutes and the resulting supernatant discarded. Five ml of sodium acetate were added to each sample and the tubes shaken until the pellet went back into solution/resuspended. Following a centrifugation of 15 minutes the supernatant was removed and another 5.0 ml sodium acetate added to the samples. After 15 minutes of centrifugation the supernatant was removed and 5.0 ml of ethanol (70%) were added to each sample. The tubes were spun for 5 minutes, the supernatant discarded and the tubes placed upside-down on a paper towel and the RNA pellet dried for 30 minutes. DEPC treated water was added (300-600 µl) and the tubes centrifuged for one minute at 4°C and 10000 rpm. The tubes were then

placed in a water bath set at 65°C, for 5 minutes. The contents of the tubes were transferred into 2 ml screw cap tubes, and 0.1 volume sodium acetate and 2.5 volumes of ethanol added to each sample. The tubes were then closed, well mixed and placed at -70°C overnight. The following day, samples were centrifuged for 15 minutes at 4°C, 10000 rpm. With a filter tip pipette, the ethanol was removed and discarded into a beaker, and one ml of ethanol (70%) was added to the sample. The tubes were well shaken by hand and then centrifuged for 15 minutes. The ethanol was removed with a pipette and tubes allowed to dry upside down for ~10 minutes. DEPC water (300 µl) was added to samples, which were subsequently placed in a water bath set at 65°C, for 5 minutes to resuspend the RNA pellet. Tubes were then centrifuged for 1 minute, and the samples were prepared for quantification by spectrophotometry.

4.7.5 Gel preparation

Two types of gels were used in this study.

4.7.5.1 Agarose gels

The required amount (depending on the percentage required) of agarose was weighed, Tris-acetate-EDTA (TAE) and/or Tris-borate-EDTA (TBE) buffer added, microwaved until molten, mixed and microwaved again until boiling. The gel was allowed to cool down to ~60°C then cast into a gel tray to a height of 0.5-0.75 cm.

4.7.5.2 Formaldehyde agarose gels

Formaldehyde is a commonly used denaturant of RNA. Although it is a suspected liver carcinogen, formaldehyde can be easily and safely manipulated in a fume hood.

Formaldehyde was added to the gel in order to maintain the denatured state of RNA during electrophoresis.

1) 1.2% gel. An amount of 1.2 g of agarose was weighed; 87 ml of DEPC treated water added to it, then microwaved. The solution was allowed to cool down and 10 ml 3-[N-morpholino] propanesulfonic acid (MOPS) added to it. In a fume hood, 3.0 ml of formaldehyde (37%) were added to the gel and then poured into the casting tray. The gel was allowed to set, then submerged into 1xMOPS.

2) 1% gels. An amount of 0.5 g of agarose was weighed; 42.25 ml of DEPC treated water added to it, then microwaved. The solution was allowed to cool down, then 5.0 ml 10xMOPS and 2.75 ml formaldehyde (37%) were added, the contents were mixed and poured into the tray. The gel was allowed to set, then submerged into chilled (4°C) running buffer (1XMOPS).

4.7.6 Sample preparation

4.7.6.1 DNA

To the samples, loading buffer (see App. 8.3.12-1) was added prior to electrophoresis in order to increase its density. In addition it contains a visible dye which allows the direction of the electrophoresis to be monitored.

4.7.6.2 RNA

In order to ensure that the RNA migrates only with respect to molecular weight, samples of RNA were denatured with both formaldehyde and formamide before electrophoresis.

1) To the RNA samples, the following were added: 2.5µl MOPS, 4.5 µl formaldehyde 37% and 12.5 µl formamide. Instead of adding one component at a time, a master mix solution was prepared, mixed and dispensed into the 1.5 ml microcentrifuge tubes containing the samples. The tubes were left for 15 minutes in a water bath set at 55°C, then placed on ice until required for loading on the gel. Prior to loading on the gel, loading buffer (see App. 8.3.12-2) was added. This protocol was used for experiment 9.

2) To 5.7 µl RNA sample, the following were added: 1.0 µl 10x MOPS buffer, 3.3 µl of formaldehyde and 10.0 µl of formamide. The sample tubes were heated at 55°C for 15 minutes to denature secondary structures within the RNA and chilled on ice prior to gel loading. Before loading the formaldehyde gel the gel loading buffer was added. This protocol was used for experiment 10.

4.7.7 Ladders used

Different ladders were used throughout the molecular studies, depending on whether the sample was DNA or RNA.

4.7.7.1 DNA

Two types of DNA Ladders were used:

1) Low DNA mass ladder (GibcoBRL, 200 µl)

Electrophoresis of 4.0 µl of DNA Mass Ladder results in bands containing 10, 20, 40, 80, 120, and 200 ng of DNA, with fragment sizes of 100, 200, 400, 800, 1200 and 2000 bp respectively.

2) DNA Molecular Weight Marker II (Boehringer Mannheim)

There are 50 μg with a concentration of 250 μg/ml, showing 8 fragments ranging from 0.12-23.1kbp.

4.7.7.2 RNA

Three types of RNA ladders were utilized:

1) RNA Ladder 0.24- 9.5 kb (GIBCO BRL, 75 μg. 75 μl)

This ladder was used for agarose gel electrophoresis. One 3.0 μl application of ladder contains approximately 0.5 μg of each RNA component (0.24, 1.4, 2.4, 4.40, 7.5, 9.5 kb).

2) RNA Markers (mouse) G319A 6761701, Promega Madison WI USA

Two μl of this RNA ladder (50 μg 1mg/ml) gives 9 bands with a range of sizes from 281-6583 bp. This marker was used with agarose gels for photography purposes.

3) RNA molecular weightmarker III, DIG-labelled (0.3 -1.6kb), Boehringer Mannheim GmbH

This marker was used with formaldehyde gels. One application of approximately 10 ng/μl molecular weight marker III gives five fragments as follows: 0.3, 0.4, 0.6, 1.0 and 1.6 kb.

4.7.8 Electrophoresis

Gel electrophoresis involved the loading of a small sample of DNA and/or RNA into a preformed well in the gel, which was then subjected to an electric current in a horizontal tank, via an ionic buffer solution (TAE of TBE), for a set amount of time depending on the agarose concentration, gel size, voltage applied and type of nucleic

acid sample. This technique effectively separates fragments of DNA and/or RNA on the basis of molecular size. As the electric field was applied across the gel, DNA and/or RNA, which are negatively charged at neutral pH, migrated towards the anode.

The rate of migration is dependent on:

- the molecular size of the nucleic acids (larger molecules migrate more slowly)
- the agarose concentration of the gel (there is a linear relationship between agarose concentration and efficient range of separation)
- voltage (at low voltage the rate of migration is proportional to the voltage applied). To obtain the maximum resolution of DNA fragments greater than 2 kb in size, agarose gels should be run at no more than 5.0 v/cm.
- the composition of the electrophoretic buffer (in buffers of high ionic strength electric conductance is very efficient)

The running electrophoresis parameters included:

Setting voltage: 120V or 100V

Time 1.5 h or 1 h,

However this depended on the electrophoresis system used.

4.7.9 Staining procedure

A stock solution of 10.00 mg/ml ethidium bromide was prepared and not more than 0.5 μg/ml used for staining. Ethidium bromide was added to distilled water, then the gel placed into the solution and left in the dark for 15 minutes. Ethidium bromide is a very powerful mutagen used for visualization of nucleic acids in gels under UV light. Consequently contaminated buffers and gels were treated as toxic waste.

4.7.10 Photodocumentation of electrophoresis gels

Photographs of the gels were obtained by using UV illumination, due to the fluorescent yield ethidium bromide/DNA or RNA complexes emit. The gels were placed onto the transilluminator in connection with a camera (Kaiser RA 1 Germany) and a gel documentation system IS-500 Flowgen (Alpha Innotech Corporation). Images were captured digitally, saved and printed on thermal paper using a Seikosha digital printer.

4.7.11 Quantitation of nucleic acid samples

Two methods were used for quantification of nucleic acid samples.

4.7.11.1 Spectrophotometric method

The reading was performed using a spectrophotometer (Beckman DU - 65) and Nucleic Acid Soft-Pac™ Module. Nucleic acid quantitation was accomplished by taking advantage of the absorbance of UV light by both DNA and RNA. This was used to derive not only information about the concentration of the sample but also its purity. Quantitation of nucleic acid samples in this fashion was predicted on the fact that nucleic acids absorb light maximally at 260 nm. This measurement permitted the direct calculation of the concentration in a sample:

[DNA] μg/ml = A_{260} x 50 x dilution

[RNA] μg/ml = A_{260} x 44.19 x dilution

where: A_{260} = absorbance at 260 nm
50 = extinction coefficient of DNA
44.19 = extinction coefficient of RNA
dilution = dilution factor

Calculation of the A_{260}: A_{280} ratio provided a reasonable estimate of the purity of the preparation. A pure sample of DNA had an A_{260}: A_{280} ratio of 1.8 ± 0.05; a pure sample of RNA had an A_{260}: A_{280} ratio of 2 ± 0.05.

4.7.11.2 Non-spectrophotometric method

This method involved the comparison of the samples with a DNA and/or RNA mass ladder on an agarose gel. The evaluation was done visually and with the aid of the image analysis system, estimating the molecular size of DNA and/or RNA by comparing the migration of the bands with that of size standards separated on the same gel.

4.7.12 Probes used for hybridization - details

In an investigation involving hybridization between complementary molecules, the selection of the type of nucleic acid probe best suited for a particular application is just as important as the methodology by which hybridization events will be quantitated and localized. The role of the probe is to hybridize to every complementary sequence in a hybridization reaction mixture. Nucleic acid probes have numerous applications, one of which is that they can be used to detect quantitative or qualitative changes in gene expression. A nucleic acid probe has a label incorporated into the molecule, which will permit its detection (localization and quantitation) at the conclusion of the experiment. One question is whether to use radioactive isotopes or not? A more pressing one is when can nonisotopic labelling and detection by chemiluminescence replace the use of radiolabelling and autoradiography? In general, chemiluminescence technologies are completely compatible with the Northern blot analysis and dot blot analysis.

Whereas it is true that the kinetic behaviour of some families of nucleic acid molecules can be predicted with a fair degree of accuracy, knowledge of the

parameters that directly influence hybrid formation is invaluable in fine-tuning the conditions of hybridization. This becomes a paramount issue when using probes that are not exactly complementary to the target (not an exact match), or when using a nucleic acid probe to screen blots or libraries for evolutionary relatedness. It should be recalled that temperature and salt concentration are the two variables most often adjusted in order to modify the specificity of hybridization (= stringency), when such modifications become necessary.

4.7.12.1 Probes obtained from John Innes Centre, Norwich

The probes (β-tubulin, *Tri5* and *Tri6*) were generated in Norwich, by PCR amplification of the three sequences from genomic DNA of *F. sporotrichioides*. They were produced and supplied by Dr. Paul Nicholson, Cereals Research Laboratory. The primer sequences are given in Table 4.5. β-tubulin and *Tri5* probes were received at first as band fragments in an agarose gel and later, the probes β-tubulin, *Tri5* and *Tri6* were received as PCR products.

Table 4.5 Probes obtained from John Innes Centre, Norwich - primer names and sequences

Gene target of primers	Primer name	Primer sequence
β - tubulin	*Bt2a*	GGTAACCAAATCGGTGCTGCTTTC
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC
	B531	GACTGACCGAAAACGAAGTTG
Tri 5	*Tri5F*	AGCGACTACAGGCTTCCCTC
	Tri5R	AAACCATCCAGTTCTCCATCTG
Tri 6	*T6FSp*	CATGCCAAGGACTTTGTCCC
	T6RSp	CACTTGTGTATCCGCCTATAGTG

4.7.12.2 Probes obtained from the Mycotoxin Research Unit, National Centre for Agricultural Utilization Research, USA

Probes were received in a plasmid vector (see Table 4.6) with *Tri4* host as *E. coli* (XLI Blue) and *Tri6* host as *E. coli* (INVαF1) on LB+ampicillin slopes. They were produced and supplied by Dr. Thomas Hohn.

Table 4.6 Plasmid probes obtained from the Mycotoxin Research Unit, National Centre for Agricultural Utilization Research, USA - plasmid names and restriction enzyme sites for subsequent probe isolation

Gene probe	Plasmid name	Restriction enzyme sites
Tri4	pTRI4script (CDS for TRI4 cDNA) Cloned in pCR-Script SK+ (Stratagene)	PvuII
Tri5	pTS22-1*	XhoI-SacI (insert size 1100 bp)
Tri6	pTRI6HisI (CDS for TRI6)	EcoRI-BamHI (insert size 641 bp)

4.7.12.3 Probe obtained from *Fusarium sporotrichioides*

Fusarium sporotrichioides mycelia was collected (see 4.7.3.1) and used for the preparation of the ribosomal DNA probe employed in Northern blot hybridization.

4.7.13 Probes - preparation

Several approaches were used in order to bring the probes to a "working" state.

4.7.13.1 Probes obtained from Norwich

β-tubulin and *Tri5* probes were initially received as band fragments in an agarose gel (see 4.7.12.1). Purification of DNA bands from agarose was done using the WIZARD™ PCR Prep Purification kit from Promega (see 4.7.13.5-1). The probes (β-tubulin, *Tri5* and *Tri6*) received later as PCR products, were resuspended in TE buffer to a concentration of 10 ng/μl, heated for an hour in a dry block at 50°C, aliquoted and stored at -20°C until required.

4.7.13.2 Probes obtained from USA

E. coli containing the plasmid probes received from the USA (see 4.7.12.2) were streaked onto Petri dishes (9 cm) containing LB medium+ampicillin and transferred into the incubator set at 37°C, for overnight. Also, LB medium (2.00 ml) was inoculated with bacterial colonies and subjected to vigorous shaking overnight, at 37°C, followed by plasmid purification. One approach of probe preparation employed direct PCR (see 4.7.13.4-2 and 3) and an alternative method involved indirect PCR (see 4.7.13.5, 6 and 7).

4.7.13.3 Probe obtained from *Fusarium sporotrichioides*

A small amount of frozen *F. sporotrichioides* mycelia was added to 200 μl of sterile water and mixed using a pestle in the microcentrifuge tube. Then, 50 μl Chelex resin (20% in water) was added to the mixture and boiled for 10 minutes. The tube was then centrifuged for 30 seconds. For the preparation of the probe, 5.0 μl were added to 95 μl of PCR reaction mix, using the primers ITS1 and ITS4 (White et al., 1990) for the specific amplification of the ITS region of ribosomal DNA. The PCR product was loaded on a TAE gel (1.4%) and the band of interest purified with the DNAce purification system, Bioline Kit (see 4.7.13.5-3).

4.7.13.4 Polymerase chain reaction (PCR)

PCR was used to rapidly amplify specific DNA sequences from complex mixtures of nucleic acids. The basic procedure relies on the ability of thermostable DNA polymerase to replicate a specific DNA sequence through many cycles of denaturation, annealing, and strand elongation using short oligonucleotides as primers.

In order to perform PCR, the following components (see Table 4.7) were compiled.

Table 4.7 Components and concentrations needed for a 50 μl PCR reaction mix

Components	Vol (μl)	Conc in 50μl
dNTP mix	5.0	200 μM
5% NP40	0.5	
5%Tween 20	0.5	
Cresol	5.0	
25mM MgCl2	5.0	
H_2O ultrapure	24.25	
Taq polymerase	0.25	2.5 units
10xPCR buffer	5.0	
Template	2.0	
Primer Forward	1.25	100 nM
Primer Reverse	1.25	100 nM

1) PCR with specific primers

First of all, PCR with specific primers was used in order to quickly amplify the amount of probe received from Norwich. The protocol used for the relevant probes is shown in Table 4.8.

Table 4.8 PCR cycling parameters used with the specific primers recommended for β-tubulin, *Tri5* and *Tri6* probes

Probe/Primers	PCR parameters		
	Temperature(°C)	Time (minutes)	No. of cycles
β-tubulin	95	2.0	
Bt 2a and *B531*	95	0.3	
	60	0.3	} 30
	72	0.45	
	72	10.0	
Tri5	95	2.0	
Tr5F and *R*	95	0.3	
	66	0.2	}5
	72	0.45	
	95	0.3	
	64	0.2	}5
	72	0.45	
	95	0.3	
	62	0.2	}30
	72	0.45	
	72	5.0	
Tri6	95	2.0	
T6Fsp and *T6Rsp*	95	0.3	
	55	0.3	}35
	72	0.45	
	72	10.0	

2) PCR - T3/ T7 (promoters)

Another approach to PCR techniques was the use of this PCR Primer Set - Stratagene 302001 (5 µM working stock). Two µl of the working stock were used for every 50 µl reaction. As a template, a small sample of growing bacterial cells of *E. coli* was used. The T3/ T7 are universal primers, which flank the probe insert within the plasmid and can therefore be used to amplify the cloned insert.

Table 4.9 Details of PCR amplification protocol utilised when using *E. coli* and T3/T7 promoter

Probe/Primers	Temperature (°C)	Time (minutes)	No. of cycles
E. coli	94	5	
T3/T7	55	5	
	72	1.5	} 35
	94	0.45	
	55	0.45	
	72	10	

3) PCR - M13 forward and reverse

M13 was also used to amplify probe sequences. As DNA template, a small sample of growing bacterial cells (*Tri4*, *Tri5* and *Tri6*) was used. The protocol used is identical to the one used for T3/T7.

4) PCR for the *Fusarium sporotrichioides* ITS probe

ITS probes (see 4.7.12.3 and 4.7.13.3) were used to validate Northern blot signals obtained after hybridization with trichothecene gene probes.

Table 4.10 Details of PCR protocol utilized when using *Fusarium sporotrichioides* and ITS1 and ITS4

Template/Primers	Temperature (°C)	Time (minutes)	No. of cycles
F.sporotrichioides	94	1	
ITS1 and ITS4	52	0.45	}30
	72	1	
	72	10	

4.7.13.5 Cloning of probes

TA Cloning® KIT (Invitrogen®) was used to clone the probes in order to obtain a larger quantity from a limited amount of starting material. This method also allowed for the purification of the resultant probe. *Taq* polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The linearized vector supplied in this kit has single 3' deoxythymidine (T) residue. This allows PCR inserts to ligate efficiently with the vector. Several steps were involved in this procedure, namely production of PCR product, cloning into pCR 2.1, transformation and analysis and details are presented in Appendices (section 8.4).

4.7.13.6 Preparation of cloned probes

Extraction and purification of plasmid DNA

This method involves 3 steps, namely: growth of bacterial culture, harvesting and lysis of bacteria and purification of plasmid DNA, and details are presented in Appendices (section 8.5).

The quantity and quality of purified plasmids was verified by gel electrophoresis (see 4.7.8). The cloned insert was removed by restriction enzyme digest (see 4.7.13.7).

4.7.13.7 Digestion of DNA with restriction enzymes

Restriction enzymes bind specifically to, and cleave, double-stranded DNA at specific sites within or adjacent to a particular site of the DNA known as recognition sequence. Some restriction enzymes cut and leave "sticky" ends (containing overhanging bases) which are of great importance in cloning work as the sticky ends provide the preliminary structure for inserting plasmids or gene sequences. After cutting or "restricting" the DNA into a series of a specific restriction enzyme it is possible to separate the fragments and importantly, derive an estimate of their size by gel electrophoresis.

Digestion of the plasmid DNA is required to separate the probe DNA sequence from the plasmid DNA.

For a 10 µl reaction:

1.0 µl Buffer

0.8 µl Enzyme (for single digest and 0.4 µl when double digest)

4.0 µl Plasmid (purified vector containing probe sequence)

4.2 µl H_2O (ultra pure)

For a 50 µl reaction mix, 42 µl Sample, 5.0 µl Buffer and 3.0 µl Enzyme (or 1.5 µl when double digest) were used.

Table 4.11 Particulars of restriction enzymes and reaction buffers used for DNA digestion

Probe	Enzyme (10U/µl)	Reaction buffer
Tri 4	*Pvu* II	REACT® 6
Tri 5	*Xho* I *Sst* I	REACT® 2
Tri 6	*Eco*R I *Bam*H I	REACT® 3

The tubes were then incubated at 37°C in a water bath overnight.

An agarose gel (0.8%) using 1xTAE buffer was prepared and DNA mass ladder (12 µl Low DNA mass ladder + 10 µl loading buffer) and samples (50 µl +10 µl loading buffer) were loaded onto the gel, and run at 120V. The gel was stained for 15 minutes in ethidium bromide (see Plate 5.20) then visualized on the transilluminator. The bands of interest were cut and transferred into pre-weight microcentrifuge tubes awaiting the purification of DNA from the agarose gel (see 4.7.13.9). After purification the probe was labelled (see 4.7.14 -2).

4.7.13.8 Purification of DNA from agarose gels

Several protocols of DNA purification from agarose gel were employed. These include the following kits: WIZARD™ PCR Prep Purification kit, QIAEX II Gel Extraction Kit and DNAce purification system. Details of protocols are presented in Appendices (section 8.6).

4.7.13.9 Purification of plasmid DNA using InViSorb™ DNA Extraction Kit (Bioline)

This kit was used for extraction and purification of DNA from TAE agarose gels, TBE agarose gels or aqueous solutions, and the details are presented in Appendices (section 8.7).

The yield and purity of the extracted DNA can be verified on an agarose gel using standard control samples or by measuring the UV absorption spectrum.

4.7.13.10 QIAquick PCR Purification Kit Protocol (Qiagen Ltd.)

This protocol is designed to purify single- or double-stranded PCR products ranging from 100 bp to 10 kb from primers, nucleotides, polymerases, and salts, using QIAquick spin columns in a microcentrifuge. Details of the protocol are presented in Appendices (section 8.8).

4.7.14 Probe labelling

The nonradioactive DIG system uses digoxigenin, a steroid hapten, to label DNA, RNA or oligonucleotides for hybridization and subsequent colour or luminescence detection. For DNA labelling, DIG is coupled to dUTP via an alkali-labile ester-bond. The use of the alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for rehybridization experiments with a second DIG-labelled probe. DNA probes, labelled with DIG-11-dUTP, alkali-labile cannot be denatured by alkali treatment (NaOH), but must be denatured by incubation in a boiling water bath.

There were two ways of probe labelling used. DIG labelling by PCR and DIG labelling by random priming (using commercial kits).

4.7.14.1 DIG labelling by PCR

Polymerase chain reaction is a good method for probe synthesis. In the presence of the appropriate precursor, molecules are labelled as they are being synthesized. The protocol employed the use of Digoxigenin-11-dUTP alkali labile (25 nmoles in 25 µl). When performing PCR and labelling in the same time, 5.0 µl of water were replaced by 5.0 µl dUTP (7.0 µl dUTP in 1ml water).

4.7.14.2 Labelling by random priming

Random priming is a type of primer extension in which a mixture of small oligonucleotide sequences, acting as primers, anneal to heat-denatured double stranded template.

There were two commercial kits employed in probe labelling, namely: DIG High Prime DNA Labelling and Detection Starter Kit II and *Gene Images* random prime labelling module (Fluorescein).

1) DIG High Prime DNA Labelling and Detection Starter Kit II (Boehringer Mannheim GmbH)

DIG (Boehringer Mannheim) is widely used as a nucleic acid label, and supports chemiluminescent detection applications. DNA probes were synthesized by random primed labelling with DIG-dUTP (DIG-11-dUTP). The resulting DIG-labelled molecules function as hybridization probes in much the same manner as any other probe. DIG labelling supports posthybridization detection by chemiluminescence. Following the posthybridization stringency washes, DIG-labelled probes are detected

by enzyme-linked immunoassay, using an antibody conjugate (anti-DIG-alkaline phosphatase). The emission of light or the formation of precipitate is mediated by dephosphorylation of a substrate compatible with the method of detection.

DIG-labelled DNA probes are generated with DIG-High Prime according to the random primed labelling technique. DIG-High Prime is a specially developed reaction mixture containing all reagents necessary for random primed labelling, premixed in an optimized 5x concentrated reaction buffer. The use of premixed DIG-High Prime reduces pipetting steps and increases yield, reproducibility and convenience.

The labelling protocol was performed as follows: the template DNA (1μg) was added to sterile water to a final volume of 16 μl into a 1.5 ml microcentrifuge tube. Heating in a boiling water bath for 10 minutes, and quickly chilling on ice/ethanol bath denatured DNA. DIG-High Prime (4.0 μl) was added to the reaction tube followed by mixing and centrifuging. The reaction tube was incubated in a water bath set at 37°C. The following day, the reaction was stopped by heating at 65°C in a water bath for 10 minutes.

The quantification of labelling efficiency was done using DIG Quantification and DIG Control Teststrips.

The principle of quantification of labelling efficiency is the following. A series of dilutions of DIG-labelled DNA was applied to the marked squares on the DIG quantification teststrips whilst DIG control teststrips are pre-loaded with defined dilutions of a control DNA (300, 100, 30, 10 and 3 pg) and are used as standards. The teststrips are then subjected to immunological detection with anti-digoxigenin-AP conjugate and the premixed stock solution of NBT/BCIP. Following a shortened detection protocol, the results can be seen after approximately 30 minutes. The small format of the teststrips allows a significant reduction of the test solutions and reagents.

The labelled DNA was applied in a 1.0 μl spot onto the marked squares of a DIG quantification test strip and was air-dried for 2 min.

For each detection series, 5 microcuvettes (Sarstedt No. 67742, 10x4x45 mm) were prepared and labelled 1-5.

To vial 1: 2 ml of blocking solution were added.
To vial 2: 2 ml of antibody solution (1:2000 in blocking solution) were added.
To vial 3: 2 ml of maleic acid buffer were added.
To vial 4: 2 ml of detection buffer were added.
To vial 5: 2 ml of colour-substrate solution were added.

The prepared teststrips (one quantification teststrip and one control teststrip positioned back to back in the microcuvettes) were dipped in the prepared solutions in the following sequence and for the given times (see Table 4.12). Between steps, the excessive liquid was allowed to drip onto a paper tissue.

Table 4.12 Succession of detection steps and time allocated for each one

Succession of steps	Vial number	Detection steps	Time (min)
1	1	Blocking	2
2	2	Antibody washing	3
3	1	Blocking	1
4	3	Washing	1
5	4	Equilibration	1
6	5	Colour reaction (in the dark)	5-30

The colour reaction was stopped after a maximum of 30 minutes (extending the colour reaction for more than 30 min increases the background), by briefly rinsing the teststrips in water and then air-drying on Whatman 3MM paper, in the dark.

The evaluation of results was as follows: after 5-10 min of the colour reaction the 30 pg spot should be visible and after 30 min incubation, the complete result is available and the 3.0 pg spot should be visible on the control strip. The evaluation of the DIG-labelled DNA in the squares of the quantification teststrip was established by comparing the colour intensity with the control teststrip, the amount being estimated by taking into account the dilution steps.

2) *Gene Images* random prime labelling module (Fluorescein) (Amersham International)

Fluorescein is a hapten that can be incorporated by standard probe synthesis reactions. Fluorescein-labelled nucleotides, fluorescein-11-dUTP (Fl-dUTP), can be used to generate continuously labelled or end-labelled probes. Following hybridization, antibodies specific for fluorescein are used to localize hybridization events. The anti-fluorescein antibodies have been modified, existing as a conjugate with horseradish peroxidase, a chemistry that supports luminol-based chemiluminescence. It is also possible to make use of the intrinsic fluorescence of the hapten to monitor the labelling of the probe.

This protocol is designed to label nucleic acid probes by random prime labelling. Nonamers of random sequence are used to prime DNA synthesis on a denatured DNA template in a reaction catalyzed by the (exonuclease-free) Klenow fragment of E. coli DNA polymerase I. Fluorescein-11-dUTP (Fl-dUTP) partially replaces dTTP in the reaction so that a fluorescein-labelled probe is generated. There is net synthesis of probe in this reaction, with up to 350 ng probe synthesized from 50 ng template.
The preparation of the labelled probe was performed as follows:
The DNA to be labelled was diluted to a concentration of 2-25 ng/µl in distilled water. The following tubes, belonging to the kit were placed on ice: nucleotide mix, primers and water leaving the enzyme at -20°C until required.

The DNA was denatured (in a volume of 20 µl) by heating for 5 minutes in a boiling water bath, then chilled on ice.

To a 1.5 ml microcentrifuge tube, placed on ice, the followings were added:

Water to a final reaction volume of 50 µl	x µl
Nucleotide mix	10 µl
Primer	5 µl
Denatured DNA	50 ng
Enzyme solution (Klenow) 5 units/µl	1 µl

The contents of the tube were mixed gently by pipetting up and down, then the tube spun briefly.

The reaction mix was incubated in a water bath set at 37°C, overnight.

To monitor incorporation, a rapid labelling assay was used. A series of dilutions of 5x nucleotide mix in TE buffer were prepared (1/5, 1/10, 1/25, 1/50, 1/100, 1/250 and 1/500). Five µl of the labelled probe and 5.0 µl of the 1/5 dilution of nucleotide mix (negative control) were spotted on a strip of Hybond™-N+, placed on a non-absorbent backing (sterile plastic Petri dish). The liquid was allowed to be absorbed but not to dry and the strip was then washed with gentle agitation in excess pre-heated (at 60°C) 2xSSC for 15 minutes. A reference strip was also prepared by dotting 5.0 µl of each nucleotide mix dilution, except the 1/5, on to a separate strip of Hybond™-N+. This reference strip was wrapped in Saran Wrap™, stored at -20°C and reused whenever a comparison was needed.

The reference and the washed strips were placed on a piece of Whatman 3MM paper lightly moistened with TE buffer and taken into the dark room. Both strips were visualized (sample side down) on an UV transilluminator.

For a hard copy of the results the strips were photographed (see 4.7.10).

4.7.15 Dot blotting

Boehringer Mannheim's Nylon Membranes (Hybond™-N+) positively charged were used for the transfer because it is known to give best results. This membrane has an optimal charge density, allowing it to bind the nucleic acid tightly without producing background. The nylon membranes are also specifically tested with the DIG-system to ensure optimal signal-to noise ratio. Nitrocellulose membranes are not recommended in combination with the DIG-System. They can only be used when colorimetric detection will be performed and no stripping and reprobing is planned.

4.7.15.1 DNA

The denatured probes (*Tri5* and *Tri6*) were spotted in 1 and 2 µl onto the membrane (Hybond™-N+) which was then wrapped in cling film and fixed (see 4.7.17 Membrane fixation).

4.7.15.2 RNA

Before proceeding to Northern blot analysis, a dot blot analysis is worthwhile in order to assess the system for mRNAs of interest. This simple technique allowed the detection of relative amounts of given RNA. Dot blot analysis yields purely quantitative data.

Different concentrations of control RNA (DIG-labelled control RNA, 100 µg/ml, Boehringer Mannheim) and RNA samples were spotted using 1 µl volumes onto the membrane (Hybond™-N+) which was then wrapped in cling film in order to be fixed (see 4.7.17.2).

4.7.16 Northern blotting

The Northern blot analysis is one of the standard techniques by which RNA molecules are evaluated by size and abundance. Northern blot is the transfer of electrophoretically separated RNAs from a gel to a filter membrane for subsequent fixation and hybridization to a specific probe. Because the RNA is transferred in exactly the same configuration as it was separated in the gel, hybridization signals generated by Northern blot analysis provide a qualitative and quantitative profile of the sample under investigation.

On top of a plastic tray (Kartell 30x15x2h cm), a glass plate (20/20 cm) was placed. Chromatographic paper (3MM), cut bigger than the gel size was folded around the glass plate. 20xSSC buffer was poured into the tray, enough to reach the half of the tray's height. Using a sterile Pasteur pipette, the chromatographic paper was wetted with the buffer taken from the tray then a sterile pipette was rolled forward-backward / left-right in order to remove the air bubbles formed in between the paper and the glass. The gel of interest was taken from its tray, turned upside down and the corner on the side of the first lane was cut with a clean razor blade. Again, a sterile pipette was rolled onto the gel in order to remove the air bubbles. The edges of the gel were sealed with parafilm so that the transfer of buffer will be made only through the gel. The gel was measured with a ruler and a piece of membrane (Hybond™-N+) was cut to the exact size. The membrane was briefly immersed into DEPC treated water, then placed on top of the gel, cutting the same corner as a sign of the first lane. On top of the membrane two layers of Whatman paper with the same size as the gel were placed. The layers of chromatographic paper were wetted with the buffer from the tray using a Pasteur pipette and the air bubbles removed in the same fashion as before. Absorbent paper was cut in order to fit the size of the gel and stacked on top of the chromatographic paper up to 2.0 cm high when dry. On top of the absorbent paper another piece of glass similar to the first one placed on the tray was used. On top of the glass a weight was placed. This arrangement was left like this overnight. The following day, the weight from the glass was lifted, the stack of absorbent paper disposed-of and the stack of 2 layers of chromatographic paper-membrane-gel was

turned upside down so the gel would be on top. Then with a pencil, the wells were marked through the gel onto the membrane. The gel was stained in ethidium bromide and examined on the transilluminator in order to check the transfer and the membrane was rinsed in 20xSSC then wrapped in cling film and fixed (see 4.7.17). After fixing, the membrane was placed in the fridge until needed for hybridization.

4.7.17 Membrane fixation

There were two methods used for fixing the membranes: alkaline method and UV irradiation.

4.7.17.1 Alkaline

The alkaline method involved dipping the membrane placed onto Whatman paper into a tray containing NaOH 0.05M, rotating it gently for 5 minutes, then transferring it into the second tray containing 10 ml 20xSSC and 90 ml DEPC water, where it was left for 2 minutes. The membrane was then wrapped in cling film and stored in the fridge.

4.7.17.2 UV irradiation

This method involved crosslinking by UV irradiation, using a transilluminator. The membrane wrapped in cling film was placed sample side down on the transilluminator. When RNA was bound to the membrane, this was left onto the transilluminator for 10 min and when DNA was bound to the membrane, this was left for 2 minutes in order to fix the DNA and/or RNA irreversibly.

The second method was preferred due to better results.

4.7.18 Hybridization with non-radioactive probes

Hybridization between nucleic acid probe and target sequences consists of three major steps, namely prehybridization, hybridization and posthybridization washes.

4.7.18.1 Prehybridization

Before hybridization, filters were subjected to a prehybridization incubation, for two reasons: (1) in order to equilibrate the filter in a buffer identical or similar to the one to be used for the actual hybridization, and 2) in order to block the filter paper completely. Blocking means covering the entire surface of the filter that is not occupied by experimental RNA samples, with sheared, denatured (heterologous) DNA from a species unrelated to the biological origin of the RNA; usually salmon sperm DNA or calf thymus DNA is selected. This step is very important so that in the actual hybridization, probe molecules do not stick in a nonspecific fashion over the surface of the filter paper, which would cause unacceptable levels of background. Prehybridization was done using two different solutions, depending on the kit used for hybridization. Firstly, DIG Easy Hybridization solution was employed when using DIG High Prime DNA Labelling and Detection Starter Kit II in conjunction with DIG Wash and Block Buffer Set and secondly, Hybridization Buffer was used as recommended, when using *Gene Images* kit. Prehybridization solution was prewarmed to the temperature of hybridization and blots were prehybridized for at least 30 minutes.

4.7.18.2 Hybridization

Double-stranded probes were denatured before use in hybridization. This was achieved by placing the microcentrifuge tube containing the probe in boiling water for 10 minutes followed by a quick transfer on ice. After prehybridization, an aliquot of the pre-hybridization solution was removed from the hybridization tube and added

to the denatured probe, mixed well avoiding foaming (bubbles may lead to background) and transferred back into the hybridization tube, avoiding direct contact with the membrane. The tube was then inverted gently in order for the probe to disperse in the hybridization buffer, without formation of foam, and hybridization began. Hybridization was carried out in 50 ml Greiner tubes, in a hybridization oven (Hybaid Micro-4) with horizontal rotating action, overnight.

Some factors that influence the rate, specificity, fidelity and probable utility of hybridization probes include: temperature, ionic strength, pH, organic solvents (such as formamide), guanine and cytosine (G+C) content of probe, probe length, probe concentration, probe complexity, the degree of complementarity between probe and target sequences, and the viscosity of the system. The influence of each of these variables is dependent on the physical state of the nucleic acid molecules involved: solution hybridization (Nuclease Protection Assay analysis) versus mixed phase hybridization (Northern blot, in which the target sequences are immobilized on a solid support).

The conditions given in kits are stringent conditions, applicable if probe and target have 100% homology and a G plus C content of about 50%.

- **Temperature**

Perhaps the most frequently manipulated variable, which can either promote or prevent hybridization, is the temperature of the system. A range of temperatures had been used for hybridization performed throughout this study.

- **Ionic strength**

Stringency is dramatically influenced by increasing or decreasing the amount of salt in a hybridization buffer. The hybridization buffers used were prepared as recommended in the relevant kit.

- **pH**

One of the factors that profoundly influences the stability of double-stranded molecules is the pH of the environment. Most hybridization reactions are conducted at near neutral pH: alkaline pH buffers promote duplex dissociation, and highly acidic pH buffers may well result in depurination of both probe and target molecules.

- **Probe length**

The shorter the probe, the more rapidly hybridization occurs and the more discriminating the probe becomes. Moreover, the shorter the probe, the more influential many of the other variables become.

- **Guanine and cytosine (G+C content)**

The stability of a hybrid molecule is profoundly influenced by the base composition of the molecules involved. Because three hydrogen bonds exist naturally between guanine (G) and cytosine (C), GC-rich duplexes are thermodynamically more stable than adenine/thymine (AT)-rich duplexes, since only two hydrogen bonds exist naturally between them.

- **Mismatching**

Many of the recommended hybridization conditions that accompany filter membranes and molecular biology kits assume a perfect or nearly perfect match between probe and target sequences. Under stringent conditions, the most rapid duplexes to form manifest exact complementarity between probe and target.

- **Probe complexity**

With respect to hybridization, the complexity of the probe is the length of different probe sequences in the hybridization buffer that could potentially base-pair to complementary target molecules. In most cases, only a single type of probe would be used. With respect to hybridization, probe complexity is an important parameter; for example hybridization with a combination of two or more probes at inappropriate conditions may cause the probes to hybridize to one another rather than to the target RNA present on the membrane.

- **Viscosity**

The original studies of the effect of viscosity on nucleic acid hybridization showed a reduction in the renaturation rate as viscosity increased. One frequently used component in hybridization buffers is the anionic polymer dextran sulfate. Although dextran sulfate contributes to the viscosity of the milieu, the rate of hybridization is accelerated because the volume of the hybridization buffer occupied by dextran sulfate effectively concentrates the probe. A similar phenomenon occurs with the inclusion of Denhardt's solution in various hybridization solutions. It is an excellent alternative to dextran sulfate and alleviates the severe background problems frequently associated with dextran sulfate in hybridization recipes.

The stringency at which the hybridization and posthybridization washes are conducted must be attuned to accommodate the chemistry of the polynucleotides involved.

One of the fundamental drawbacks of filter-based hybridizations is that complete hybridization is not guaranteed, because some of the membrane-bound target sequences may not be fully accessible to probe sequences. Theoretically, complete hybridization is difficult, if not impossible.

1) DIG High Prime DNA Labelling and Detection Starter Kit II

DIG Easy Hyb, a ready-to-use, non-toxic hybridization solution, especially developed for DIG hybridization was used. DIG Easy Hyb (2.5 ml/100 cm^2 membrane) was pre-heated to the required temperature and added, together with the denatured probe (5-25 ng/ml) to the membrane.

2) The *Gene Images* CDP - *Star* Detection protocol

Following labelling, the probe can be denatured and used directly in hybridization. An advantage of using fluorescein-labelled probes is that it is generally unnecessary to remove unincorporated Fl-dUTP prior to use of the probe in hybridization. In addition, fluorescein-labelled DNA is stable under standard hybridization conditions and the stringency of hybridization can be controlled either with temperature or salt concentration.

The required volume of hybridization buffer (0.125 ml/cm^2 =10 ml) was pre-heated to the appropriate temperature and the blots placed in the buffer and left to pre-hybridize for at least 1 hour with constant gentle agitation (in the oven).

The amount of probe needed (10 ng/ml max) was transferred to a clean microcentrifuge tube using sterile pipette tips with filter. If the probe volume was less than 20 µl, it was brought up to this with DEPC water. The microcentrifuge tube was placed in boiling water for 5 min and then snap cooled on ice. The denatured probe was centrifuged briefly, then added to the pre-hybridization buffer avoiding the direct contact with the blot, mixed gently, transferred back into the hybridization oven and left rotating overnight.

For subsequent reactions, pre-hybridization was done using the hybridization buffer. For the hybridization step, the buffer was replaced with the hybridization buffer+probe used previously and stored at –20 °C. Prior to adding it to the pre-hybridized blot, the hybridization buffer+probe had to be defrosted, left in boiling water for 10 minutes and snap cooled on ice.

4.7.18.3 Posthybridization Stringency Washes

Depending on the nature of the probe and the stability of the hybrid, the investigator may have to adjust the exact posthybridization washing conditions for each probe. It is very important to keep in mind that in posthybridization washes, stringency is a function of ionic strength, temperature, and time; thus, it may not be suitable to cut down on the washing time by increasing the stringency of the washes.

Several combinations of temperature between the primary and secondary wash were used.

1) DIG High Prime DNA Labelling and Detection Starter Kit II + DIG Wash and Block Buffer Set

- **Primary wash buffer**

2xSSC+0.1%SDS was pre-heated to the required temperature, blots transferred into the solution (20 ml/time) and washed twice for 5 minutes.

- **Secondary wash buffer**

0.2xSSC+0.1%SDS or 0.5xSSC+0.1%SDS were pre-heated to the required temperature, blots transferred into the solution and washed twice for 15 minutes.

2) The *Gene Images* CDP - *Star* Detection protocol

- **Primary wash buffer**

1xSSC+0.1%SDS was pre-heated to the required temperature, then the blot transferred into the solution (using 2-5 ml/cm^2 of membrane=25 ml) and washed for 15 minutes with gentle agitation on a horizontal shaker.

- **Secondary wash buffer**

0.1xSSC+0.1%SDS was pre-heated to the required temperature, blots transferred into it and washed for 15 minutes with gentle agitation on a horizontal shaker.

4.7.18.4 Blocking, antibody incubation and washes

1) DIG High Prime DNA Labelling and Detection Starter Kit II

After one minute equilibration with washing buffer (25 ml), to the membrane transferred into a clean tube, 50 ml of blocking solution (10 ml maleic acid: 10 ml blocking solution: 30 ml DEPC water) were added. The tube was left rotating in the oven at room temperature for 30 minutes.

Anti-DIG-AP conjugate (5 µl) was added to 50 ml blocking solution and mixed. Twentyfive ml of this mixture replaced the blocking solution and was left one hour rotating in the oven, at room temperature.

After antibody binding, the membrane was washed 2x15 minutes in 25 ml wash buffer followed by equilibration.

2) The *Gene Images* CDP - *Star* Detection protocol

The blots were incubated with gentle agitation for 1 hour at room temperature in approximately 0.75-1.0 ml/cm^2 (50 ml/ membrane) of a 1 in 10 dilution of a liquid blocking agent in Buffer A.

The anti-fluorescein-AP conjugate was diluted 5000-fold in freshly prepared 0.5% bovine serum albumin in buffer A. The blots were incubated in this conjugate (0.3 ml/cm^2 of membrane) with gentle agitation at room temperature for 1 hour.

The unbound conjugate was removed by washing the blots 3x10 minutes in 0.3% Tween 20 in buffer A at room temperature with gentle agitation. An excess volume is again used (2-5 ml/cm^2).

The excess washing buffer was drained off from the blots by touching the corner of the blot against the box used for washing and then placed sample side up on a transparent sheet.

4.7.19 Signal generation and detection

4.7.19.1 DIG High Prime DNA Labelling and Detection Starter Kit II

The membrane with nucleic acids side facing up was placed on a sheet of transparent acetate and 0.5 ml CSPD®, ready to use solution added to it. Immediately, the membrane was covered with the second sheet of transparent acetate and the substrate was spread evenly in the same time removing the airbubles formed, using a roller After 5 minutes incubation at room temperature, the membrane was transferred using clean forceps onto cling film, wrapped in it, then incubated for 15 minutes at 37°C in order to enhance the luminescent reaction. In the dark room, the wrapped blot was placed in a cassette with sample side up and a film (Lumi-Film, Chemiluminescent Detection Film, Boehringer Mannheim) was placed on top of the blot. The cassette was closed, kept at room temperature, and the exposure time decided according to signal intensity. Luminescence continues for at least 24 hours and signal intensity increase during the first hours.

4.7.19.2 The *Gene Images* CDP - *Star* Detection protocol

Consequently, exposures to film made in the first few hours after addition of the substrate to a membrane will produce a much stronger signal than previously.

Following hybridization, this detection module allows detection of hybrids (after a blocking step) by incubation with an anti-fluorescein alkaline phosphatase (AP) conjugate. After washing off the excess conjugate, probe-bound AP is used to catalyze light production by enzymic decomposition of a stabilized dioxetane substrate.

The detection reagent was pipetted onto the blots (30-40 µl/cm^2= 1980 µl / membrane) and the blot covered with another transparent sheet. All air bubbles were removed. After 5 minutes the blot was transferred using forceps onto a piece of cling film (Saran). In the dark room, the wrapped blot was placed in a cassette with sample side up and a film (Lumi-Film, Chemiluminescent Detection Film, Boehringer Mannheim) was placed on top of the blot. The cassette was closed and the exposure time decided according to signal intensity, taking into account the following aspects. The light output increases rapidly during the first few hours. After this time, the light output will be more stable, making it easier to judge the optimum exposure time. The light output will begin to decline 2-3 days after addition of the detection reagent so exposure times will have to be adjusted accordingly.

4.7.20 Chemiluminescence film development

Films were developed manually, in the dark room. The film was dipped in developer solution (Kodak GBX) for 2 minutes, washed in water for 2 minutes, fixed (Kodak GBX) for 2 minutes then rinsed in water for 2 minutes and dried. In order to see the signals clearly, a light box was used.

4.7.21 Documentation of films

Films were scanned using Hewlett Packard ScanJet 3c in connection with a Dell computer and saved for future reference.

4.7.22 Stripping and reprobing

In order to reuse the blots they were stripped. For this, SDS buffer had to be brought to the boiling point into a treated beaker and poured onto the membrane which was placed in a treated glass tray. The membrane was incubated for 10 minutes, with agitation, followed by a 5 minutes wash with washing buffer. The membrane was then ready for prehybridization. Stripping processes tend to damage the target to some extent as well as remove the previous probe. Therefore, a maximum of three strippings per blot were performed.

4.8 Statistical analysis

Analysis of variance for a factorial design was performed using Minitab and Genstat, significant main effects and interactions being established by F tests. Significant differences between treatment means were determined using t tests according to standard procedures (Mead R, Curnow RN, 1983).

Analysis of variance for multifactorial experiments was done using the statistical programme "MINITAB" for the majority of experiments in the present study.

Analysis of variance for experiments 5, 6 and 7 was performed using the statistical programme "GENSTAT", in order to accommodate the unusual arrangement of treatments.

General linear modelling, using "Minitab" was applied in experiment 14, due to loss of one sample.

Chapter 5

Results

5 Results

5.1 Experiment 1 : The effects of medium, pH, temperature and carbendazim on fungal growth and mycotoxin production

A preliminary experiment containing three parts (A, B and C) was conducted using *Fusarium sporotrichioides* as inoculum.

5.1.1 Experiment 1A : The effects of medium type, pH and temperature at different stages of growth

Colony growth and TLC analysis of mycotoxins produced by *F. sporotrichioides* grown on yeast extract sucrose agar (YES) with or without trace elements, different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) are presented in Tables 5.1-5.6. In general, irrespective of time, pH or temperature, *F. sporotrichioides* colony growth on YES without trace elements was similar to the colony growth on YES with trace elements. It is clear from Tables 5.3-5.6 that T-2 is the major toxin produced under these conditions, followed by NEO. There was no ZEN being produced and DAS was present only when *F. sporotrichioides* was grown on YES without trace elements at pH 7.0, under two temperatures, 13 and 18°C respectively. Furthermore, DAS was present only when the mycelial side (M/S) of the plug was developed. pH 3.0, was the least favourable for mycotoxin production, while pH 5.4 and 7.0 were the most propitious values for toxin production. In general, the mycelial side of the plugs analysed, gave the positive sign for presence of mycotoxins.

Irrespective of pH value, the 18°C temperature gave the highest number of positive values for toxin production, followed by 13°C and 25°C.

Table 5.1 Experiment 1A : *Fusarium sporotrichioides* colony growth (cm) on YES media without trace elements, with different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) and two time points

YES media				
Time (days)	pH	Colony growth (cm)		
		Temperature (°C)		
		13	18	25
8	3.0	2.5	2.5	3.0
	5.4	5.0	6.0	4.5
	6.4	3.5	4.0	4.5
	7.0	6.0	6.0	6.5
28	3.0	3.0	3.0	3.5
	5.4	8.5	8.5	5.0
	6.4	8.5	8.5	5.0
	7.0	8.5	8.5	8.5

Table 5.2 Experiment 1A : *Fusarium sporotrichioides* colony growth (cm) on YES media with trace elements, different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) and two time points

YES media + trace elements				
Time (days)	pH	Colony growth (cm)		
		Temperature (°C)		
		13	18	25
8	3.0	1.8	2.3	2.0
	5.4	5.0	5.0	4.5
	6.4	3.5	5.5	4.5
	7.0	5.5	5.0	5.5
28	3.0	2.5	2.5	2.3
	5.4	8.5	8.5	5.5
	6.4	5.5	8.5	5.5
	7.0	8.5	8.5	5.5

Table 5.3 Experiment 1A : Production of mycotoxins (ZEN, T-2, DAS and NEO) by *Fusarium sporotrichioides*, grown on YES without trace elements, with different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) 8 days after inoculation

pH of YES	Plug Side	Temperature (°C)											
		13				18				25			
		Mycotoxin production											
		ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO
3.0	A/S	-	-	-	-	-	-	-	-	-	-	-	-
	M/S	-	-	-	-	-	-	-	-	-	-	-	-
5.4	A/S	-	+	-	-	-	+	-	-	-	+	-	-
	M/S	-	+	-	-	-	+	-	-	-	+	-	-
6.4	A/S	-	-	-	-	-	-	-	-	-	-	-	-
	M/S	-	-	-	-	-	-	-	-	-	-	-	-
7.0	A/S	-	+	-	+	-	+	-	-	-	+	-	+
	M/S	-	+	-	+	-	+	-	+	-	+	-	+

A/S = agar side of the plug
M/S = mycelial side of the plug
+ = presence of toxin
- = absence of toxin

Table 5.4 Experiment 1A : Production of mycotoxins (ZEN, T-2, DAS and NEO) by *Fusarium sporotrichioides*, grown on YES with trace elements and different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) 8 days after inoculation

pH of YES+	Plug Side	Temperature (°C)											
		13				18				25			
		Mycotoxin production											
		ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO
3.0	A/S	-	-	-	-	-	-	-	-	-	+	-	-
	M/S	-	-	-	-	-	-	-	-	-	+	-	-
5.4	A/S	-	+	-	-	-	+	-	-	-	-	-	-
	M/S	-	+	-	-	-	+	-	-	-	+	-	-
6.4	A/S	-	-	-	-	-	-	-	-	-	-	-	-
	M/S	-	-	-	-	-	+	-	-	-	-	-	-
7.0	A/S	-	-	-	-	-	+	-	-	-	+	-	-
	M/S	-	+	-	+	-	+	-	+	-	+	-	+

YES+=YES with trace elements
A/S = agar side of the plug
M/S = mycelial side of the plug
+ = presence of toxin
- = absence of toxin

Table 5.5 Experiment 1A : Production of mycotoxins (ZEN, T-2, DAS and NEO) by *Fusarium sporotrichioides*, grown on YES without trace elements, with different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) 28 days after inoculation

pH of YES	Plug Side	Temperature (°C)											
		13				18				25			
		Mycotoxin production											
		ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO
3.0	A/S	-	-	-	-	-	-	-	-	-	-	-	-
	M/S	-	-	-	-	-	-	-	-	-	-	-	-
5.4	A/S	-	+	-	-	-	+	-	-	-	-	-	-
	M/S	-	+	-	-	-	+	-	-	-	-	-	-
6.4	A/S	-	+	-	-	-	+	-	-	-	-	-	-
	M/S	-	+	-	-	-	+	-	-	-	-	-	-
7.0	A/S	-	+	-	+	-	+	-	+	-	-	-	-
	M/S	-	+	+	+	-	+	+	+	-	-	-	-

A/S = agar side of the plug
M/S = mycelial side of the plug
+ = presence of toxin
- = absence of toxin

Table 5.6 Experiment 1A : Production of mycotoxins (ZEN, T-2, DAS and NEO) by *Fusarium sporotrichioides*, grown on YES with trace elements and different pH values (3.0, 5.4, 6.4 and 7.0), under three temperatures (13, 18 and 25°C) 28 days after inoculation

pH of YES+	Plug Side	Temperature (°C)											
		13				18				25			
		Mycotoxin production											
		ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO	ZEN	T-2	DAS	NEO
3.0	A/S	-	-	-	-	-	-	-	-	-	-	-	-
	M/S	-	-	-	-	-	-	-	-	-	-	-	-
5.4	A/S	-	+	-	-	-	+	-	-	-	-	-	-
	M/S	-	+	-	-	-	+	-	-	-	-	-	-
6.4	A/S	-	-	-	-	-	+	-	+	-	+	-	-
	M/S	-	-	-	-	-	-	-	+	-	+	-	+
7.0	A/S	-	-	-	-	-	+	-	-	-	+	-	+
	M/S	-	+	-	-	-	-	-	-	-	+	-	+

YES+=YES with trace elements
A/S = agar side of the plug
M/S = mycelial side of the plug
+ = presence of toxin
- = absence of toxin

5.1.2 Experiment 1B : The effects of carbendazim and temperature

Results from part (B) of the preliminary experiment, are presented in Tables 5.7 and 5.8. Irrespective of fungicide concentration, *F. sporotrichioides* colony growth on YES increased with time. In general, irrespective of time, colony diameters decreased with the increase in fungicide level.

Irrespective of fungicide concentration, T-2 toxin was the major trichothecene toxin produced, followed by NEO (Table 5.8). ZEN appeared to be present only in one sample (0.1 μg/ml, M/S). NEO production was visualized mostly when agar side (A/S) was developed. Therefore, a decision was taken to extract the entire colony for mycotoxin analysis.

Table 5.7 Experiment 1B : *Fusarium sporotrichioides* colony growth (cm) on YES containing carbendazim (added as Bavistin) at 0.0, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/ml at 25°C and 2, 4, 7 days after inoculation

Carbendazim level (μg/ml)	Time (days)		
	2	4	7
0.0 (without ethanol)	2.9	6.4	7.9
0.0 (with ethanol)	2.7	5.4	8.5
0.1	2.9	5.8	8.5
0.5	2.9	5.2	7.5
1.0	2.8	5.1	7.2
5.0	2.9	5.0	7.1
10.0	3.0	5.1	7.7

Table 5.8 Experiment 1B : Mycotoxin production by *Fusarium sporotrichioides* grown on YES containing different levels of carbendazim (added as Bavistin)

Carbendazim level (μg/ml)	Plug side	Mycotoxin production		
		ZEN	T-2	NEO
0.0 (without ethanol)	A/S	-	+	+
	M/S	-	+	+
0.0 (with ethanol)	A/S	-	+	+
	M/S	-	+	-
0.1	A/S	-	+	+
	M/S	+	+	-
0.5	A/S	-	+	+
	M/S	-	+	-
1.0	A/S	-	+	+
	M/S	-	+	+
5.0	A/S	-	+	+
	M/S	-	+	-
10.0	A/S	-	+	+
	M/S	-	+	-

5.1.3 Experiment 1C : The effects of medium type and temperature shock at different stages of growth

Results of mycotoxin production by *F. sporotrichioides* grown on YES and PDA for 5 days at 25°C, followed by a transfer of cultures to 11°C until the completion of experiment, are presented in Table 5.9. T-2 toxin and NEO were the trichothecene toxins produced under these conditions. Irrespective of time, T-2 toxin and NEO were produced on YES as well as PDA media. In three instances, T-2 and/or NEO production was visible only from the development of A/S.

Table 5.9 Experiment 1C : Mycotoxin production by *Fusarium sporotrichioides* grown on YES and PDA under the 25-11°C temperature regime and six time points after inoculation

Time (days)	Plug side	Mycotoxin production			
		T-2		NEO	
		Media		Media	
		YES	PDA	YES	PDA
8	A/S	+	+	+	+
	M/S	+	+	-	-
11	A/S	+	+	+	+
	M/S	+	+	+	+
13	A/S	+	+	+	+
	M/S	+	-	+	+
15	A/S	+	+	+	+
	M/S	+	+	+	+
19	A/S	+	+	+	+
	M/S	+	+	+	+
22	A/S	+	+	+	+
	M/S	+	+	+	+

Due to ease of preparation, consistent composition between batches and commercial availability, PDA was chosen as solid media for following experiments.

A bigger difference between temperatures was desired and much of the literature contains data derived from temperature 23-28°C, therefore 25°C was the chosen upper temperature. For temperature shock investigations, the lower limit was chosen as 11°C. A reasonable production was obtained for T-2 toxin and NEO at 25°C.

Although there were more positive indications of mycotoxin production at pH 7.0 (Tables 5.2-5.5) compared to pH 5.4, there were inconsistent patterns between A/S and M/S samples in mycotoxin production at the higher pH. Furthermore, parallel (unpublished) studies in the department had shown a more diverse array of mycotoxin production in *F. sporotrichioides* at pH 5.6 on PDA than in the pH 7.0 YES media used in experiment 1 (Tables 5.2-5.5). This greater diversity with PDA also emerged in studies with fungicides. Thus, in Table 5.8, only two mycotoxins

appeared irrespective of carbendazim level whereas unpublished experiments within the department demonstrated the occurrence of T-2 toxin, HT-2 toxin, DAS, NEO and ZEN on PDA. Supplementation of media with trace elements resulted in no difference in fungal growth. However, mycotoxin production was marginally inferior in the presence of trace elements. Finally, the results of experiment 1C (Table 5.9) showed PDA to be similar to YES in terms of mycotoxin production. For these reasons PDA at pH 5.6, unsupplemented with trace elements, was used in all subsequent experiments with solid media. An additional advantage was the ease of preparation, consistent composition between batches/ reproducibility between standard batches and commercial availability of PDA.

5.2 Experiment 2 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

Colony diameters measured 2, 4, 5 and 28 days after inoculation of PDA with agar plugs of *Fusarium* species and the effects of carbendazim are shown in Table 5.10. Statistical analysis indicated that there were no significant ($P>0.05$) differences in colony diameters between control and control+ethanol. Furthermore, the addition of 0.1 and 1.0 μg carbendazim/ml PDA did not affect colony diameter, but 10 μg and 100 μg/ml PDA were lethal. Plates with viable colonies were transferred after 5 days incubation at 25°C, to 11°C for a further period of 23 days, when the experiment was terminated and the entire colonies with PDA extracted and analysed for mycotoxins. At this point all viable colonies grew to the full diameter of the plate. It is clear that fungicide concentration of up to 1.0 μg/ml had no significant ($P>0.05$) effect on radial growth from a very early stage of the experiment. The results in Table 5.11 show that ZEN was produced in three out of four replicates of *F. sporotrichioides* grown on PDA containing 1.0 μg carbendazim/ml, whereas at lower levels of fungicide, fewer replicates of the fungus produced ZEN. In controls all replicates of

F. sporotrichioides produced T-2 and NEO at all levels of fungicide up to and including 1.0 μg/ml PDA.

Quantitative data on mycotoxin production by *F. sporotrichioides* are shown in Table 5.12. No significant differences (P>0.05) were observed in ZEN production at the three fungicide levels, despite the earlier results indicating greater number of replicates producing ZEN at 1.0 μg carbendazim/ml PDA (Table 5.11).

T-2 toxin production, on the other hand, was slightly increased by carbendazim added at 0.1 μg/ml PDA, but production declined when the fungicide level was increased from 0.1 μg to 1.0 μg/ml PDA. Production of NEO was not significantly affected (P>0.05) by fungicide level.

Table 5.10 The effects of carbendazim[a] (added as Bavistin) on colony diameters (cm) of *Fusarium sporotrichioides* 2, 4, 5 and 28 days[b] after inoculation of PDA media with peripheral plugs from 5 day old cultures

Carbendazim level (μg/ml)	Time after inoculation (days)[b]			
	2	4	5	28
0.0 (Control)	3.8	7.1	7.7	8.5
0.0 (Control+Ethanol)	3.7	7.1	7.8	8.5
0.1	3.5	7.1	8.0	8.5
1.0	3.7	7.4	8.3	8.5
10.0	0.0	0.0	0.0	0.0
100.0	0.0	0.0	0.0	0.0
SEM (df=9)	0.04	0.12	0.18	

[a]The fungicide was dissolved in ethanol prior to addition to PDA media
[b]Temperature was maintained at 25°C, for the first 5 days and reduced to 11°C thereafter until termination of the experiment at 28 days

Table 5.11 Experiment 2 : The number of replicates (out of 4) showing the presence of three mycotoxins from *Fusarium sporotrichioides* in the absence or presence of the fungicide, carbendazim[a] (added as Bavistin)

Carbendazim level (μg/ml)	Toxin production		
	ZEN	T-2	NEO
0.0 (Control)	1/4	4/4	4/4
0.0 (Control+Ethanol)	0	4/4	4/4
0.1	1/4	4/4	4/4
1.0	3/4	4/4	4/4
10.0	-	-	-
100.0	-	-	-

[a]The fungicide was dissolved in ethanol prior to addition to PDA media
[b]For explanation of abbreviations, see List of abbreviations

Table 5.12 Experiment 2 : Production of three mycotoxins (ZEN, T-2 toxin and NEO) by *Fusarium sporotrichioides* in the absence or presence of the fungicide, carbendazim [a] (added as Bavistin)

Carbendazim level (μg/ml)	Mycotoxin production (μg/ml) [b]		
	ZEN	T-2	NEO
0.0 (Control)	0.94	19.06	6.25
0.0 (Control with ethanol)	0.00	9.69	2.25
0.1	0.00	19.37	2.25
1.0	0.94	11.87	8.12
Mean	0.47	15.00	4.72
SEM (df=9)	1.39	5.99	2.16
LSD (P<0.05)	4.45	19.14	6.91

[a] The fungicide was dissolved in ethanol prior to addition to PDA media.
[b] For explanation of abbreviations, see List of abbreviations

5.3 Experiment 3 : The effects of carbendazim (added as Bavistin), temperature regime and time on pigmentation, fungal growth and mycotoxin production

Colony growth was significantly (P<0.001) influenced by time, temperature, and fungicide concentration, and a consistent difference in colour was observed (see Plate 5. 1), between cultures kept at 25°C compared to 25-11°C temperature regime. In general, irrespective of time or temperature, colony growth decreased with the increase in fungicide level and irrespective of temperature regime and fungicide concentration, colony diameters increased with time.

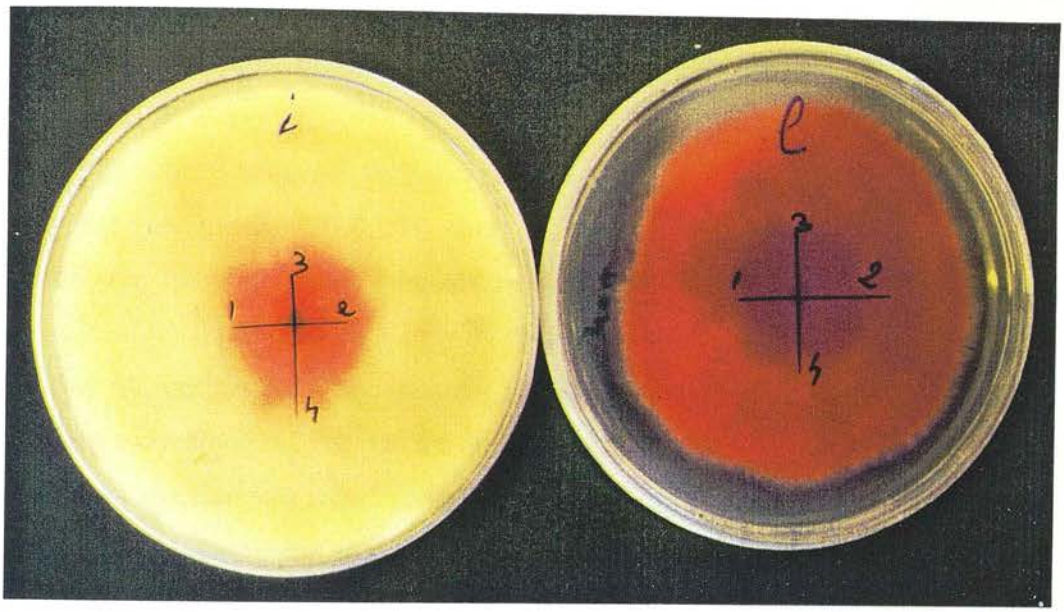

Plate 5.1 Experiment 3: Reverse of *Fusarium sporotrichioides* colonies grown on PDA with carbendazim (added as Bavistin) at 5.0 μg/ml, under two temperature regimes, namely 25°C (left) and 25-11°C (right)

Production of ZEN, T-2 toxin and NEO was significantly influenced by time ($P<0.001$), whereas temperature ($P<0.01$) and concentration of fungicide induced significant ($P<0.05$) effects only for ZEN and T-2 toxin synthesis. Irrespective of fungicide concentration and temperature regime, T-2 toxin and ZEN increased with time (Table 5.16 and 5.14 respectively), while NEO production reached a peak at 14 days, followed by a decrease in toxin production by 26 days of colony growth (Table 5.18). While ZEN production increased with the decrease in temperature (Table 5.15), T-2 toxin production decreased under the 25-11°C temperature regime (Table 5.17). Irrespective of temperature regime, production of ZEN and T-2 toxin followed a distorted pattern with the increase in fungicide concentration, in the sense that there was no clear increase or decrease with the increase in fungicide level (Table 5.15 and 5.17).

The interaction between time and temperature had a significant influence on ZEN ($P<0.01$) and T-2 toxin ($P<0.001$) production.

Time and concentration interaction significantly influenced growth and T-2 toxin production ($P<0.05$).

The interaction between temperature and fungicide concentration exerted an influence on growth ($P<0.001$) and T-2 toxin production ($P<0.01$), and the interaction between time, temperature and concentration of fungicide had a significant ($P<0.001$) influence only on colony growth.

At 5µg/ml, carbendazim significantly ($P<0.05$) increased T-2 toxin production in cultures maintained at 25°C. On the other hand, in cultures in the 25-11°C regime, T-2 toxin production was significantly ($P<0.05$) reduced by carbendazim at 5.0 µg/ml, whereas ZEN production was reduced by fungicide at levels of as low as 1.0 µg/ml ($P<0.05$). Taken together with previous results (Table 5.12) it is concluded that T-2 toxin production is increased with carbendazim application. In contrast, ZEN production is reduced with this fungicide.

Table 5.13 Experiment 3 : The effects of carbendazim (added as Bavistin) on colony diameters (cm) of *Fusarium sporotrichioides* grown on PDA, under two temperature regimes, namely 25°C and 25-11°C respectively

Carbendazim level (µg/ml)	Colony age (days)							
	2	4	6 1st extraction		14 2nd extraction		26 3rd extraction	
			Temperature regime (°C)		Temperature regime (°C)		Temperature regime (°C)	
			25	25-11	25	25-11	25	25-11
0.0	2.11	5.26	7.10	6.65	8.50	8.50	8.50	8.50
1.0	2.29	5.24	7.00	6.55	8.50	8.50	8.50	8.50
2.5	2.38	5.27	6.90	6.00	8.50	7.00	8.50	8.50
5.0	2.24	5.38	8.5	5.40	8.50	6.12	8.50	8.50
7.5	2.20	4.86	6.90	5.00	8.50	5.50	8.50	4.75
10.0	1.28	2.03	2.25	1.30	4.60	2.15	6.50	2.20
SEM (df= 1)			0.35					
LSD (P<0.05)			1.82					

Table 5.14 Experiment 3 : ZEN production (mg/ml) by *Fusarium sporotrichioides* under the influence of carbendazim (added as Bavistin) levels of 1.0, 2.5, 5.0, 7.5 and 10.0 μg/ml, at two temperature regimes, namely 25°C and 25-11°C respectively, after 6, 14, and 26 days of culture growth

Carbendazim level (μg/ml)	Temperature regime (°C)								Grand mean
	25				25 - 11				
	Time (days)			Mean 1	Time (days)			Mean 2	
	6	14	26		6	14	26		
0.0	0.02	0.36	0.56	0.31	0.00	0.34	1.29	0.65	0.48
1.0	0.02	0.20	0.56	0.26	0.00	0.03	0.80	0.28	0.27
2.5	0.03	0.00	0.23	0.09	0.00	0.54	1.09	0.54	0.31
5.0	0.02	0.17	0.59	0.26	0.00	0.47	0.45	0.31	0.28
7.5	0.01	0.37	0.08	0.15	0.00	0.37	1.16	0.51	0.33
10.0	0.01	0.07	0.05	0.04	0.00	0.05	0.06	0.04	0.04
Grand mean	0.02	0.19	0.34	0.18	0.00	0.30	0.81	0.39	0.29
SEM (df=35)	0.16			0.09	0.16			0.09	
LSD (P<0.05)	0.45			0.26	0.45			0.26	

Table 5.15 Experiment 3 : ZEN production, a summary table extracted from Table 5.14

Carbendazim level (μg/ml)	Mean 1	Mean 2	Grand mean
0.0	0.31	0.65	0.48
1.0	0.26	0.28	0.27
2.5	0.09	0.54	0.31
5.0	0.26	0.31	0.28
7.5	0.15	0.51	0.33
10.0	0.04	0.04	0.04
Grand mean	0.18	0.39	0.29
SEM (df=35)	0.09		
LSD (P<0.05)	0.26		

Table 5.16 Experiment 3 : T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* under the influence of carbendazim (added as Bavistin) levels of 1.0, 2.5, 5.0, 7.5 and 10.0 μg/ml, at two temperature regimes, namely 25°C and 25-11°C respectively, after 6, 14, and 26 days of culture growth

Carbendazim level (μg/ml)	Temperature regime (°C)								Grand mean
	25				25-11				
	Time (days)			Mean 1	Time (days)			Mean 2	
	6	14	26		6	14	26		
0.0	10.18	35.38	9.81	18.46	4.34	15.15	46.05	21.85	20.15
1.0	7.85	14.90	17.05	13.27	11.19	4.31	31.58	15.69	14.48
2.5	11.78	23.89	18.34	18.00	1.04	7.00	39.58	15.88	16.94
5.0	14.61	41.90	32.83	29.78	5.20	13.31	11.54	10.02	19.90
7.5	7.66	34.49	11.88	18.01	0.00	11.54	17.51	9.68	13.84
10.0	3.42	35.27	9.24	15.98	1.36	4.63	2.88	2.96	9.47
Grand mean	9.25	30.97	16.53	18.92	3.87	9.32	24.86	12.68	15.80
SEM (df=35)	5.72			3.30	5.72			3.30	
LSD (P<0.05)	16.50			9.53	16.50			9.53	

Table 5.17 Experiment 3 : T-2 toxin production, a summary table extracted from Table 5.16

Carbendazim level (μg/ml)	Mean 1	Mean 2	Grand mean
0.0	18.46	21.85	20.15
1.0	13.27	15.69	14.48
2.5	18.00	15.88	16.94
5.0	29.78	10.02	19.90
7.5	18.01	9.68	13.84
10.0	15.98	2.96	9.47
Grand mean	18.92	12.68	15.80
SEM (df=35)	3.30		
LSD (P<0.05)	9.53		

Table 5.18 Experiment 3 : NEO production (mg/ml) by *Fusarium sporotrichioides* under the influence of carbendazim (added as Bavistin) levels of 1.0, 2.5, 5.0, 7.5 and 10.0 μg/ml, at two temperature regimes, namely 25°C and 25-11°C respectively, after 6, 14, and 26 days of culture growth

Carbendazim level (μg/ml)	Temperature regime (°C)								Grand mean
	25				25-11				
	Time (days)			Mean 1	Time (days)			Mean 2	
	6	14	26		6	14	26		
0.0	6.51	6.08	7.42	6.67	1.28	18.52	12.20	10.67	8.67
1.0	0.55	6.44	10.37	5.79	0.00	6.79	8.25	5.01	5.40
2.5	6.39	5.68	4.85	5.64	0.39	7.49	8.46	5.45	5.54
5.0	0.60	29.33	10.26	13.40	2.09	9.34	1.12	4.18	8.79
7.5	0.90	18.84	13.07	10.93	1.61	10.68	1.87	4.72	7.82
10.0	1.03	3.94	5.79	3.59	0.31	2.87	0.18	1.12	2.35
Grand mean	2.66	11.72	8.63	7.67	0.95	9.28	5.35	5.19	6.43
SEM (df=35)	4.63			2.67	4.63			2.67	
LSD (P<0.05)	13.36			7.71	13.36			7.71	

Table 5.19 Experiment 3 : NEO production, a summary table extracted from Table 5.18

Carbendazim level (μg/ml)	Mean 1	Mean 2	Grand mean
0.0	6.67	10.67	8.67
1.0	5.79	5.01	5.40
2.5	5.64	5.45	5.54
5.0	13.40	4.18	8.79
7.5	10.93	4.72	7.82
10.0	3.59	1.12	2.35
Grand mean	7.67	5.19	6.43
SEM (df=35)	2.67		
LSD (P<0.05)	7.71		

5.4 Experiment 4 : The effects of difenoconazole (added as Plover) and temperature regime on fungal growth and mycotoxin production

Results for this experiment are presented in Tables 5.20-5.26.

Temperature had a highly significant ($P<0.001$) effect on colony growth (Table 5.21), ZEN and T-2 toxin production (Tables 5.22 and 5.23) and a significant effect on HT-2 ($P<0.05$) and NEO ($P<0.01$) production (Tables 5.25 and 5.26). In time, the lower temperature regime led to smaller colonies of *F. sporotrichioides* grown on PDA with different levels of difenoconazole (Table 5.20). Colony growth (Table 5.21) as well as HT-2 toxin production (Table 5.25) were reduced with the decrease in temperature, while ZEN (Table 5.22) and T-2 toxin production (Table 5.23) increased at 25-11°C temperature regime.

Concentration of fungicide was highly significant ($P<0.001$) for colony growth (Table 5.21), ZEN, T-2 toxin and NEO production (Tables 5.22, 5.23 and 5.26 respectively). Irrespective of temperature there was a decrease in ZEN, T-2 toxin and NEO production with the increase in fungicide concentration (Tables 5.22, 5.23 and 5.26 respectively). HT-2 toxin production increased with the increase in concentration of fungicide at 25°C (Table 5.25).

The interaction between temperature and concentration of fungicide had a highly significant ($P<0.001$) effect on colony growth and T-2 toxin production and a significant ($P<0.05$) effect on HT-2 toxin production. Irrespective of temperature regime, there was a decrease in colony growth with the increase in fungicide concentration while irrespective of fungicide concentration, there was a decrease in colony growth at 25-11°C temperature regime. In general, irrespective of fungicide concentration there was an increase in T-2 toxin production with the reduction in temperature while HT-2 toxin production decreased at 25-11°C temperature regime.

Table 5.20 Experiment 4 : Growth of *Fusarium sporotrichioides* on PDA with difenoconazole (added as Plover) concentrations of 0.1, 1.0, 10.0 and 100.0 μg/ml, under two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)					
	25			25-11		
	Colony age (days)			Colony age (days)		
	3	5	28	3	5	28
0.0	5.37	8.00	8.00	5.50	8.00	8.00
0.1	3.60	5.77	8.00	3.47	5.55	7.87
1.0	0.87	1.80	7.90	0.90	1.80	6.25
10.0	0.50	0.60	5.30	0.50	0.50	1.32
100.0	0.50	0.52	4.37	0.50	0.50	2.25
Mean	2.17	3.34	6.71	2.17	3.27	5.14

Table 5.21 Experiment 4 : The effects of difenoconazole (added as Plover) levels of 0.1, 1.0, 10.0 and 100.0 μg/ml on colony diameters (cm) of *Fusarium sporotrichioides* after 28 days of growth under two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	8.00	8.00
0.1	8.00	7.90
1.0	7.90	6.20
10.0	5.30	1.30
100.0	4.40	2.20
SEM (df= 27)	0.36	
LSD (P<0.05)	1.05	

Table 5.22 Experiment 4 : ZEN production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with difenoconazole (added as Plover) levels of 0.1, 1.0, 10 and 100 μg/ml, at two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	1.41	1.48
0.1	0.87	1.03
1.0	0.00	1.28
10.0	0.00	0.05
100.0	0.02	0.06
Mean	0.46	0.78
SEM (df=27)	0.29	
LSD (P<0.05)	0.84	

Table 5.23 Experiment 4 : T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with difenoconazole (added as Plover) levels of 0.1, 1.0, 10 and 100 μg/ml, at two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	3.74	13.99
0.1	2.87	15.57
1.0	1.15	12.31
10.0	2.83	0.52
100.0	2.91	0.55
Mean	2.70	8.59
SEM (df=27)	1.23	
LSD (P<0.05)	3.58	

Table 5.24 Experiment 4 : DAS production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with difenoconazole (added as Plover) levels of 0.1, 1.0, 10 and 100 μg/ml, at two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	2.75	5.09
0.1	1.10	5.95
1.0	1.43	3.11
10.0	1.24	0.43
100.0	3.93	0.68
Mean	2.09	3.05
SEM (df=27)	1.54	
LSD (P<0.05)	4.49	

Table 5.25 Experiment 4 : HT-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with difenoconazole (added as Plover) levels of 0.1, 1.0, 10 and 100 μg/ml, at two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	2.38	2.74
0.1	3.12	0.93
1.0	0.90	1.07
10.0	4.15	0.17
100.0	20.44	0.03
Mean	6.20	0.99
SEM (df=27)	3.24	
LSD (P<0.05)	9.43	

Table 5.26 Experiment 4 : NEO production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with difenoconazole (added as Plover) levels of 0.1, 1.0, 10 and 100 μg/ml, at two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	12.08	15.29
0.1	18.55	15.29
1.0	18.01	8.93
10.0	9.23	1.26
100.0	7.69	1.45
Mean	13.11	8.44
SEM (df=27)	2.49	
LSD (P<0.05)	7.27	

5.5 Experiment 5 : The effects of carbendazim (added as Bavistin and/or pure substance) and time on growth and mycotoxin production

Results for experiment 5 are presented in Tables 5.27-5.31. Table 5.32 illustrates an overall picture of analysis of variance (ANOVA) for the current experiment.

In general, time had a significant effect on colony growth ($P<0.05$) and DAS ($P<0.01$) production, and a highly significant ($P<0.001$) effect on T-2 and HT-2 toxin production, while fungicide application had a significant ($P<0.05$) effect only on colony growth and HT-2 toxin production. Colony diameters generally increased with time irrespective of fungicide type or concentration, while application of fungicides led to smaller colony diameters compare to *F. sporotrichioides* colonies grown on PDA without fungicides (Table 5.27). T-2 toxin and DAS production increased with time (Tables 5.28 and 5.29) while HT-2 toxin production reached a peak at 14 days followed by a decrease in toxin production by 29 days of growth

(Table 5.30). Application of fungicides increased HT-2 toxin production irrespective of fungicide type and concentration.

The interaction between time and fungicide application had a significant ($P<0.01$) effect only on HT-2 toxin production. With the exception of a decrease in HT-2 toxin

Table 5.27 Experiment 5 : *Fusarium sporotrichioides* colony growth (cm) on PDA with carbendazim levels of 0.1 and 1.0 μg/ml, added as Bavistin (B) or as pure substance (C), at 25°C and three time points

Carbendazim level (μg/ml)	Age of colony (days)			Mean
	7	14	29	
0.0	7.90	8.15	8.40	8.15
0.1 B	8.20	8.40	8.40	8.33
1.0 B	7.10	8.20	7.20	7.50
0.1 C	8.40	8.40	8.40	8.40
1.0 C	3.60	5.90	6.50	5.33

Table 5.28 Experiment 5 : T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with carbendazim added as Bavistin (B) or as the pure substance (C), at 25°C and three time points

Carbendazim level (μg/ml)	Age of colony (days)			Mean
	7	14	29	
0.0	1.69	1.56	3.51	2.25
0.1 B	1.55	0.37	2.63	1.52
1.0 B	3.31	1.96	2.66	2.64
0.1 C	3.92	1.38	2.46	2.59
1.0 C	1.87	2.34	11.21	5.14

Table 5.29 Experiment 5 : DAS production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with carbendazim added as Bavistin (B) or the pure substance (C), at 25°C and three time points

Carbendazim level (µg/ml)	Age of colony (days)			Mean
	7	14	29	
0.0	0.37	1.21	1.94	1.17
0.1 B	0.35	1.91	2.12	1.46
1.0 B	1.45	2.74	2.45	2.21
0.1 C	1.17	1.99	1.42	1.53
1.0 C	0.47	3.16	5.26	2.96

Table 5.30 Experiment 5 : HT-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with carbendazim added as Bavistin (B) or as the pure substance (C), at 25°C and three time points

Carbendazim level (µg/ml)	Age of colony (days)			Mean
	7	14	29	
0.0	3.83	3.60	1.44	2.96
0.1 B	2.97	6.04	0.61	3.21
1.0 B	2.40	13.65	2.22	6.09
0.1 C	0.00	9.39	1.53	3.64
1.0 C	8.57	9.92	2.20	6.90

Table 5.31 Experiment 5 : NEO production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with carbendazim added as Bavistin (B) or as the pure substance (C), at 25°C and three time points

Carbendazim level (µg/ml)	Age of colony (days)			Mean
	7	14	29	
0.0	5.10	11.25	7.65	8.00
0.1 B	2.79	22.41	6.17	10.46
1.0 B	12.91	17.29	2.98	11.06
0.1 C	66.74	13.41	3.32	27.82
1.0 C	5.15	19.99	13.82	12.99

Table 5.32 Experiment 5 : Variance ratios (F) and probabilities (P) from Analysis of Variance

ANOVA	Degrees of Freedom	Growth		T-2		DAS		HT-2		NEO	
		F	P	F	P	F	P	F	P	F	P
Time	2	4.67	0.028	18.59	<0.001	8.88	0.003	44.44	<00.02	3.03	NS
Control	1	6.77	0.021	2.00	NS	3.34	NS	6.70	0.021	2.07	NS
Time*control	2	0.42	NS	0.59	NS	0.20	NS	7.24	0.007	0.96	NS
Fungicide	1	16.23	0.001	15.44	0.002	0.94	NS	0.80	NS	4.19	NS
Concentration	1	55.99	<0.001	16.38	0.001	6.66	0.022	19.72	<0.001	2.28	NS
Time*fungicide	2	2.11	NS	7.04	0.008	0.62	NS	0.57	NS	4.02	0.042
Time*concentration	2	3.73	0.05	8.70	0.004	1.64	NS	1.96	NS	3.93	0.044
Fungicide*concentration	1	18.36	<0.001	2.48	NS	0.65	NS	0.07	NS	2.68	NS
Time*fungicide*concentration	2	2.76	NS	17.14	<0.001	3.28	NS	11.71	0.001	8.91	0.003

See legend on following page for further description of factors

Time=influence of time

Control=comparison between control versus all (any) fungicides

Time*control=influence of time on fungicide or no fungicide

*fungicide=comparison between fungicides

*concentration=comparison between concentrations

Time*fungicide=interaction between time and fungicide type

Time*concentration=interaction between time and fungicide concentration

Fungicide*concentration=interaction between fungicide type and concentration of fungicide

Time*fungicide*concentration=interaction between time, fungicide type and concentration of fungicide

production at 7 days when fungicide application was involved, there was a general increase in toxin production at 14 and 29 days of growth compared to HT-2 toxin obtained from colonies grown on PDA. HT-2 toxin production obtained from control colonies decreased with time while the toxin obtained from colonies grown under the influence of fungicide reached a peak at 14 days of growth followed by a decrease by 29 days of incubation (Table 5.30).

Fungicide type had a highly significant ($P<0.001$) effect on colony growth (Table 5.27) and a significant ($P<0.01$) effect on T-2 toxin production (Table 5.28). Colony growth was significantly smaller when carbendazim was added to PDA as pure substance (98%) than when it was added as Bavistin. On the contrary, T-2 toxin production was significantly higher when carbendazim was added to PDA as pure substance.

Concentration of fungicides had a highly significant ($P<0.001$) effect on colony growth, T-2 and HT-2 toxin production and a significant ($P<0.05$) effect on DAS production. While colony diameter decreased with the higher level of fungicide (1.0 μg/ml), the production of T-2, DAS and HT-2 toxin increased with the increase in fungicide concentration.

Interaction between time and fungicide type was significant for T-2 ($P<0.01$) and NEO ($P<0.05$) production. Irrespective of fungicide type, T-2 toxin production decreased at 14 days followed by an increase by 29 days of incubation. NEO production from colonies grown under the influence of carbendazim decreased with time while NEO production from colonies grown on PDA with Bavistin reached a peak at 14 days of growth and decreased by 29 days of growth.

Interaction between time and concentration of fungicides was significant ($P<0.05$) for colony growth, T-2 toxin ($P<0.01$) and NEO ($P<0.05$) production. Irrespective of time, colony growth was significantly smaller at the higher concentration (1.0 μg/ml) of fungicide. At 7 days of growth, T-2 toxin and NEO production showed a decrease in toxin production with the increase in fungicide level while at 14 and 29 days of growth, T-2 toxin and NEO expressed increased levels with the increase in fungicide concentration.

Interaction between fungicide type and concentration was significant ($P<0.001$) only for colony growth. Irrespective of fungicide type, colony growth was higher at the lower concentration (0.1 μg/ml) of fungicide compared to 1.0 μg/ml and irrespective of concentration of fungicide, colony growth was higher when carbendazim was applied to PDA media as Bavistin than when added as pure substance (carbendazim 98% purity).

The interaction between time, fungicide type and concentration was highly significant ($P<0.001$) for T-2 and HT-2 toxin and significant ($P<0.01$) for NEO production.

5.6 Experiment 6 : The effects of carbendazim (added as Bavistin and/or pure substance), fungal interaction and time on fungal growth and mycotoxin production

Results for experiment 6 are presented in Tables 5.33-5.37. Table 5.38 illustrates the overall picture of analysis of variance (ANOVA) for the present experiment.

In general, time was highly significant ($P<0.001$) for colony growth and significant ($P<0.05$) for ZEN production. Application of fungicide exerted a highly significant ($P<0.001$) effect on colony growth and a significant effect on T-2 toxin ($P<0.05$) and NEO ($P<0.01$) production. Colony growth as well as ZEN production (Table 5.33) increased with time. While colony growth decreased with fungicide application, T-2 toxin and NEO production increased with application of fungicides.

Fungicide type had a highly significant ($P<0.001$) effect on colony growth and a significant ($P<0.01$) effect on HT-2 toxin production. Colony diameter was significantly smaller for colonies grown on PDA with carbendazim added as pure substance (98%) compared to colonies grown on PDA with carbendazim added as Bavistin). However, HT-2 toxin production was significantly increased when carbendazim as pure substance was employed (Table 5.36).

Colony growth was significantly ($P<0.001$) influenced when *F. sporotrichioides* was grown together with *A. ochraceus* than when it was growing alone. Production of ZEN ($P<0.05$), T-2 toxin ($P<0.001$) and NEO ($P<0.01$) was also influenced when fungal interaction was involved. Irrespective of time, fungicide type and concentration, growth of *F.sporotrichioides* colonies was significantly smaller when cultured together with *A. ochraceus*. ZEN and T-2 toxin registered a decline in production (Table 5.33 and 5.34) while NEO increased its production when *F. sporotrichioides* was grown together with *A. ochraceus* (Table 5.37).

Table 5.33 Experiment 6 : Colony growth(1) (cm) and ZEN production(2) (mg/ml) by *Fusarium sporotrichioides* grown on PDA with fungicide carbendazim added as Bavistin (B) or as the pure substance (C), alone or in combination with *Aspergillus ochraceus*, at 25°C and three time points

(1)

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	8.4	8.4	8.4	8.4
0.1 B	8.4	8.4	8.2	8.3
1.0 B	8.4	8.2	8.3	8.3
0.1 C	7.9	8.4	8.4	8.2
1.0 C	2.4	4.6	6.3	4.4
Fusarium sporotrichioides /Aspergillus ochraceus				
0.0	6.6	6.5	7.0	6.7
0.1 B	6.1	6.5	6.9	6.5
1.0 B	6.1	6.6	5.3	6.0
0.1 C	6.5	5.9	6.6	6.3
1.0 C	2.2	5.4	6.0	4.5

(2)

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	0.0	0.0	0.0	0.0
0.1 B	0.0	0.0	0.0	0.0
1.0 B	0.0	0.0	0.23	0.08
0.1 C	0.0	0.0	0.29	0.10
1.0 C	0.0	0.0	0.0	0.0
Fusarium sporotrichioides /Aspergillus ochraceus				
0.0	0.0	0.08	0.0	0.03
0.1 B	0.0	0.0	0.0	0.0
1.0 B	0.0	0.0	0.0	0.0
0.1 C	0.0	0.03	0.0	0.02
1.0 C	0.0	0.0	0.0	0.0

Table 5.34 Experiment 6 : T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with fungicide carbendazim added as Bavistin (B) or as the pure substance (C), alone or in combination with *Aspergillus ochraceus*, at 25°C and three time points

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	1.10	2.49	0.84	1.48
0.1 B	7.42	12.69	8.37	9.49
1.0 B	1.40	3.90	2.79	2.70
0.1 C	7.46	5.08	2.60	5.05
1.0 C	0.69	2.73	5.46	2.96
Fusarium sporotrichioides/ Aspergillus ochraceus				
0.0	3.04	1.95	2.74	2.58
0.1 B	1.49	1.34	2.70	1.84
1.0 B	3.14	0.86	2.24	2.08
0.1 C	0.80	5.09	1.63	2.50
1.0 C	0.43	2.44	3.32	2.07

Table 5.35 Experiment 6 : DAS production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with fungicide carbendazim added as Bavistin (B) or as the pure substance (C), alone or in combination with *Aspergillus ochraceus*, at 25°C and three time points

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	0.24	0.26	0.15	0.22
0.1 B	0.13	0.16	0.22	0.17
1.0 B	0.84	0.81	0.56	0.73
0.1 C	0.52	0.26	0.29	0.35
1.0 C	0.33	0.27	5.41	2.00
Fusarium sporotrichioides/ Aspergillus ochraceus				
0.0	0.56	0.29	0.39	0.41
0.1 B	1.48	0.12	0.19	0.60
1.0 B	0.29	0.08	0.38	0.25
0.1 C	0.24	0.12	0.21	0.07
1.0 C	1.11	0.31	0.33	0.58

Table 5.36 Experiment 6 : HT-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with fungicide carbendazim added as Bavistin (B) or as the pure substance (C), alone or in combination with *Aspergillus ochraceus*, at 25°C and three time points

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	0.22	0.48	0.33	0.35
0.1 B	0.13	0.13	0.09	0.12
1.0 B	0.00	0.48	0.22	0.23
0.1 C	0.24	0.48	1.41	0.71
1.0 C	0.07	0.25	1.15	0.49
Fusarium sporotrichioides/ Aspergillus ochraceus				
0.0	0.00	0.04	1.80	0.61
0.1 B	0.08	0.09	0.09	0.09
1.0 B	0.27	0.12	0.43	0.27
0.1 C	1.10	0.73	1.42	1.08
1.0 C	0.93	0.77	0.12	0.61

Table 5.37 Experiment 6 : NEO production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with fungicide carbendazim added as Bavistin (B) or as the pure substance (C), alone or in combination with *Aspergillus ochraceus*, at 25°C and three time points

Carbendazim level (μg/ml)	Time (days)			Mean
	7	15	28	
Fusarium sporotrichioides				
0.0	0.31	0.54	0.29	0.38
0.1 B	0.53	1.37	0.11	0.67
1.0 B	1.00	0.46	0.21	0.56
0.1 C	0.36	0.52	0.80	0.56
1.0 C	0.11	0.28	0.79	0.39
Fusarium sporotrichioides/ Aspergillus ochraceus				
0.0	0.13	0.17	0.42	0.24
0.1 B	0.44	2.80	0.23	1.15
1.0 B	0.70	0.88	1.13	0.90
0.1 C	2.34	0.06	0.53	0.97
1.0 C	0.78	0.96	0.32	0.68

Concentration of fungicides had a highly significant ($P<0.001$) influence on colony growth and T-2 toxin production. Colony growth as well as T-2 toxin production was significantly smaller when the higher concentration (1.0 μg/ml) of fungicide was employed.

Interaction between time and fungicide type was highly significant ($P<0.001$) for colony growth and significant ($P<0.01$) for NEO production. Irrespective of time, colony growth was smaller when carbendazim was utilized as pure substance compared to carbendazim added as Bavistin.

Table 5.38 Experiment 6 : Variance ratios (F) and probabilities (P) from Analysis of Variance

ANOVA	DF	Growth		ZEN		T-2		DAS		HT-2		NEO	
		F	P	F	P	F	P	F	P	F	P	F	P
Time	2	9.86	<0.001	4.61	0.017	1.79	NS	1.10	NS	2.89	NS	2.22	NS
Control	1	23.21	<0.001	0.27	NS	6.22	0.018	0.63	NS	0.03	NS	7.50	0.010
Time*control	2	1.73	NS	2.62	NS	0.39	NS	0.29	NS	1.31	NS	0.72	NS
Fungicide type	1	61.17	<0.001	0.21	NS	2.5	NS	1.06	NS	10.85	0.002	1.44	NS
Fungal species	1	69.50	<0.001	6.08	0.019	27.4	<0.001	1.50	NS	0.57	NS	7.57	0.010
Concentration	1	73.46	<0.001	0.21	NS	16.51	<0.001	2.83	NS	0.35	NS	2.18	NS
Time*fungicide type	2	12.49	<0.001	0.06	NS	0.02	NS	1.72	NS	0.72	NS	7.34	0.002
Time*fungal species	2	0.28	NS	7.48	0.002	0.50	NS	2.15	NS	1.43	NS	1.25	NS
Fungicide type*fungal species	1	10.44	0.003	0.01	NS	4.67	0.038	1.30	NS	0.51	NS	0.06	NS
Time*concentration	2	6.94	0.003	0.06	NS	3.02	NS	1.74	NS	0.32	NS	2.40	NS
Fungicide type*concentration	1	50.68	<0.001	7.94	0.008	3.26	NS	1.84	NS	2.28	NS	0.03	NS
Fungal species*concentration	1	4.44	0.043	0.01	NS	15.08	<0.001	2.62	NS	0.08	NS	0.21	NS
Time*fungicide type*fungal species	2	0.18	NS	0.16	NS	4.88	0.014	1.67	NS	1.69	NS	8.08	0.001
Time*fungicide type*concentration	2	12.63	<0.001	6.54	0.004	2.98	NS	1.28	NS	0.61	NS	10.70	<0.001
Time*fungal species*concentration	2	2.60	NS	0.16	NS	1.73	NS	1.25	NS	0.26	NS	1.31	NS
Fungicide type*fungal species*concentration	1	12.01	0.002	6.08	0.019	5.80	0.022	0.07	NS	0.23	NS	0.00	NS
Time*fungicide type*fungal species*concentration	2	0.73	NS	7.48	0.002	0.94	NS	3.05	NS	0.65	NS	3.66	0.037

See legend on following page for further description of factors

Time=influence of time
Control=control versus all fungicides
Time*control=influence of time on fungicide and no fungicide
*fungicide type=comparison between fungicides
*fungal species=comparison between fungal species
*concentration=comparison between concentrations
Time*fungicide type=influence of time on fungicide type
Time*fungal species=influence of time on fungal species
*fungicide type*fungal species=fungicide type versus fungal species
Time*concentration=influence of time on concentration
*fungicide type*concentration=fungicide type versus concentration
*fungal species*concentration=fungal species versus concentration
Time*fungicide type*fungal species=influence of time on fungicide type and fungal species
Time*fungicide type*concentration=influence of time on fungicide type and concentration
Time*fungal species*concentration=influence of time on fungal species and concentration
*fungicide type*fungal species*conc=fungicide type versus fungal species versus concentration
Time*fungicide type*fungal species*conc=influence of time on fungicide type versus fungal species versus concentration

ZEN production was the only toxin influenced ($P<0.01$) by the interaction between time and fungal species.

Interaction between fungicide type and fungal species was significant for colony growth ($P<0.01$) and T-2 toxin ($P<0.05$) production. Irrespective of fungicide type, colony growth was smaller when *F. sporotrichioides* was grown in combination with *A. ochraceus*. Similarly, irrespective of fungal species, colony growth was smaller when carbendazim as a pure substance was involved in media preparation. Irrespective of fungicide type, T-2 toxin production was reduced when *F. sporotrichioides* was grown together with *A. ochraceus*. However, when *F. sporotrichioides* was grown alone, T-2 toxin production was reduced when carbendazim added as pure substance was involved, whereas in combination with *A.ochraceus*, T-2 toxin production by *F. sporotrichioides* increased with the addition of carbendazim as pure substance.

Interaction between time and concentration of fungicide was significant ($P<0.01$) only for colony growth. Irrespective of time, colony growth was smaller when the higher concentration (1.0 μg/ml) of fungicides was used, where irrespective of concentration of fungicide employed, colony growth increased with time.

The interaction between fungicide type and concentration was highly significant ($P<0.001$) for colony growth and significant ($P<0.01$) for ZEN production. Irrespective of fungicide type, colony growth was reduced at the higher concentration (1.0 μg/ml) whilst irrespective of fungicide concentration, colony growth was reduced when carbendazim was utilized as pure substance.

The interaction between fungal species and concentration was significant ($P<0.05$) for colony growth and highly significant ($P<0.001$) for T-2 toxin production. Irrespective of fungal species, colony growth as well as T-2 toxin production decreased when higher concentration of fungicide was used and in the same time, irrespective of concentration of fungicide, colony growth and T-2 toxin production decreased when *F. sporotrichioides* was grown together with *A. ochraceus*.

Interaction between time, fungicide type and fungal species was significant ($P<0.05$) for T-2 toxin and highly significant ($P<0.001$) for NEO production. Irrespective of time and fungicide type, T-2 toxin production decreased when *F.sporotrichioides*

was grown in combination with *A. ochraceus*. NEO production on the other hand, did not follow the same pattern. Irrespective of time and fungal species, T-2 toxin production decreased when carbendazim was utilized as pure substance for *F. sporotrichioides* grown alone. T-2 toxin production produced by *F. sporotrichioides* grown together with *A. ochraceus* decreased with the use of carbendazim at 7 days of incubation followed by an increase in production at 15 and 28 days with NEO production pattern being vice versa.

The interaction between time, fungicide type and concentration of fungicide was highly significant ($P<0.001$) for colony growth and NEO production and significant ($P<0.01$) for ZEN production. Irrespective of time and fungicide type, colony growth was smaller at the higher concentration (1.0 μg/ml) and irrespective of time and fungicide concentration, colony growth was smaller when carbendazim was used as pure substance in the media. When *F. sporotrichioides* was grown alone, ZEN production was increased by the use of carbendazim as pure substance towards the end of the time course while when grown in combination with *A. ochraceus*, it increased slightly at 15 days followed by a decline in ZEN production by 28 days of growth (Table 5.33).

The interaction between fungicide type, fungal species and concentration of fungicide was significant for colony growth ($P<0.01$), ZEN ($P<0.05$) and T-2 toxin ($P<0.05$) production. Irrespective of fungicide type and fungal species, colony growth was reduced at the higher level of fungicide (1.0 μg/ml). At the lower level of fungicide, irrespective of fungicide type there was a decrease in colony growth when *F. sporotrichioides* was grown together with *A. ochraceus*. At the higher level (1.0 μg/ml) of fungicide, colony growth decreased when *Fusarium* was grown in combination on media with carbendazim added as Bavistin and increased when grown in combination on media with carbendazim as pure substance. Irrespective of fungicide type and concentration, T-2 toxin production decreased when *F. sporotrichioides* was grown in combination with *A. ochraceus*. T-2 toxin and ZEN production generally decreased with the increase in fungicide concentration.

The interaction between time, fungicide type, fungal species and concentration of fungicide was significant for ZEN ($P<0.01$) and NEO ($P<0.05$) production.

5.7 Experiment 7 : The effects of fungicide combinations, temperature and time on pigmentation and mycotoxin production

5.7.1 Colour development

The reverse of *Fusarium sporotrichioides* colonies grown on PDA with ethanol (see Plate 5.2.-1), PDA with carbendazim (see Plate 5.3.-1) and PDA with different combinations of fungicides, namely carbendazim+propiconazole (see Plate 5.4.-1), carbendazim+maneb (see Plate 5.5.-1) and carbendazim+maneb+tridemorph (see Plate 5.6.-1), at two levels (0.1 and 1.0 μg/ml), under two temperature regimes (25-11°C and 25°C) were photographed, due to an interest in factors involved in different colour development according to the temperature treatment used. Fluorescent staining of hyphae, using calcofluor white (see 4.6) was employed for studies of hyphal growth under these conditions. The results obtained are presented in the following plates: control colonies are represented by Plate 5.2.2, 3 and for colonies grown on PDA with carbendazim alone (see Plate 5.3.2-5) or mixed with propiconazole (see Plate 5.4.2-5), maneb (see Plate 5.5.2-5) and maneb+tridemorph (see Plate 5.6.2-5).

Macroscopic analysis shows that there is an obvious temperature effect on colony aspect in terms of pigmentation, with a pink coloration for colonies that suffered a temperature shock from 25-11°C and a yellow coloration for colonies maintained at 25°C throughout the experiment (see Plates 5.2-5.6 -1). Also, temperature had an effect on colony diameter, with smaller colonies at 25-11°C temperature regime (see 5.2 -5.6 -1).

Concentration of fungicides had an effect on colony growth with smaller diameters present at levels of 1.0 μg/ml PDA (see Plates 5.3-5.6 -1).

The combination between temperature regime and fungicide concentration had a striking effect resulting in colonies grown at 25-11°C and 1.0 μg/ml being half the size of the ones grown at 25°C and 1.0 μg/ml. The exception is the combination

between carbendazim+maneb+tridemorph (see Plate 5.6-1) where the radial growth between colonies grown at 25-11°C and 1.0 µg/ml are similar to the ones grown at 25°C.

At microscopic level, the results obtained show differences between the two temperature regimes in terms of hyphal growth, with thin, long cells, less branched hyphae at 25-11°C compared to increased hyphal diameter, swelling, more branching at 25°C (see Plate 5.2 -2 and 3).

The temperature effect was visible even in the presence of fungicides: carbendazim (see Plate 5.3.2-5), carbendazim+propiconazole (see Plate 5.4.2-5), carbendazim+maneb (see Plate 5.5.2-5) and carbendazim+maneb+tridemorph (see Plate 5.6.2-5). Increased fluorescence of the hyphal wall indicates an accumulation of chitin.

Overall, the presence of fungicides combined with the higher temperature regime had a more marked effect on hyphal aspect in terms of hyphal diameter (see Plate 5.6.-3), swollen tips (see Plate 5.3.-3), branching (see Plate 5.6.-3), rounded cells (see Plate 5.6.-5), distortionate growth (see Plate 5.5.-3), orientation (see Plate 5.5.-3) and cytoplasm organization (see Plate 5.4.-5).

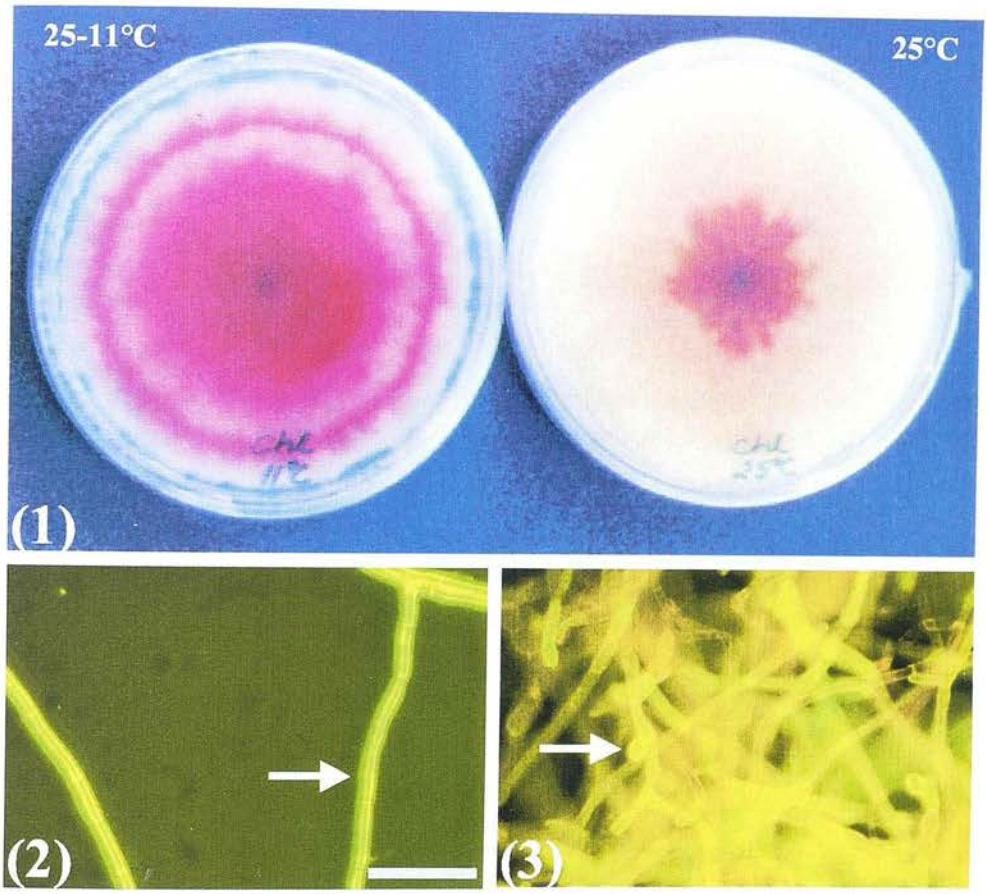

Plate 5.2 Experiment 7 : *Fusarium sporotrichioides* colonies (reverse,-1) grown on PDA, under two temperature regimes namely 25-11°C (left) and 25°C (right). (2) and (3) represent calcofluor white stained hyphae. The bar represents 5 μm.

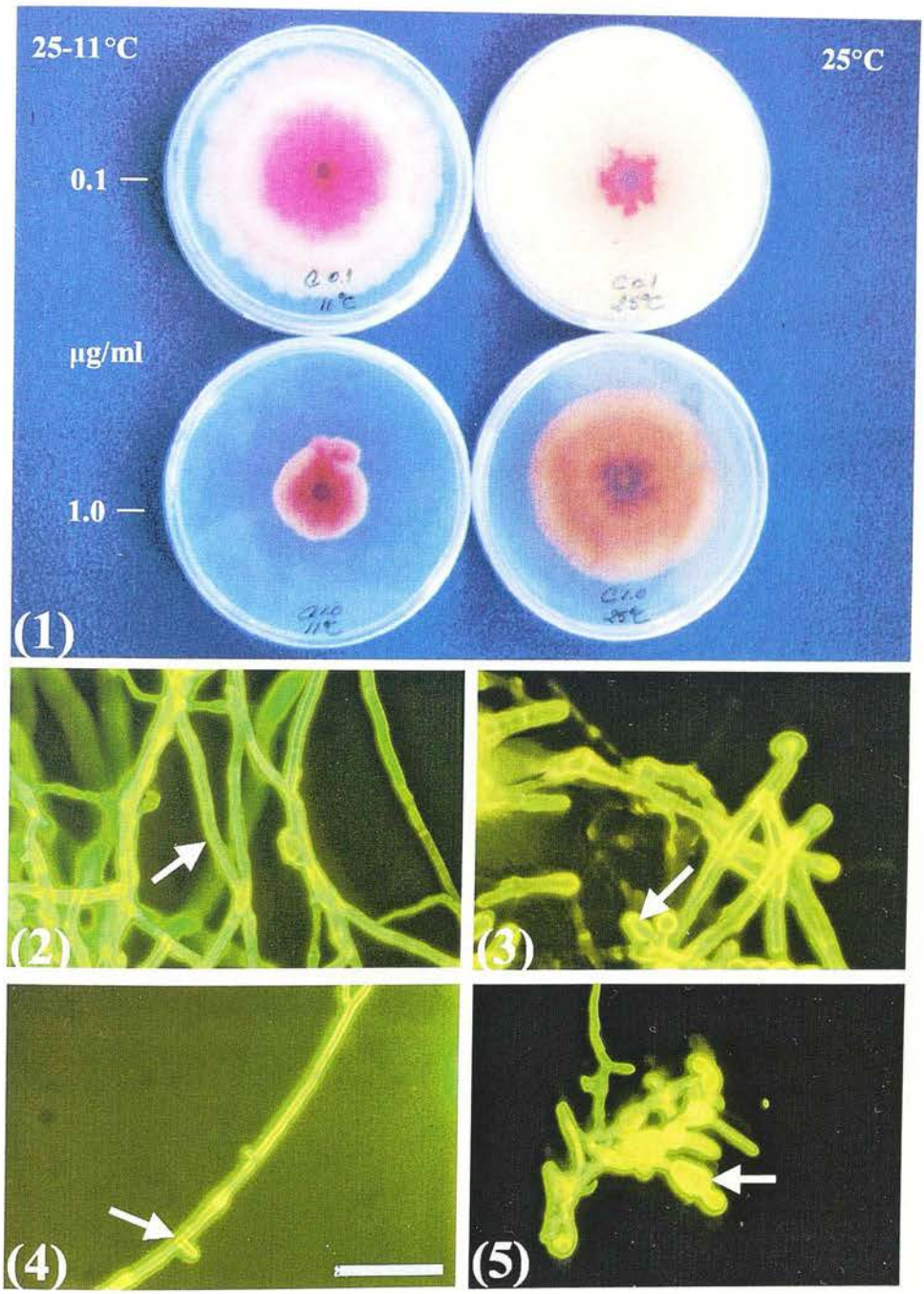

Plate 5.3 Experiment 7 : *Fusarium sporotrichioides* grown on PDA containing carbendazim at two levels 0.1 (1, 2, 3) and 1.0 (1, 4, 5) µg/ml, under two temperature regimes namely 25-11°C (left) and 25°C (right). (2), (3), (4) and (5) represent calcofluor white stained hyphae. The bar represents 5 µm.

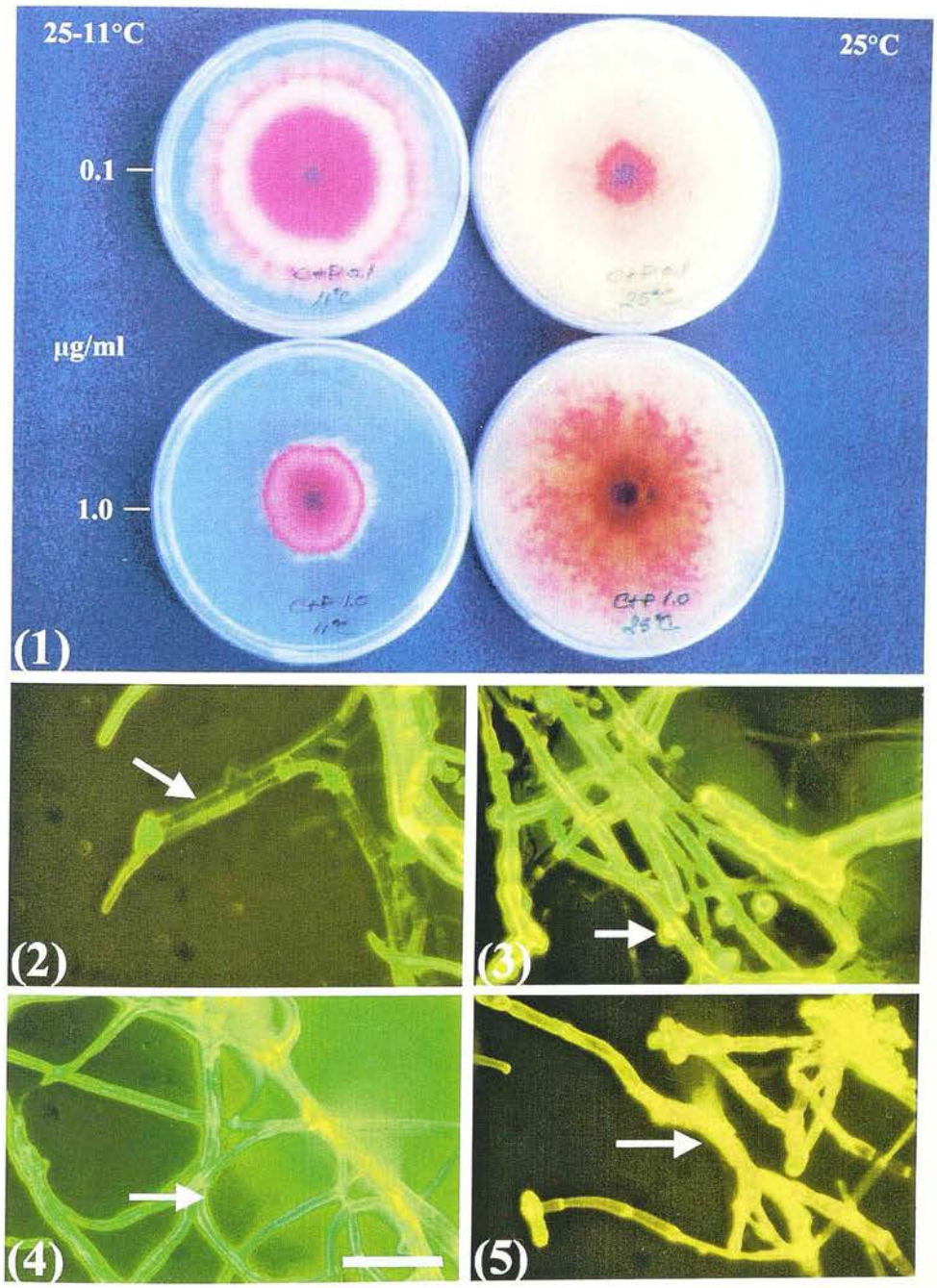

Plate 5.4 Experiment 7 : *Fusarium sporotrichioides* grown on PDA containing a mixture of fungicides carbendazim+propiconazole at two levels 0.1 (1, 2, 3) and 1.0 (1, 4, 5) µg/ml, under two temperature regimes namely 25-11°C (left) and 25°C (right). (2), (3), (4) and (5) represent calcofluor white stained hyphae. The bar represents 5 µm.

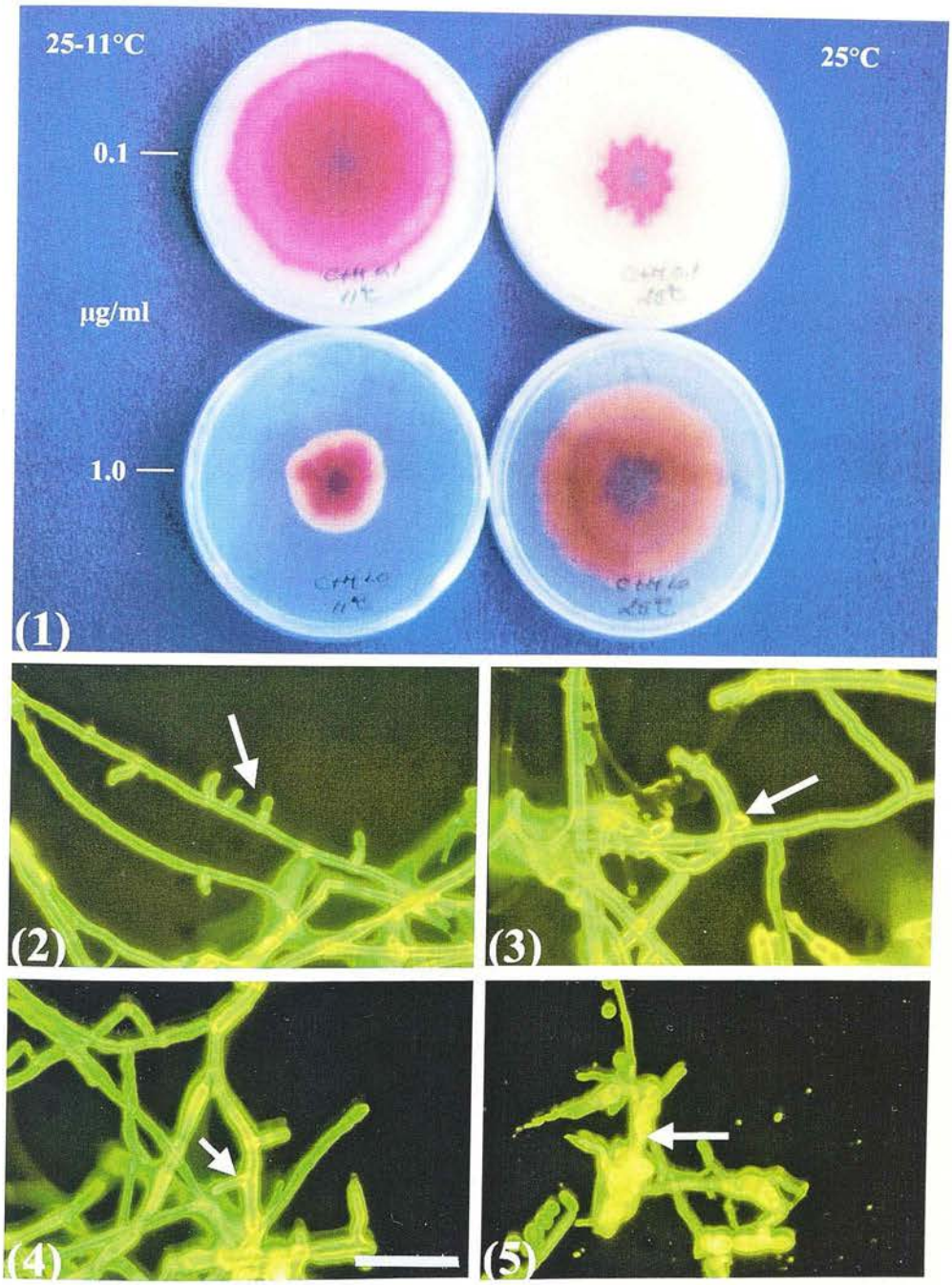

Plate 5.5 Experiment 7 : ***Fusarium sporotrichioides*** **grown on PDA containing a mixture of fungicides carbendazim+maneb at two levels 0.1 (1, 2, 3) and 1.0 (1, 4, 5) µg/ml, under two temperature regimes namely 25-11°C (left) and 25°C (right). (2), (3), (4) and (5) represent calcofluor white stained hyphae. The bar represents 5 µm.**

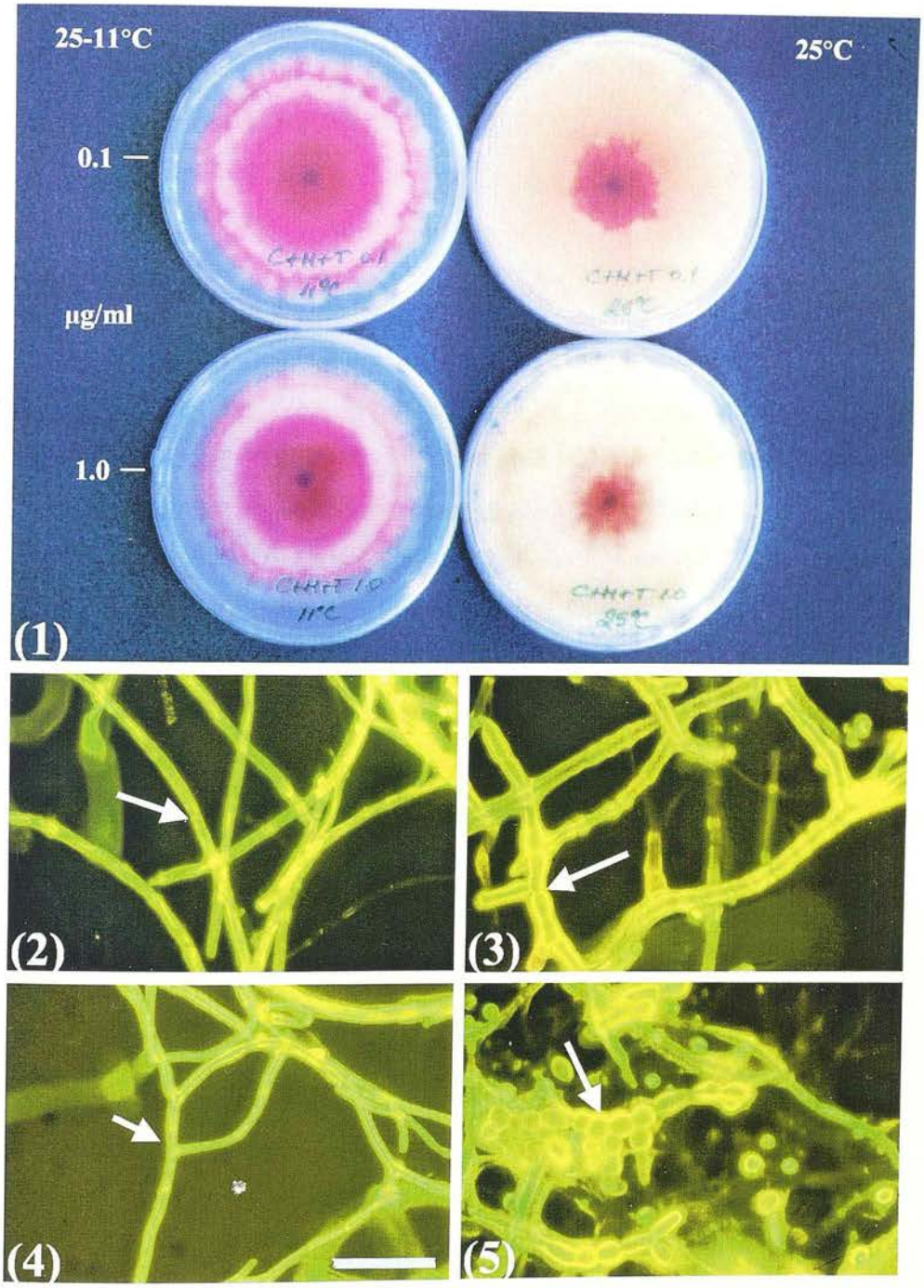

Plate 5.6 Experiment 7 : ***Fusarium sporotrichioides*** **grown on PDA containing a mixture of fungicides carbendazim+maneb+tridemorph at two levels 0.1 (1, 2, 3) and 1.0 (1, 4, 5) µg/ml, under two temperature regimes namely 25-11°C (left) and 25°C (right). (2), (3), (4) and (5) represent calcofluor white stained hyphae. The bar represents 5 µm.**

5.7.2 Mycotoxin production

Results for experiment 7 are presented in Tables 5.39-5.43. Table 5.44 illustrates an overall picture of ANOVA results.

5.7.2.1 Main effects

In general, time and temperature regime had a significant effect ($P<0.001$) on production of ZEN, T-2, DAS, HT-2 and NEO by *F. sporotrichioides* (Table 5.44). ZEN, DAS, HT-2 and NEO production increased with time (Tables 5.39, 5.41, 5.42 and 5.43 respectively) compared to T-2 toxin production which reached a maximum at 14 days followed by a fall in production by 25 days of colony growth (Table 5.40). The decrease in temperature from 25-11°C led to an increased production of ZEN, DAS and NEO (Tables 5.39, 5.41 and 5.43 respectively) and a decrease in T-2 and HT-2 toxin production (Tables 5.40 and 5.42). Application of fungicides had a highly significant ($P<0.001$) effect on T-2 toxin and DAS production. While ZEN and T-2 production increased with fungicide application, DAS, HT-2 and NEO production decreased.

5.7.2.2 Interaction effects

The interaction between time and temperature had a highly significant ($P<0.001$) effect on the production of ZEN, T-2, DAS and HT-2 and a significant effect ($P<0.05$) on NEO production (Table 5.44).

Time effect on fungicide application versus control had a significant effect on T-2 ($P<0.05$) and DAS ($P<0.001$), with an increase in T-2 toxin production and a decrease in DAS production when application of fungicides was employed. Temperature regime effect on fungicide versus control had a highly significant effect only on T-2 toxin production, with an increase in toxin production at 25-11°C in control colonies and a decrease in T-2 toxin production at 25-11°C when colonies of *F. sporotrichioides* were grown under the influence of fungicides.

Concentration of fungicide had a highly significant ($P<0.001$) effect on ZEN, DAS, HT-2 and NEO production with a decrease in mycotoxin production when the higher concentration (1.0 μg/ml) of fungicide was used.

Fungicide type had a highly significant ($P<0.001$) effect on T-2 toxin production and a significant ($P<0.05$) effect on ZEN and DAS production. Mycotoxin production was increased when carbendazim was used in combination with maneb, propiconazole and maneb+tridemorph than when carbendazim was used alone. Furthermore, ZEN production decreased when tridemorph was used in combination with maneb and carbendazim compared with carbendazim+maneb combination, while T-2 and DAS production increased under the same conditions.

The interaction between time and temperature regime had a significant ($P<0.01$) effect on T-2 toxin and DAS production. In control cultures kept at 25°C T-2 toxin production decreased with time while in control cultures kept at 25-11°C temperature regime, T-2 toxin production reached a peak at 14 days of age followed by a decline in toxin production by 25 days of growth.

When application of fungicides was involved, irrespective of temperature regime, T-2 toxin production reached a peak at 14 days followed by a decrease in toxin production by 25 days of growth. DAS production decreased slightly at 14 days in colonies kept at 25°C followed by an increase in toxin production by 25 days of growth, compared to colonies kept at 25-11°C temperature regime which expressed an increase in DAS production with time.

Table 5.39 Experiment 7 : ZEN production (mg/ml) by *Fusarium sporotrichioides* grown on PDA, containing two levels (0.1 and 1.0 μg/ml) of carbendazim alone or in combination with propiconazole, maneb and maneb+tridemorph, under two temperature regimes, namely 25°C and 25-11°C, and at three time points

Fungicide level (μg/ml)	Temperature (°C)							
	25				25-11			
	Time (days)			Mean	Time (days)			Mean
	7	14	25		7	14	25	
0.0	0.0	0.56	0.59	0.38	0.00	0.00	1.80	0.60
0.1 C	0.0	0.35	1.05	0.47	0.00	0.26	0.60	0.29
1.0 C	0.0	0.00	0.00	0.00	0.00	0.00	1.60	0.53
0.1 CP	0.0	1.08	1.41	0.83	0.00	0.00	3.22	1.07
1.0 CP	0.0	0.89	0.00	0.30	0.00	0.00	1.15	0.38
0.1 CM	0.0	1.06	2.12	1.06	0.00	0.33	3.05	1.13
1.0 CM	0.0	0.00	0.00	0.00	0.00	0.35	2.59	0.98
0.1 CMT	0.0	0.72	1.39	0.70	0.00	0.00	1.73	0.58
1.0 CMT	0.0	0.00	0.00	0.00	0.00	0.00	2.16	0.72

C=carbendazim
CP=carbendazim+propiconazole
CM=carbendazim+maneb
CMT=carbendazim+maneb+tridemorph

Table 5.40 Experiment 7: T-2 production (mg/ml) by *Fusarium sporotrichioides* grown on PDA, containing two levels (0.1 and 1.0 μg/ml) of carbendazim alone or in combination with propiconazole, maneb and maneb+tridemorph, under two temperature regimes, namely 25°C and 25-11°C, and at three time points

Fungicide level (μg/ml)	Temperature (°C)							
	25				25-11			
	Time (days)			Mean	Time (days)			Mean
	7	14	25		7	14	25	
0.0	9.04	3.15	0.61	4.27	5.02	15.37	10.04	10.14
0.1 C	10.98	9.73	1.80	7.50	6.78	12.73	10.86	10.12
1.0 C	11.37	27.94	3.01	14.11	3.38	6.06	6.35	5.26
0.1 CP	26.59	38.58	1.62	22.26	12.67	20.77	12.91	15.45
1.0 CP	24.13	37.38	1.81	21.11	6.23	13.53	9.00	9.59
0.1 CM	20.85	27.50	1.28	16.54	14.08	20.32	20.45	18.28
1.0 CM	27.73	38.23	4.44	23.47	8.33	7.92	6.09	7.45
0.1 CMT	33.33	36.69	1.31	23.78	8.96	19.22	15.52	14.57
1.0 CMT	22.93	22.45	1.50	15.63	9.71	19.31	18.44	15.82

C=carbendazim
CP=carbendazim+propiconazole
CM=carbendazim+maneb
CMT=carbendazim+maneb+tridemorph

Table 5.41 Experiment 7: DAS production (mg/ml) by *Fusarium sporotrichioides* grown on PDA, containing two levels (0.1 and 1.0 μg/ml) of carbendazim alone or in combination with propiconazole, maneb and maneb+tridemorph, under two temperature regimes, namely 25°C and 25-11°C, and at three time points

Fungicide level (μg/ml)	Temperature (°C)							
	25				25-11			
	Time (days)			Mean	Time (days)			Mean
	7	14	25		7	14	25	
0.0	0.20	0.17	1.23	0.53	0.17	1.29	1.58	1.01
0.1 C	0.01	0.06	0.35	0.14	0.45	1.18	1.13	0.92
1.0 C	0.33	0.38	0.20	0.30	0.14	0.25	0.30	0.23
0.1 CP	0.15	0.00	0.17	0.11	0.16	0.76	1.26	0.73
1.0 CP	0.14	0.52	0.19	0.28	0.00	0.34	0.67	0.34
0.1 CM	0.26	0.01	0.00	0.09	0.30	1.07	1.60	0.99
1.0 CM	0.26	0.01	0.14	0.14	0.01	0.35	0.41	0.26
0.1 CMT	0.21	0.25	0.11	0.19	0.01	0.93	1.73	0.89
1.0 CMT	0.01	0.005	0.13	0.05	0.005	1.00	1.83	0.94

C=carbendazim
CP=carbendazim+propiconazole
CM=carbendazim+maneb
CMT=carbendazim+maneb+tridemorph

Table 5.42 Experiment 7 : HT-2 production (mg/ml) by *Fusarium sporotrichioides* grown on PDA, containing two levels (0.1 and 1.0 μg/ml) of carbendazim alone or in combination with propiconazole, maneb and maneb+tridemorph, under two temperature regimes, namely 25°C and 25-11°C, and at three time points

Fungicide level (μg/ml)	Temperature (°C)							
	25				25-11			
	Time (days)			Mean	Time (days)			Mean
	7	14	25		7	14	25	
0.0	0.09	0.14	0.30	0.18	0.01	0.17	0.17	0.12
0.1 C	0.01	0.10	0.66	0.26	0.01	0.06	0.08	0.05
1.0 C	0.00	0.00	0.50	0.17	0.00	0.00	0.06	0.02
0.1 CP	0.10	0.31	0.35	0.25	0.005	0.00	0.45	0.15
1.0 CP	0.00	0.12	0.58	0.23	0.00	0.00	0.09	0.03
0.1 CM	0.05	0.30	0.47	0.27	0.07	0.01	0.31	0.13
1.0 CM	0.00	0.00	0.28	0.09	0.00	0.00	0.05	0.02
0.1 CMT	0.00	0.11	0.54	0.22	0.00	0.07	0.31	0.13
1.0 CMT	0.00	0.13	0.30	0.14	0.00	0.00	0.10	0.03

C=carbendazim
CP=carbendazim+propiconazole
CM=carbendazim+maneb
CMT=carbendazim+maneb+tridemorph

Table 5.43 Experiment 7 : NEO production (mg/ml) by *Fusarium sporotrichioides* grown on PDA, containing two levels (0.1 and 1.0 μg/ml) of carbendazim alone or in combination with propiconazole, maneb and maneb+tridemorph, under two temperature regimes, namely 25°C and 25-11°C, and at three time points

Fungicide level (μg/ml)	Temperature (°C)							
	25				25-11			
	Time (days)			Mean	Time (days)			Mean
	7	14	25		7	14	25	
0.0	0.34	0.40	1.04	0.59	0.38	0.91	1.15	0.81
0.1 C	0.31	0.34	1.05	0.57	0.55	0.99	0.78	0.77
1.0 C	0.38	0.29	0.41	0.36	0.18	0.23	0.31	0.24
0.1 CP	0.35	0.62	0.89	0.62	0.42	0.53	1.40	0.78
1.0 CP	0.20	0.82	0.54	0.52	0.21	0.33	0.63	0.39
0.1 CM	0.47	0.71	0.67	0.62	0.52	1.10	1.67	1.10
1.0 CM	0.12	0.28	0.33	0.24	0.31	0.25	0.31	0.29
0.1 CMT	0.22	0.48	0.63	0.44	0.32	0.66	1.53	0.84
1.0 CMT	0.25	0.35	0.50	0.37	0.22	0.79	1.92	0.98

C=carbendazim
CP=carbendazim+propiconazole
CM=carbendazim+maneb
CMT=carbendazim+maneb+tridemorph

Table 5.44 Experiment 7 : Variance ratios (F) and probabilities (P) from Analysis of Variance

ANOVA	DF	ZEN		T-2		DAS		HT-2		NEO	
		F	P	F	P	F	P	F	P	F	P
Time	2	55.64	<0.001	62.17	<0.001	57.12	<0.001	110.23	<0.001	40.76	<0.001
Temperature	1	6.55	0.013	21.03	<0.001	130.60	<0.001	53.33	<0.001	16.73	<0.001
Control	1	0.17	NS	23.54	<0.001	27.36	<0.001	0.01	NS	2.78	NS
Time*temperature	2	20.90	<0.001	53.79	<0.001	39.98	<0.001	18.82	<0.001	4.25	0.019
Time*control	2	0.10	NS	4.02	0.024	9.60	<0.001	3.10	0.053	0.54	NS
Temperature*control	1	0.04	NS	13.43	<0.001	0.02	NS	2.76	NS	0.00	NS
Concentration	1	11.79	0.001	3.48	NS	16.42	<0.001	25.34	<0.001	29.85	<0.001
Fungicide	3	2.97	0.040	13.04	<0.001	3.04	0.037	1.17	NS	1.72	NS
Time*temperature	2	0.02	NS	6.36	0.003	5.69	0.006	1.24	NS	1.66	NS
Time*concentration	2	4.88	0.011	0.07	NS	2.06	NS	3.81	0.028	2.63	NS
Temperature*concentration	1	6.16	0.016	8.08	0.006	29.28	<0.001	0.00	NS	3.79	NS
Time*fungicide	6	1.51	NS	2.18	NS	3.48	0.006	0.56	NS	2.27	0.05
Temperature*fungicide	3	0.51	NS	1.57	NS	4.81	0.005	1.02	NS	4.42	0.008
Concentration*fungicide	3	1.13	NS	0.90	NS	1.21	NS	1.13	NS	5.85	0.002
Time*temperature*concentration	2	2.27	NS	1.04	NS	2.31	NS	3.44	0.039	0.29	NS
Time*temperature*fungicide	6	1.05	NS	2.02	NS	3.58	0.005	3.55	0.005	3.44	0.006
Time*concentration*fungicide	6	1.36	NS	1.18	NS	1.08	NS	0.81	NS	1.17	NS
Temperature*concentration*fungicide	3	1.13	NS	7.28	<0.001	7.94	<0.001	1.19	NS	1.81	NS
Time*temperature*concentration*fungicide	6	0.81	NS	0.85	NS	0.89	NS	3.73	0.004	1.58	NS

See legend on following page for further description of factors.

Time= time influence over all variables
Temperature= temperature influence over all variables
Control= no fungicide versus any fungicide
Time*temperature= interaction between time and temperature
Time*control= time effect on fungicide versus no fungicide
Temperature*control= temperature effect on fungicide versus no fungicide
Concentration= difference (comparison) between concentrations
Fungicide= difference (comparison) between fungicide types
Time*temperature*control= interaction between time and temperature - effect on fungicide versus no fungicide
Time*concentration= interaction between time and concentration of fungicides
Temperature*concentration= interaction between temperature and concentration of fungicide
Time*fungicide= interaction between time and fungicide type
Temperature*fungicide= interaction between temperature and fungicide type
Concentration*fungicide= interaction between concentration of fungicide and fungicide type
Time*temperature*concentration= interaction between time, temperature and concentration of fungicide
Time*temperature*fungicide= interaction between time, temperature and fungicide type
Time*concentration*fungicide= interaction between time, concentration and fungicide type
Temperature*concentration*fungicide= interaction between temperature, concentration and fungicide type
Time*temperature*concentration*fungicide= interaction between time, temperature, concentration and fungicide type

The interaction between time and concentration of fungicides was significant ($P<0.05$) only for ZEN and HT-2 toxin production. Irrespective of concentration, ZEN and HT-2 toxin production increased with time. However, the lower level of fungicide, 0.1 μg/ml, led to a higher level of mycotoxin production than 1.0 μg/ml. Interaction between temperature regime and concentration of fungicide had a significant effect on ZEN ($P<0.05$), T-2 ($P<0.01$) and DAS ($P<0.001$) production. At concentration of 1.0 μg/ml, there was an increase in ZEN production with the decrease in temperature, yet 0.1 μg/ml level of fungicides led to a higher production of ZEN irrespective of temperature regime. T-2 toxin production decreased with the decrease in temperature, irrespective of fungicide level, although taken separately, there was an increase in T-2 toxin production at 1.0 μg/ml compare to 0.1 μg/ml at 25°C in contrast to a decrease in T-2 toxin production at 1.0 μg/ml compared to 0.1 μg/ml under the 25-11°C temperature regime. DAS production increased with the decrease in temperature at both levels of fungicide, namely 0.1 and 1.0 μg/ml. However, at 25°C, DAS was produced in a higher concentration at 1.0 μg/ml and at 25-11°C temperature regime, DAS production was higher at 0.1 μg/ml.

Interaction between time and fungicide type had a significant influence on DAS ($P<0.01$) and NEO ($P<0.05$) production. Irrespective of fungicide type, DAS and NEO production increased with time.

Temperature and fungicide type interaction had a significant ($P<0.01$) effect on DAS and NEO production. The lower temperature regime, 25-11°C led to increased DAS and NEO production irrespective of the fungicide type involved.

The interaction between concentration of fungicide and fungicide type had a significant effect ($P<0.01$) only on NEO production. At 0.1 μg/ml, *F. sporotrichioides* colonies grown on PDA with carbendazim+maneb and carbendazim+propiconazole produced more NEO than the ones grown on PDA with carbendazim only, whereas colonies grown on PDA containing carbendazim+ maneb+tridemorph produced slightly less NEO compare to carbendazim.

The interaction between time, temperature regime and concentration of fungicide had an effect ($P<0.05$) on HT-2 toxin production. Irrespective of fungicide concentration

HT-2 toxin production increased with time, and irrespective of time, the lower level of fungicide (0.1 μg/ml) led to higher levels of HT-2 toxin.

The interaction between time, temperature regime and fungicide type had a significant effect ($P<0.01$) on DAS, HT-2 and NEO production.

Interaction between temperature regime, concentration and fungicide type had a significant ($P<0.001$) effect on T-2 and DAS production.

The interaction between time, temperature regime, concentration and fungicide type had a significant ($P<0.01$) effect only on HT-2 toxin production.

5.8 Experiment 8 : The effects of difenoconazole (added as Plover) and time on fungal growth and mycotoxin production in liquid culture

It was established at the completion of this experiment, that fungal growth (mass) in control cultures was of 0.5 g, a finding of utmost importance for experimental design involving RNA extraction. TLC analyses of mycotoxin production indicated that, T-2 toxin and DAS were present in all samples (at 24 and 48 hours of growth), being more concentrated in extracts obtained from cultures with difenoconazole levels of 0.0 (control) and 0.1 μg/ml GYEP, with NEO showing only in extracts from cultures grown on 0.0 and 0.1 μg difenoconazole/ml GYEP at 48 hours (Figure 5.1 and 5.2 respectively). It was also established that trichothecene mycotoxins were produced under this conditions (liquid media, shaking, 25°C under the influence of fungicide difenoconazole).

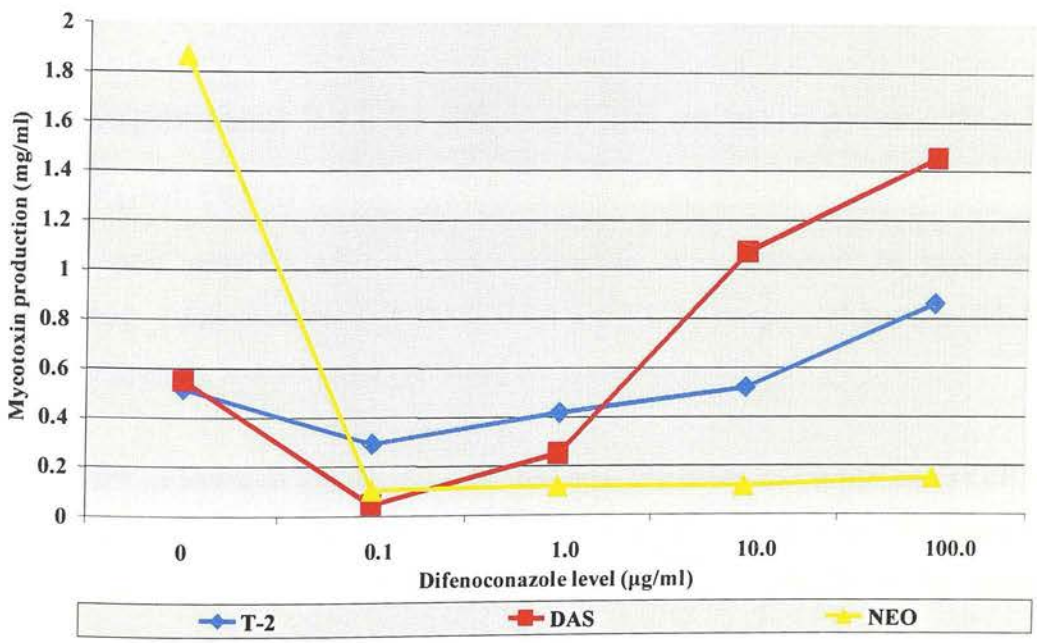

Figure 5.1 **Experiment 8 : Mycotoxin production (T-2, DAS and NEO) by *Fusarium sporotrichioides* grown in GYEP, with different levels of difenoconazole, at 25°C and 24 hours of growth**

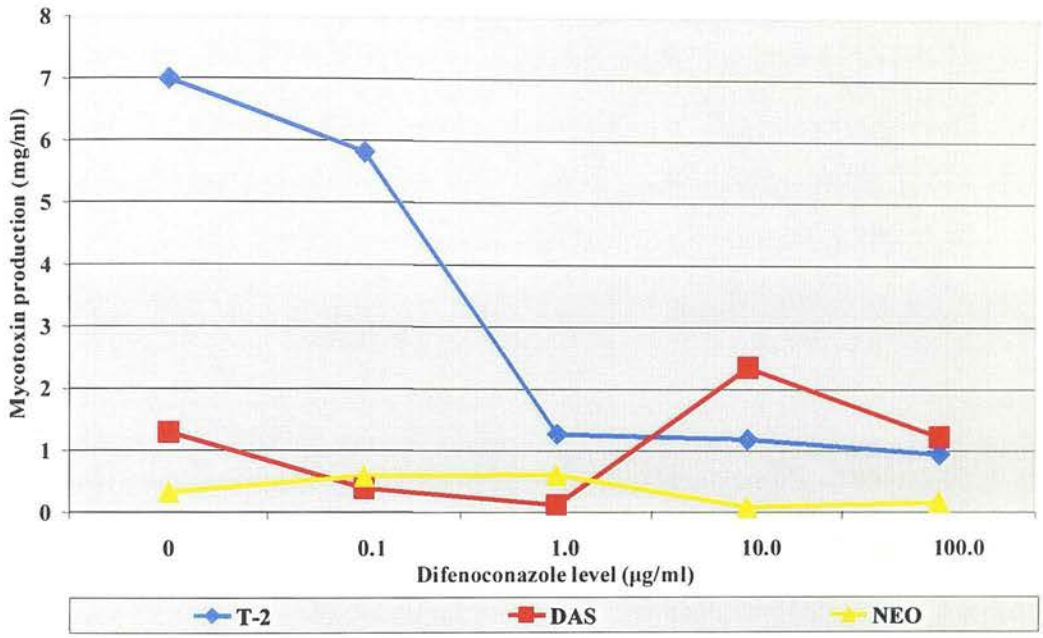

Figure 5.2 **Experiment 8 : Mycotoxin production (T-2, DAS and NEO) by *Fusarium sporotrichioides* grown in GYEP with different levels of difenoconazole, at 25°C and 48 hours of growth**

5.9 Experiment 9 : The effect of time on toxin production in liquid culture

The filtrates resulting after mycelial separation, were analysed for mycotoxin production. Trichothecenes T-2, DAS, HT-2 and NEO were present starting with 48 hours of growth, and the results obtained are presented in Figure 5.3.

Mycelium collected at 24, 48, 72, 120, 168 and 264 hours of growth on GYEP at 25°C was utilized for RNA extraction. Mycelium collected at 6 and 12 hours of growth was insufficient for RNA extraction therefore it was abandoned. The results from the RNA extraction are depicted on Plate 5.7.

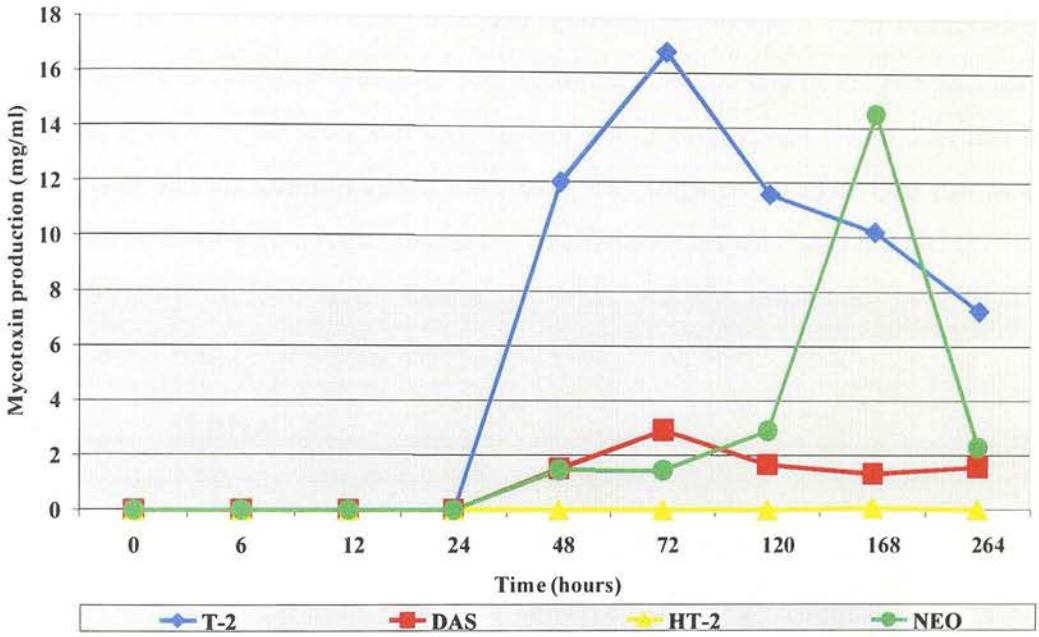

Figure 5.3 Experiment 9 : Mycotoxin production (T-2, DAS, HT-2 and NEO) by *Fusarium sporotrichioides* grown on GYEP, at 25°C

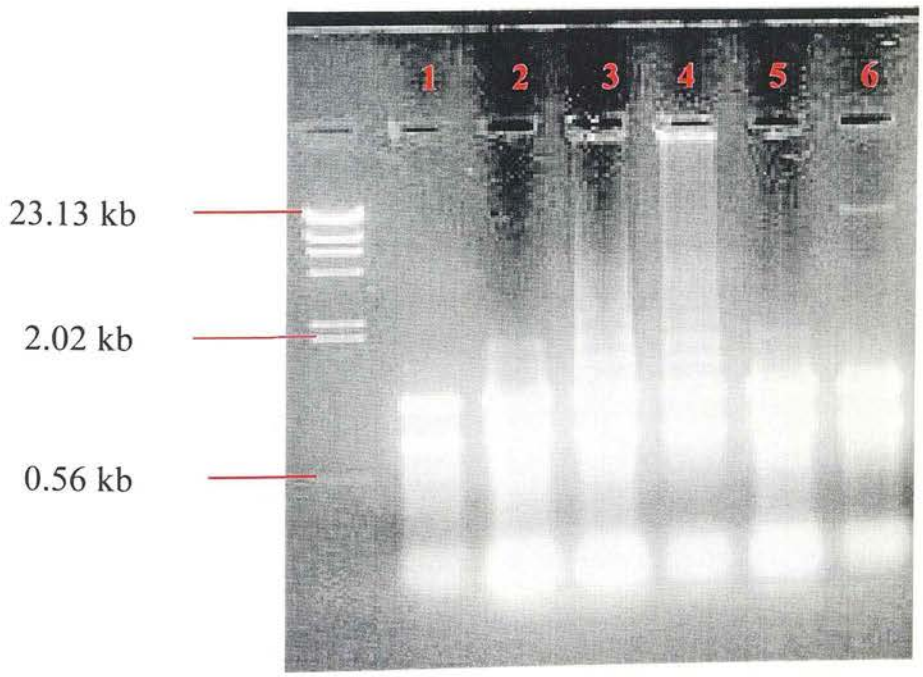

Plate 5.7 Experiment 9 : Total RNA extracted from mycelia of *Fusarium sporotrichioides*. Lanes 1-6 relate to 24, 48, 72, 120, 168 and 264 hours of growth.

After spectrophotometer reading and adjustment to 10 μg/5μl, a 1.2% formaldehyde agarose gel was prepared with four sets of samples. At the end of the run, one set of samples (gel) was cut from the rest, stained and photographed (Plate 5.8) and the other three sets of samples (gels) were used for Northern transfer. One membrane was used in conjunction with radioactive labelled probes (see Plates 5.9 and 5.10), in the laboratory in John Innes Centre, Norwich, and the other two were used in combination with DIG-labelled probes (see Plate 5.15), in Edinburgh.

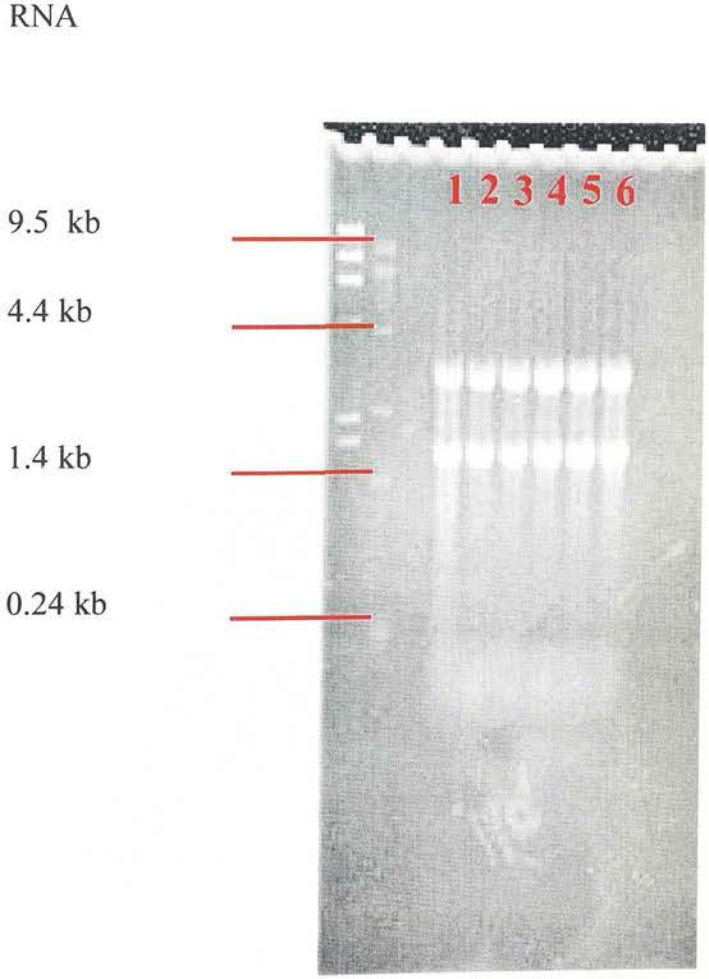

Plate 5.8 Experiment 9 : Formaldehyde agarose gel with a set of total RNA samples from *Fusarium sporotrichioides*, identical to those transferred onto nylon membranes. Lanes 1-6 relate to 24, 48, 72, 120, 168 and 264 hours of growth.

Three sets of samples (gels) were transferred onto Nylon membranes. One membrane was hybridized using the radioactive probes, β-tubulin and *Tri5* (courtesy of Dr. Lesley Boyd, John Innes Centre) and the other two membranes were hybridized using DIG-labelled probes obtained from Norwich as PCR products and prepared in Edinburgh. The results of the hybridizations using radioactive probes are presented in Plate 5.9 for β-tubulin and Plate 5.10 for *Tri5*. Hybridization obtained with β-tubulin shows a slight variation in the amount of RNA in each lane, with less RNA in lanes 1, 5 and 6 (24, 168 and 264 h respectively). Hybridization obtained with *Tri5* shows an apparent signal in lanes 3 and 4 (72 and 120 h).

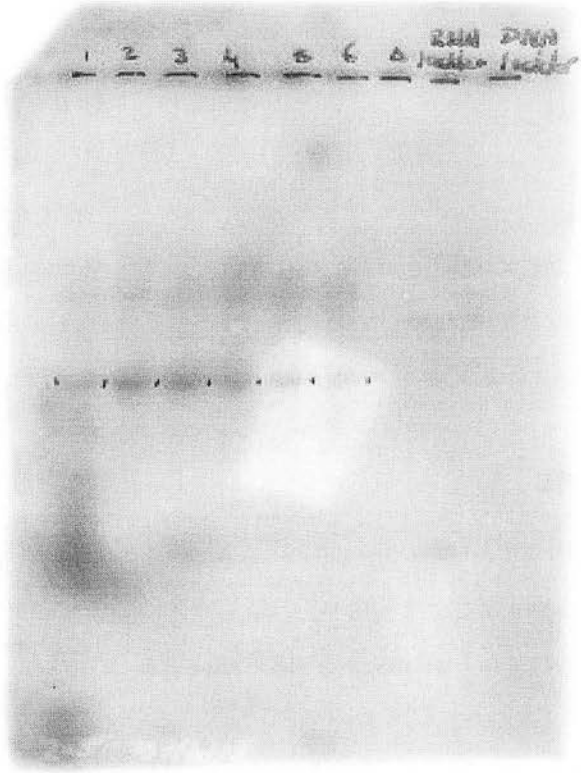

Plate 5.9 Experiment 9 : Northern blot hybridized with β-tubulin radioactively labelled probe. The film was exposed for ten days prior to development. Lanes 1-6 relate to 24, 48, 72, 120, 168 and 264 hours growth of *Fusarium sporotrichioides* respectively.

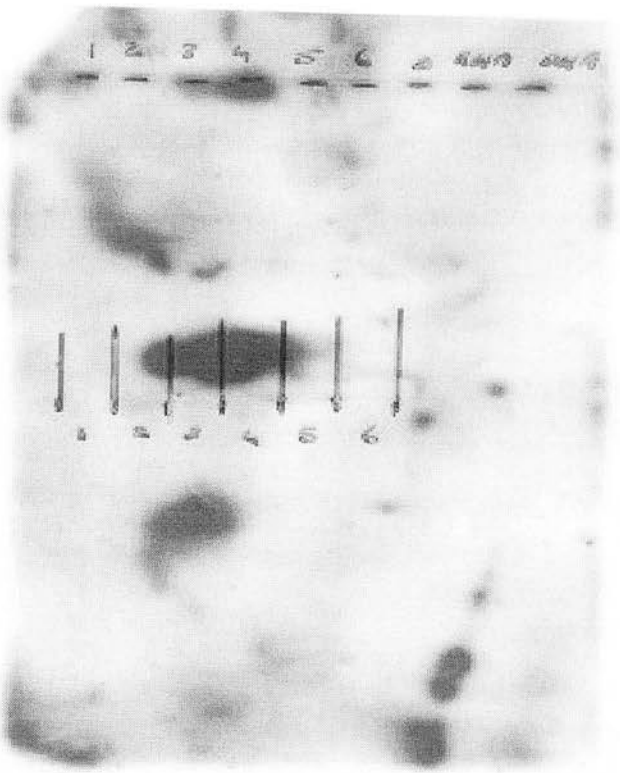

Plate 5.10 Experiment 9 : Northern blot hybridized with *Tri5* radioactively labelled probe. The film was exposed for 8 days prior to development. Lanes 1-6 relate to 24, 48, 72, 120, 168 and 264 hours growth of *Fusarium sporotrichioides*.

The first attempt to probe preparation in Edinburgh was to purify β-tubulin and *Tri5* from agarose gel using WIZARD™ PCR Prep Purification kit (Promega) (see 4.7.12.1 and 4.7.13.5 -1). Good results were not obtained even when the purification method (see 4.7.13.8 -1) was improved. The new PCR products received, β-tubulin, *Tri5* and *Tri6* respectively, were first amplified by PCR using specific primers (see 4.7.13.4 -1) in order to produce more material to work with (see 4.7.13.1 -2), then labelled. The first attempt to DIG labelling was done by PCR (see 4.7.14.1) with no consistent results. Consequently, a commercial kit for DIG labelling by random priming was employed (see 4.7.14.2 -1). DIG labelling was successful when using DIG High Prime DNA Labelling and Detection Starter Kit II. Results of DIG labelling (see 4.7.14.2 -1) efficiency are presented in the following plates.

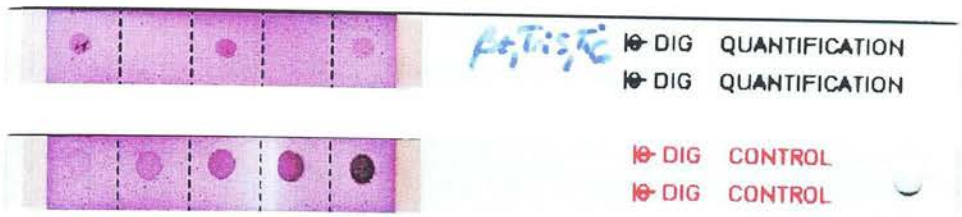

Plate 5.11 Experiment 9 : Example of quantification of labelling efficiency for β-tubulin, *Tri5* and *Tri6* DIG-labelled probes using test strips. The control teststrips coated with positively charged nylon membrane, are loaded with DIG labelled control DNA in the quantities 3, 10, 30, 100 and 300 pg.

Several combinations of parameters were used for hybridization of the remaining two Northern blots with DIG labelled DNA probes (see Table 5.45) but no distinct signal was obtained even after 24 hours film exposure.

Table 5.45 Experiment 9 : Combinations of hybridization parameters used for Northern blots probed with DIG labelled β-tubulin, *Tri5* and *Tri6*

Succession of steps	Parameters (°C) and signal detection	
Prehybridization	50	50
Hybridization	50	50
Washes - primary	R.t.	R.t.
Washes - secondary	42	R.t.
Detection		
β-tubulin	No distinct signal	No distinct signal
Tri5	No distinct signal	No distinct signal
Tri6	No distinct signal	No distinct signal

R.t. = room temperature

Due to lack of distinct signals on the Northern blots, and the number of times a membrane can be stripped and reprobed a series of dot blots were prepared.

A DNA:DNA dot blot was carried out in order to establish the binding efficiency of the probes (see 4.7.15.1) to themselves. The DNA:DNA dot blot was hybridized with *Tri5* and *Tri6* at 42°C using DIG Easy Hyb followed by room temperature washes. The probes hybridized successfully and consequently DNA:RNA dot blots were prepared (see 4.7.15.2). The blots were hybridized (see 4.7.18.2 -1) with *Tri5* and *Tri6* using several combinations of hybridization parameters (see Table 5.46), in order to find the optimum parameters. The RNA samples correspond to the time course (24, 48, 72, 120, 168 and 264 hours of growth) that constitutes experiment 9. The results obtained using 50°C for prehybridization and hybridization steps and room temperature for posthybridization washes, are presented in Plates 5.12, 5.13 and 5.14.

Table 5.46 Experiment 9 : Combinations of hybridization parameters used for the DNA:RNA dot blots

Hybridization parameters (°C) and Signal	**Probe**						
	β-tubulin	***Tri5***		***Tri6***			
Prehybridization	50	50	60	50	50	68	55
Hybridization	50	50	60	50	50	68	55
Posthybridization washes	R.t.	R.t.	R.t.	R.t.	42	R.t.	R.t.
Signal	Present in all samples	Present in all samples	No signal	Present in all samples	No signal	No signal	No signal

R.t.= room temperature

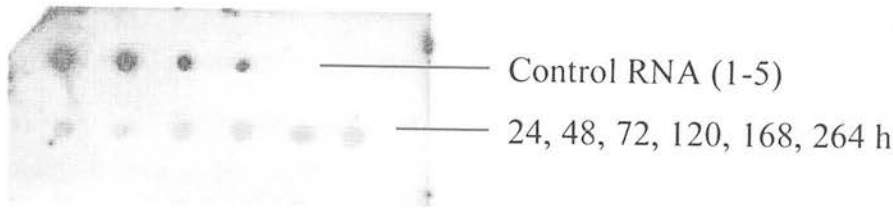

Plate 5.12 Experiment 9 : Dot blot hybridization of RNA obtained from *Fusarium sporotrichioides* with β-tubulin DIG-labelled DNA probe. Prehybridization and hybridization at 50°C followed by room temperature posthybridization washes and overnight film exposure. Concentrations of control RNA: 500, 50, 5, 0.5 and 0.05 ng/μl (1-5 respectively).

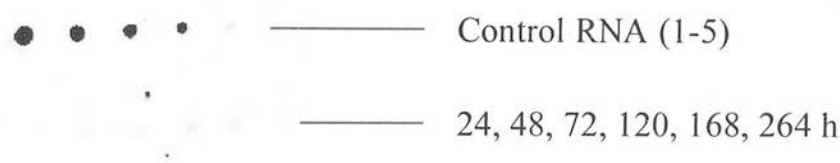

Plate 5.13 Experiment 9 : Dot blot hybridization of RNA obtained from *Fusarium sporotrichioides* with *Tri5* DIG-labelled DNA probe. Prehybridization and hybridization at 50°C followed by room temperature posthybridization washes and one hour film exposure. Concentrations of control RNA included: 500, 50, 5, 0.5 and 0.05 ng/μl (1-5 respectively).

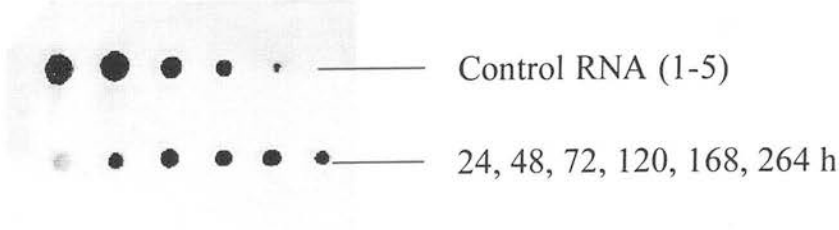

Plate 5.14 Experiment 9 : Dot blot hybridization of RNA obtained from *Fusarium sporotrichioides* with *Tri6* DIG-labelled DNA probe. Prehybridization and hybridization at 50°C followed by room temperature posthybridization washes and one hour film exposure. Concentrations of control RNA included: 500, 50, 5, 0.5 and 0.05 ng/μl (1-5 respectively).

After the series of dot blot trials, another Northern blot was prepared using the remaining of the time course samples (Exp. 9), fixed using UV cross-linking rather than alkaline fixation of membrane and hybridized without delay, using the parameters delimited by the DNA:RNA dot blots.

Table 5.47 Experiment 9 : Details of hybridization parameters and detection used with the Northern blot

Hybridization parameters (°C) and Detection	Probe		
	β-tubulin	*Tri5*	*Tri6*
Prehybridization	50	50	50
Hybridization	50	50	50
Posthybridization washes	R.t.	R.t.	R.t.
Signal detection	2.5 h and O.n.	2.5 h	2.5 h and O.n.

R.t.= room temperature
O.n.= overnight

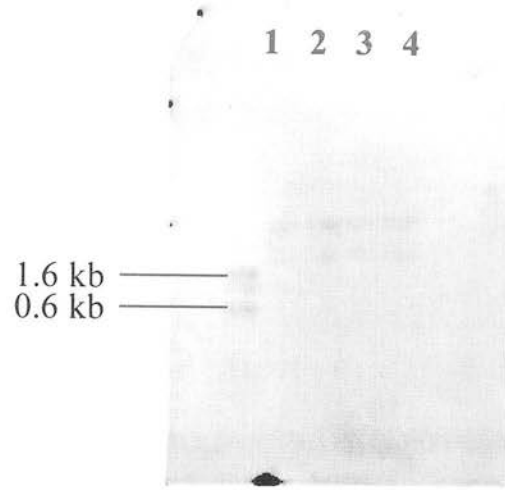

Plate 5.15 Experiment 9 : Northern blot hybridized with β-tubulin DIG-labelled probe. Prehybridization and hybridization at 50°C followed by room temperature posthybridization washes and 2.5 hours film exposure. Lanes 1-4 relate to 48, 72, 120 and 168 hours growth of *Fusarium sporotrichioides* respectively.

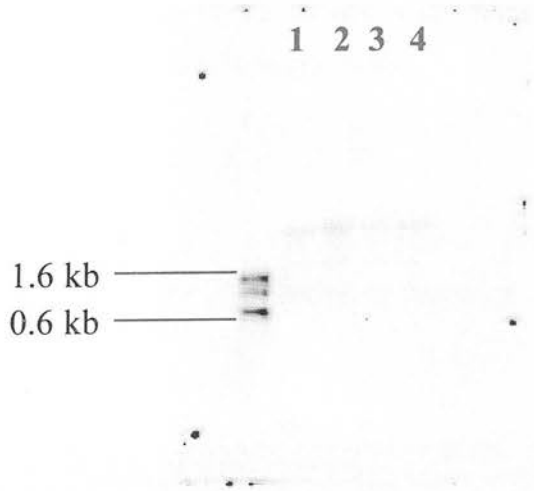

Plate 5.16 Experiment 9 : Northern blot hybridized with *Tri5* DIG-labelled probe. Prehybridization and hybridization at 50°C followed by room temperature washes and 2.5 hours film exposure. Lanes 1-4 relate to 48, 72, 120 and 168 hours growth of *Fusarium sporotrichioides* respectively.

Signals were obtained in Northern blots hybridized with β-tubulin, *Tri5* and *Tri6*. However, the signals obtained were not the expected RNA transcript size and distribution. The bands may correspond to ribosomal RNA subunits as observed in the formaldehyde agarose gel (see Plate 5.8).

5.10 Experiment 10 : The effects of carbendazim and time on gene expression and mycotoxin production

Results of mycotoxin analysis from this experiment are presented in Tables 5.48-5.50. Time, fungicide concentration as well as the interaction between time and concentration of fungicide had a highly significant ($P<0.001$) effect on T-2 toxin, DAS and NEO production. Irrespective of fungicide concentration T-2 toxin and NEO production increased with time while DAS production reached a peak at 72 hours of growth followed by a decline by 264 hours of growth. Irrespective of time, T-2 toxin, DAS and NEO generally decreased with the increase in fungicide concentration (Table 5.48, 5.49 and 5.50 respectively).

Table 5.48 Experiment 10 : T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on GYEP with different levels of carbendazim, at six time points

Carbendazim level (μg/ml)	Time (hours)						Mean
	30	36	48	72	120	264	
0.0	0.0	0.45	8.66	16.50	20.90	18.58	10.85
1.0	0.0	0.11	4.01	4.29	2.11	4.89	2.57
2.0	0.0	0.0	0.0	0.18	0.16	0.10	0.07
3.0	0.0	0.0	0.0	0.15	0.22	0.15	0.09
Mean	0.0	0.14	3.17	5.28	5.85	5.93	3.39

Table 5.49 Experiment 10 : DAS production (mg/ml) by *Fusarium sporotrichioides* grown on GYEP with different levels of carbendazim, at six time points

Carbendazim level (µg/ml)	Time (hours)						Mean
	30	36	48	72	120	264	
0.0	0.0	0.35	2.45	2.25	2.64	1.28	1.49
1.0	0.0	0.0	0.80	1.13	0.45	0.68	0.51
2.0	0.0	0.0	0.0	0.0	0.0	0.0	0.00
3.0	0.0	0.0	0.0	0.0	0.0	0.02	0.003
Mean	0.0	0.09	0.81	0.84	0.77	0.49	0.50

Table 5.50 Experiment 10 : NEO production (mg/ml) by *Fusarium sporotrichioides* grown on GYEP with different levels of carbendazim, at six time points

Carbendazim level (µg/ml)	Time (hours)						Mean
	30	36	48	72	120	264	
0.0	0.0	0.06	3.10	3.12	7.41	8.16	3.64
1.0	0.0	0.0	0.84	0.92	0.47	1.55	0.63
2.0	0.0	0.0	0.0	0.06	0.04	0.01	0.02
3.0	0.0	0.0	0.0	0.06	0.08	0.07	0.03
Mean	0.0	0.015	0.98	1.04	2.0	2.45	1.08

Fusarium sporotrichioides mycelium grown on GYEP with different levels of carbendazim, was collected at different time points (20, 24, 30, 36, 48, 72, 120 and 264 hours) and prepared for RNA extraction. The total RNA resulted, is presented in Plate 5.17.

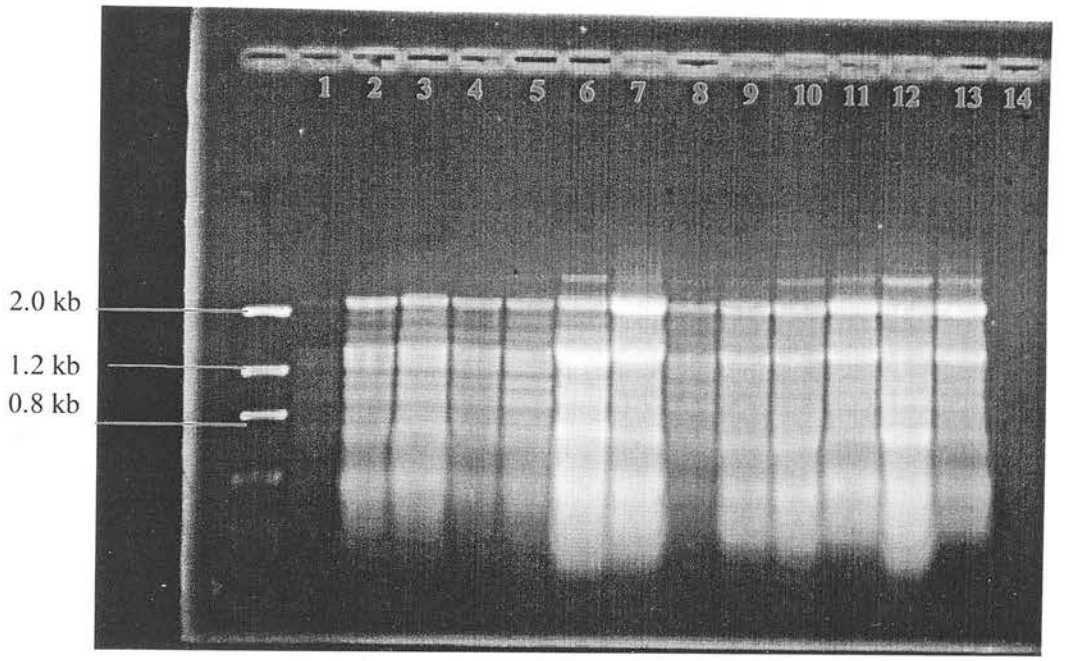

Plate 5.17 Experiment 10 : Total RNA extraction from samples collected at 20, 24, 30, 36, 48, 72, 120 and 264 hours. Details of RNA samples sequence shown on Plate 5.17 are represented in Table 5.51.

Table 5.51 Succession of total RNA samples pictured in Plate 5.17 and their details

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Level of carbendazim	0	0	0	1.0	0	1.0	0	1.0	0	1.0	0	1.0	0	1.0
Time (h)	20	24	30	30	36	36	48	48	72	72	120	120	264	264

After visualizing the RNA on the gels, each set of samples (replicates) was split into two groups: first part of the time course (samples 1-7, 20-48h respectively) and second part of the time course (samples 8-14, 48-264h respectively). For each group of samples four gels were prepared. One was used for photography purposes and the other three transferred onto Nylon membranes.

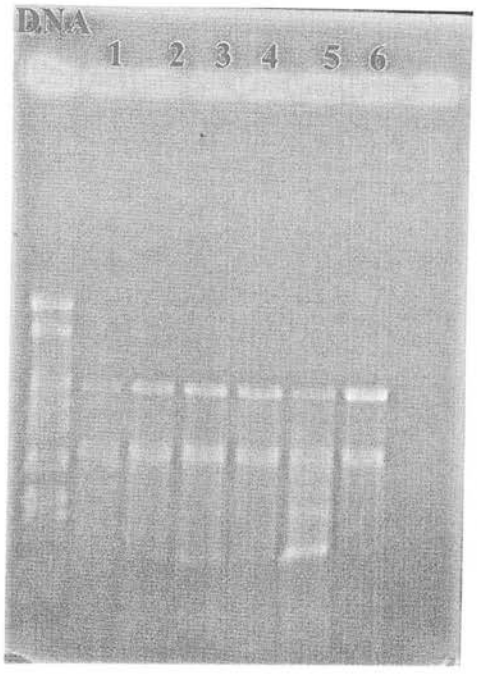

Plate 5.18 Experiment 10 : Gel with total RNA samples from time course, identical to the gels for Northern transfer and probing. Lanes 1-6 relate to 24, 48, 72, 120, 168 and 264 h of growth respectively.

Several trials of DIG labelling during PCR were tested. When using PCR and DIG labelling, a colony or dilution of plasmid preps was used as template DNA, different *Taq* polymerases were tried due to inconsistent results, T3/T7 and M13 forward/reverse (see 4.7.13.1 -2, and -3 respectively). Probe production, using plasmid as a template for PCR yielded inconsistent results. These inconsistent /variable results were always obtained irrespective of PCR primers used. Therefore, probe production was performed by an alternative method, indirect PCR, which consisted of isolation and labelling of the gene probe from a digested plasmid preparation (see 4.7.13.5-7). Probe labelling was performed using the two commercial kits employed in this study (see 4.7.14.2 -1 and 2). Better results were obtained when fluorescent labelling and CSPD/ CDP-Star detection were employed. Results of purified plasmids containing *Tri4*, *Tri5* and *Tri6* probe inserts, overnight

digest reactions, purification of gene fragment from agarose gel and probe labelling (fluorescein) are presented in the following plates.

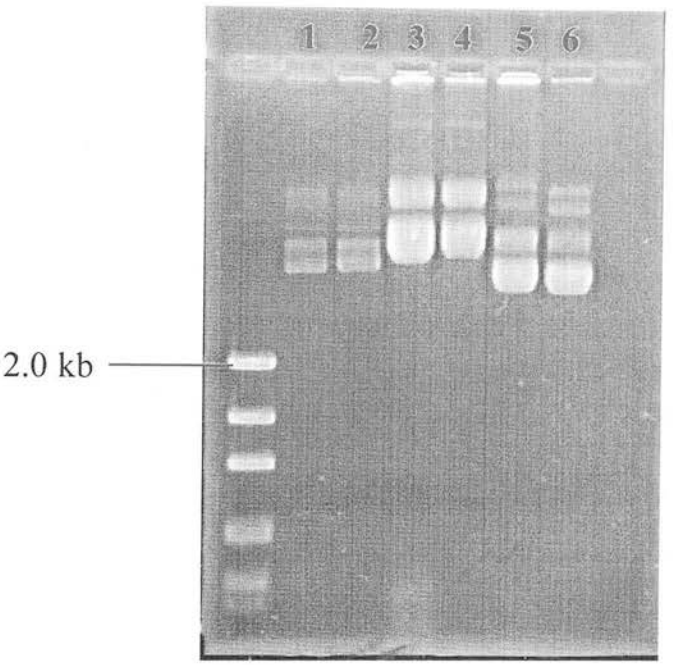

Plate 5.19 Plasmids containing *Tri4* (1, 2), *Tri5* (3, 4) and *Tri6* (5, 6) probe inserts

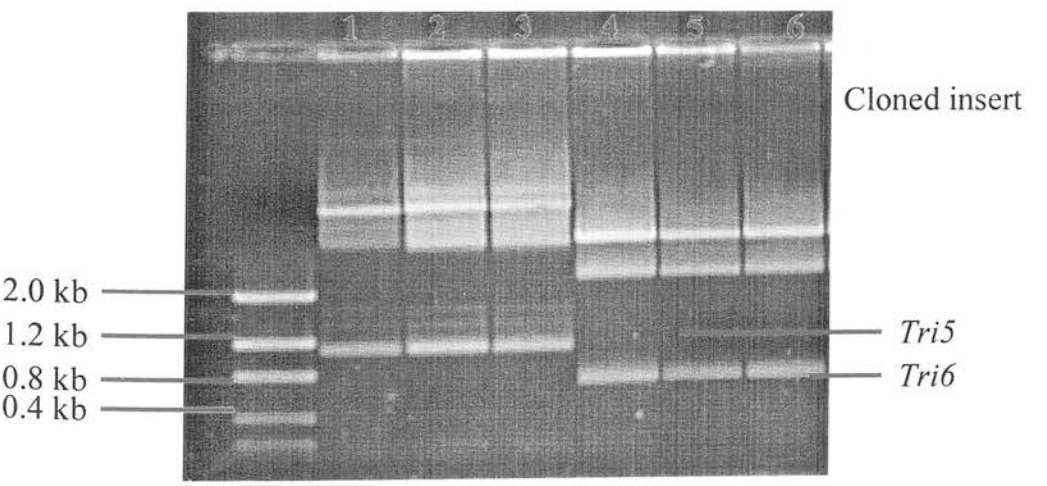

Plate 5.20 Products of overnight restriction enzyme digest reactions for *Tri5* (1-3) and *Tri6* (4-6)

Digestion of the *Tri4* plasmid was problematic due to lack of sequence information and difficulty in digestibility of the plasmid. Digest of *Tri4* with *Pvu* II produced multiple faint bands. Consequently, a single band could not be isolated for this probe.

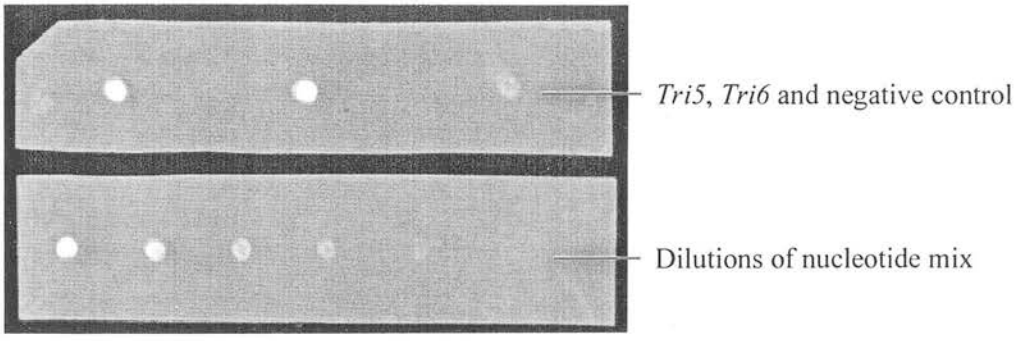

Plate 5.21 Fluorescent labelling of *Tri5* and *Tri6*. Seven dilutions of 5xnucleotide mix in TE buffer were prepared and used as follows: dilution 1/5 as a negative control and subsequent dilutions (1/10, 1/25, 1/50, 1/100, 1/250 and 1/500 respectively) to verify labelling efficiency.

Several combinations of hybridization parameters were evaluated (see Table 5.52) in order to find the optimum combination.

Table 5.52 Experiment 10 : Combinations of hybridization parameters used for Northern blots with *Tri5* and *Tri6* random prime labelled DNA probes obtained from the USA and *Fusarium sporotrichioides* fluorescein labelled rDNA probe prepared in Edinburgh

Probe	*Tri5*			*Tri6*			*F. sporotrichioides*
Prehybridization (°C)	65	50	50	65	50	50	50
Hybridization (°C)	65	50	50	65	50	50	50
Posthybridization washes (°C)	65	50	R.t.	65	50	R.t.	R.t.
Film exposure	1h, O.n., 2days	O.n., 1h, 3days	O.n., 3days	1h, O.n., 2days	O.n, 1h, 3days	O.n.	O.n., 1-2h

O.n. = overnight
R.t. = room temperature

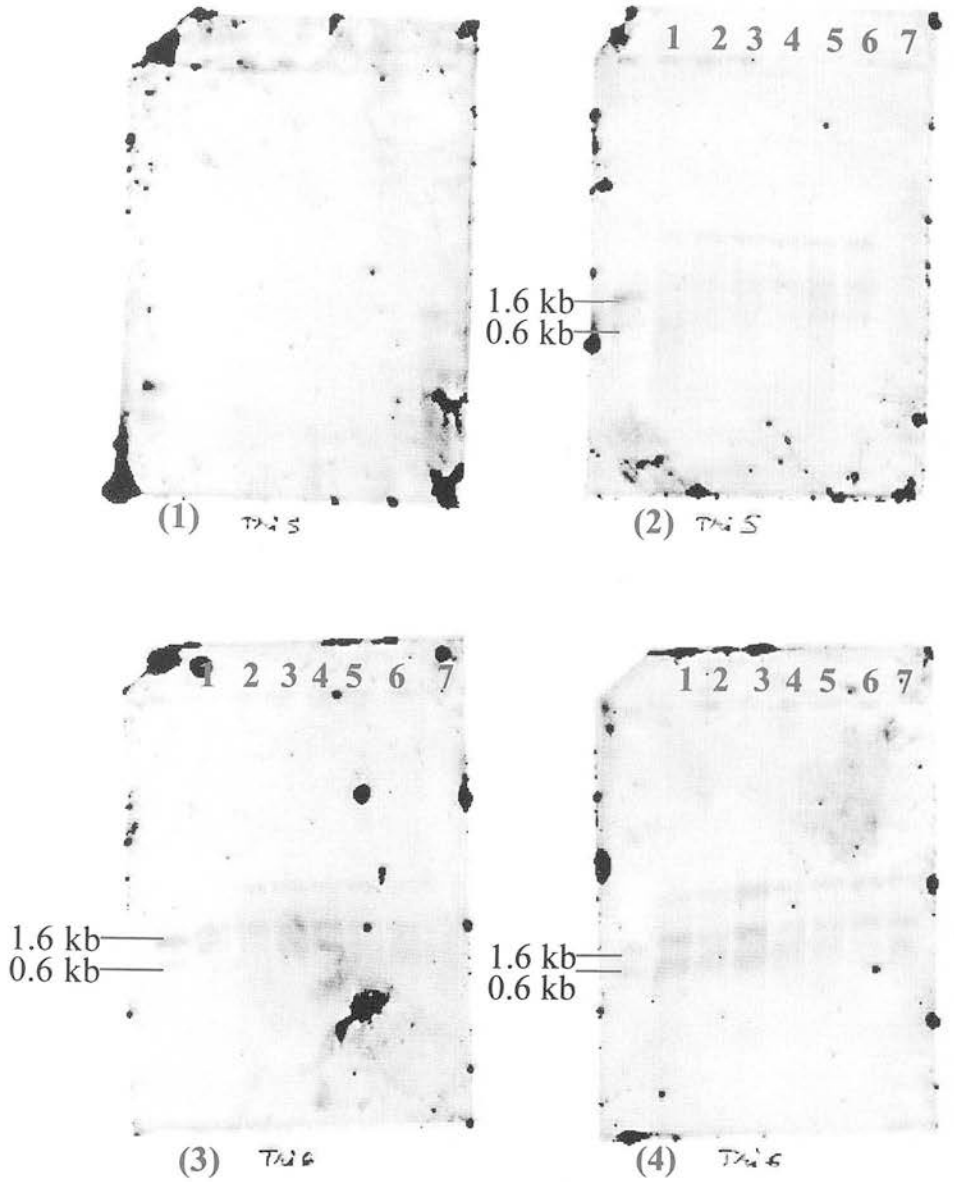

Plate 5.22 Experiment 10 : Northern blots hybridized with *Tri5* and *Tri6*. Prehybridization, hybridization and posthybridization washes at 50°C, with overnight film exposure. (1) first set of replicates, first part of time course (20, 24, 30, 36 and 48 h); (2) first set of replicates, second part of time course (48, 72, 120 and 264 h); (3) second set of replicates, first part of time course; (4) second set of replicates, second part of time course. Blots (1) and (2) were hybridized with *Tri5* while (3) and (4) were hybridized with *Tri6*.

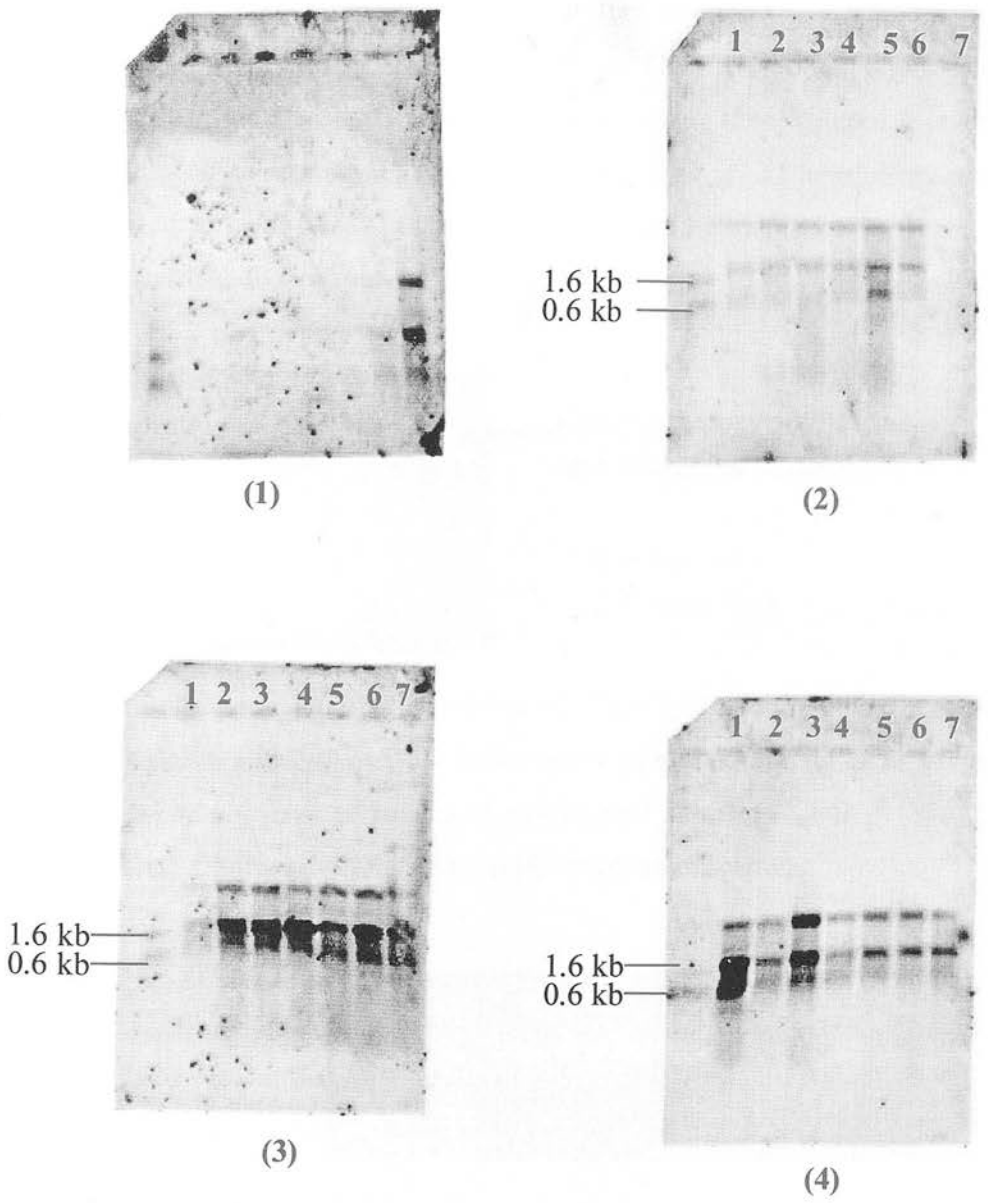

Plate 5.23 Experiment 10 : Northern blots hybridized with *Tri5* and *Tri6*. Prehybridization and hybridization at 50°C with room temperature posthybridization washes and 3 days film exposure. (1) first set of replicates, first part of time course (20, 24, 30, 36 and 48 h); (2) first set of replicates, second part of time course (48, 72, 120 and 264 h); (3) second set of replicates, first part of time course; (4) second set of replicates, second part of timecourse. Blots (1) and (2) were hybridized with *Tri5* while (3) and (4) were hybridized with *Tri6*.

The hybridization signals are similar to those obtained in Experiment 9 (see 5.9). An overlapping of ribosomal RNA with the signals of interest was suspected. In an attempt to differentiate the two signals, hybridization of the Northern blots with fluorescein-labelled ribosomal DNA probe obtained from *F. sporotrichioides* (see 4.7.12.3) was performed. Results of probe labelling and hybridization are presented in plate 5.24 and 5.25 respectively.

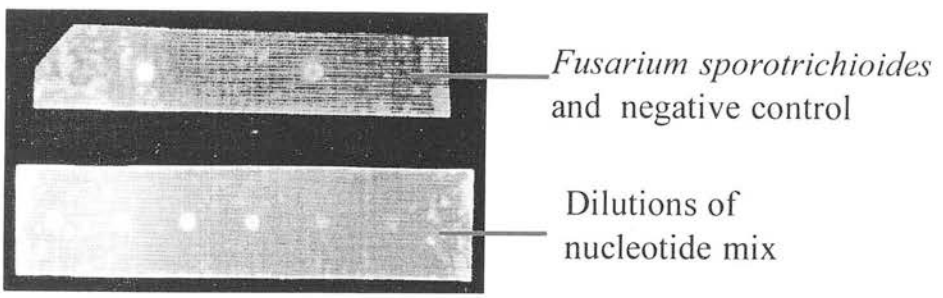

Plate 5.24 Fluorescent labelling of rDNA of *Fusarium sporotrichioides*. Seven dilutions of 5xnucleotide mix in TE buffer were prepared and used as follows: dilution 1/5 as a negative control and subsequent dilutions (1/10, 1/25, 1/50, 1/100, 1/250 and 1/500 respectively) to verify labelling efficiency.

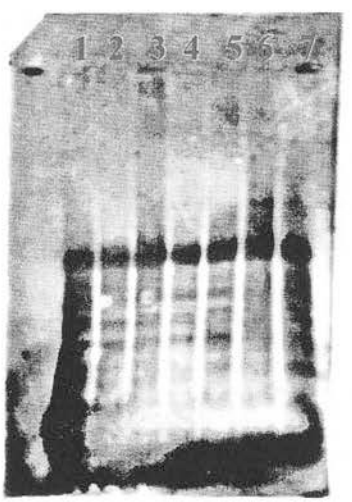

Plate 5.25 Northern blot hybridized with fluorescein labelled rDNA from *Fusarium sporotrichioides*. Prehybridization and hybridization at 50°C followed by posthybridization washes at room temperature and 2 hours film exposure.

Due to the abundance of bands present and position of bands of interest on the Northern blots, it was difficult to accomplish this objective. An alternative method to overcome this problem is discussed (see 6.10). To summarize what has been done for trichothecene gene expression studies, Table 5.53 was created.

Table 5.53 Overview of elements employed in trichothecene gene expression studies

Process	Kit used	Probes				Exp. No.
		Norwich		USA	*F. sp*	
		βtubulin *Tri5*	βtubulin *Tri5* *Tri6*	*Tri4* *Tri5* *Tri6*		
Purification	WIZARD PCR Prep purif. Kit					9
	- without vacuum manifold	+				
	- with vacuum manifold	+				
	- improved method	+				
	QIAEX II agarose gel extraction			+		10
	DNAce purification system			+	+	10
	InViSorb DNA extraction Kit			+		10
	QIAquick PCR purification Kit			+		10
PCR			+	+	+	9, 10
Cloning	TA Cloning KIT			+		10
Labelling	Radioactive (^{32}P)	+				9
	DIG High Prime DNA labelling and detection starter Kit II	+	+	+		9, 10
	Gene Images random prime labelling module			+	+	10
Detection	DIG High Prime DNA labelling and Detection Starter Kit II					
	Gene Images CDP - *Star* detection			+	+	10
Dot blot						9
DNA:DNA			+			
DNA:RNA		+	+	+	+	
Northern blot		+	+	+	+	9, 10

Note: Exp.9 = time course
Exp.10 = time course with fungicide

5.11 Experiment 11 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

Results obtained for this experiment are presented in Tables 5.54-5.56. Colony growth was significantly ($P<0.01$) influenced by temperature regime and concentration of fungicide and similarly, ZEN production ($P<0.05$). Irrespective of temperature regime, colony growth generally decreased with the increase in fungicide concentration while irrespective of fungicide concentration, colony growth was smaller at the lower temperature (Table 5.54). ZEN production increased with the decrease in temperature irrespective of fungicide concentration and generally, increased with the increase in fungicide concentration, irrespective of temperature (Table 5.55).

The interaction between temperature regime and concentration of fungicide had a significant ($P<0.05$) effect only on colony growth.

Table 5.54 Experiment 11 : The effects of carbendazim (added as Bavistin) on development of *Fusarium culmorum* colonies grown on PDA, under two temperature regimes, namely 25°C and 25-11°C after 29 days

Carbendazim level (µg/ml)	Temperature regime (°C)	
	25	25-11
0.0	8.50	8.50
1.0	6.75	8.00
2.5	8.50	8.00
5.0	7.90	3.50
7.5	7.05	4.95
10.0	6.75	2.05
Mean	7.57	5.83
SEM (df=11)	0.78	
LSD (P<0.05)	2.45	

Table 5.55 Experiment 11 : ZEN production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels of carbendazim (added as Bavistin), under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (µg/ml)	Temperature regime (°C)	
	25	25-11
0.0	0.00	0.09
1.0	0.00	0.01
2.5	0.00	0.32
5.0	0.08	0.67
7.5	0.002	0.54
10.0	0.59	0.53
Mean	0.112	0.36
SEM (df=11)	0.08	
LSD (P<0.05)	0.25	

Table 5.56 Experiment 11 : 3-ADON production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels of carbendazim (added as Bavistin), under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (µg/ml)	Temperature regime (°C)	
	25	25-11
0.0	0.00	0.07
1.0	0.00	0.13
2.5	0.87	1.09
5.0	0.42	0.13
7.5	1.09	1.07
10.0	0.00	0.12
Mean	0.40	0.43
SEM (df=11)	0.27	
LSD (P<0.05)	0.85	

5.12 Experiment 12 : The effects of carbendazim (added as Bavistin) and temperature regime on fungal growth and mycotoxin production

Similar to *F. sporotrichioides*, colour differences between colonies grown under two temperature regimes were also observed for *F. culmorum* employed in this experiment. Thus, a yellow pigmentation developed when *F. culmorum* colonies were kept at 25°C and a red pigmentation, when colonies were kept under 25-11°C temperature regime. Plate 5.26 illustrates the colour difference between *F. culmorum* colonies grown on PDA with levels of carbendazim (added as Bavistin) at 0.0, 0.1, 1.0, 10.0, 100.0 µg/ml, under two temperature regimes, 25°C and 25-11°C respectively.

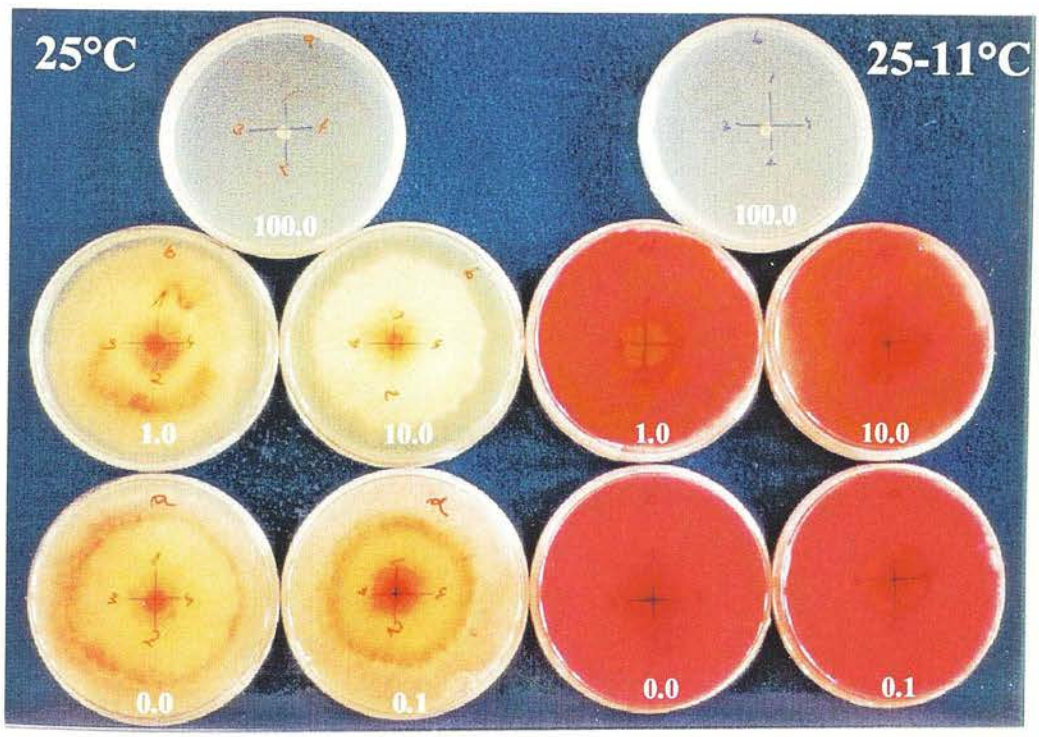

Plate 5.26 Experiment : Reverse of *F. culmorum* colonies grown on PDA with carbendazim (added as Bavistin) at 0.0, 0.1, 1.0, 10.0, 100.0 µg/ml, under two temperature regimes, namely 25°C (left) and 25-11°C (right) after 27 days

Mycotoxin analysis results obtained for this experiment are presented in Tables 5.57-5.60. Temperature regime had a highly significant ($P<0.001$) effect on ZEN and DON production and a significant ($P<0.05$) effect on 3-ADON production by *F. culmorum*. Irrespective of temperature regime, colony growth decreased with the increase in fungicide concentration.

Colony growth was significantly ($P<0.001$) influenced by concentration of carbendazim. Concentration of fungicide had also a highly significant ($P<0.001$) effect on ZEN, DON and 3-ADON production and, similarly, the interaction between temperature regime and fungicide concentration produced an equal effect. Irrespective of fungicide concentration, ZEN production increased with the decrease in temperature (Table 5.58), while DON and 3-ADON production decreased with the decrease in temperature (see Tables 5.59 and 5.60 respectively). Irrespective of temperature regime, ZEN, DON and 3-ADON production reached a peak at 10.0 μg/ml.

Table 5.57 Experiment 12 : The effects of carbendazim (added as Bavistin) on development of *Fusarium culmorum* colonies grown on PDA, under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	5.00	4.65
0.1	4.50	4.00
1.0	5.13	3.90
10.0	3.80	4.37
100.0	0.00	0.00
Mean	3.69	3.38
SEM (df=27)	0.40	
LSD (P<0.05)	2.10	

Table 5.58 Experiment 12 : ZEN production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels of carbendazim, under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	0.00	0.45
0.1	0.03	0.44
1.0	0.00	0.35
10.0	0.00	0.61
100.0	0.00	0.00
Mean	0.01	0.37
SEM (df=27)	0.05	
LSD (P<0.05)	0.26	

Table 5.59 Experiment 12 : DON production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels of carbendazim, under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	1.57	0.00
0.1	0.00	0.00
1.0	1.98	0.00
10.0	2.88	0.00
100.0	0.00	0.00
Mean	1.29	0.00
SEM (df=27)	0.24	
LSD (P<0.05)	1.24	

Table 5.60 Experiment 12 : 3-ADON production(mg/ml) by *Fusarium culmorum* grown on PDA with different levels of carbendazim, under two temperature regimes, namely 25°C and 25-11°C

Carbendazim level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	3.29	1.22
0.1	0.20	2.71
1.0	4.01	0.73
10.0	3.55	2.35
100.0	0.00	0.00
Mean	2.21	1.40
SEM (df=27)	0.5	
LSD (P<0.05)	1.06	

5.13 Experiment 13 : The effects of bromoxynil + ioxynil + mecoprop (added as Swipe) and temperature regime on mycotoxin production

Toxin analysis has been performed at the completion of the experiment, which lasted one month. The results obtained show that the production of mycotoxins was significantly (P<0.001) influenced by temperature regime; while zearalenone (ZEN) production increased with the decrease in temperature (Table 5.61), deoxynivalenol (DON) and 15-acetyldeoxynivalenol (15-ADON) production decreased with the decrease in temperature (Tables 5.62 and 5.64 respectively).

There is a consistent effect of increase in mycotoxin production when using the herbicide level of 0.1 μg/ml. For example, under the 25°C temperature regime, in the case of DON and 15-ADON there is a four fold increase between 0.1μg/ml and control, for 3-ADON there is a twenty fold increase between 0.1μg/ml and control. At the next level of herbicide used in this experiment, 1.0μg/ml, 15-ADON increased by a factor of six compared with the control. Under the 25-11°C temperature regime,

ZEN production is more than one fold increased at 0.1μg/ml compared with the control. In conclusion, there is an overall effect of 4-6 fold increase in mycotoxin production with the use of low levels of herbicide. It has to be mentioned that at the highest level of fungicide (100 μg/ml) used in this experiment mycotoxin production was reduced but not eliminated.

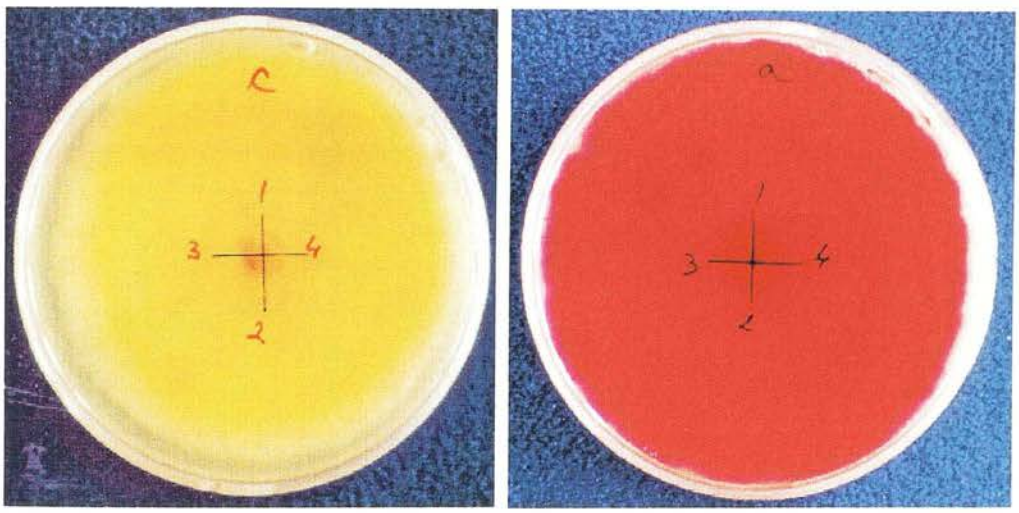

Plate 5.27 Experiment 13 : Reverse of *F. culmorum* colonies grown on PDA at two temperature regimes, namely 25°C (left) and 25-11°C (right) at 33 days of colony growth

Table 5.61 Experiment 13 : ZEN production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels (0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml) of herbicide Bromoxynil-Ioxynil-Mecoprop (added as Swipe), under two temperature regimes, namely 25°C and 25-11°C

Herbicide level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	0.00	0.28
0.1	0.00	0.36
1.0	0.00	0.42
10	0.00	0.40
100	0.00	0.21
Mean	0.00	0.33
SEM (df=27)	0.06	
LSD (P<0.05)	0.16	

Table 5.62 Experiment 13 : DON production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels (0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml) of herbicide Bromoxynil-Ioxynil-Mecoprop (added as Swipe), under two temperature regimes, namely 25°C and 25-11°C

Herbicide level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	1.71	0.00
0.1	6.91	0.00
1.0	3.19	0.00
10	1.78	0.00
100	2.36	0.00
Mean	3.19	0.00
SEM (df=27)	1.08	
LSD (P<0.05)	3.15	

Table 5.63 Experiment 13 : 3-ADON production by *Fusarium culmorum* grown on PDA with different levels (0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml) of herbicide Bromoxynil-Ioxynil-Mecoprop (added as Swipe), under two temperature regimes, namely 25°C and 25-11°C

Herbicide level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	0.69	1.88
0.1	16.35	1.51
1.0	1.22	1.35
10	1.83	0.84
100	1.49	0.68
Mean	6.63	1.25
SEM (df=27)	4.78	
LSD (P<0.05)	13.92	

Table 5.64 Experiment 13 : 15-ADON production (mg/ml) by *Fusarium culmorum* grown on PDA with different levels (0.0, 0.1, 1.0, 10.0 and 100.0 μg/ml) of herbicide Bromoxynil-Ioxynil-Mecoprop (added as Swipe), under two temperature regimes, namely 25°C and 25-11°C

Herbicide level (μg/ml)	Temperature regime (°C)	
	25	25-11
0.0	1.11	0.00
0.1	5.22	0.00
1.0	6.41	0.00
10	0.00	0.00
100	5.87	0.00
Mean	3.72	0.00
SEM (df=27)	1.39	
LSD (P<0.05)	4.06	

5.14 Experiment 14 : The effects of difenoconazole (added as Plover) and temperature regime on fungal growth and mycotoxin production

Mycotoxin analysis results obtained from this experiment are presented in Tables 5.65-5.67.

Temperature regime had a highly significant ($P<0.001$) effect on 3-ADON production and a significant ($P<0.01$) effect on ZEN production. Colony growth (Table 5.65) as well as ZEN (Table 5.66) and 3-ADON production (Table 5.67) generally decreased with the decrease in temperature.

Concentration of fungicide had a highly significant ($P<0.001$) effect on growth and 3-ADON production. Irrespective of temperature regime, colony growth and 3-ADON production decreased with the increase in fungicide concentration (Tables 5.65 and 5.67).

Interaction between temperature regime and fungicide concentration was highly significant ($P<0.001$) for colony growth and 3-ADON production.

Table 5.65 Experiment 14 : The effect of difenoconazole (added as Plover) on development of *Fusarium graminearum* colonies (cm), grown on PDA, under two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (µg/ml)	Temperature regime (°C)		Mean
	25	25 -11	
0.0	8.4	8.4	8.4
0.1	8.4	8.4	8.4
1.0	7.42	8.4	7.91
10.0	8.17	3.87	6.02
100.0	5.1	5.25	5.17
Mean	7.5	6.9	7.2
SEM(df=26)	0.57		
LSD (P<0.05)	3.03		

Table 5.66 Experiment 14 : ZEN production (mg/ml) by *Fusarium graminearum* grown on PDA with different levels of difenoconazole (added as Plover), under two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)		Mean
	25	25 -11	
0.0	0.05	0.00	0.02
0.1	0.04	0.04	0.04
1.0	0.03	0.02	0.02
10.0	0.09	0.00	0.04
100.0	0.04	0.03	0.03
Mean	0.05	0.02	0.03
SEM(df=26)	0.015		
LSD (P<0.05)	0.061		

Table 5.67 Experiment 14 : 3-ADON production (mg/ml) by *Fusarium graminearum* grown on PDA with different levels of difenoconazole, under two temperature regimes, namely 25°C and 25-11°C

Difenoconazole level (μg/ml)	Temperature regime (°C)		Mean
	25	25 -11	
0.0	0.63	0.12	0.37
0.1	1.67	0.22	0.94
1.0	1.48	0.10	0.79
10.0	1.58	0.00	0.79
100.0	0.58	0.00	0.29
Mean	1.19	0.09	0.64
SEM(df=26)	0.133		
LSD (P<0.05)	0.70		

Chapter 6

Discussion

6 Discussion

6.1 Outcomes of literature review

It was clear at the outset that data on the production of different *Fusarium* mycotoxins and their global occurrence in cereals had not been reviewed for a considerable period of time and that the literature review would be a major undertaking. It is relevant and instructive to discuss the outcomes. Indications for future work, outwith the scope of this thesis, have also emerged but these aspects will be presented in a distinct section.

6.1.1 Qualitative issues

In its current format, Table 2.1 is considered to be original and particularly useful to separate type A and type B trichothecene producers. As such, construction of Table 2.1 has helped to resolve discrepancies posed by several respected authors. Thus *F. sporotrichioides* is clearly a type A trichothecene producer and the claim by Abramson *et al.* (1993) that it is also capable of synthesising DON is inconsistent with the overall pattern in Table 2.1. Furthermore evidence from the current experiments (see, for example, Tables 5.12, 5.34 and 5.50) confirm *F. sporotrichioides* to be a type A trichothecene producer. Likewise, the report by Abramson *et al.* (1993) that *F. graminearum* is an HT-2 toxin producer also appears to be anomalous. The evidence in Table 2.1 shows that this phytopathogen is overwhelmingly a type B trichothecene producer and the results of the current series of experiments confirm this conclusion. Four studies indicate that *F. poae* is both a type A and type B trichothecene producer, but the marked discrepancy between the results of Abramson *et al.* (1993) and Sugiura *et al.* (1993) requires resolution (Table 2.1). Given that *Fusarium* species identification using conventional techniques is fraught with difficulties occasional anomalies regarding the pattern of mycotoxin

production are inevitable. In this regard it is worth noting that *F. nivale* is now classified as *Microdochium nivale*. The published mycotoxin pattern of this species has been disregarded in the construction of Table 2.1 as subsequent studies (Briere *et al.*, 1999, personal communication) suggested the absence of trichothecene formation in different strains of *M. nivale*. The implications of the data in Table 2.1 were considered to be original and important enough to warrant participation of the present author in a review publication (D'Mello *et al.*, 1997).

6.1.2 Quantitative aspects

It was deemed necessary to determine the extent to which the qualitative data in Table 2.1 translated into the practical issue of mycotoxin contamination of cereal grains and animal feed. Global trichothecene distribution had previously been reviewed by Scott (1989) and Yoshizawa (1991) but considerable data had since accumulated on these and the more novel mycotoxins such as fumonisins. In the meantime, the question of co-contamination had also gained momentum. An updated survey was clearly justified and a publication duly appeared (Placinta *et al.*, 1999). That review showed that the qualitative diversity, as represented by the data in Table 2.1, was amply expressed in terms of natural contamination of cereal grains with the *Fusarium* mycotoxins (see also Table 2.2 and 2.3). Global aspects were emphasized as were issues relating to co-contamination. For example, high incidence rates of contamination with DON and NIV were reported for maize samples in New Zealand. In Poland, unacceptably high values (up to 927 mg/kg) for DON were recorded for maize grain and cobs. Potentially harmful levels of DON (up to 40 mg/kg) were also observed in wheat produced in Germany, Poland, Japan, New Zealand, USA, Canada and Argentina. Samples of barley grain in Norway, Japan and USA were found with DON levels of up to 71 mg/kg. In the Norwegian study oat samples were also contaminated with DON at levels ranging from 7-62 mg/kg grain. Abnormally high concentrations of both NIV and ZEN were recorded for some Japanese barley samples (up to 26 and 15 mg/kg, respectively) and for maize produced in New

Zealand (up to 7 and 10.5 mg/kg, respectively). Other trichothecenes such as 3-acetyl DON, DAS, T-2 toxin and HT-2 toxin were also found in cereals and animal feed in both temperate and tropical countries. In Uruguay all samples of maize-based animal feeds tested were positive for FB_1. However, highest FB_1 values were observed in South Africa for compound feed (11000 μg/kg), and in Thailand and China for maize (18800 and 25970 μg/kg, respectively). In a study of Argentinian maize, FB_2 was the major fumonisin at values of up to 11300 μg/kg. An alarming feature of several surveys was that in the tropics in particular, several *Fusarium* mycotoxins co-occurred with each other and with AFB_1, an *Aspergillus* compound sharing carcinogenic properties with fumonisins (Placinta *et al.*, 1999; see also Tables 2.2 and 2.3).

6.1.3 Mode of action of fungicides: new proposals

Examination of the literature pertaining to the control of FHB and other diseases of cereals indicated only a partial explanation for the mode of action of fungicides. Thus tebuconazole, recommended for control of FHB, acts as a demethylation inhibitor of ergosterol biosynthesis in fungi. Carbendazim is also used for cereals to control mildew, septoria and rusts; it acts by disrupting tubulin integrity in fungi. It is clear that fungicides have been designed to disrupt primary metabolism of fungi and efficacy has been assessed in terms of control of infection and cereal diseases. Consequently, the effects on mycotoxin biosynthesis have been a subsidiary issue. In retrospect, this approach is flawed as shown by disappointing fungicide efficacy in the control of trichothecene contamination of grain (Table 2.4). However, a re-assessment of published data (Figure 2.5) indicates that some fungicides may exert a direct and more profound effect on mycotoxin biosynthesis than on primary metabolism as exemplified by mycelial growth. Thus, dicloran elicits an immediate effect on ZEN production, while the effect on growth is less pronounced. Effects of this type may explain why, on occasion (Boyacioglu *et al.*, 1992), propiconazole and thiabendazole are individually more effective in reducing DON contamination of

grain than in alleviating infection by *F. graminearum* (Table 2.4). The direct effects of fungicides on mycotoxin formation, as opposed to the effects on primary metabolism, have not been addressed in any previous publications and are depicted in Figure 6.1. This model refers to fungicide effects on both trichothecenes and ZEN with the potential for application to other mycotoxins. Fungicides may exert effects on mainstream reactions such as cyclizations which, for example, are at

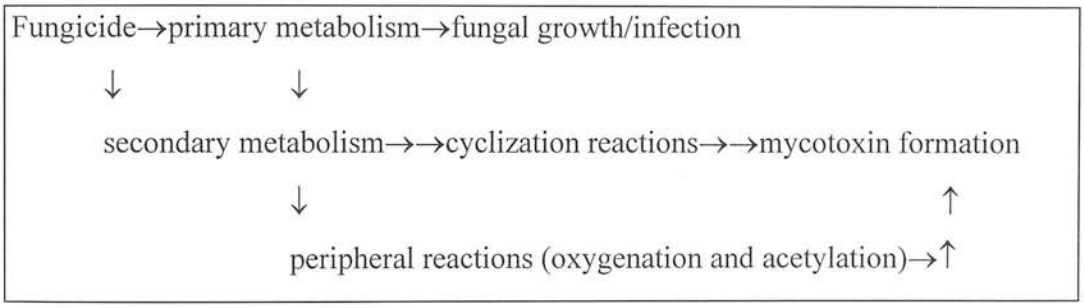

Figure 6.1 A proposed model for the action of fungicides on mycotoxin biosynthesis

the core of trichothecene biosynthesis (Figure 6.1). In addition, other fungicides may target peripheral pathways involved in the ordered sequence of oxygenation and acetylation reactions.

Despite environmental concerns, the commercial manufacture of fungicides is destined to continue. It is therefore imperative that future design of anti-fungals should be undertaken with the view to controlling both crop diseases and the accompanying and continuing risks of mycotoxin production (Tables 2.2 and 2.3). It is hoped that the model in Figure 6.1 will provide the basis for innovative processes in the development of more effective fungicides.

6.1.4 Toxicology

No review of *Fusarium* mycotoxins would be complete without reference to the adverse effects caused by these diverse compounds. Although incidental to the main thrust of this thesis, toxicological aspects were reviewed as part of the work within the departmental team. The present author has contributed to a publication on the animal welfare, health and productivity implications of *Fusarium* mycotoxins (D'Mello *et al.*, 1999). Table 2.6 has been derived from that paper. The toxicological basis of mycotoxin-induced syndromes has been formalized. More importantly, novel features have been introduced in the interpretation of syndromes associated with trichothecenes and ZEN. The toxicological issues of additivity, synergism and potentiation were also addressed. The carcinogenic potential of certain *Fusarium* mycotoxins is now an emerging issue in human health.

6.2 *Fusarium* morphology

Marked effects were noted in terms of pigmentation and hyphal structure following temperature and/or fungicide treatments.

6.2.1 Pigmentation

Pigment development appears to be important for pathogenicity in certain fungi. A rule-of-thumb based on colour differences of *Fusarium* colonies is proposed. The present results suggest that the striking differences in pigmentation might be associated with the pattern of mycotoxin production. Thus yellow is possibly indicative of the presence of T-2 toxin, HT-2 toxin, DON, 15-ADON and 3-ADON while pink/red coloration might suggest the production of ZEN (see for example Plates 5.2 and 5.26 and Tables 5.40 and 5.58), assumption made on the basis of grand means. This rule-of-thumb, if sufficiently developed, might find application in field assessments of toxigenic potential in cases of cereal diseases such as FHB.

6.2.2 Fluorescence microscopy

As previously indicated, ultrastructural changes in hyphae have been reported in fungi following exposure to fungicides. The current studies extend the evidence, obtained with fluoresence microscopy, to include other morphological features. Thus, isolates of *F. sporotrichioides* treated with carbendazim alone (Plate 5.3) or with different combinations of propiconazole (Plate 5.4), maneb (Plate 5.5) and tridemorph (Plate 5.6) showed hyphal features such as swollen tips, distortionate growth and cytoplasmic abnormalities. Furthermore, other effects induced by temperature regimes were also observed (Plate 5.2) and these included the development of thin elongated hyphal cells with less branching. These differences were not apparent in the macroscopic results for linear growth. As far as is known, these temperature effects have not been recorded before for the major *Fusarium* phytopathogens. The implications of these changes for mycotoxin production have yet to be determined but the present studies suggest that there is potential for further exploration.

Marked effects were noted in terms of pigmentation and hyphal development following temperature and/or fungicide treatments.

The linear growth rate appears the same for 25-11°C and 25°C but in terms of hyphal growth there is a dramatic difference between the two temperature regimes with thin, elongated, less branched hyphae at 25-11°C compared to 25°C.

Fungicides combined with the higher temperature showed more marked effects than at the lower temperature, with an increase in hyphal wall fluorescence indicating an accumulation of chitin and/or chitosan. As a result of carbendazim addition alone (see Plates 5.3.-5) and/or in combination with propiconazole (see Plates 5.4.-5), maneb (see Plates 5.5.-5) and maneb+tridemorph (see Plates 5.6.-5) at 1.0 µg/ml PDA and 25°C temperature regime, a greater increase in the fluorescence of the hyphal walls was observed compared to 25-11°C temperature regime. An explanation for this observation might be that the cytoskeleton is more stable at 25-11°C temperature regime rather than at 25°C. The cytoskeleton might be disrupted by fungicide addition to a greater extent at 25°C than at 25-11°C. Carbendazim, a

benzimidazole fungicide is known to bind to tubulin and prevent mitosis (Uesugi, 1998). If chitin synthesis was relatively inhibited, accumulation of chitin in the unelongated cell walls could occur. Uncoordinated synthesis of chitin could also result from an alteration in the transport of vesicles and an accumulation of chitin in the cytoplasm (Plates 5.4.-5). More fluorescence at branching point at the opposing wall was observed (see for example Plate 5.5-3).

Propiconazole (azole fungicide) is known (Sisler, 1996) to inhibit ergosterol biosynthesis (the C-14-demethylation reaction). Striking morphological abnormalities are produced in a number of fungal species by ergosterol biosynthesis inhibitor fungicides.

Maneb, a dithiocarbamate fungicide, is known (Hewitt, 1998) to inhibit spore germination and mycelial growth.

Tridemorph, a morpholine fungicide, is known (Hewitt, 1998) to act primarily on sterol biosynthesis. Sterols are known to be major structural components of cell membranes where they may contribute to their selective permeability properties and modify membrane fluidity. Inhibition of biosynthesis of sterols is detrimental to fungal growth and development and it is found that interference with sterol biosynthesis results in cell or hyphal membranes becoming disorganised and losing their ability to regulate the movement of substances in and out of the cell or hyphae (Gooday, 1995).

Multiple interactions between various factors could lead to these results; therefore, further investigations are required in order to elucidate this phenomenon.

6.2.3 Mycotoxin profiles

The preliminary studies (Tables 5.2-5.5 and Tables 5.8 and 5.9) examined the validity of using direct TLC analysis of agar culture plugs to establish mycotoxin profiles in *Fusarium* species. The results in this thesis indicate that this method only detected a limited number of mycotoxins and this was not consistent between surfaces of the plugs tested or between experiments in the preliminary series.

6.3 Dynamics of *Fusarium* mycotoxin synthesis

The relationship between fungal growth and mycotoxin production is well established and the present results confirm that mycotoxin yield is proportionate to growth. For example, growth and ZEN production proceeded in parallel over time (Figure 6.2). The data in Figure 6.2 represents a combination of values calculated from the 25°C results of experiment 3 (Tables 5.13 and 5.14). However, the relationship varies with the mycotoxin under investigation. Thus, also in experiment 3, growth was only marginally enhanced at 14 days of incubation but T-2 toxin production increased three-fold (Figure 6.2).

The dynamics of *Fusarium* mycotoxin production has been reviewed (section 2.4.1). In particular, the reasons for the decline in mycotoxin synthesis with time have been discussed. The current experiments confirm this decline for T-2 toxin production in solid (Figure 6.2A) and in broth (Figure 6.2B) cultures of *F. sporotrichioides*. However, the reduction is most clearly seen at an incubation temperature of 25°C (Table 5.16) whereas for the 25-11°C regime the effect is possibly delayed due to sub-optimal growth rates. In the case of NEO, synthesis also declined with time but only in one experiment (Table 5.31). These effects of time and temperature may be important in practical risk assessment. Thus, Scott *et al.* (1984) reported a pre-harvest decline in DON concentrations in winter wheat naturally infected with FHB and suggested this effect to be the result of reaction with plant components, or metabolism by plant enzymes or bacterial degradation. The current findings (Figure 6.2), however, indicate that the reduction may be independent of plant or bacterial intervention and may be a function of metabolism by the *Fusarium* pathogen itself. With the advent of molecular biology techniques it may be possible to resolve the underlying mechanisms by investigating effects on synthetic and degradative pathways.

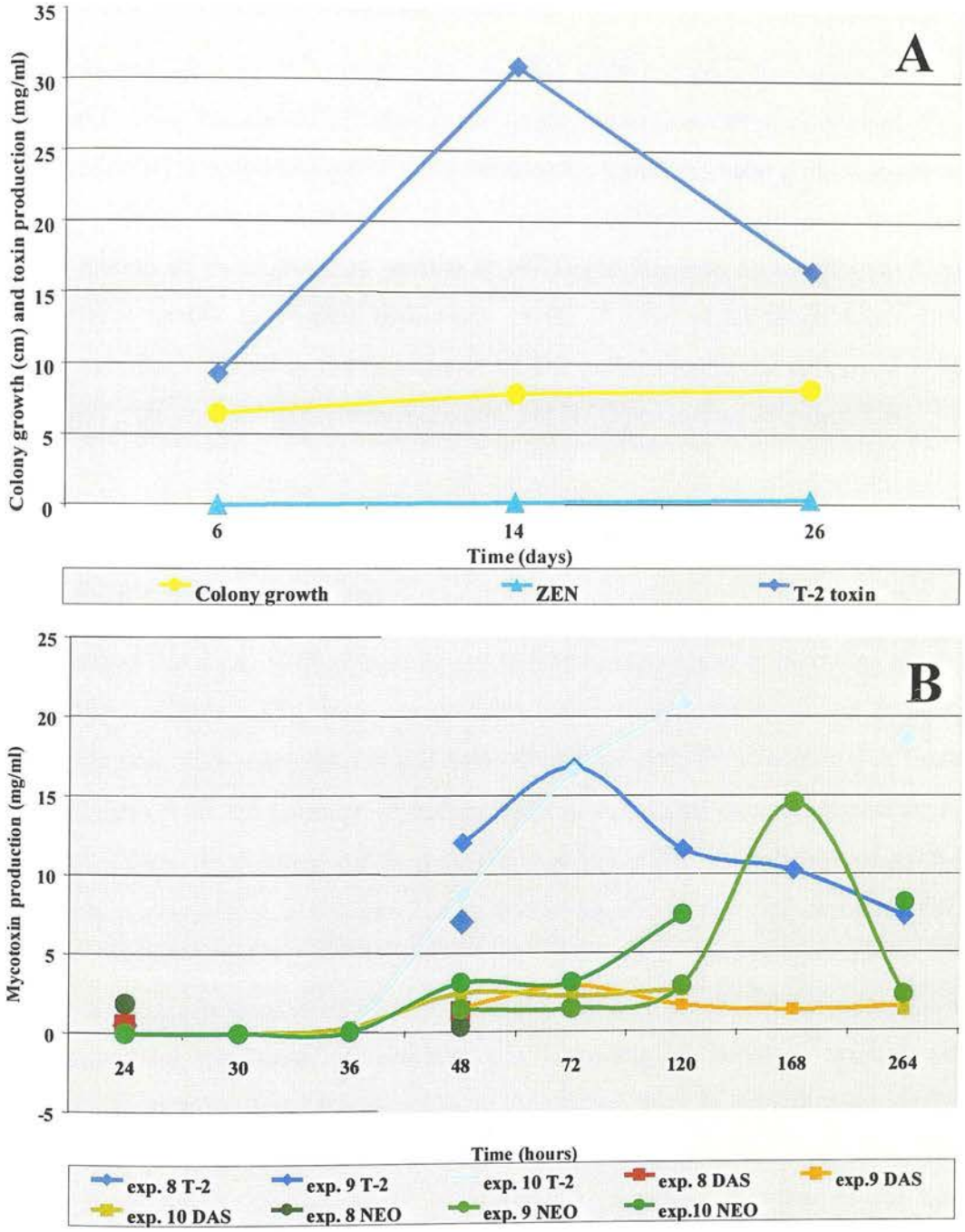

Figure 6.2 Colony growth (cm) on PDA and production (mg/ml) of ZEN and T-2 toxin by *Fusarium sporotrichioides* at 6, 14 and 26 days of growth and 25°C (A) and production (mg/ml) of mycotoxins by *Fusarium sporotrichioides* grown in GYEP at 25°C, and different time points (B)

6.4 Trichothecene metabolism

Kotsonis and Ellison (1975) established that T-2 toxin preceded formation of HT-2 toxin in *F. poae*, but the factors affecting this pathway have not been elaborated. This may be merely a manifestation of a time-induced reaction but such a view might be over-simplistic. Temperature and type and dose of fungicide may also exert an effect while species of *Fusarium* may well be an additional factor to be considered. Thus the present results show little production of HT-2 toxin in liquid cultures of *F. sporotrichioides* following the appearance of T-2 toxin whereas the pattern of DAS synthesis over time coincides with T-2 toxin, but NEO peaks later (Figure 6.2 B).

6.5 Fungal competition

The outstanding features of mycotoxin production in experiment 6, involving fungal competition (Table 5.38), were the complex interactions between fungal species, fungicide type and concentration and time. It is clear that, for example, T-2 toxin enhancement with the addition of carbendazim at 0.1 μg/ml occurs only when *F. sporotrichioides* is cultured on its own and that the effect is abolished when the fungus is grown with *A. ochraceus*. It may also be significant that Ramakrishna *et al.* (1996) demonstrated the persistence of T-2 toxin production when *F. sporotrichioides* was cultured with *Hyphopichia burtonii*. It should be pointed out that under the conditions of experiment 6 *A. ochraceus* failed to produce its mycotoxins in the form of the ochratoxins. Additional work is needed to assess the effects of a toxigenic strain of *A. ochraceus* on *Fusarium* mycotoxin production. The question of fungal competition is of practical significance in view of the co-occurrence of fungi in cereal grains at harvest.

6.6 Temperature effects

The results of six of the current experiments (Figure 6.3) now firmly establish that ZEN production in all three *Fusarium* species studied was sensitive to temperature, with higher production in the 25-11°C regime. These observations are entirely consistent with those of Merino *et al.* (1993). However, the lack of effect of temperature on ZEN production in *F. graminearum* (Figure 6.3) suggests that this phytopathogen responds in a different manner to the other two species examined. The discrepancies highlighted in the literature review between the work of Merino *et al.* (1993) and Milano and Lopez (1991) still remain unresolved.

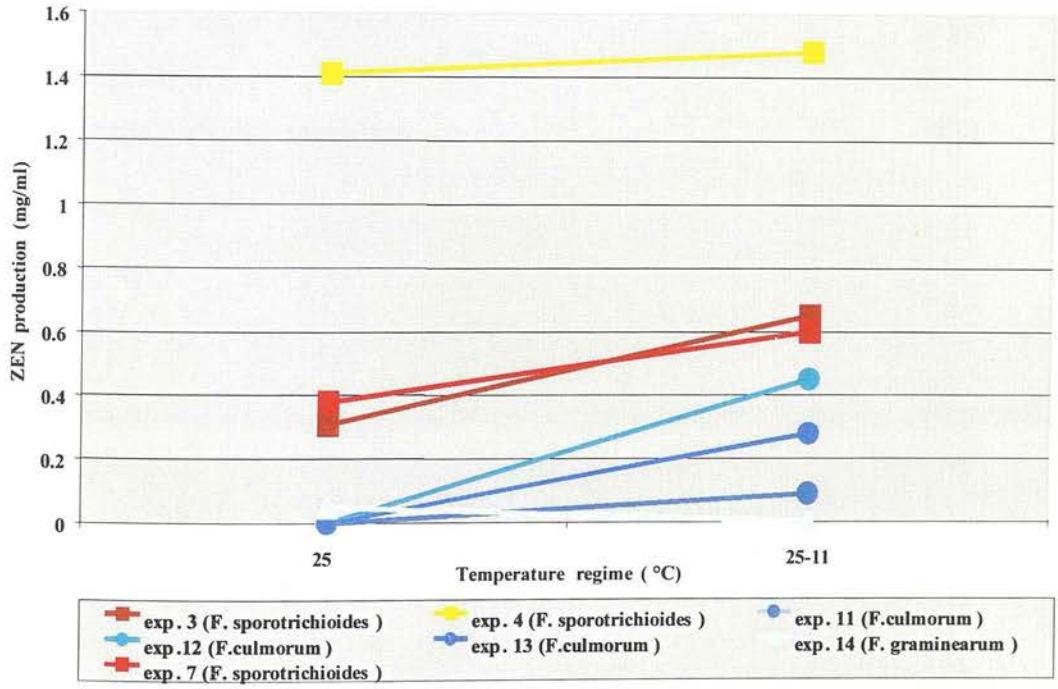

Figure 6.3 ZEN production (mg/ml) by *Fusarium* species at two temperature regimes, namely 25°C and 25-11°C

Contrasting effects of temperature were observed in production of type A trichothecenes (Figures 6.4-6.7). In general, synthesis of HT-2 toxin and NEO is

favoured by the higher temperature whereas DAS production maximizes at 25-11°C. It would appear that temperature effect on the pattern and levels of trichothecene production are influenced by fungicide type. The abbreviations below are designed to serve as a legend for all figures relating to pesticide applications. The interaction between temperature and fungicide type on biosynthesis of individual trichothecenes will be discussed below.

Pesticide	Abbreviation
Bavistin	B
Carbendazim	C
Carbendazim +propiconazole	CP
Carbendazim+maneb	CM
Carbendazim+maneb+tridemorph	CMT
Difenoconazole	D
Bromoxynil+ioxynil+mecoprop	BIM

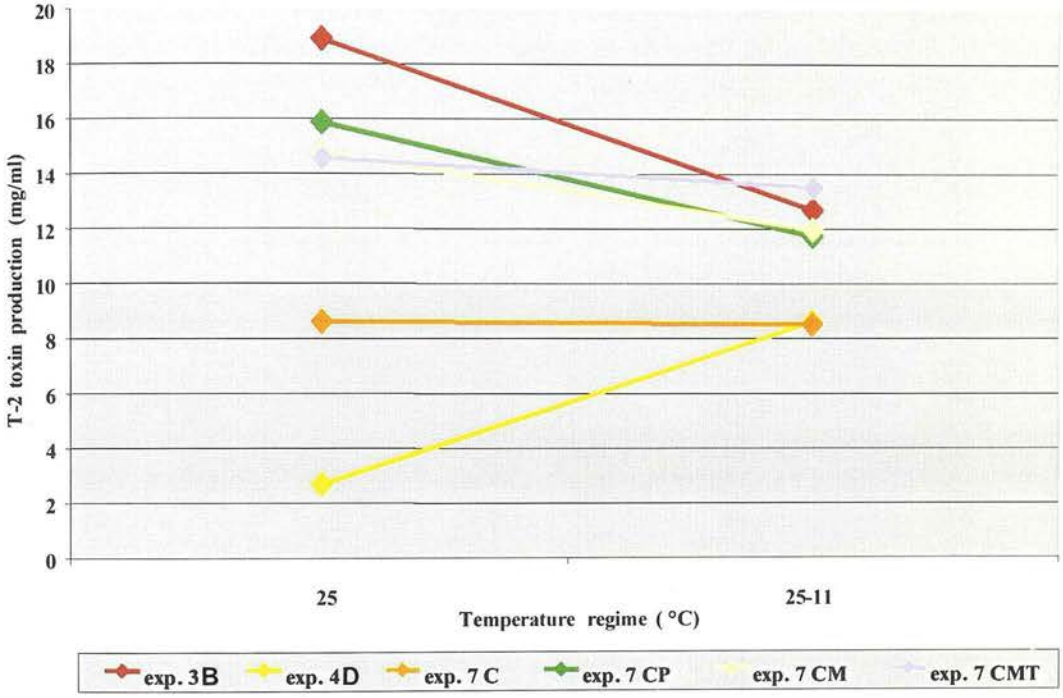

Figure 6.4 T-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with different fungicides, at two temperature regimes, namely 25°C and 25-11°C

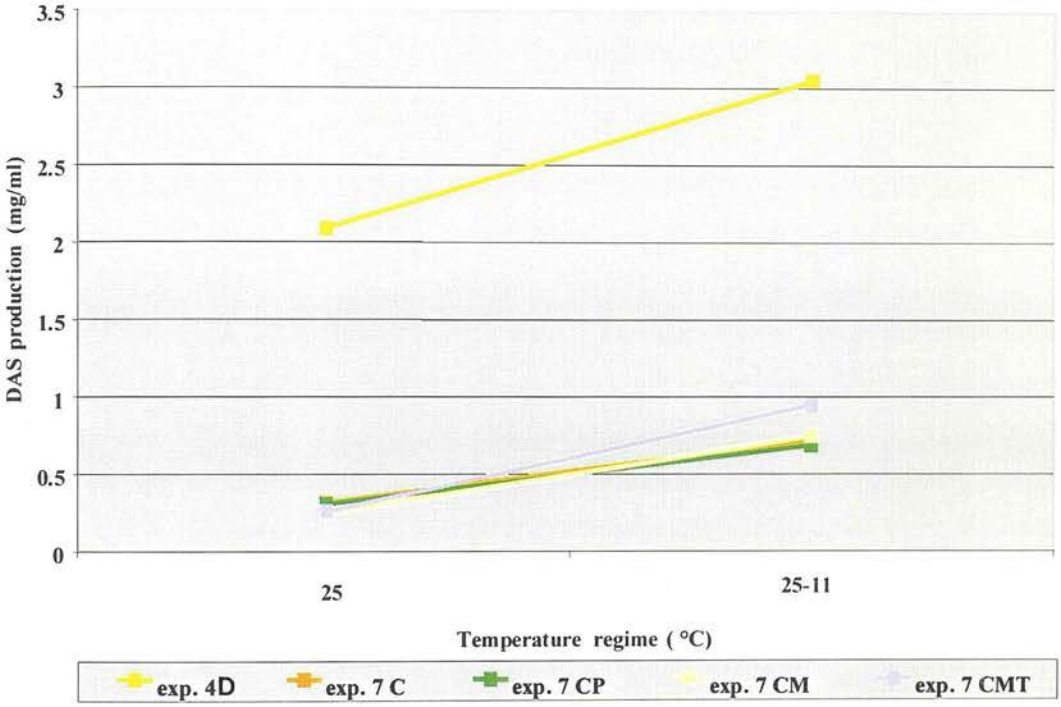

Figure 6.5 DAS production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with different fungicides, at two temperature regimes, namely 25°C and 25-11°C

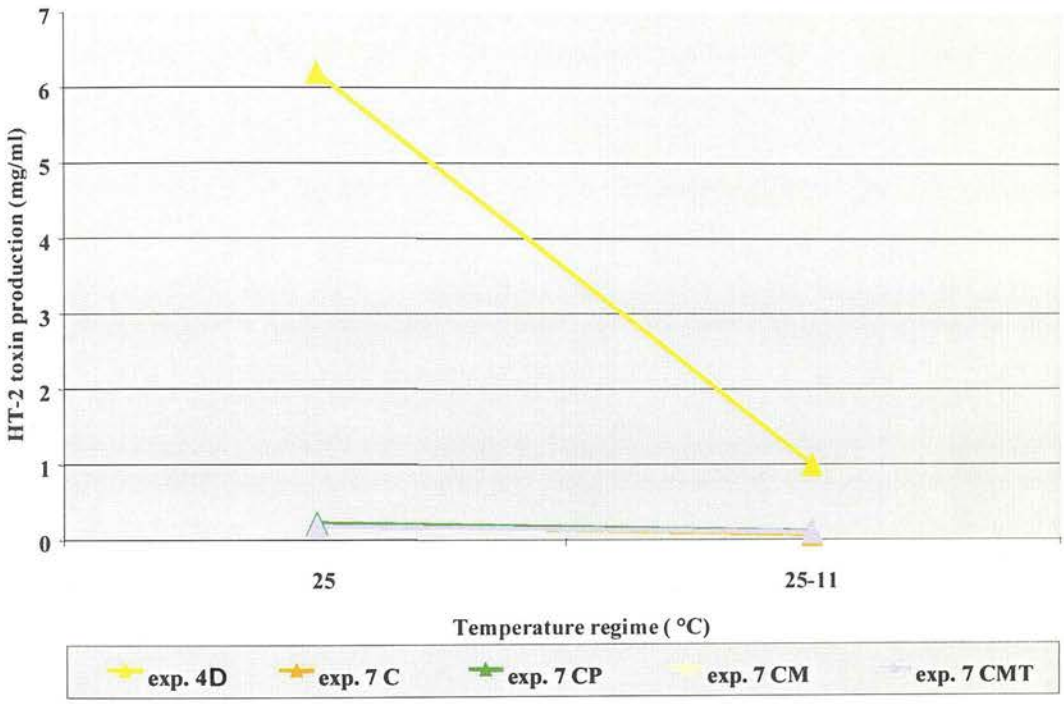

Figure 6.6 HT-2 toxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with different fungicides, at two temperature regimes, namely 25°C and 25-11°C

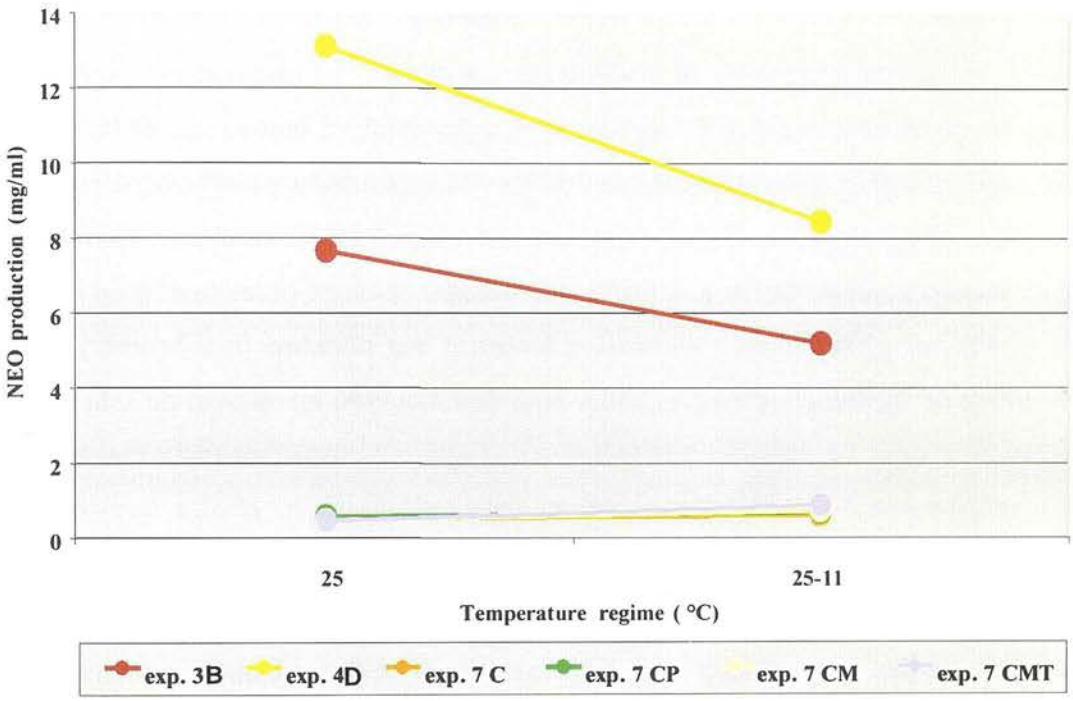

Figure 6.7 NEO production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with different fungicides, at two temperature regimes, namely 25°C and 25-11°C

6.7 Pesticide efficacy

It is apparent from the present studies that pesticide efficacy for mycotoxin control was influenced by a number of factors. Efficacy was affected by pesticide type and dose. In addition, type, form and combinations of fungicides exerted marked effects on mycotoxin production. As mentioned above, fungal competition also affected efficacy.

6.7.1 Effects of pesticide type and dose

Pesticide enhancement of mycotoxin production in *Fusarium* species was not restricted to that caused by fungicides. A novel finding in the present study with *F. culmorum* was the demonstration of 3-ADON enhancement induced by the herbicide combination of bromoxynil, ioxynil and mecoprop added as Swipe (Table 5.63). It would be of interest to examine whether this effect is a universal phenomenon with other herbicides in common use in cereal production. Additionally, the effects of herbicides on mycotoxin production in type A trichothecene producers is worthy of further investigation.

The overall effects of fungicides used in the current series of experiments are presented in Figure 6.8. A two-dimensional displacement in response is apparent. Firstly, distinct dose-related patterns in the enhancement of production of specific mycotoxins may be discerned. The second facet includes effects caused by different types and forms of fungicides. At around the 0.1 μg/ml concentration, production of two trichothecenes is stimulated by carbendazim, difenoconazole and combinations of carbendazim with propiconazole or of carbendazim, maneb and tridemorph. At the 100 μg/ml dose, enhanced production of a second set of trichothecenes is apparent only with difenoconazole. Production of ZEN also appears to be a function of fungicide type and dose. These patterns, derived largely from statistically significant data are sufficiently important to warrant detailed analysis as discussed below.

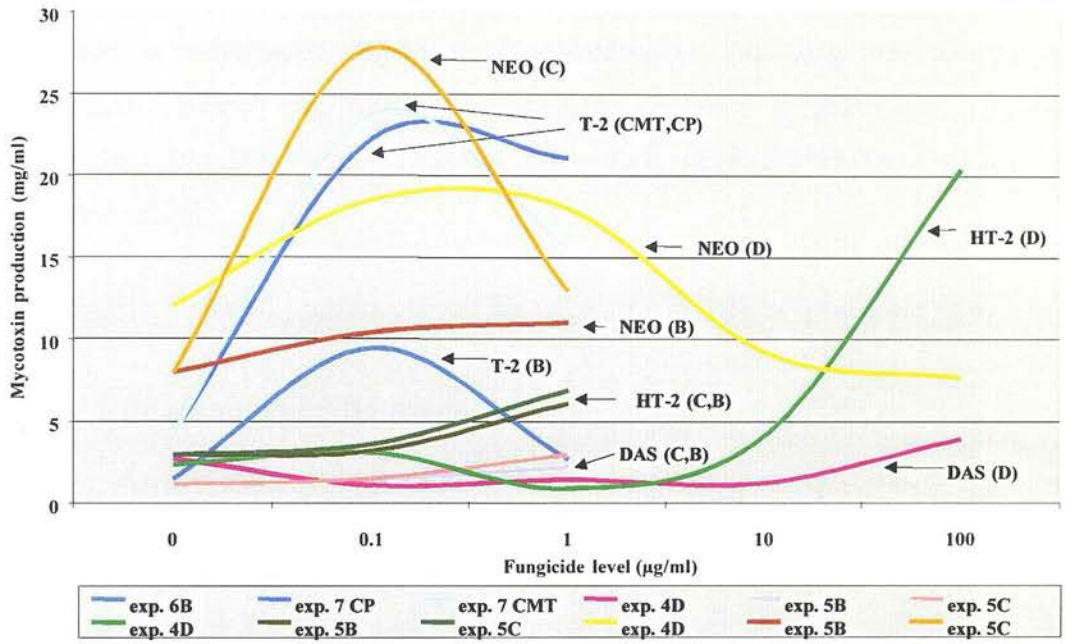

Figure 6.8 Mycotoxin production (mg/ml) by *Fusarium sporotrichioides* grown on PDA with various concentrations (µg/ml) of different fungicides at 25°C

6.7.2 Effects of fungicide form

It will have been noted that NEO production was not stimulated by fungal exposure to Bavistin, whereas carbendazim acted in a stimulatory manner. It is suggested that this discrepancy is due to fungicide form since Bavistin is the proprietary form of carbendazim. The mechanism for this difference in activity remains unresolved but resides in variations in solubility and dispersal of the two forms.

6.7.3 Effects of fungicide combinations

It will be recalled that the magnitude of enhancement of T-2 toxin synthesis was different between experiments (Figure 6.8). Thus in experiment 6, stimulation was

less than in experiment 7. This difference may be attributed to the effects of fungicide combinations. Mixtures of carbendazim and propiconazole or of carbendazim, maneb and tridemorph stimulated T-2 toxin production to a greater extent than Bavistin alone. However, the effects of fungicide form cannot be excluded entirely.

6.7.4 Effects on specific mycotoxins

6.7.4.1 Zearalenone

Pesticide efficacy for ZEN control in the three species of *Fusarium* used in the present studies is presented in Figure 6.9. A marked difference is seen for the two temperature regimes used. Thus, in the 25-11°C regime, overall efficacy of pesticides is consistently lower than at 25°C. At the higher doses, efficacy is superior at 25°C than in the 25-11°C regime.

6.7.4.2 T-2 toxin

Moss and Frank (1985) demonstrated a marked enhancement of T-2 toxin production in *F. sporotrichioides* treated with tridemorph. The present results for T-2 toxin production in the presence of fungicides were alluded to in Figure 6.8 but a more complete presentation is provided in Figure 6.10 which also compares the effect of the two temperature regimes. It is clear from Figure 6.10 that Bavistin and carbendazim and mixtures including tridemorph and other fungicides may also significantly ($P<0.05$ or better) stimulate T-2 toxin production. Thus mixtures of carbendazim and propiconazole or carbendazim plus maneb or carbendazim plus maneb plus tridemorph all enhanced T-2 toxin formation (see data for experiment 7 in Figure 6.10). However, the efficacy of particular fungicides for T-2 toxin regulation is determined by interactions with temperature. For example, as shown in

Figure 6.4, Bavistin was more effective at 25-11°C than at 25°C while difenoconazole was more effective at 25°C.

6.7.4.3 Diacetoxyscirpenol (DAS)

Examination of Figure 6.11 indicates a temperature x fungicide interaction on DAS production. At 25°C, there is an overall theme of enhancement in DAS biosynthesis with increasing levels of various fungicides. However, at 25-11°C, fungicide efficacy progressively increases with fungicide concentration. The particular effects of difenoconazole are worth noting (Figures 6.5 and 6.11).

6.7.4.4 HT-2 toxin

An even clearer picture of the unique behaviour of difenoconazole is seen in its effects on HT-2 toxin production (Figure 6.12). At 25°C there is once again an overall theme of enhancement of HT-2 toxin production with increasing concentrations of fungicides, whereas efficacy is substantially improved in the 25-11°C regime. A substantive finding, not previously noted, is that difenoconazole failed to stimulate T-2 toxin formation at 25°C but was capable of transforming it to HT-2 toxin and then stimulating the production of the latter product.

6.7.4.5 Neosolaniol (NEO)

Unique effects have been recorded for NEO which deserve comment. Neosolaniol production was stimulated in the presence of carbendazim and of difenoconazole in *F. sporotrichioides* cultured at 25°C (Figure 6.13A). These effects were diminished in the 25-11°C regime (Figure 6.13B). The interaction between temperature regime and fungicide type has already been shown in Figure 6.7 which again portrays the

unique position of difenoconazole in mycotoxin regulation. The results for NEO now give a comprehensive picture of the effects of fungicides on type A trichothecenes and have not been published by other authors.

6.7.4.6 Deoxynivalenol (DON) and 3-ADON

An amalgamation of all data on these type B trichothecenes is presented in Figure 6.14. The major theme is the general inefficacy of pesticides to control synthesis of these mycotoxins. This observations are in agreement with those published for a variety of other fungicides in field and *in vitro* studies (Table 2.4). However, a striking feature of the results in Figure 6.14 is the consistent enhancement in production of the two type B trichothecenes which reaches significance ($P<0.05$) for 3-ADON.

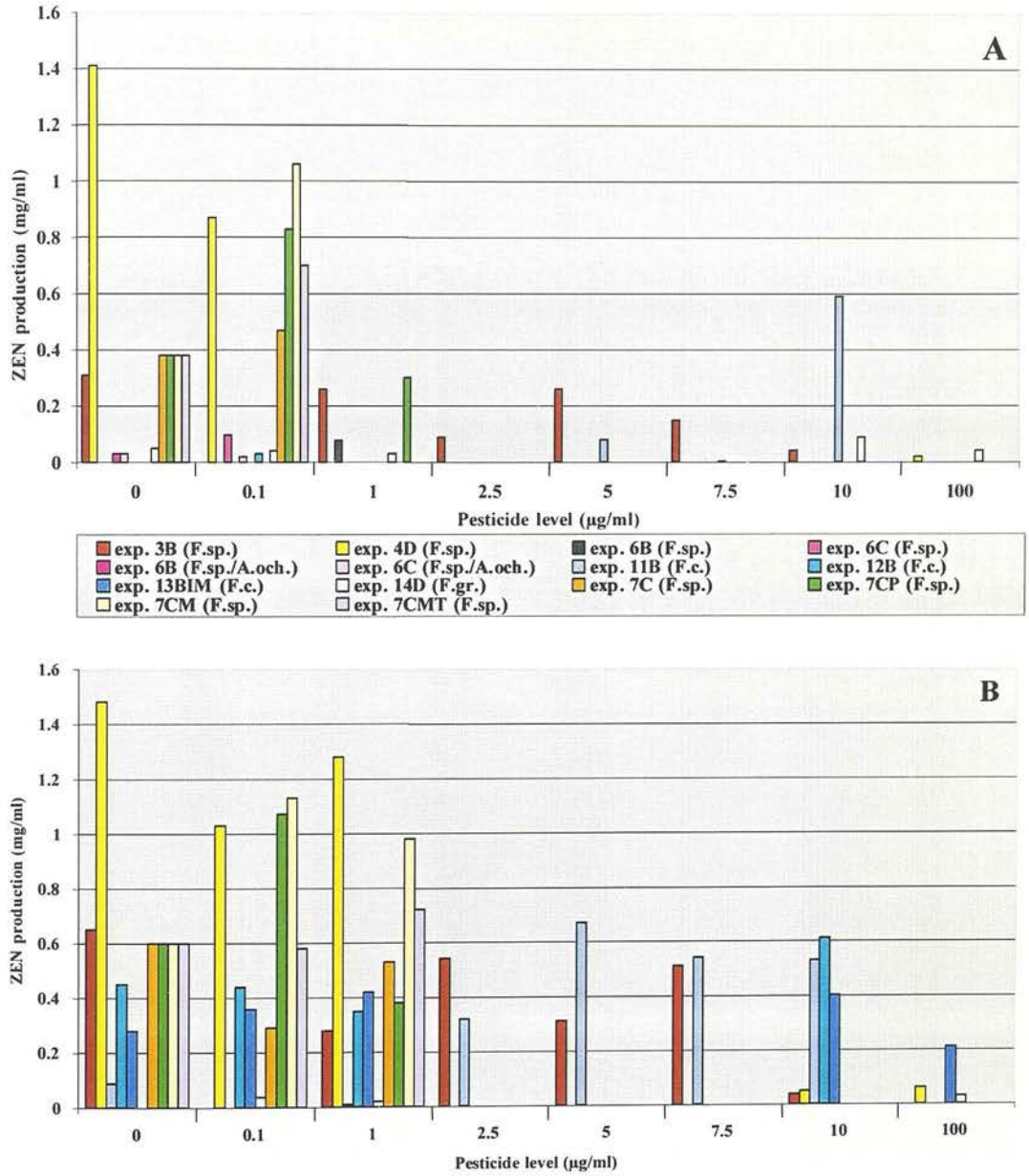

Figure 6.9 ZEN production (mg/ml) by *F. sporotrichioides* under the influence of various concentrations (µg/ml) of different pesticides at two temperature regimes, namely 25°C (A) and 25-11°C (B)

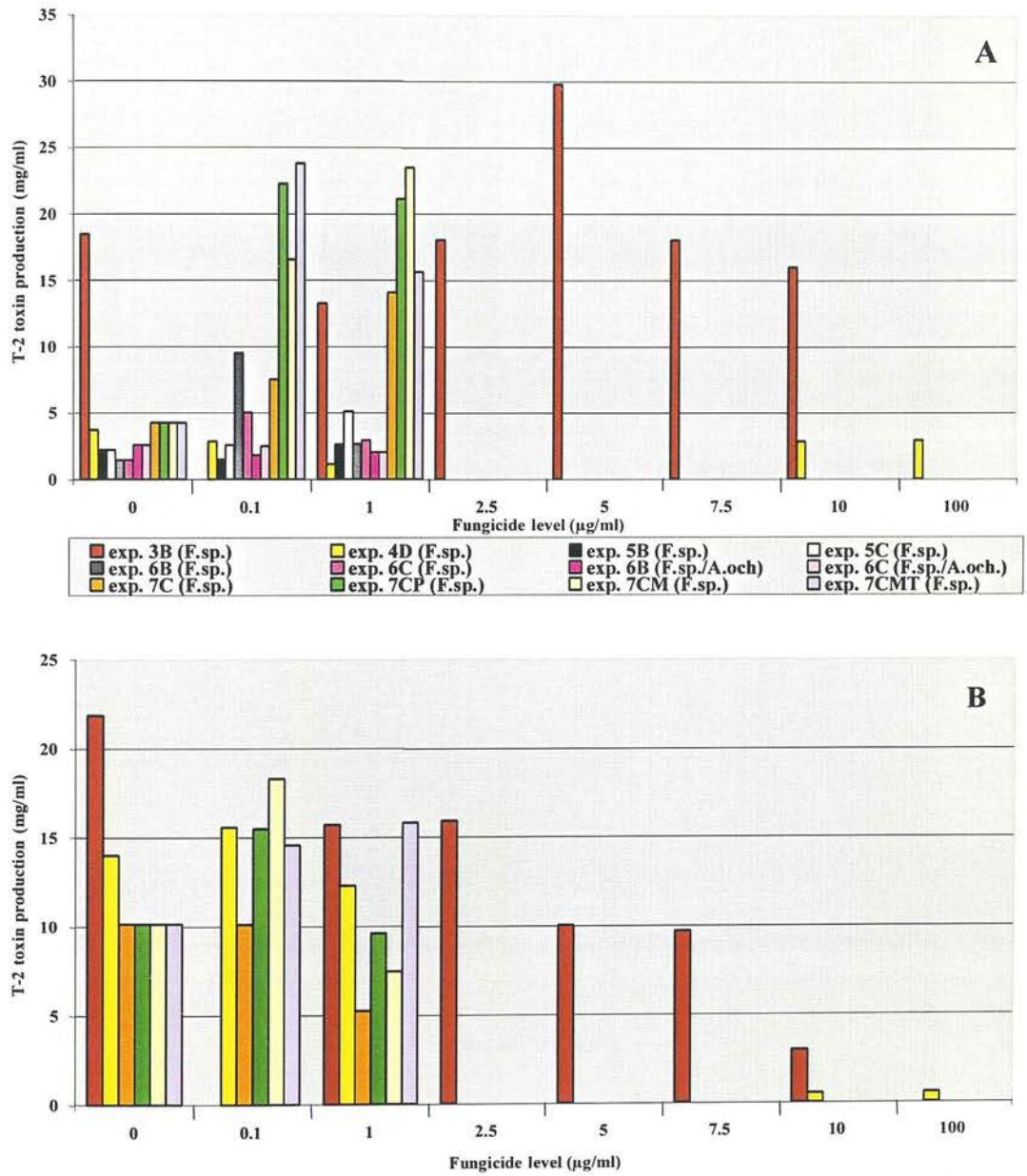

Figure 6.10 T-2 toxin production (mg/ml) by *Fusarium sporotrichioides*, under the influence of various concentrations (µg/ml) of different fungicides, at two temperature regimes, namely 25°C (A) and 25-11°C (B)

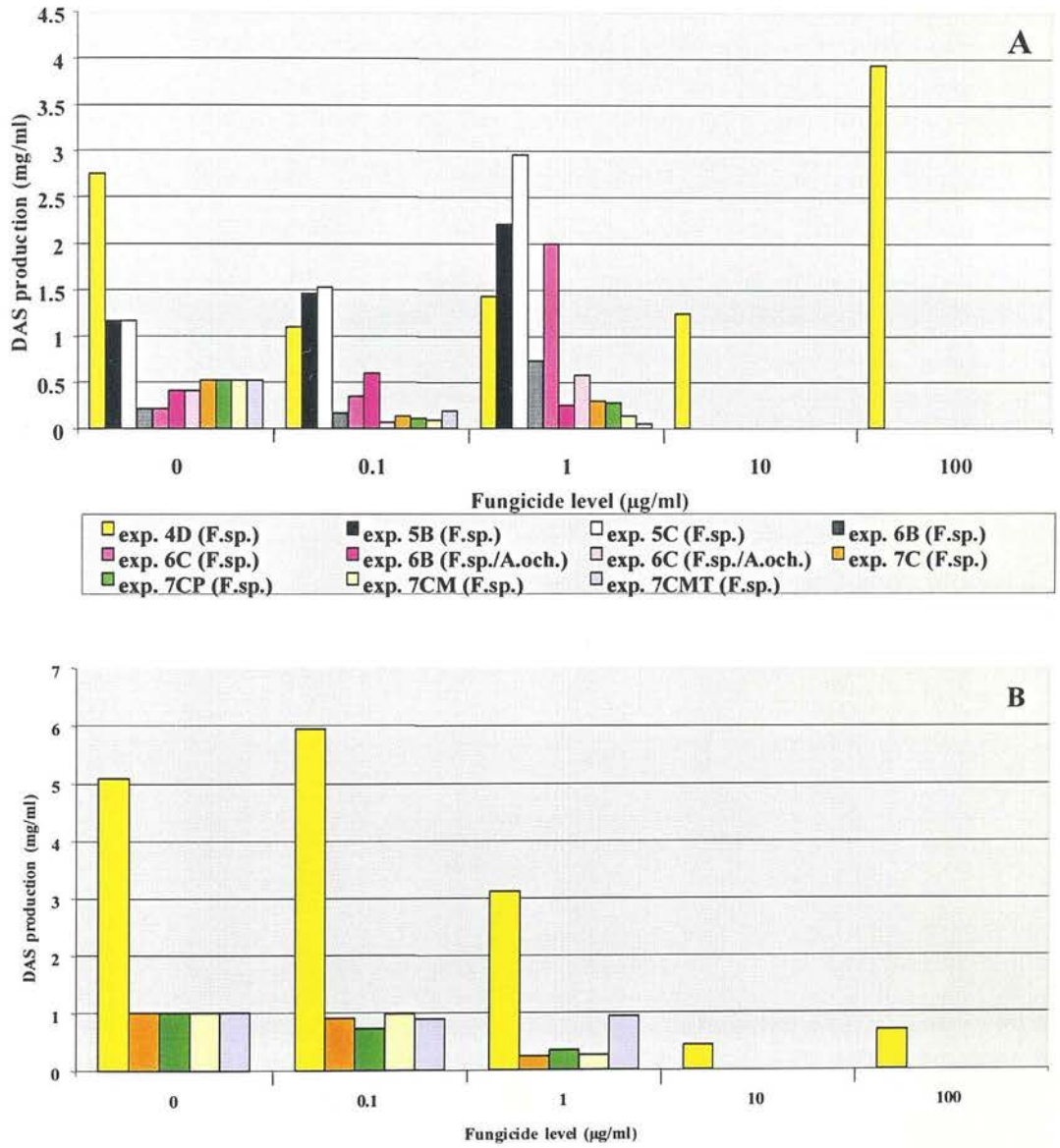

Figure 6.11 DAS production (mg/ml) by *F. sporotrichioides* under the influence of various concentrations (µg/ml) of different fungicides at two temperature regimes, namely 25°C (A) and 25-11°C (B)

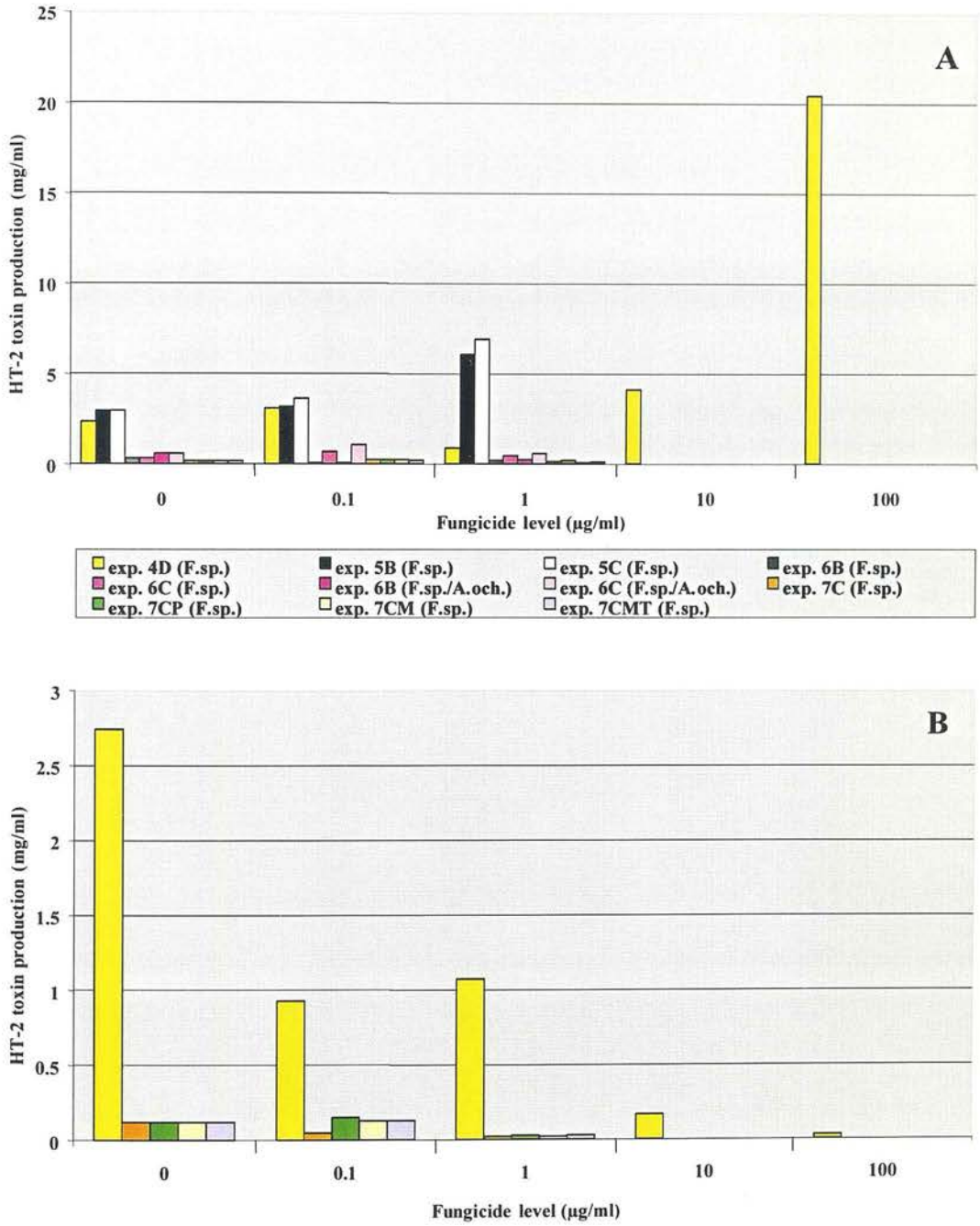

Figure 6.12 HT-2 toxin production (mg/ml) by *Fusarium sporotrichioides* under the influence of various concentrations (µg/ml) of different fungicides at two temperature regimes, namely 25°C (A) and 25-11°C (B)

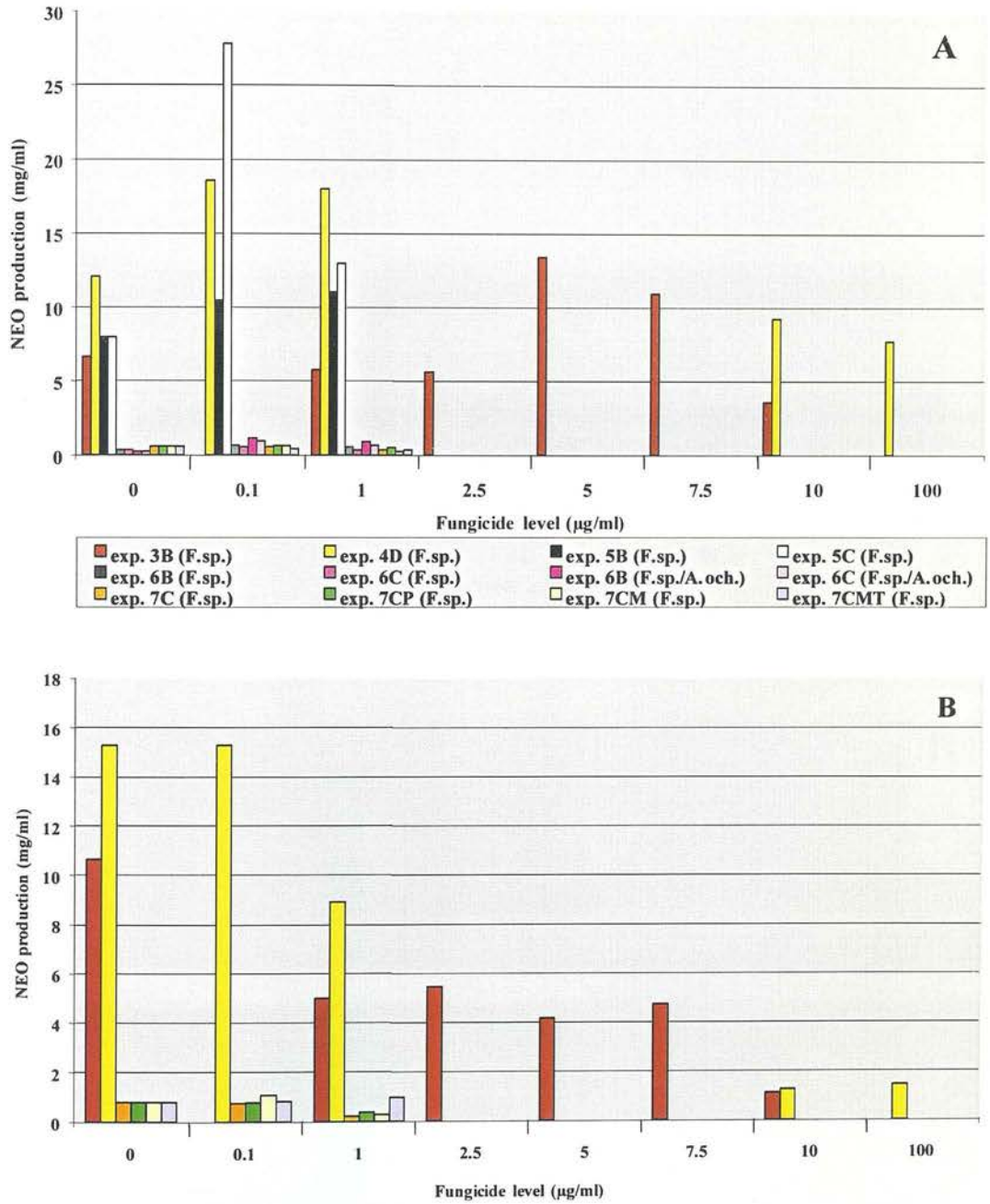

Figure 6.13 NEO production (mg/ml) by *Fusarium sporotrichioides* under the influence of various concentrations (µg/ml) of different fungicides at two temperature regimes, namely 25°C (A) and 25-11°C (B)

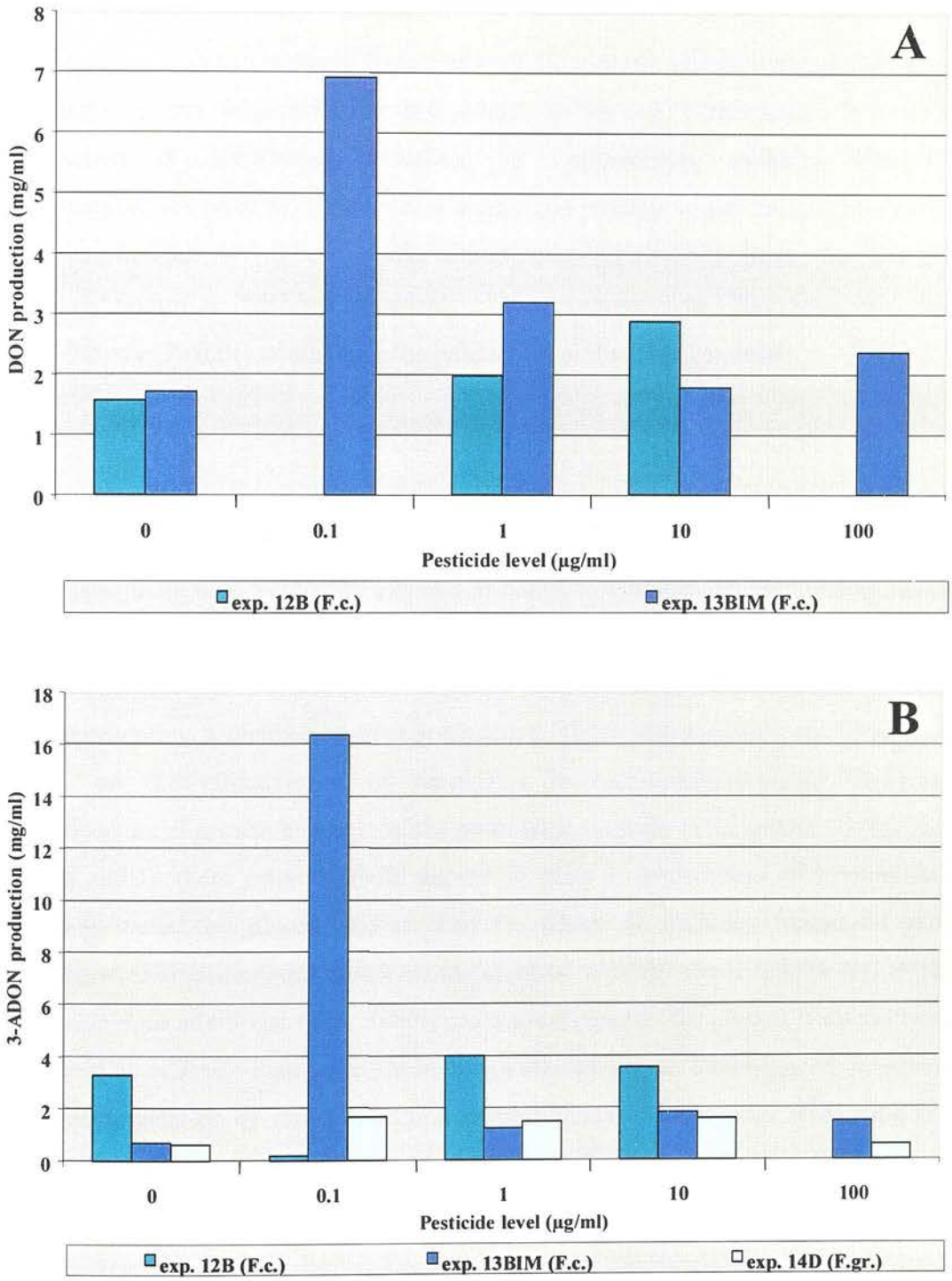

Figure 6.14 Effect of various concentrations (µg/ml) of different pesticides on production (mg/ml) of DON (A) and of 3-ADON (B) by *Fusarium culmorum* and *Fusarium graminearum* cultured at 25°C

6.7.5 Fungicide-induced metabolism of trichothecenes

A consistent feature emerging from some of the current experiments on fungicide treatment is the stimulation in mycotoxin production. Specifically, T-2 toxin production is significantly enhanced by carbendazim addition. However, examination of Figure 6.11 indicates a substantive finding which has not previously been noted. Thus, it will be seen that difenoconazole fails ($P>0.05$) to stimulate T-2 toxin formation at 25°C but is capable of transforming it to HT-2 toxin and then significantly ($P<0.01$) stimulating the production of the latter product.

6.7.6 Fungicide efficacy: a classification

It is appropriate here to provide a synthesis based on the data obtained in the current programme of research and on the evidence presented in Tables 2.4 and 2.5 and Figures 2.5 and 2.6. A tentative classification of fungicide efficacy is shown in Table 6.1 representing a distillation of this evidence. Three major groups are envisaged, based on the effectiveness of fungicides to completely control FHB/grain infection/fungal growth and mycotoxin production on grain or in culture. Fungicides which satisfy these criteria would appear in class I, while those of intermediate efficacy would be placed within class II. Class III includes fungicides with performance characteristics which are inconsistent with the above criteria and which may also pose additional risks. Subdivisions are suggested for classes II on the basis that partial efficacy may arise through growth-dependent inhibition of *Fusarium* phytopathogens or by direct reduction of trichothecene formation. With class IIA fungicides, if fungal growth or infection is not totally inhibited then residual mycotoxin production may be retained with the potential to contaminate cereal grains.

Table 6.1 Proposed classification of fungicide efficacy[a]

Class	Descriptor	Examples of fungicides	Mycotoxins affected	Conditions
I	Effective	Maneb	ZEN[b]	Field trial
IIA	Partially effective [growth-dependent inhibition; mycotoxin residues possible]	Thiophanate-methyl	DON[c] and NIV[d]	Field trial
		Prochloraz	3-ADON[e]	*In vitro*
		Vinclozolin	ZEN	*In vitro*
IIB	Partially effective [direct inhibition of mycotoxin synthesis; disease/infection/fungal growth possible]	Thiabendazole	DON	Field trial
		Difenoconazole	3-ADON	*In vitro*
		Dicloran	DAS	*In vitro*
IIIA	Ineffective	Propiconazole	DON	Field trial
		Morpholines	3-ADON	*In vitro*
IIIB	Stimulatory and inducing transformation	Tebuconazole with triadimenol	NIV	Field trial
		Tridemorph	T-2 toxin	*In vitro*
		Tebuconazole	3-ADON	*In vitro*
		Difenoconazole	3-ADON	*In vitro*
		Carbendazim (pure)	T-2 toxin	*In vitro*[f]
		Carbendazim (Bavistin)	T-2 toxin	*In vitro*[g]
		Carbendazim+ propiconazole	T-2 toxin	*In vitro*[f]
		Carbendazim+maneb	T-2 toxin	*In vitro*[f]
		Carbendazim+maneb+ tridemorph	T-2 toxin	*In vitro*[f]
		Carbendazim (pure)	NEO	*In vitro*[h]
		Difenoconazole	HT-2 toxin	*In vitro*[i]

[a] for control of disease/grain infection/fungal growth and inhibition of mycotoxin production
[b] zearalenone
[c] deoxynivalenol
[d] nivalenol
[e] 3-acetyl deoxynivalenol
[f] experiment 7
[g] experiment 3
[h] experiment 5
[i] experiment 4

With class IIB fungicides, trichothecene production in *Fusarium* species may be prevented but FHB or infection may not be suppressed fully and cereal yield may be compromised. Class III subdivisions are proposed on the basis that inefficacy may be compounded by the propensity of certain fungicides to stimulate fungal growth and/or trichothecene production (Table 2.5). With both class II and III fungicides there is an additional risk that *Fusarium* phytopathogens may develop resistance and overall efficacy might be further prejudiced.

Inclusion of maneb as a class I fungicide (Table 6.1) is tentative as data for *Fusarium* infection of grain was not presented (Draughon and Churchville, 1985; Table 2.4). In addition its inhibitory action towards FHB and trichothecene production remains untested. Furthermore, it should be noted that maneb is a non-systemic protectant fungicide previously recommended for prevention of FHB but only in combination with Zn (UK Pesticide Guide, 1995). It will also be noted that the systemic compound, tebuconazole, specifically recommended for FHB has been excluded from class I and indeed is designated as a class IIIB fungicide. The evidence presented by Suty *et al.* (1996) was accorded low weighting as it was based on an unpublished commercial report not subjected to peer review or to statistical analysis. Propiconazole is designated a class IIIA fungicide due residual trichothecene production even in the two studies with beneficial outcomes (Table 2.4). Two additional investigations showed no control of trichothecene formation by propiconazole (Martin and Johnston, 1982; Milus and Parsons, 1994).

Examination of Table 6.1 shows that most if not all of the fungicides tested fall in Class IIIB in that they either stimulate mycotoxin production directly or after transformation into another active fungal metabolite.

6.8 Practical implications

The present *in vitro* studies support field observations of the low efficacy of fungicides for *Fusarium* mycotoxin control. The current findings suggest that the complex interactions involving fungicides, temperature and duration of exposure to

these factors may be critical in the timing of fungicide applications in the field. However, before application of the current findings more research is required to establish comparability with doses used in field trials and with fungicide residues in cereal plants. Furthermore, the issue of fungicide resistance and the implications for mycotoxin production are, at present, unknown quantities, underlining the need for additional research.

6.9 Trichothecene gene expression studies

There was no distinct signal corresponding to the trichothecene gene transcripts obtained after Northern blot hybridization (Exp.9) with DIG labelled β-tubulin, *Tri5* and *Tri6* probes. It was initially thought that the alkaline fixation of the Northern blots was inefficient or the length of time between Northern transfer and hybridization was considerably long and therefore a clear signal could not be achieved because of RNA degradation on the membrane. Dot blots with samples from time course (Exp. 9) were prepared and subjected at first, to similar hybridization conditions, followed by an increase in hybridization temperature from 50 to 55, 60 and 68°C. With the exception of one combination of parameters where 42°C was used for the posthybridization washes, the rest were done at room temperature. Once more, no specific signals were obtained unless the hybridization temperature was 50°C followed by room temperature posthybridization washes. Results from Exp. 9 show that using the DIG labelled β-tubulin, *Tri5* and *Tri6* probes for dot blot hybridization, the signals detected were very similar and furthermore, an increase in hybridization temperature to 55, 60 or 68°C resulted in no signal present. It was a case of all samples showing a signal or none. Due to the fact that from Northern blots more information is obtained regarding the position of the signal, another Northern blot with samples from the time course was prepared and fixed, this time using UV cross-linking. This time, clear signals were visualized after 2.5 and overnight film exposure. This signals appeared to be present at the same level on the Northern blot after hybridization with any of the probes (β-tubulin, *Tri5* and *Tri6*).

There was a combination of factors that lead to the decision of obtaining alternative probes. This included: potential lack of sequence homology between probe sequence and test host specificity.

The next time course experiment (see Experiment 10) involved the presence of the fungicide carbendazim, as part of this study was aimed at investigating the influence of fungicides on trichothecene gene expression by *Fusarium sporotrichioides*. The same pattern of banding as in previous experiment was observed. However the hybridization signals were stronger in this experiment possibly due to the use of different probes, UV fixation of the blots, fluorescent labelling versus DIG labelling. The use of fluorescent labelling and detection kit proved to be more sensitive than the DIG labelling and detection starting kit. The hybridization signals obtained were thought to correspond to rRNA. Therefore a rDNA probe specific to *F. sporotrichioides* was constructed. This probe was then hybridized to Northern blots carrying RNA from experiment 10. Due to abundance of signals present and their position on the Northern blot it was difficult to distinguish the true signals from the rRNA signals.

6.10 Future work

F. poae is a common phytopathogen of cereals worldwide but it remains to be positively established as to whether it is a producer of both types of trichothecenes (A and B).

In addition, T-2 toxin to HT-2 toxin conversion in *F. poae* should be compared with that in *F. sporotrichioides* under different temperature and fungicide regimes.

The structural changes in hyphal development caused by temperature and fungicides is worthy of further investigation using more sophisticated techniques such as electron microscopy.

The issue of fungal interactions can be taken further by examining competing colonies of toxigenic species. The interface between competing colonies (see Plate 6.1) appears to be an area of potential interest. The clear droplets present at the area of contact between *Fusarium sporotrichioides* and *Aspergillus ochraceus* (see Plate 6.1-2) developed an intense coloration with time (see Plate 6.1-3), an observation which was confined to this area only. It would be interesting to investigate and compare mycotoxin production around the contact area with the opposite side of the colonies.

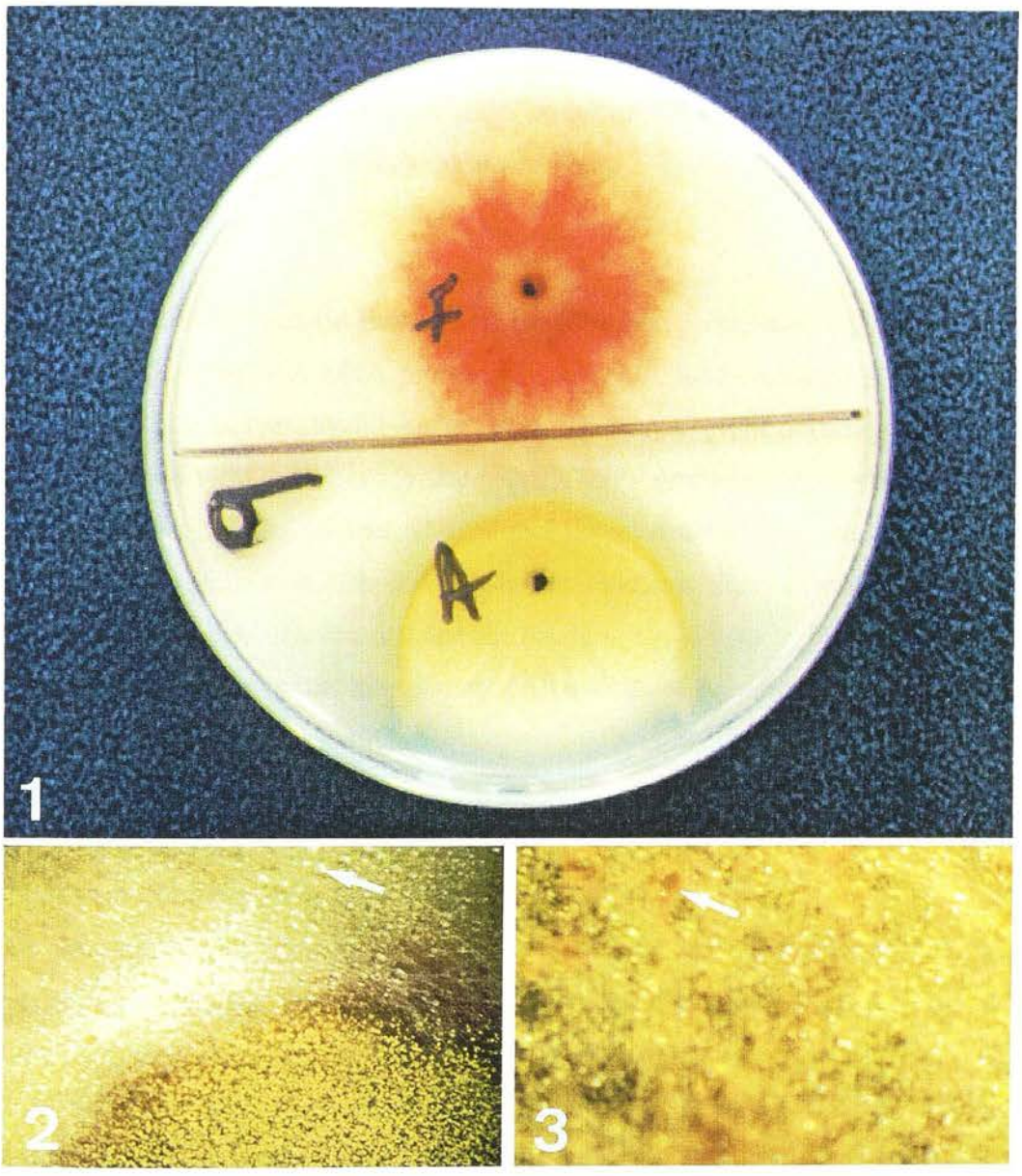

Plate 6.1 Reverse of *Fusarium sporotrichioides* (F) and *Aspergillus ochraceus* (A) colonies grown on PDA containing carbendazim at 0.1 μg/ml and 25°C (1). (2) and (3) represent the contact area (obverse) between the competing fungi *Fusarium sporotrichioides* and *Aspergillus ochraceus* under the microscope.

Alternative approaches could be adopted in order to overcome the limitations encountered in the trichothecenes gene expression studies. Three possibilities are presented as follows: preparation of RNA probes, radioactive labelling versus DIG labelling and alternative methods to Northern blots.

1) RNA probes

The cloning kit was used (in Experiment 10) for probe preparation with the thought that if hybridization with DNA probes will not yield good results, a possibility of producing RNA probes would be at hand. The RNA probes were going to be produced by *in vitro* transcription using the T7/SP6 promoters present in pCR 2.1 plasmid vector. It is known that RNA probes demonstrate stronger signals and less non-specific hybridization than DNA probes on Northern blots. Therefore the use of a RNA probe would be recommended for future work. However, blots probed with RNA are problematic in that they can not be stripped and reprobed effectively due to the strength of RNA:RNA hybrids.

2) DIG labelling versus radioactive labelling (P^{32})

The development of DIG labelling and detection methods is laborious and time consuming. Establishing the optimum parameters requires plenty of RNA sample for trials and time, considering that the evaluation of results obtained from any combination of parameters is available after three or more days of work. An alternative method worth trying is radioactive labelling (P^{32}). The positive aspects of radioactive labelling are: it is more effective, quicker to establish the optimum parameters, it requires less handling, the detection of the bound probe is done directly, it is more reliable and sensitive than DIG labelling. The negative aspect of it is the health hazard although good monitoring systems can be provided and an estimation of the degree of contamination given as compared to the DIG system where there is no such control measure available.

3) Alternative method to Northern blots

Although the Northern blot analysis generically provides detailed information about the natural size and abundance of particular transcripts, the sensitivity and the resolution of this technique are often compromised by one or a combination of the following factors: inability to load large amounts of RNA on a gel, inefficient transfer from gel to blotting membrane, degradation of RNA during transfer, non-specific cross-hybridization between probe and transcripts exhibiting limited homology. Moreover, one of the fundamental drawbacks of filter-based hybridizations is that complete hybridization is not guaranteed, because some of the membrane-bound target sequences may not be fully accessible to probe sequences. Theoretically, complete hybridization is difficult, if not impossible. An alternative approach that can overcome some of the problems associated with Northern blots is the nuclease protection assay, which is an extremely sensitive and precise method for extracting, quantifying and characterizing specific messenger RNA (mRNA). This assay offers 10 to 50 times the sensitivity of Northern blots and enables the use of multiple probes (as many as 12 have been used simultaneously) in a single assay. The hybridization reaction occurs in a solution (not on a solid support, i.e. nylon membrane) allowing complete hybridization of the probe to the mRNA target. After hybridization, remaining single-stranded probe RNA and unhybridized sample RNA are removed by digestion with a mixture of ribonucleases A and T1 (RPA), or S1 nuclease (S1 assay). Then, in a single step, the nucleases are inactivated and the remaining hybrids precipitated. The products are separated on a denaturing or native polyacrylamide gel and visualized by autoradiography. Or, if a nonradioactive probe is used, the separated probe fragments are transferred from the gel to a membrane. The membrane is then subjected to a nonisotopic probe detection protocol. In this approach, the formation of hybrid duplexes between probe and target RNA is promoted under highly stringent hybridization, in solution. Hybridization is immediately followed by exposure to a single-strand specific nuclease that has virtually no affinity for DNA:RNA or RNA:RNA hybrids. Thus, any probe sequences or target RNA that do not participate in duplex formation are digested; protected fragments are then recovered by ethanol precipitation, and the size and

quantity of these hybrid molecules are deduced by gel electrophoresis and concomitant signal intensity. It has been reported that as little as 100 femtograms of target RNA can be quantitated after a 3-day X-ray exposure using high specific active probes. Any transcript that can be studied quantitatively by standard Northern blot analysis is easily detectable by a sensitive nuclease protection assay. This is especially convenient when working with small amounts of RNA from several different samples. A variety of nucleases and approaches are used to quantify and characterize specific messages in greater detail than can be derived from Northern blot analysis.

Chapter 7

Conclusions

7 Conclusions

We may identify four classes of conclusions: 1) substantive, incorporating definitive, noteworthy or novel findings unique to the present research programme with potential impact for new developments; 2) secondary, based on speculative issues; 3) confirmatory findings; and 4) new hypothesis.

7.1 Substantive findings

Contamination of cereal grains with *Fusarium* mycotoxins is a global and continuing issue and is likely to remain so until a systematic elucidation of controlling factors has been accomplished.

An important observation at the commencement of the research programme examined the validity of using direct TLC analysis of agar culture plugs to establish mycotoxin profiles in *Fusarium* species. The results in this thesis indicate that this method only detected a limited number of mycotoxins and this was not consistent between surfaces of the plugs tested or between experiments in the preliminary series.

A noteworthy observation was that HT-2 toxin production from T-2 toxin was not time-related as claimed by other workers. There may be a real species differences in this respect between *F. sporotrichioides* and *F. poae*.

Mixtures of carbendazim and propiconazole or carbendazim plus maneb or carbendazim plus maneb plus tridemorph all enhanced T-2 toxin formation.

The fungicide-induced enhancement of mycotoxin production has now been extended, for the first time, to HT-2 toxin and NEO.

A substantive finding, not previously noted, is that difenoconazole failed to stimulate T-2 toxin formation at 25°C but was capable of transforming it to HT-2 toxin and then stimulating the production of the latter product.

Difenoconazole appears to be a fungicide in a class of its own in that DAS and NEO production are consistently higher than for a large majority of other fungicides tested.

NEO production was not substantially affected by fungal exposure to Bavistin, whereas carbendazim acted in a stimulatory manner. It is suggested that this discrepancy is due to fungicide form, an effect not previously reported.

A novel finding has been a consistent indication of reduced efficacy of pesticides for the control of ZEN in the 25-11°C regime compared to the constant incubation at 25°C.

The effects of incubation temperature on T-2 toxin production is dependent upon fungicide type whereas in the case of HT-2 toxin, DAS and NEO, there is a clear effect of temperature.

Contrasting effects were observed in fungicide efficacy for the control of HT-2 toxin in the two temperature regimes. At 25°C, stimulation occurred at the 100 µg/ml dose but in the 25-11°C regime, HT-2 toxin production was almost eliminated at the same concentration.

It is suggested that the complex interactions involving fungicides, temperature and duration of exposure to these factors may be critical in the timing of fungicide applications in the field.

A new classification for fungicide efficacy is proposed, based on the capacity to completely control FHB/grain infection/fungal growth and mycotoxin production on grain or in culture. Three classes have been identified. Most fungicides appear in the

Class III group, incorporating fungicides that are either ineffective or capable of stimulating trichothecene production.

Although maneb has been identified as a Class I fungicide from evidence in the literature, its high efficacy is not conferred to mixtures incorporating this fungicide.

A novel finding in the present study with *F. culmorum* was the demonstration of 3-ADON enhancement induced by the herbicide combination of bromoxynil, ioxynil and mecoprop added as Swipe.

A radical re-design of pesticides is proposed to incorporate efficacy for regulation of mycotoxin biosynthesis.

7.2 Secondary findings

A rule-of-thumb based on colour differences of *Fusarium* colonies is proposed. The present results suggest that the striking differences in pigmentation might be associated with the pattern of mycotoxin production. Thus yellow is possibly indicative of the presence of T-2 toxin, HT-2 toxin, DON, 15-ADON and 3-ADON while pink/red coloration might suggest the production of ZEN. This rule-of-thumb, if sufficiently developed, might find application in field assessments of toxigenic potential in cases of cereal diseases such as FHB.

The lack of efficacy of propiconazole to regulate trichothecene production has been extended to instances where it is combined with other fungicides.

7.3 Confirmatory findings

Present results with *F. sporotrichioides* confirm for only the second time in recent years that this phytopathogen is a consistent producer of ZEN.

Biosynthesis of ZEN is clearly temperature-sensitive but there may be species differences regarding this effect.

The enhancement effect of fungicides on T-2 toxin production has now been extended to carbendazim.

7.4 New hypothesis

Trichothecene gene expression was investigated. It was hypothesized that there could be an effect of carbendazim on trichothecene gene expression either quantitatively, qualitatively or both. Probes to *Tri4*, *Tri5* and *Tri6*, and the control RNAs β-tubulin and rDNA from *F. sporotrichioides* were used. However, no firm conclusions could be drawn for gene expression under the influence of carbendazim. For the probes used under the conditions tested no distinct expression pattern could be found for the *Tri5* and *Tri6* genes. Probes utilized did not generate a hybridization signal which could be attributed to the gene products of *Tri5* and *Tri6*. No conclusions could be drawn on expression of these genes either with or without fungicide. The transcripts may well be present but masked on the blot at the same position as rRNA. Increasing the stringency of hybridization did not allow differentiation, nor did the hybridization of *F. sporotrichioides*-specific rDNA. In time, further improvements to the methods used as discussed above, should make it possible to determine how fungicide applications interact with specific steps in trichothecene biosynthesis.

The current studies on gene expression indicate future potential for elucidating the biochemical mechanisms underlying trichothecene biosynthesis as affected by

factors such as incubation temperature and fungicide applications. Such an approach might lead to more effective pesticides.

Chapter 8

Appendices

8 Appendices

8.1 Publications

8.1.1 Paper 1 "Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens"

European Journal of Plant Pathology **104:** 741–751, 1998.

Mini review

Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens

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Abstract

The major mycotoxigenic species of *Fusarium* and *Aspergillus* phytopathogens have been identified in this review. Since fungicides are widely used to control crop diseases caused by these fungi, it is pertinent to assess efficacy with respect to mycotoxin production. In both laboratory studies with pure cultures of phytopathogens and field trials with crop plants, the overall evidence concerning the effectiveness of fungicides is contradictory and in certain cases somewhat unexpected. In particular, at sub-lethal doses of a number of fungicides including carbendazim, tridemorph, difenoconazole and tebuconazole with triadimenol, mycotoxin production from *Fusarium* phytopathogens may increase. Furthermore, the efficacy of propiconazole and thiabendazole in the control of deoxynivalenol production from *F. graminearum* is not consistent. Evidence has been presented to suggest, for the first time, that fungicide-resistance in *F. culmorum* may be accompanied by a more persistent pattern of mycotoxin production. The limited evidence on the effects of fungicides on mycotoxin production in *Aspergillus* species is also conflicting. Under laboratory conditions, miconazole and fenpropimorph have been shown to increase aflatoxin production from *A. parasiticus*. Moreover, fenpropimorph increased production of the more toxic aflatoxin B_1. Since fungal infection of plant products is often preceded by insect damage, there is interest in the effectiveness of insecticides to reduce infestation, infection and mycotoxin contamination. Additionally, insecticides may be effective in their own right, causing a direct effect on mycotoxin synthesis. The bulk of the evidence relates to effects on aflatoxin (AF) components B_1, B_2, G_1 and G_2. Under laboratory conditions, AFB_1 production was most resistant to inhibition by insecticides, followed by AFG_1, AFG_2 and AFB_2. This pattern of inhibition was particularly consistent for the organophosphorus insecticides. In one field study, Bux and carbaryl were considerably more effective than naled in reducing AFB_1 contamination of maize kernels. It is concluded that if pesticide control is to be more effective in the future, additional criteria may be required in developing evaluation protocols for candidate compounds. In particular, the issue of fungicide-resistance in relation to mycotoxin production needs to be addressed in a concerted programme of research. Additionally, the potential of breeding and selecting cultivars resistant to disease caused by toxigenic fungi needs to be exploited in a parallel search for an environmentally acceptable solution to the question of mycotoxin contamination of plant products.

Abbreviations: NEO – neosolaniol; DAS – diacetoxyscirpenol; DON – deoxynivalenol; 3-ADON – 3-acetyl DON; 15-ADON – 15-acetyl DON; NIV – nivalenol; ZEN – zearalenone; FB_1, FB_2, FB_3 – fumonisin mycotoxins B_1, B_2, B_3; AFB_1, AFB_2, AFG_1, AFG_2 – aflatoxins B_1, B_2, G_1, G_2; OA, OB – ochratoxins A, B; CS, RS – control and resistant strains of *Fusarium culmorum*; YES – yeast extract-sucrose, PDA – potato dextrose agar; IMI – International Mycological Institute; PCR – polymerase chain reaction.

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Introduction

Economic losses arising from crop diseases caused by phytopathogenic fungi are principally associated with yield reductions. However, crop quality and safety may also be adversely affected, undermining both consumer confidence and profitability to the producer. An important feature of safety centres on the extent of contamination of plant-derived foods and animal feed with the secondary metabolites of phytopathogenic fungi, particularly mycotoxins. These compounds are endowed with toxic properties towards humans and other animals, causing a wide range of acute and chronic effects collectively known as mycotoxicoses. Some mycotoxins bear structural analogy with or act as host-specific or non-specific phytotoxins (D'Mello and Macdonald, 1998). In chemical terms mycotoxins are a diverse group of compounds produced by an equally wide range of fungi. However, the synthesis of a particular mycotoxin is invariably confined to a small number of fungal species and may even be strain-specific. Many plant pathogenic species of *Fusarium*, *Aspergillus*, *Alternaria* and *Penicillium* produce the most important mycotoxins of concern in human and animal health (D'Mello et al., 1997a; Smith, 1997; Panigrahi, 1997; Abramson, 1997). Following infection with these fungi, seeds, nuts and fruit regularly become contaminated with mycotoxins which may directly or indirectly enter the human food chain. Forages infected with certain species of *Acremonium*, *Phomopsis* and *Pithomyces* may also contain mycotoxins associated with disorders in farm animals, particularly ruminants (D'Mello and Macdonald, 1997). Mycotoxins commonly occur in the spores of fungi, including those of *Stachybotrys* and *Alternaria* and inhalation therefore represents another route of entry into the body, adding an environmental health dimension to the risks posed by these substances.

Although mycotoxins have been linked with many human and animal disorders, it is their carcinogenic potential which has evoked particular concern (Smith, 1997). In addition, recent findings implicating mycotoxins in neurotoxic, hepatotoxic and immunosuppressive conditions have sustained the impetus for monitoring and remediation. There is now ample evidence indicating that certain mycotoxins are associated with reproductive dysfunction in animals but the implications for human fertility have yet to be addressed (D'Mello and Macdonald, 1997). Since the contamination of primary foods and animal feeds with mycotoxins represents an unacceptable hazard to human and animal health, measures are in force in many countries to monitor and regulate levels of these compounds (van Egmond and Dekker, 1996).

The synthesis of mycotoxins is determined by an elaborate array of factors, broadly classified into physical, biological and chemical, and by interactions involving these factors. Time, environmental temperature, humidity and physical damage caused by insect infestation are primary factors which interact in complex ways to induce mycotoxin synthesis. In general the fungi associated with disease in cereal plants and grain legumes are also endowed with the capacity to produce the major mycotoxins. Consequently, when pesticides are used to arrest the predisposing factors of insect infestation and fungal disease, the implications for mycotoxin production need to be considered.

In this review we compare the overall efficacy of fungicides and insecticides to control mycotoxin production in *Fusarium* and *Aspergillus* fungi, taking into account recent work on fungicide resistance, persistence of critical mycotoxins and alternative strategies for control of these mycotoxins. Particular attention is given to *Fusarium* and *Aspergillus* fungi due to the economic and health implications of their respective mycotoxins. In addition, virtually all studies comparing the effectiveness of pesticides to control mycotoxins have been conducted with these two genera of fungi, and a review is now opportune.

Toxigenic species of *Fusarium* and *Aspergillus*

The major toxigenic species of these genera are presented in Table 1. The natural occurrence of mycotoxins from *Fusarium* species is conventionally associated with temperate foods, since these fungi require somewhat lower temperatures for growth and mycotoxin production than the aflatoxigenic *Aspergillus* species described below. Extensive data exist to indicate the global scale of contamination of cereal grains with a number of *Fusarium* mycotoxins (Scott, 1989), of which the most important for human and animal health are the trichothecenes, zearalenone, moniliformin and the fumonisins (D'Mello et al., 1997a). The trichothecenes are subdivided into four basic groups, with types A and B representing the most important components. The synthesis of the two types of trichothecenes appears to be characteristic for a particular *Fusarium*

Table 1. Toxigenic species of *Fusarium* and *Aspergillus*

Fungal species	Mycotoxins[1,2]
F. culmorum	Deoxynivalenol (DON), 3-acetyl DON, 15-acetyl DON, nivalenol (NIV), fusarenon X (FX), Zearalenone (ZEN)
F. graminearum (*Gibberella zeae*)	DON, 15-ADON, NIV, FX, ZEN
F. sporotrichioides	T-2 toxin, HT-2 toxin, neosolaniol (NEO), diacetoxyscirpenol (DAS), FX, ZEN
F. poae	T-2 toxin, HT-2 toxin, NIV, DAS, FX
F. moniliforme (*Gibberella fujikuroi*)	Fumonisins, moniliformin, fusarin C
F. oxysporum	Moniliformin, wortmannin, fusaric acid, sambutoxin
F. sambucinum	Sambutoxin
A. flavus	Aflatoxins, cyclopiazonic acid
A. parasiticus	Aflatoxins
A. ochraceus	Ochratoxins

[1] Type A trichothecenes: T-2 toxin, HT-2 toxin, NEO, DAS.
[2] Type B trichothecenes: DON, 3-acetyl DON, 15-acetyl DON, NIV, FX.

species (Table 1). A common feature of many *Fusarium* species is their ability to synthesise zearalenone (ZEN), and its co-occurrence with certain trichothecenes raises important issues regarding additivity and/or synergism in the aetiology of mycotoxicoses in humans and animals. The secondary metabolism of *F. moniliforme* is also important in this respect since it is capable of producing at least three mycotoxins: the fumonisins (FB_1, FB_2 and FB_3), moniliformin and fusarin C. Certain strains of two *Aspergillus* species are capable of synthesising a family of related mycotoxins comprising: aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2 respectively; Smith, 1997). Another species of *Aspergillus* produces ochratoxins A and B (OA and OB), a property shared with certain *Penicillium* fungi.

Role of *Fusarium* and *Aspergillus* fungi in phytopathology

Toxigenic fungi were considered to belong to two groups: 'field' (or pathogenic) and 'storage' or (saprophytic) but this distinction is perhaps over-simplistic and possibly counter-productive since virtually all the major toxigenic fungi in Table 1 are common pathogens of economically important plants. Scab of wheat, also known as fusarium head (ear) blight, has been caused by *F. graminearum* in epidemics in Canada and USA (Wiersma et al., 1996; Schaafsma et al., 1993; Miller et al., 1985) and by *F. culmorum* in Poland (Perkowski et al., 1996) and the Netherlands (Snijders and Perkowski, 1990). Other *Fusarium* species, e.g. *F. poae* and *F. avenaceum*, have also been implicated and Sturz and Johnston (1983) cautioned that initial colonisation by these fungi prior to ear emergence may encourage subsequent infection by end-of-season species such as *F. graminearum* and *F. culmorum*. Other cereal diseases have been associated with some of these pathogens. Thus, crown rot of wheat is attributed to *F. graminearum* (Wildermuth and McNamara, 1994) and ear rot of maize is linked with *F. graminearum* and *F. moniliforme* (Schaafsma et al., 1993).

Although *A. flavus* and *A. parasiticus* are commonly associated with the production of aflatoxins during storage the ultimate source of these fungi is likely to be soil from fields previously used for maize and other crops (Shearer et al., 1992). In addition, however, *A. flavus* has been implicated in aspergillus ear and kernel rot of maize (Campbell and White, 1995a).

A relationship between fungal infection and mycotoxin production was confirmed in the studies of Brown et al. (1995) who showed a direct correlation between colonisation with *A. flavus* and AFB_1 contamination of maize kernels. Susceptible genotypes of maize yielded grain with high levels of AFB_1 compared to kernels from resistant varieties.

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Efficacy of fungicides

Since fungicides are widely used to control crop diseases, it is pertinent to consider the effects of these agents on mycotoxin production. In both laboratory studies with pure cultures of pathogens and field trials with crop plants the resulting evidence concerning the effectiveness of fungicides is contradictory and in certain cases somewhat unexpected. In a number of instances, fungicide concentrations tested in laboratory studies were in excess of maximum solubility levels in aqueous media and, therefore, the interpretation of results is not straightforward.

Effects on Fusarium *mycotoxins*

Hasan (1993) showed that dicloran, iprodione and vinclozolin each inhibited DAS and ZEN synthesis in *F. graminearum*, but efficacy depended upon dosage and the mycotoxin in question. Dicloran eliminated DAS production at 500 μg/ml, but only 250 μg/ml was sufficient to prevent ZEN synthesis. Vinclozolin arrested DAS production at 250 μg/ml whereas levels up to 500 μg/ml markedly reduced but failed to eliminate ZEN. With all these fungicides, mycelial growth was reduced but not totally inhibited, even at 500 μg/ml. These results may be compared with earlier observations indicating greater efficiency of maneb (at 50 μg/g) in preventing ZEN production in maize kernels inoculated with *F. graminearum*. In a concurrent series with a broth culture of this fungus, growth virtually ceased on addition of maneb at 50 μg/ml medium (Draughon and Churchville, 1985).

A comprehensive series of separate experiments with benomyl, thiabendazole, prochloraz, tebuconazole, tridemorph and fenpropimorph indicated that the two morpholine fungicides had no appreciable effects on the production of 3-ADON by *F. graminearum* in pure culture (Matthies and Buchenauer, 1996). The remaining fungicides inhibited 3-ADON synthesis to the same extent or marginally more than mycelium growth provided that a level of 1 μg/ml medium was used for benomyl, thiabendazole or tebuconazole and 0.005 μg/ml for prochloraz. Tebuconazole (0.1 μg/ml) induced an almost 4-fold increase in 3-ADON production, whilst another triazole, difenoconazole (0.1 μg/ml), increased 3-ADON production in *F. culmorum* cultures maintained at 25°C but not when incubated at 11°C (D'Mello et al., 1997b). Radial growth at 25°C was unaffected by this level of fungicide. At 1 μg/ml, difenoconazole reduced growth by 9% at 25°C but 3-ADON synthesis was inhibited altogether.

Increased mycotoxin production following exposure to sub-lethal doses of fungicides has also been demonstrated with *F. sporotrichioides*. Moss and Frank (1985) observed that low concentrations of tridemorph (6–8 μg/ml) enhanced growth marginally, but consistently, yet considerably inhibited synthesis of T-2 toxin and DAS. However, at tridemorph levels of 30–50 μg/ml, growth was reduced by 50% but T-2 toxin production was stimulated five-fold. In addition, sub-lethal concentrations of carbendazim influenced production of T-2 toxin and other mycotoxins; the effects were dependent upon fungicide dose and temperature (Placinta et al., 1996). At 25°C, carbendazim (5 μg/ml) markedly enhanced T-2 toxin concentrations, and also induced smaller increases in NEO production without affecting radial growth. However, in replicate cultures transferred to 11°C, carbendazim caused progressive reductions in both mycotoxins. At the highest levels of carbendazim (10 μg/ml) synthesis of these mycotoxins and ZEN was reduced to low but measurable levels irrespective of incubation temperature. These observations correlated with lower fungal growth. Thiabendazole (100 μg/ml) on the other hand, was highly effective as a suspension applied to maize in inhibiting growth and T-2 toxin production in *F. tricinctum* (Gabal, 1987).

Several field trials conducted to assess the effect of fungicides on mycotoxin control have yielded conflicting results. Draughon and Churchville (1985) reported that maneb prevented ZEN production in maize following inoculation of corn ears with *F. graminearum* 20 days after silk development, whereas grain from untreated ears accumulated ZEN up to 168 mg/kg. These results correlated well with their laboratory findings on the effectiveness of maneb. By contrast, Suty et al. (1996) reviewed data showing that following natural *Fusarium* infection of wheat, products containing tebuconazole markedly reduced DON levels in the grain. The carbendazim precursor fungicide, thiophanate-methyl, was investigated over three seasons in 1982, 1983 and 1987 in order to determine the effects on the incidence of fusarium ear blight in wheat and barley and the extent of contamination of kernels with DON and NIV (Ueda and Yoshizawa, 1988). Fungicide application at ear emergence and at flowering reduced disease damage to the crop, and mycotoxin contamination of the harvested grain decreased to non-detectable

levels in a few instances. The levels of DON in grain were highly correlated with NIV concentrations irrespective of fungicide applications. However, seasonal variations were observed and thiophanate-methyl was ineffective, in terms of mycotoxin control, for one cultivar of wheat and barley in the 1983 harvest. Further evidence of variable efficacy depending upon fungicide type and time of application were provided by the studies of Boyacioglu et al. (1992) who examined the effects of triadimefon, propiconazole and thiabendazole on DON in a wheat crop inoculated with *F. graminearum*. Triadimefon reduced DON concentrations in grain by 65% when the fungicide was applied prior to inoculation, but the effectiveness increased to 78% when triadimefon was applied 2 days after inoculation. For propiconazole applied at the same times, efficiency was 34 and 78% respectively, but for thiabendazole, efficacy of DON control was highest (83%) when applied prior to inoculation and lower (16%) when applied 2 days after inoculation. Thiabendazole had no effect on fungal infection at either application stage.

The opposite end of the spectrum, as regards fungicide efficacy under field conditions, is represented by the observations of Milus and Parsons (1994) who examined the effects of a wide range of fungicides on the control of fusarium ear blight of wheat and DON contamination of harvested grain. Disease incidence and DON concentrations in grain remained totally unaffected by fungicide applications. It is possible that differences in the timing and number of inoculations and fungicide applications as well as environmental factors may have contributed to the discrepancy between these observations and those of Boyacioglu et al. (1992). However, the theme of increased mycotoxin production has emerged from another source (Gareis and Ceynowa, 1994). In their studies, application of Matador (a mixture of tebuconazole and triadimenol) to winter wheat inoculated with *F. culmorum* reduced head blight but increased NIV content of harvested grain 16-fold. Thus, results of both laboratory studies and field trials show that some fungicides may reduce but not eliminate trichothecene production and at worst mycotoxin synthesis may indeed be enhanced.

Effects on Aspergillus *mycotoxins*

The limited evidence on fungicide efficacy in the regulation of *Aspergillus* mycotoxins is also conflicting. The studies reviewed here all refer to laboratory assessments using isolates cultured in different media or on cereal grains. Substantial reductions in AFB_1 contamination was reported by Gabal (1987) on treatment of whole maize with thiabendazole, but fungicide level was an important factor determining effectiveness. Rama Devi and Polassa (1984) showed that carbendazim, added as Bavistin, stimulated growth of *A. flavus* and *A. ochraceus* when added to a liquid medium at levels of up to 100 µg/ml, with AFB_1 and ochratoxin production remaining at control values, but at levels of 2–3 mg/ml both growth and mycotoxin production were totally inhibited. The low solubility of carbendazim in aqueous media (8 µg/ml at pH 7) makes these results difficult to explain. However, the effect of fungicide on mycotoxin production may not correspond proportionately with growth inhibition. For example, El-Kady et al. (1993) observed that at relatively low levels (10 µg/ml) of a carboxin-captan fungicide mixture, growth of *A. flavus* in a liquid medium was reduced by only 4% after 12 days of incubation, but aflatoxin synthesis was suppressed by 100%. At the same concentration of a mixture of the protectant organophosphorus fungicide, tolclofos-methyl, with thiram (Rizolex-T) fungal growth marginally increased, AFB_1 and AFB_2 remained at control levels, but AFG_1 and AFG_2 production decreased by at least 50%.

Although the fungicides cited above were effective to varying degrees in their mycotoxin-inhibiting potential, other fungicides are known to enhance total aflatoxin production and alter the relative proportions of the component aflatoxins. Sub-inhibitory levels of miconazole stimulated total aflatoxin production in *A. parasiticus* on four different substrates (Buchanan et al., 1987). Peptone-mineral salts substrate promoted low levels of aflatoxin, but a 4-fold increase in synthesis of the mycotoxin occurred in the presence of miconazole (0.1 µM). This theme was confirmed and developed further in the studies of Badii and Moss (1988). Incubation of *A. parasiticus* for 8 days in a liquid medium containing fenpropimorph (250 µg/ml) stimulated AFB_1 and AFG_1 production to 267 and 367 µg/flask from control levels of 75 and 167 µg/flask respectively for similar rates of fungal growth. The ratio of the two mycotoxins had shifted in favour of the more toxic AFB_1. Other studies have confirmed the persistence of AFB_1 synthesis in the presence of fungicide (Fernando and Bean, 1986). In different isolates of *A. parasiticus* and *A. flavus*, tricyclazole reduced production of all four aflatoxins in a

dose-related manner but detectable levels of AFB_1 persisted even at the highest concentration (100 μg/ml).

Pre-harvest occurrence of aflatoxins in peanuts is common (Anderson et al., 1996) and the fungi responsible have also been associated with aspergillus ear rot in maize (Campbell and White, 1995a). However, the effects of fungicides in the control of aflatoxin contamination have not been investigated in field trials. Consequently, it is not possible to corroborate the laboratory findings reviewed above with field data. It is likely that future research on the control of mycotoxins from *Aspergillus*, and indeed *Fusarium*, species will focus on the development of disease-resistant genotypes of crop plants. Such an approach would be consistent with the aims of good environmental practice and, in addition, might address the issue of fungicide resistance in plant pathogenic fungi.

Fungicide resistance

Both laboratory and field studies have underlined the ineffectiveness of some fungicides in controlling mycotoxin production by *Fusarium* and *Aspergillus* phytopathogens. Indeed, there is some evidence that fungicide use may be associated with increased synthesis of critical mycotoxins, and it is conceivable that fungicide resistance might contribute to increases in mycotoxin production. In the first study of its kind, D'Mello et al. (1997c) investigated the production of 3-ADON in a difenoconazole-resistant strain of *F. culmorum*. Resistance was developed in our laboratory using the following procedure. A freeze-dried culture of *F. culmorum* 309344 from IMI was re-suspended in Ringers solution and grown on potato dextrose agar (PDA) in 9 cm Petri dishes which were then incubated at 25°C until growth was established. Peripheral plugs from these colonies were used to prepare 5d-old cultures also on PDA. A plug was removed from these 5d-old cultures and placed centrally on to 20 ml PDA containing difenoconazole at 100 μg/ml. The fungicide was added as Plover (Ciba Agriculture), containing 250 g active ingredient/l. Prior to addition, Plover was diluted in ethanol. Several inoculated plates prepared in this manner were incubated at 25°C for 21 days, after which time they were stored at 4°C for approximately 11 months. These cultures were designated as 'resistant' for the purposes of our investigations since on further exposure to difenoconazole, these cultures had superior growth in comparison with the original control cultures. Control ('sensitive') cultures were prepared and stored in an identical process, except that equivalent volumes of ethanol without fungicide were added to the PDA prior to inoculation. After storage, control and resistant cultures were used to prepare 5 d-old colonies on PDA alone for control cultures and on PDA + difenoconazole at 100 μg/ml for resistant strains. Plugs were removed as before and placed centrally on PDA containing difenoconazole at 0, 0.1, 100 and 200 μg/ml to obtain a control and resistant series in factorial combination. Inoculated Petri dishes were incubated at 25°C for 8, 15 or 22 days. Following treatment, the resistant strain (RS) continued to synthesize 3-ADON even when difenoconazole levels of 100 and 200 μg/ml media were used. In contrast, the control strain (CS) of *F. culmorum* failed to produce the mycotoxin at either of these two concentrations of difenoconazole. These differences between RS and CS cultures were apparent at each of the three times of experimental observation (8, 15 and 22 days of incubation). Statistical tests indicated that two of these specific differences were significant ($P < 0.05$), on days 8 and 15 for the 100 μg/ml level of fungicide addition.

Further definition of these strain differences emerged in an extended study (D'Mello et al., 1998) designed to examine the pattern of 3-ADON production in CS and RS cultures over a period of 57 days, using lower levels of difenoconazole so that mycotoxin synthesis could be determined at equivalent levels of growth for both strains. In the earlier study (D'Mello et al., 1997c), the RS isolates exhibited superior growth relative to the CS cultures. That this objective was achieved is apparent from a comparison of the growth and 3-ADON results in the second study (D'Mello et al., 1998). In particular, it was clear that at 21 days, there were no significant differences ($P > 0.05$) in colony diameters between the two strains following exposure to difenoconazole at 1, 2 and 4 μg/ml, but 3-ADON production (Table 2) only occurred in RS cultures, albeit at relatively low levels. Similarly, at 43 days equivalent growth was attained by both strains on exposure to the fungicide at 6 and 10 μg/ml media, but 3-ADON synthesis was only apparent with RS isolates. Further analysis of variance showed that these values for the RS cultures were significantly different from the zero values recorded for CS ($P < 0.05$), reflecting previous findings by D'Mello et al. (1997c). Moreover, there was a significant interaction ($P < 0.01$) involving strain, time and difenoconazole level in the study of D'Mello et al. (1998). Whereas, relative to basal values, the 1 μg/ml addition of difenoconazole significantly

Table 2. Effects of incubation time and difenoconazole concentrations on 3-acetyl deoxynivalenol (3-ADON) production in control (CS) and fungicide-resistant strains (RS) of *Fusarium culmorum*[1]

Difeno-conazole level (µg/ml)	Incubation time (days)											
	7		14		21		28		43		57	
	CS	RS	CS	RS	CS	RS	CS	RS	CS	RS	CS	RS
	3-ADON in culture extracts (mg/ml)											
0	1.4	1.4	6.8	13.1	10.6	10.7	6.7	5.6	11.2	5.2	10.8	10.3
1	0	0	0	0	0	0.3	2.9	1.8	2.9	4.2	5.6	8.8
2	0	0	0	0	0	0.2	0.8	0	2.2	1.4	5.9	1.0
4	0	0	0	0	0	0.6	0.6	0.4	2.0	2.9	7.1	1.2
6	0	0	0	0	0	0	0	0	0	0.3	0.9	1.5
10	0	0	0	0	0	0	0	0	0	0.2	1.0	1.4
LSD ($P < 0.05$)	3.03											

[1] From D'Mello et al. (1998).

depressed 3-ADON production in CS cultures at 43 and 57 days ($P < 0.01$ or better), this level of fungicide failed ($P > 0.05$) to reduce mycotoxin levels in RS cultures at the same stages of the experiment (Table 2). Some anomalous results have, however, appeared in this study. Thus, the depression in 3-ADON production was greater with the RS than with the CS cultures at 57 days when both were subjected to the 2 µg/ml addition of difenoconazole (Table 2). However, this discrepancy does not invalidate the main thrust of the observations concerning 3-ADON production in the two strains of *F. culmorum* (D'Mello et al., 1997c, 1998).

The production of trichothecene mycotoxins in *Fusarium* species occurs in a systematic process of oxygenations and esterifications. There is particular interest in the role of esterases not only in biosynthesis but also in the metabolism of trichothecenes in animals. It is theoretically possible to use esterases to distinguish between different species, toxigenic groups and races of *Fusarium*. That such an approach is feasible has been indicated by the recent results of Baayen et al. (1997) who reported that isolates from different vegetative compatibility groups of *F. oxysporum* had unique esterase profiles. In a second experiment, D'Mello et al. (1998) demonstrated that total esterase production increased progressively over time and at higher levels for RS from day 14 onwards to the extent that by day 35 the difference between RS and CS was significant ($P < 0.05$). Although the increase with time occurred in both strains, this enhancement appeared significantly earlier (at 14 days) for RS but delayed (until 21 days) for the CS cultures ($P < 0.05$). It is premature to attempt to correlate total esterase production with 3-ADON concentrations since such comparisons would only be valid for the specific esterase isoenzymes involved in trichothecene biosynthesis. With this objective in mind, further studies are now in progress to examine the profile differences for esterase isoenzymes in the two strains, but the potential for diagnostic use is clear if the results of the second experiment of D'Mello et al. (1998) are considered in conjunction with those of Baayen et al. (1997). These results are of practical importance since *F. culmorum* has been associated with fusarium head blight and mycotoxin contamination of wheat kernels (Snijders and Perkowski, 1990). Although difenoconazole is not intended for the control of fusarium head blight, the efficacy of this fungicide towards *Septoria tritici* and rust diseases and its relative persistence implies that any residues may affect the secondary metabolism of other fungi. *Fusarium* species commonly occur on ears in the field even in the absence of overt symptoms of disease (D'Mello et al., 1993). Furthermore, it is possible that there may be cross-resistance with other fungicides such as epoxiconazole, tebuconazole, thusilazole or prochloraz. Consequently, our results may have more universal implications for mycotoxin production in *Fusarium* species which have developed resistance to demethylation inhibitors in general. Although this would be of more practical significance, as yet there appears to be no evidence of resistance to demethylation inhibitors in field populations of *Fusarium culmorum*.

On the basis of the results presented by D'Mello et al. (1997c, 1998) there appears to be substantive evidence

that difenoconazole-resistance in *F. culmorum* may accelerate the onset of 3-ADON production following further exposure to the fungicide. In addition, although difenoconazole-induced suppression of 3-ADON synthesis does occur, this is less pronounced in RS than in CS cultures. New data also demonstrates consistently higher production of esterases in RS cultures, but the significance for toxigenicity and pathogenicity needs to be addressed.

Effects of insecticides

Since fungal infection of grain, nuts and fruit is often preceded by physical damage caused by insect invasion, much effort has been expended on the potential of insecticides to reduce infestation, infection and, therefore, mycotoxin contamination from 'storage' fungi such as the *Aspergilli*. Such a strategy offers the advantage of dual-function insecticides, potentially contributing to lower overall pesticide use. However, insecticides may be effective in their own right. In pure culture studies with *Aspergillus parasiticus*, uncomplicated by insect infestation, dichlorvos, landrin, malathion and diazinon significantly inhibited production of AFB_1 in a dose-dependent manner (Draughon and Ayres, 1981). AFB_1 inhibition was greater than the reduction in growth of the fungus; with dichlorvos at 100 mg/l of culture broth, fungal growth declined by 29% whereas AFB_1 synthesis was reduced by 99%. At this concentration, diazinon increased mycelial growth by 19% but AFB_1 production was still reduced by 23%. At 100 mg/l, naled inhibited growth totally, but not at 10 mg/l at which level it precipitated a 68% reduction in AFB_1 synthesis, but dichlorvos was still more effective, causing a 92% inhibition of AFB_1 production. AFB_2 levels were reduced below detection levels in all insecticide-treated cultures. At sub-lethal levels of insecticide, dichlorvos was more effective than naled in reducing production of AFG_1 and AFG_2. In general, AFB_1 synthesis was most resistant to inhibition by insecticides, followed by AFG_1, AFG_2 and AFB_2. This pattern of inhibition was particularly consistent for the organophosphorus insecticides (Draughon and Ayres, 1981).

In field studies, naled reduced AFB_1 concentrations in harvested kernels after application to a maize crop which had been artificially inoculated with *A. parasiticus* (Draughon et al., 1983). However, Bux and carbaryl were considerably more effective than naled, whereas in laboratory studies with both cultures naled exhibited higher efficacy. In the uninoculated maize crop, Bux and carbaryl were again highly effective in reducing natural production of AFB_1 while naled was virtually inactive.

Production of OA and OB by *A. ochraceus* in yeast extract-sucrose (YES) medium and in maize kernels can be reduced in a dose-dependent manner by application of dichlorvos at levels of up to 300 mg/l broth or per kg corn (Wu and Ayres, 1974). Mycelial growth, measured in YES cultures, remained at 80% of control values at the highest level of dichlorvos addition but OA and OB levels were reduced to, respectively, 21 and 11% of control values.

Naled has also been found to be effective in reducing ZEN levels in pure cultures of *Fusarium graminearum* in liquid media or on maize kernels when the insecticide has been applied as a liquid preparation or as fumigant at concentrations of 30 and 100 µl/l (Berisford and Ayres, 1976). Production of ZEN was completely inhibited only when naled was applied prior to inoculation of the culture media. When applied to 12-day or older cultures, naled did not inhibit ZEN synthesis but in 3 to 9-day cultures ZEN production was reduced by 45–92% when the insecticide was applied at 10–100 µl/l.

Biocontrol

Lack of confidence with respect to the efficacy of pesticides in the control of mycotoxin contamination, particularly following fusarium head blight induction (Milus and Parsons, 1994), has led to a quest for alternative methods. There is increasing optimism that exploitation of disease resistance in different plant genotypes offers considerable potential as an environmentally acceptable technique to control mycotoxin contamination of primary foodstuffs, and the limited evidence is encouraging. Among early studies, the evidence of Miller et al. (1985) marked a defining point in the application of this strategy. A single isolate of *F. graminearum* was used to experimentally infect spring wheat, rye and triticale cultivars. In genotypes resistant to infection, low kernel concentrations of DON (mean, 0.6 mg/kg) were recorded, while in susceptible cultivars much higher values (mean, 10.2 mg/kg) were found. Miller et al. (1985) suggested that resistant cultivars may be endowed with factors that prevent synthesis and/or promote degradation of the mycotoxin. It is also conceivable that resistant cultivars may simply prevent growth of the pathogen. Indeed, the following

evidence is indicative of resistance directed at the fungus itself rather than at the synthesis of mycotoxins. Consequently, reduced infection can be correlated with lower disease incidence which in turn may lead to reduced mycotoxin levels in grain of resistant genotypes. Marked effects were reported by Snijders and Perkowski (1990) who investigated the relationship between head blight induced by three pathotypes of *F. culmorum* in ten wheat genotypes and DON levels in harvested grain. Head blight incidence caused by the most virulent strain of the pathogen ranged from 2% for one wheat genotype to 62.5% for another, with the values for DON being 4.6 and 37.0 mg/kg grain, respectively. Overall, for the three pathotypes and ten genotypes, there was a linear correlation between incidence of head blight and DON contamination of grain. Work in Canada has further underlined genotype differences in fusarium head blight susceptibility and mycotoxin contamination of wheat (Wong et al., 1995). Canadian cultivars susceptible to the disease caused by *F. culmorum* had DON levels ranging from 17 to 121 mg/kg grain, while the same cultivars infected with *F. graminearum* yielded grain with DON concentrations in the range 6–53 mg/kg as well as higher levels of 15-ADON. In contrast, Chinese cultivars infected with these pathogens produced grain with DON levels of 0–10 and 0–6 mg/kg, respectively and low levels of NIV.

Investigations with fusarium ear rot of maize indicate the potential for wider application of resistant hybrids as a means of reducing mycotoxin contamination of plant products (Schaafsma et al., 1993). In one study, a resistant maize hybrid artificially inoculated with *F. graminearum* yielded kernels with DON concentrations ranging from 0.4 to 80.4 mg/kg, while in kernels from a susceptible line values of 1.3–313 mg/kg were found. Levels for ZEN were 0–0.1 mg/kg in the resistant hybrid and 0–8.4 in the susceptible cultivar. These results correlated with differences in ear rot ratings for the two hybrids.

It has been suggested that the most effective control of aspergillus ear and kernel rot in maize centres on the use of genetically resistant hybrids (Campbell and White, 1995b). This approach should also result in reduced contamination of kernels with aflatoxins. Evidence from plot experiments with artificially inoculated maize plants tends to justify the validity of such a strategy. In one study, susceptible lines yielded kernels with AFB_1 contents of up to 943 μg/kg, whereas resistant hybrids had around half the level of ear rot and AFB_1 levels typically in the range 3–21 μg/kg. Screening peanut genotypes for resistance to *Aspergillus* fungi is also being undertaken with a view to reducing mycotoxin contamination of kernels (Anderson et al., 1996).

Another method of biocontrol, based on the use of atoxigenic fungi, deserves mention as it amplifies ecological interactions with the potential for exploitation in the future. Garber and Cotty (1997) showed that co-inoculation of developing cotton bolls with a toxigenic and an atoxigenic strain of *A. flavus* reduced sclerotia formation and synthesis of aflatoxins. Lower toxicity may be expected not only from reduced aflatoxin production but also from decreased levels of other sclerotial metabolites.

Diagnostic issues

Conventional methods for the detection of toxigenic fungi are based on a combination of culturing isolates on specialised nutrient media to induce sporulation followed by microscopic examination of spores and conidia for characteristic morphological features. Such procedures are laborious, time-consuming and sometimes inconclusive. Consequently, there is considerable interest in the application of molecular biology techniques for rapid and positive identification of toxigenic fungi and already the polymerase chain reaction (PCR) has been shown to offer potential to accurately detect and identify several cereal pathogens directly from plant material. A laboratory-based PCR method has recently been developed to identify *F. poae*, *F. culmorum*, *F. graminearum*, *F. avenaceum* and *Michrodochium* (*Fusarium*) *nivale* in cereal grains. Indeed, a PCR diagnostic test for *Fusarium* on wheat is available from ADGEN at SAC (Stevenson, 1997). PCR methodology requires just 48 h to provide accurate results, whereas conventional procedures may take up to 21 days to yield somewhat ambiguous results. As presently designed, the PCR method is based on electrophoresis, but there is potential to develop alternative multi-well plate techniques to facilitate kit-based applications for use under field conditions.

Another method with some potential for species identification involves esterase profiling (Baayen et al., 1997). Although attention has focused on *F. oxysporum* and *A. parasiticus*, based on this evidence, there is now scope for investigating esterase patterns in other toxigenic species of *Fusarium*. D'Mello et al. (1998)

have correlated total esterase production with fungicide insensitivity in *F. culmorum*, but more recent unpublished data from our laboratory indicate that species differences may also be of diagnostic value. *F. sporotrichioides*, with the confirmed capacity to yield T-2 toxin, HT-2 toxin, NEO and DAS under our conditions, contained at least six different esterase isoenzymes, whereas *F. culmorum*, which produces only 3-ADON had just two esterase bands.

Conclusions

Evidence has been presented which highlights the overall ineffectiveness of pesticides to control mycotoxin production in *Fusarium* and *Aspergillus* fungi. In retrospect this conclusion is not entirely inconsistent with the notion that fungicides were designed for use against diseases and not necessarily against the mycotoxins associated with some of these diseases. If chemical control is to succeed in the future, additional criteria may need to be introduced into evaluation protocols for candidate pesticides. An invidious feature of fungicide application at sub-lethal doses emanates from persistence of mycotoxin production, occasionally at elevated levels. For example, increased persistence and changes in the pattern of 3-ADON production have been observed in a strain of *F. culmorum* resistant to the fungicide, difenoconazole. Development of fungicide resistance occurs readily in *Fusarium* phytopathogens but the effects on mycotoxin production need to be addressed in a more deliberate and systematic manner, given these findings on 3-ADON. Of the aflatoxins, AFB_1 production occurs more persistently in the presence of sub-lethal doses of fungicides and insecticides. This is of particular concern in view of the potency of AFB_1 as a carcinogen. Nevertheless, the concept that insecticides may exert a dual role in both insect and mycotoxin control may need further exploration to enable overall reductions in pesticide use. However, in terms of an environmentally acceptable alternative, there is considerable potential in breeding or selecting cultivars of crop plants which are resistant to those fungal diseases that are associated with mycotoxin contamination.

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8.1.2 Paper 2 "A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins"

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A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins

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Abstract

From a global perspective, three classes of *Fusarium* mycotoxins may be considered to be of particular importance in animal health and productivity. Within the trichothecene group, deoxynivalenol (DON) is widely associated with feed rejection in pigs, while T-2 toxin can precipitate reproductive disturbances in sows. Another group comprising zearalenone (ZEN) and its derivatives is endowed with oestrogenic properties. The third category includes the fumonisins which have been linked with specific toxicity syndromes such as equine leukoencephalomalacia (ELEM) and porcine pulmonary oedema. Many toxigenic species of *Fusarium* are also common pathogens of cereal plants, causing diseases such as head blight of wheat and barley and ear rot of maize. Consequently, when cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins and that these may subsequently be transferred to compound feeds. The surveillance of grain and animal feed for the occurrence of *Fusarium* mycotoxins continues to attract worldwide attention and has been the subject of extensive investigations over recent years. For example, high incidence rates of contamination with DON and another trichothecene, nivalenol (NIV), have been reported in maize samples in New Zealand. In Poland, unacceptably high values (up to 927 mg/kg) for DON were recorded for maize grain and cobs. Potentially harmful levels of DON (up to 40 mg/kg) were also observed in wheat produced in Germany, Poland, Japan, New Zealand, USA, Canada and Argentina. Samples of barley grain in Norway, Japan and USA were found with DON levels of up to 71 mg/kg. In the Norwegian study oat samples were also contaminated with DON at levels ranging from 7 to 62 mg/kg grain. Abnormally high concentrations of both NIV and ZEN have been observed in some Japanese barley samples (up to 26 and 15 mg/kg, respectively), and in maize produced in New Zealand (up to 7 and 10.5 mg/kg, respectively). Other trichothecenes such as 3-acetyl DON, diacetyoxyscirpenol (DAS),

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Abbreviations: NEO, neosolaniol; DAS, diacetoxyscirpenol; DON, deoxynivalenol; 3-ADON, 3-acetyl DON; 15-ADON, 15-acetyl DON; NIV, nivalenol; ZEN, zearalenone; FB_1, FB_2, FB_3, fumonisin mycotoxins B_1, B_2, B_3; ELEM, equine leukoencephalomalacia; ELISA, enzyme-linked immunosorbent assay; AFB_1, aflatoxin B_1

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T-2 toxin and HT-2 toxin have also been found in cereals and animal feed in both temperate and tropical countries. In Uruguay all samples of maize-based animal feeds tested were positive for fumonisin B_1 (FB_1). However, highest FB_1 values were observed in South Africa for compound feed (11 000 μg/kg), and in Thailand and China for maize (18 800 and 25 970 μg/kg, respectively). In a study of Argentinian maize, FB_2 was the major fumonisin at values of up to 11 300 μg/kg. An alarming feature of several surveys is that in the tropics in particular, several *Fusarium* mycotoxins may co-occur with each other and with aflatoxin B_1, an *Aspergillus* compound sharing carcinogenic properties with fumonisins. It is concluded that, although sample size has been small in a number of surveys, there is nevertheless unequivocal evidence of global contamination of cereal grains and animal feed with several trichothecenes, ZEN and fumonisins. Furthermore, it is clear that legislation for the control of these mycotoxins in animal feed is now overdue and that further work is required to exploit cereal genotypes that are resistant to diseases caused by toxigenic *Fusarium* phytopathogens.

Keywords: *Fusarium* sp., trichothecenes; Zearalenone; Fumonisins; Toxicity; Cereal grains; Co-occurrence; Control strategies

1. Introduction

Cereal grains and associated by-products constitute important sources of energy and protein for all classes of farm livestock. When cereal grains and animal feed are colonised by moulds there is a significant risk of contamination with the secondary metabolites of these fungi. A number of these fungal compounds are endowed with toxic effects towards animals and human beings and are accorded the collective term mycotoxins. Many species of *Fusarium, Aspergillus, Penicillium* and *Alternaria* are not only recognised plant pathogens but are also sources of the important mycotoxins of concern in animal and human health (Abramson, 1997; D'Mello et al., 1997; Panigrahi, 1997; Smith, 1997). Traditionally, mycotoxigenic fungi have been considered to fall into two groups: 'field' (or plant pathogenic) and 'storage' (or saprophytic) fungi. However, in the case of *Aspergillus flavus*, for example, this distinction is academic since this fungus is associated with aspergillus ear rot and kernel rot of maize (Campbell and White, 1995) and also colonises stored grain when factors such as temperature and water activity are optimised (Smith, 1997). Forages infected with certain species of *Acremonium, Phomopsis* and *Pithomyces* may also contain mycotoxins capable of precipitating specific disorders in farm livestock, particularly ruminants (D'Mello and Macdonald, 1997). In addition, mycotoxins occur in the spores of many fungi, including *Stachybotrys* and *Alternaria* and inhalation therefore represents another route of entry into the body. Considerable attention has been given to the distribution and toxicology of the aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* due in particular to the carcinogenic potential of these mycotoxins, to the extent that legislation is now in place in many countries to control levels of contamination (Smith, 1997). It is apparent, however, that the *Fusarium* mycotoxins are also widely distributed, some with carcinogenic properties and others of well-defined toxicology for farm livestock. The occurrence of *Fusarium* trichothecene mycotoxins in cereals and animal feed has been summarised by Scott (1989) and Yoshizawa (1991), but considerable data has since been published on these

and more novel mycotoxins such as the fumonisins and there is now a clear need for an updated review. Recent advances in analytical methodology further justify the need for compilation of new data. Enzyme-linked immunosorbent assays (ELISA) have been developed for analysis of *Fusarium* mycotoxins but in global terms, high-pressure liquid chromatography and gas chromatography with mass spectrometry have now emerged as the methods of choice, largely replacing earlier techniques based on thin-layer chromatography (Wang et al., 1995a, b; Yamashita et al., 1995). Co-occurrence of *Fusarium* mycotoxins has also become a significant issue with complex and, thus far, indeterminate implications for animal health and welfare. In this paper we identify the primary *Fusarium* mycotoxins of relevance to livestock production on a global scale. In addition, we present quantitative evidence of worldwide contamination of cereal grains and animal feed with these mycotoxins.

2. *Fusarium* mycotoxins

Mycotoxins from *Fusarium* species have traditionally been associated with temperate cereals, since these fungi require somewhat lower temperatures for growth and mycotoxin production than the aflatoxigenic *Aspergillus* species. However, extensive data exists to indicate the global scale of contamination of cereal grains with a number of *Fusarium* mycotoxins (Muller and Schwadorf, 1993; Chulze et al., 1996; Viquez et al., 1996). *Fusarium* species synthesise a wide range of mycotoxins of diverse structure and chemistry (Flannigan, 1991). The most important from the point of view of animal health and productivity are the trichothecenes, zearalenone, moniliformin and the fumonisins (D'Mello et al., 1997). The trichothecenes are subdivided into four basic groups, with types A and B representing the most important members. The type A trichothecenes include T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS), while type B trichothecenes include deoxynivalenol (DON, also known as vomitoxin) and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV) and fusarenon-X. The synthesis of the two types of trichothecenes appears to be characteristic for a particular *Fusarium* species. For example, production of type A trichothecenes predominates in *F. sporotrichioides* and possibly also *F. poae*, whereas synthesis of type B trichothecenes occurs principally in *F. culmorum* and *F. graminearum*. In addition, within-species chemotypes are discernable. Thus, Perkowski et al. (1997) identified two chemotypes of *F. graminearum*, one producing NIV and DON and the other elaborating a mixture of DON and both its acetylated derivatives.

A common feature of many *Fusarium* species is their ability to synthesise zearalenone (ZEN), and its co-occurrence with certain trichothecenes raises important issues regarding additivity and/or synergism in the aetiology of mycotoxicoses in animals. Furthermore, ZEN (previously known as F-2 toxin) may occur in the form of four hydroxy derivatives.

With regard to the co-occurrence of *Fusarium* mycotoxins, the secondary metabolism of *F. moniliforme* is particularly relevant since it has been associated with the production of at least three mycotoxins, namely the fumonisins, moniliformin and fusarin C. The fumonisins (FB_1, FB_2 and FB_3) are regarded as unique in that they are also structural

congeners of the host-specific AAL-toxins produced by *Alternaria alternata*, the causative agent of alternaria stem canker of tomato. In addition, however, *F. moniliforme* and *F. proliferatum* have recently been linked with the natural co-contamination of maize with FB_1, and two relatively novel mycotoxins, fusaproliferin and beauvericin (Ritieni et al., 1997). Moniliformin is also synthesised by *F. oxysporum* which, in addition, is a recognised source of the mycotoxins wortmannin and fusaric acid (D'Mello et al., 1997). The diversity of *Fusarium* mycotoxins is further illustrated by the production of fusarochromanones (TDP-1, TDP-2 and TDP-6) by *F. equiseti* which also synthesises several trichothecenes as well as ZEN (Flannigan, 1991; D'Mello et al., 1997).

3. Toxicity and syndromes

As with other bioactive compounds, the *Fusarium* mycotoxins are endowed with both acute and chronic aspects of toxicity. It is generally accepted that among the trichothecenes, type A members are more toxic than those in the type B category. Thus, Leeson et al. (1995) listed LD_{50} values, determined with young chickens, of 2–5.9 mg/kg body weight for DAS and 3.6–5.25 mg/kg body weight for T-2 toxin. In contrast, a single LD_{50} value of 140 mg/kg body weight was cited for DON.

Interest in the toxicology of *Fusarium* mycotoxins for farm animals is set to continue, not so much for the acute effects as for the chronic syndromes reported on a worldwide basis. In addition, the recent association of FB_1 with carcinogenesis in human beings has increased concern over the possibility that *Fusarium* mycotoxins may be transferred into milk, eggs and meat.

In Japan, several cases of mycotoxicoses in animals and human beings have been attributed to consumption of cereals contaminated with DON and NIV (Yoshizawa, 1991). Chronic exposure of farm animals to DON is also an important issue in Canada, USA and continental Europe. Studies with pigs indicate that DON is a potent feed intake and growth inhibitor, the levels of reduction typically being of the order of 20% and 13%, respectively, for a dietary concentration of 4 mg DON/kg. The feed rejection and emetic syndromes are aptly embodied in the alternative term for DON, namely 'vomitoxin'. Ruminants, on the other hand, are considerably more tolerant to DON, as exemplified by the lack of effect on feed intake and milk output in dairy cows (Charmley et al., 1993). As regards the other trichothecenes, Prelusky et al. (1994) point to circumstantial evidence implicating DAS and T-2 toxin in field cases of mycotoxicoses. In sows, T-2 toxin has been shown to induce infertility, and after parenteral administration during the last trimester of gestation, is able to precipitate abortion within 48 h.

Acute tests indicate that ZEN is of relatively low toxicity, with an LD_{50} value of 2–10 g/kg body weight as determined with mice (Flannigan, 1991). However, chronic investigations demonstrate that its oestrogenic properties towards mammals are a more important feature at levels as low as 1.5–3 mg/kg diet. In cows, infertility, reduced milk production and hyperoestrogenism have been associated with ZEN or with *Fusarium* species producing this mycotoxin.

Feeding corn contaminated with *F. moniliforme* is often associated with the induction of leukoencephalomalacia and acute neurotoxicity in equine species (ELEM) and with

pulmonary oedema syndrome in pigs (Prelusky et al., 1994). Cases of ELEM have been confirmed in Brazil, South Africa and USA (Marasas, 1995). ELEM is an acutely fatal neurological disorder with clinical signs such as ataxia, paresis, hypersensitivity and locomotor derangements. The porcine manifestation is characterised by pulmonary oedema as well as pancreatic and liver damage, with cases confirmed in Brazil and USA. There is little doubt now that most, if not all, of these effects are attributable to the consumption of the fumonisins.

As will be seen later in this review, co-occurrence of *Fusarium* mycotoxins in cereal grains and animal feed is a recurring feature, raising the question of interactions in the manifestation of toxicity. Although of minor toxicity at levels detected in nature, fusaric acid can enhance the activity of other *Fusarium* mycotoxins. Thus, a synergistic interaction between fusaric acid and FB_1 has been demonstrated in the fertile chicken egg. In combination, high lethality was observed whereas individually the mycotoxins had virtually no effect on mortality (D'Mello et al., 1997). Similarly, Harvey et al. (1996) demonstrated that whereas DON and FB_1 individually caused similar but marginal reductions in weight gain of pigs, a marked synergistic growth depression occurred on combined addition of the two mycotoxins. However, the effect of this combination on growth of broiler chicks appears to be additive (Kubena et al., 1997). Of greater practical significance is the co-occurrence of various *Fusarium* mycotoxins with aflatoxin B_1 (AFB_1). A synergistic interaction might be expected with respect to induction of toxicity. Limited evidence indicates that this might be the case. For example, Harvey et al. (1995) observed that a combination of aflatoxins with DAS caused a synergistic depression in growth of lambs and increase in serum activity of γ-glutamyltransferase. The toxic interaction between aflatoxins and fumonisins awaits elucidation but it is likely that animal species differences will predominate over the issue of additivity and synergism.

4. Worldwide contamination

Many of the toxigenic species of *Fusarium* described above are also major pathogens of cereal plants, causing head blight in wheat and barley, for example, and ear rot in maize. A relationship between level of crop infection with these pathogens and mycotoxin contamination of harvested grain is to be expected and this is borne out in a considerable body of evidence reviewed by D'Mello and Macdonald (1998). Thus, a striking positive correlation was presented between *F. culmorum*-induced head blight incidence in wheat and DON contamination of grain.

It is possible that fungi may be spread from one country to another with increases in global grain trade. However, in respect of *Fusarium* fungi this risk is likely to be minimal since these phytopathogens are field rather than storage organisms. On the other hand, there is now overwhelming evidence of global contamination of cereals and animal feeds with *Fusarium* mycotoxins particularly trichothecenes, zearalenone and fumonisins (Tables 1 and 2). Trade in these commodities may, therefore, contribute to the worldwide dispersal of mycotoxins. Co-contamination of maize and poultry feeds with fumonisins and aflatoxins has also been demonstrated for the first time in India (Shetty and Bhat, 1997), while in North Vietnam samples of feed-grade maize powder were found with

Table 1
Global distribution of DON, NIV and ZEN in cereal grains and animal feed (mg/kg)

Country	Cereal/feed type	DON	NIV	ZEN	References
Germany	Wheat	0.004–20.5	0.003–0.032	0.001–8.04	Muller and Schwadorf (1993)
Poland	Wheat	2–40	0.01	0.01–2	Perkowski et al. (1990)
Poland	Maize kernels	4–320			Grabarkiewicz-Szczesna et al. (1996)
	Maize cobs: axial stems	9–927			
Bulgaria	Wheat	Up to 1.8		Up to 0.12	Vrabcheva et al. (1996)
Finland	Feeds and grains	0.007–0.3		0.022–0.095	Hietaniemi and Kumpulainen (1991)
	Oats	1.3–2.6			
Norway	Wheat	0.45–4.3	Max 0.054		Langseth and Elen (1996)
	Barley	2.2–13.33	Max 0.77		
	Oats	7.2–62.05	Max 0.67		
Netherlands	Wheat	0.020–0.231	0.007–0.203	0.002–0.174	Tanaka et al. (1990)
	Barley	0.004–0.152	0.030–0.145	0.004–0.009	
	Oats	0.056–0.147	0.017–0.039	0.016–0.029	
	Rye	0.008–0.384	0.010–0.034	0.011	
South Africa	Maize	Up to 1.83	Up to 0.37		Rheeder et al. (1995)
South Africa	Cereals/animal feed			0.05–8.0	Dutton and Kinsey (1996)
India	*Paspalum palidosum*				Phillips et al. (1996)
	Straw			0.422	
	Mixed concentrate			0.843	

Philippines	Maize		0.018–0.102	0.059–0.505	Yamashita et al. (1995)
Thailand	Maize			0.923	Yamashita et al. (1995)
Korea	Barley	0.005–0.361	0.040–2.038		Ryu et al. (1996)
	Maize	Mean 0.145	Mean 0.168		
Vietnam	Maize powder	1.53–6.51	0.78–1.95		Wang et al. (1995a)
China	Maize	0.49–3.10	0.6		Wang et al. (1995b)
Japan	Wheat	0.03–1.28	0.04–1.22	0.002–0.025	Sugiura et al. (1993)
	Barley			0.010–0.658	
Japan	Wheat	0.029–11.7	0.01–4.4	0.053–0.51	Yoshizawa (1997)
	Barley	61–71	14–26	11–15	
New Zealand	Maize	Max 3.4–8.5	Max 4.4–7.0	Max 2.7–10.5	Lauren et al. (1996)
USA	Wheat	Up to 9.3			Fernandez et al. (1994)
USA	Wheat (winter), 1991	<0.1–4.9			Trucksess et al. (1995a)
	Wheat (spring), 1991	<0.1–0.9			
	Wheat, 1993	<0.5–18			
	Barley, 1993	<0.5–26			
Canada	Wheat and barley	up to 0.5		Up to 0.3	Stratton et al. (1993)
Canada	Wheat (hard)	0.01–10.5			Scott (1997)
	Wheat (soft, winter)	0.01–5.67			
	Wheat (soft, spring)	0.01–1.51			
	Maize	0.02–4.09			
Canada	Animal feeds	0.013–0.2	0.065–0.311		Abramson et al. (1997).
Argentina	Wheat	0.10–9.25			Pacin et al. (1997)
Brazil	Wheat	0.47–0.59	0.16–0.40	0.04–0.21	Furlong et al. (1995)

Table 2
Worldwide contamination of maize and animal feeds with fumonisins (μg/kg)

Country	FB_1	FB_2	FB_3	Total	References
Maize					
Benin	nd[a]-2630	nd-680		nd-3310	Doko et al. (1995)
Botswana	35–255	nd-75	nd-30	35–305	Doko et al. (1996)
Mozambique	240–295	75–110	25–50	340–395	Doko et al. (1996)
South Africa	60–70	nd	nd	60–70	Doko et al. (1996)
South Africa	max 2000				Dutton and Kinsey (1995)
Malawi	nd-115	nd-30	nd	nd-135	Doko et al. (1996)
Zambia	20–1420	nd-290		20–1710	Doko et al. (1995)
Zimbabwe	55–1910	nd-620	nd-205	55–2735	Doko et al. (1996)
Tanzania	nd-160	nd-60	nd	nd-225	Doko et al. (1996)
Honduras	68–6555				Julian et al. (1995)
Argentina	85–8791	nd-11 300	nd-3537	85-16 760	Chulze et al. (1996)
Uruguay	nd-3688				Pineiro et al. (1997)
Costa Rica	1700–4780				Viquez et al. (1996)
Italy	10–2330	nd-520		10–2850	Doko et al. (1995)
Portugal	90–3370	nd-1080		90–4450	Doko et al. (1995)
USA	nd-350				Trucksess et al. (1995b)
Vietnam	268–1516	155–401	101–268	524–2185	Wang et al. (1995a)
China	160–25 970	160–6770	110–4130	430–36 870	Wang et al. (1995b)
Philippines	57–1820	58–1210			Yamashita et al. (1995)
Thailand	63–18 800	50–1400			Yamashita et al. (1995)
Indonesia	226–1780	231–556			Yamashita et al. (1995)
Animal feed					
South Africa	4000–11 000				Dutton and Kinsey (1995)
Uruguay	256–6342				Pineiro et al. (1997)
India	20–260				Shetty and Bhat (1997)

[a] nd – not detectable.

combinations of NIV, DON, fumonisins and AFB_1 (Wang et al., 1995a). In Italy, pre-harvest maize ears were naturally contaminated with combinations of fusaproliferin, beauvericin and FB_1 (Ritieni et al., 1997).

4.1. Trichothecenes

Scott (1989) and Yoshizawa (1991) provided an exhaustive survey of the global contamination of cereal grains and animal feed with trichothecenes. Recent data confirms the widespread distribution of these mycotoxins particularly with respect to DON, NIV (Table 1). The levels of DON in Polish wheats (Perkowski et al., 1990) and Japanese barley (Yoshizawa, 1997) are worth noting. Of considerable significance are consistent reports of co-occurrence of type A and type B trichothecenes in cereal grains.

4.1.1. Europe

In one German study (Muller and Schwadorf, 1993), 79 of the 84 wheat samples analysed contained between two and six *Fusarium* mycotoxins, with 0.20 of samples co-

contaminated with DON and ZEN (Table 1). The most frequent combination included DON, 3-ADON and ZEN. T-2 and HT-2 toxins were detected at levels ranging from 0.003 to 0.250 and from 0.003 to 0.020 mg/kg, respectively, but these mycotoxins only occurred in combination with DON, NIV and ZEN.

The co-occurrence of 3-ADON with 15-ADON in Polish wheats has also been observed by Perkowski et al. (1990), although not cited by Yoshizawa (1991). A subsequent study in Poland showed four samples of wheat bran and three samples of wheat grain to be contaminated with trichothecenes (Czerwiecki and Giryn, 1994). One of the wheat bran samples contained DON, NIV, DAS and T-2 toxin as co-contaminants. However, individual levels were relatively low, at 0.05 mg/kg for DON and DAS and 0.1 mg/kg for NIV and T-2 toxin. In the Lublin region of southeastern Poland, type A trichothecene contamination of barley grain was linked with the natural incidence fusarium head blight in which the predominating organism was *F. sporotrichioides* (Perkowski et al., 1997). Of 24 barley grain samples, 12 were positive for T-2 toxin with a range of 0.02–2.4 mg/kg. In five of these samples co-contamination with HT-2 toxin occurred with a range of 0.01–0.37 mg/kg. Furthermore, two of these samples were co-contaminated with T-2 tetraol at 0.01 and 0.21 mg/kg. Maize ears may also become naturally infected with *Fusarium* pathogens. The findings of one study in Poland indicated that infection with *F. graminearum* can result in contamination of cobs simultaneously with DON and 15-ADON (Grabarkiewicz-Szczesna et al., 1996). Concentrations of DON and 15-ADON in *Fusarium*-damaged kernels ranged from 4 to 320 mg/kg and from 3 to 86 mg/kg, respectively, but the axial stems of the cobs were more heavily contaminated at 9–927 and 6–606 mg/kg, respectively (Table 1). 3-ADON was only detected in husks in the range 2–11 mg/kg, in combination with DON (1–236 mg/kg) and 15-ADON (5–218 mg/kg).

In Bulgaria, *F. graminearum* is a major pathogen of wheat and consequently grain can be expected to be contaminated with type B trichothecenes. Vrabcheva et al. (1996) recorded relatively low levels of DON (Table 1), but the incidence of contamination was 0.67 and was accompanied by co-occurrence of 3-ADON and 15-ADON each at levels of up to 0.1 mg/kg grain.

Representative samples of Finnish and imported cereals and feeds have been found to contain DON (Table 1) together with 3-ADON and ZEN (Hietaniemi and Kumpulainen, 1991). Levels of 3-ADON ranged from 0.01 to 0.6 mg/kg.

Oat grains produced in Norway by commercial growers were found to be more heavily contaminated with DON than barley or wheat kernels (Table 1) and this has been attributed to edaphic and agronomic factors and to different infection pathways by *Fusarium* pathogens (Langseth and Elen, 1996). In addition to NIV (Table 1), other contaminants included 3-ADON and fusarenon-X. For example, 0.56 of certain oat samples contained detectable quantities of 3-ADON at 0.03 mg/kg or more.

Furthermore, a study of cereals in The Netherlands (Tanaka et al., 1990) revealed the co-occurrence of DON, NIV and ZEN (Table 1). Of the 29 samples tested, 0.90 and 0.79 were positive for DON and NIV, respectively, with 0.76 containing both mycotoxins as co-contaminants.

Values for DON and other trichothecenes in Italian maize samples have been summarised by Scott (1989). In maize infected with stalk rot caused by a variety of

Fusarium species, DON levels ranging from 0.115 to 0.668 mg/kg have been found by Bottalico et al. (1989), who also commented on other surveys in northern Italy indicating high kernel contamination with DON following *Fusarium* infection.

4.1.2. South Africa

In Natal, South Africa, 417 samples comprising of maize, compound animal feeds, oilseeds and forage were examined for mycotoxins, of which 0.19 were contaminated with DON, NIV, DAS and an unknown trichothecene, but results were not quantified (Dutton and Kinsey, 1995). Subsequently Dutton and Kinsey (1996) found that 0.10 of animal feeds and cereals in Kwazulu Natal were contaminated with trichothecenes DON, NIV, DAS and T-2 toxin, but quantitative results were not presented. Earlier however, Rheeder et al. (1995) concluded that contamination of commercial maize with trichothecenes (Table 1) constituted little risk to animal health in South Africa.

4.1.3. Asia

NIV was found in 0.14 of maize samples in the Philippines (Yamashita et al., 1995) at lower levels than in other regions of Asia (Table 1). In that study, maize from Thailand and Indonesia were found to be free of trichothecenes but were contaminated with other *Fusarium* mycotoxins (Tables 1 and 2).

In Korea, Ryu et al. (1996) showed that both barley and maize harvested in 1992 were co-contaminated with DON and NIV (Table 1), with a higher incidence for NIV in barley (0.93) than in maize (0.53). However for DON, incidence of contamination was higher in maize (0.93) than in barley (0.67). In addition fusarenon-X occurred in 0.33 of maize samples within a range of 0.015–0.072 mg/kg. T-2 toxin and HT-2 toxin were below detection limits in both cereals.

The issue of co-contamination was taken a step further with a report by Wang et al. (1995a) who suggested that for the first time in North Vietnam some maize samples destined for animal feeds were found with DON, NIV, fumonisins (Tables 1 and 2) and AFB_1.

Two sets of data from Japan showed strikingly different values for trichothecene contamination of cereal grains (Table 1). The first group, based on the results of Sugiura et al. (1993) indicated relatively low levels of DON and NIV in wheat. However, a more recent set of data (Yoshizawa, 1997) indicated considerably higher values for these trichothecenes in both wheat and barley. In addition, an appreciable number of barley samples were found with 3-ADON levels of up to 18.7 mg/kg. In highly contaminated grains, a positive correlation occurred between levels of DON and its acetyl derivatives. DON levels were always higher than those of 3-ADON and 15-ADON, with ratios ranging from 2.9 to 155. Furthermore, regional differences were observed in that DON was the major contaminant in grain from northern districts of Japan, whereas in central districts NIV was the predominant trichothecene. These differences were correlated with chemotype variants of *Fusarium* species.

4.1.4. New Zealand

In New Zealand, high incidence of DON and NIV contamination of maize has been reported (Lauren et al., 1996). In 1992, all samples tested were positive for DON/NIV, this incidence declining to 0.97 in the 1994 harvest. Maximum levels of these

trichothecenes and ZEN are shown in Table 1. In 1992, 0.24 of samples had high levels of DON + NIV (in excess of 2 mg/kg) which corresponded with adverse weather conditions and the consequent delay in harvest. However, in 1994 0.60 of samples had less than 0.4 mg/kg and only 0.05 were found with DON + NIV levels in excess of 2 mg/kg. Lauren et al. (1996) suggested that pig performance would not be reduced by DON + NIV levels of less than 0.4 mg/kg, but maize grain contaminated with 2 mg/kg would exert a deleterious effect.

4.1.5. North America

Trichothecene contamination of grain and animal feeds in the United States of America and Canada is extensive and has been adequately reviewed by Scott (1989). The impetus for monitoring has continued particularly with respect to DON in wheat and barley (Table 1). In addition, regional variations in DON contamination of wheat have been observed (Fernandez et al., 1994). Highest levels in the 1991 harvest in the United States were seen in Missouri, North Dakota and Tennessee. Even higher levels of contamination of cereals with DON were seen in the 1993 harvest (Table 1; Trucksess et al., 1995a), with 0.86 of samples from Minnesota and up to 0.78 of samples from North and South Dakota containing levels in excess of 2 mg/kg.

In a study of trichothecene contamination of grain in Atlantic Canada (Table 1), DON was detected in 0.53–0.62 of samples tested. Of 55 samples, five contained T-2 toxin in the range 0.16–0.31 mg/kg, two were contaminated with HT-2 toxin at levels of 0.12 and 0.44 mg/kg, and DAS occurred in two samples, both at 0.11 mg/kg (Stratton et al., 1993). A comprehensive review of trichothecene levels in Canadian grain is now available (Scott, 1997) indicating higher values for DON in cereal grains (Table 1) than those reported by Stratton et al. (1993). In Ontario, DON incidence was consistently higher for maize than for soft wheat over a 15-year period (Scott, 1997). Of particular note is the lower levels of DON in soft spring wheats over this period (Table 1). It was concluded that DON was a frequent contaminant of Canadian grains. However, T-2 toxin and NIV were detected in these cereals at levels of 0.06–0.59 and 0.09–0.81 mg/kg, respectively.

Analysis of animal feeds in 94 cases of suspected mycotoxicosis of farm animals in western Canada, over the period 1982–1994, indicated relatively low levels of DON, NIV (Table 1) and several other trichothecenes (Abramson et al., 1997). However, in four of these cases feed refusal in pigs was linked with levels of DON ranging from 0.013 to 0.095 mg/kg feed.

4.1.6. South America

Argentinian sources of maize have been monitored for DON (Scott, 1989) but a recent analysis of 60 wheat samples indicated high incidence of contamination at over 0.93 (Pacin et al., 1997) with the levels shown in Table 1.

In Brazil, 13 out of 20 grain samples from experimental plots in wheat-producing areas of the State of Sao Paulo were free of mycotoxin contamination (Furlong et al., 1995). Relatively low levels of DON and NIV were found in positive samples (Table 1). In addition, T-2 toxin (0.04, 0.80 mg/kg) and DAS (0.60 mg/kg) occurred in three of these samples. However, the authors cautioned that their findings may not reflect the potential for contamination in other wheat-growing regions of Brazil.

4.2. Zearalenone

The predominant feature of ZEN distribution in cereal grains and animal feed is its co-occurrence with other *Fusarium* mycotoxins, including trichothecenes (Table 1). This observation is consistent with the confirmed production of ZEN by virtually all toxigenic and plant pathogenic species of *Fusarium* (D'Mello et al., 1997). The highest values for ZEN in Table 1 (11 and 15 mg/kg) relate to two barley samples from the Fukuoka region of Japan (Yoshizawa, 1997). Co-contamination of maize with ZEN, NIV, fumonisins and aflatoxins is an emerging issue in Philippines, Thailand and Indonesia (Yamashita et al., 1995) and elsewhere in the humid tropics. Consequently, although only four sets of data in Table 1 indicate serious ZEN contamination of grain and feeds, synergistic effects on animal health may arise at lower levels if ZEN occurs in combination with other mycotoxins.

An Indian source of *Paspalum palidosum* straw and mixed concentrate were reported to contain ZEN at 0.42 and 0.84 mg/kg (Phillips et al., 1996).

4.3. Fumonisins

Widespread contamination of maize and animal feed with fumonisins has recently been reported (Table 2; see also Shephard et al., 1996). In most instances the predominant fumonisin was FB_1. Highest values for FB_1 were recorded for maize samples in China (Wang et al., 1995b), where AFB_1 co-occurred in 0.85 of samples, and in Thailand (Yamashita et al., 1995). Multiple contamination of maize with fumonisins, DON, NIV and AFB_1 was also observed in north Vietnam (Wang et al., 1995a). For FB_2, highest values in maize were found in samples from Argentina (Chulze et al., 1996). In Philippines, Thailand and Indonesia, FB_1 and FB_2 occurred in over 0.50 of maize samples and, furthermore, these mycotoxins co-occurred with aflatoxins at an incidence of 0.48. In addition these fumonisins co-occurred with NIV and ZEN (Yamashita et al., 1995). Incidence rates of 0.82–1.0 were recorded for samples from Italy, Portugal, Zambia and Benin (Doko et al., 1995). These studies were extended to include maize samples from Botswana, Mozambique, South Africa, Malawi, Zimbabwe and Tanzania (Doko et al., 1996). The results of Doko et al. (1996) for maize (60–70 μg/kg) in South Africa may be compared with the much higher values of Dutton and Kinsey (1995) for maize (2000 μg/kg) and animal feed (4000–11000 μg/kg) in Natal (Table 2). In Honduras, Julian et al. (1995) detected FB_1 in all 24 samples maize tested (Table 2). Of additional concern is the recent demonstration of co-contamination of pre-harvest maize ears in Italy with FB_1, fusaproliferin and beauvericin, at 300, 500 and 520 mg/kg, respectively (Ritieni et al., 1997). The levels of FB_1 are well in excess of those shown in Table 2. In Argentina, fumonisins were detected during ear development and their occurrence was closely correlated with the natural infection with *Fusarium moniliforme* and *F. proliferatum* (Chulze et al., 1996). In Costa Rica, significant regional differences were observed in contamination of maize with FB_1 (Viquez et al., 1996). Some of the values cited in Table 2 are of toxicological significance since a level as low as 1000 μg/kg has been associated with disease syndromes in livestock (Shephard et al., 1996).

5. Control strategies

Although worldwide regulations exist to control aflatoxins in animal feed, similar legislation is not available for any of the *Fusarium* mycotoxins, including the carcinogenic fumonisins. However, in USA advisory directives exist for concentrations of DON in grains and by-products. A value of 10 mg/kg has been set for such feeds offered to cattle and chickens. For pigs the advisory level is set at 5 mg/kg (Trucksess et al., 1995a).

Once contamination has occurred in grains, a number of options are available for limiting adverse effects in livestock. Thus, DON levels have been reported to be reduced by as much as 75% during milling and other forms of physical treatment. Density segregation enables separation of mouldy grain and this technique may also result in reduced levels of trichothecenes and ZEN in the residual batch (Charmley and Prelusky, 1994). Chemical treatments involving calcium hydroxide monomethylamine, sodium bisulphite or ammonia have also been examined. However, although effective to different extents, these physical and chemical methods have yet to be applied in commercial situations.

Dilution of contaminated grain with other feed components is another option, providing that monitoring is performed before grains are incorporated into compound feeds. All too often, analyses are conducted after deleterious effects on livestock performance have been observed.

In view of the foregoing, it is axiomatic that preventive strategies are likely to be more effective than remedial measures in reducing the risk to animal health. When fungicides are used effectively to control fungal diseases of cereal plants, then the risk may be minimised. However, fungicide-resistance among *Fusarium* pathogens has been recorded. Recently, D'Mello et al. (1998) showed that development of fungicide-resistance was accompanied by a more persistent pattern of 3-ADON production in *F. culmorum*.

Selection of cultivars of cereal plants resistant to *Fusarium* pathogens is currently viewed as a viable and sustainable option for reducing mycotoxin contamination of grain (D'Mello and Macdonald, 1998). In experimental studies, exploitation of genetic resistance to fusarium head blight in wheat has been successfully used to restrict kernel contamination with DON. Similarly, selection of Chinese genotypes of wheat which are naturally resistant to this disease has also resulted in lower levels of DON in grain, compared with values for susceptible Canadian cultivars (Wong et al., 1995).

6. Conclusions

This review has provided updated confirmation of the global scale of contamination of cereal grains and animal feed with *Fusarium* mycotoxins. Despite the small sample size in several published surveys, an inescapable conclusion is that cereal grains and animal feeds may be subject to multiple contamination with *Fusarium* mycotoxins. It is clear that DON, NIV, ZEN and the fumonisins are of major concern in terms of their ubiquitous distribution and effects on animal health. It is also acknowledged that contamination is, to a significant degree, linked with specific cereal diseases caused by *Fusarium* pathogens.

High incidence rates of contamination have been observed worldwide. Thus in New Zealand, all maize samples tested in 1992 were positive for DON/NIV, while in Italy, Portugal, Zambia and Benin high proportions (0.82–1.0) of maize samples harvested in the period 1989–1992 were found to be contaminated with fumonisins. In Uruguayan maize-based animal feeds collected during 1995/1996, all 13 samples tested were positive for FB_1.

Unacceptably high values for DON have been recorded for maize kernels and cobs in Poland, associated with *F. graminearum* infection. Potentially harmful levels of DON were also reported for wheat in Germany, Poland, Japan, New Zealand, USA, Canada and Argentina and for barley in Norway, Japan and USA. Concentrations of NIV and ZEN in grains are generally low relative to those for DON. Nevertheless, above-average values for both mycotoxins have been observed in some Japanese barley samples and in maize produced in New Zealand. Of the fumonisins, FB_1 is a major contaminant of maize and animal feed in the tropics. Highest values were reported for cereal samples in China and Thailand and for animal feed in South Africa. However, in a study of Argentinian maize FB_2 was the predominant fumonisin, present at levels equivalent to those for FB_1 in animal feed.

Of particular concern is the co-occurrence of several *Fusarium* mycotoxins in the same sample of grain or animal feed, often in the presence of AFB_1. In North Vietnam, for example, some maize samples destined for animal feeds were found with DON, NIV, fumonisins and AFB_1 as co-contaminants. In Italy, pre-harvest maize ears were naturally contaminated with combinations of fusaproliferin, beauvericin and FB_1.

Introduction of legislation for the control of certain trichothecenes and fumonisins in cereals and animal feed is now overdue. However, efforts to define tolerance limits and to formulate regulatory guidelines are likely to be thwarted by multiple contamination of these commodities with different *Fusarium* mycotoxins and AFB_1. In the meantime, selection of cereal genotypes resistant to *Fusarium* diseases is clearly an option which deserves sustained commitment in the future if the control of mycotoxin contamination of grain and feeds is to succeed. Such a strategy would, in addition, be consistent with the quest for an environmentally acceptable alternative to control by fungicides.

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8.1.3 Paper 3 "Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity

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Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity

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Abstract

Trichothecenes, zearalenone (ZEN) and fumonisins are the major *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains, animal feeds and forages. Other important *Fusarium* mycotoxins include moniliformin and fusaric acid. Spontaneous outbreaks of *Fusarium* mycotoxicoses have been recorded in Europe, Asia, New Zealand and South America and, in addition, chronic exposure occurs on a regular and more widespread scale. The metabolism and adverse effects of the *Fusarium* mycotoxins are considered in this review with particular reference to recent data on specific and proposed syndromes and to interactions among co-occurring mycotoxins. Within the trichothecene group, deoxynivalenol (DON) is associated with emesis, feed refusal and depressed feed intake in pigs, while T-2 toxin and diacetoxyscirpenol (DAS) are now clearly linked with oral lesions in poultry. The gut microflora of farm livestock are able to transform DON to a de-epoxy derivative. In contrast, the ovine metabolism of ZEN results in the production of five metabolites and relatively high levels of these forms may be excreted in the urine as glucuronides. There is now undisputed evidence that ZEN and its metabolites possess estrogenic activity in pigs, cattle and sheep, but T-2 toxin has also been implicated in reproductive disorders in farm livestock. Fumonisins are positively linked with pulmonary edema in pigs, leukoencephalomalacia in equines and with deranged sphingolipid metabolism in these animals. *Fusarium* mycotoxins have also been provisionally implicated in ovine ill-thrift, acute mortality of poultry and in duodenitis/proximal jejunitis of horses. Several *Fusarium* mycotoxins may co-occur in a particular feed ingredient or in compound feedingstuffs. In general, combinations of *Fusarium*

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Abbreviations: DON, deoxynivalenol; 3-ADON, 3-acetyl DON; NIV, nivalenol; DAS, diacetoxyscirpenol; ZEN, zearalenone; FB_1, FB_2, FB_3, fumonisins B_1, B_2, B_3; MON, moniliformin; OA, ochratoxin A; LD_{50}, median lethal dose; LH, luteinizing hormone; Sa, sphinganine; So, sphingosine; ELEM, equine leukoencephalomalacia; DPJ, duodenitis/proximal jejunitis; PPE, porcine pulmonary edema

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mycotoxins result in additive effects, but synergistic and/or potentiating interactions have been observed and are of greater concern in livestock health and productivity. Synergistic effects have been reported between DON and fusaric acid; DON and fumonisin B_1 (FB_1); and DAS and the *Aspergillus*-derived aflatoxins. Limited evidence of potentiation between FB_1 and DON or T-2 toxin has also emerged recently. Additive and synergistic effects between known and unidentified mycotoxins may account for enhanced adverse effects observed on feeding *Fusarium*-contaminated diets. The potential for transmission of DON into eggs and of ZEN into porcine kidney and liver has been demonstrated. However, lactational carry-over of FB_1 appears not to occur, at least in cows and sows. It is concluded that livestock health, welfare and productivity may be severely compromised by consumption of DON, T-2 toxin, DAS, ZEN and fumonisins and by interactions among these mycotoxins. Safety of some animal products may also be at risk. Furthermore, in view of the limited options available for remediation, it is concluded that exploitation of crops resistant to *Fusarium* infection offers the most viable strategy for reducing mycotoxin contamination of grain and animal feed.

Keywords: *Fusarium* sp.; Trichothecenes; Zearalenone; Fumonisins; Livestock; Metabolism; Syndromes; Interactions; Residues; Decontamination; Amelioration

1. Introduction

There is now compelling evidence implicating the *Fusarium* mycotoxins in livestock disorders in different parts of the world. Furthermore, the risk of continuing exposure has not diminished in spite of enhanced awareness of the debilitating effects of these mycotoxins. Thus, Placinta et al. (1999) concluded that on a global scale, cereal grains and animal feed may be subject to multiple contamination with trichothecenes, zearalenone and fumonisins, the major mycotoxins of *Fusarium* fungi. Contamination of feed grains is inevitable since many toxigenic species of *Fusarium* are also common phytopathogens, producing cereal crop diseases which are difficult to control. The unexpected lack of efficacy of pesticides in general and fungicides in particular to control cereal diseases and consequent mycotoxin contamination of grain has been the focus of a major review (D'Mello et al., 1998). Among the trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) co-occur regularly throughout the world, with unacceptably high values in cereal grains in Poland, Germany, Japan, New Zealand, and the Americas (Placinta et al., 1999). Other co-occurring trichothecenes in grain and feeds included 3-acetyl DON (3-ADON), diacetoxyscirpenol (DAS), T-2 toxin and HT-2 toxin. Concentrations of zearalenone (ZEN) are generally low, but above-average values have been reported for cereal grains or animal feed in Japan, New Zealand and South Africa. Fumonisin B_1 (FB_1) is a major contaminant of maize and animal feed in many tropical countries including China, Thailand and South Africa, whereas in Argentinian maize FB_2 was the predominant form of the mycotoxin in one study.

The co-occurrence of individual trichothecenes in cereal grains and animal feed is serious enough in that it is not possible yet to quantify the extent of any resulting interactions on animal health and performance. However, of greater potential significance are widespread reports of co-contamination of grain and feeds with trichothecenes, fumonisins and the *Aspergillus*-derived aflatoxins (Placinta et al., 1999).

It is clear that chronic intake of *Fusarium* and indeed other mycotoxins by farm livestock is inevitable and there are numerous cases of suspected mycotoxicoses on a worldwide basis. In addition, specific conditions have been positively identified and there is now sufficient data to propose further syndromes arising from other *Fusarium* mycotoxins such as fusaric acid and moniliformin (MON). Both aspects are reviewed in this paper to reflect the increased impetus currently being accorded to the toxicity of a wide range of these mycotoxins. Furthermore, new data have been published on the metabolism of certain *Fusarium* mycotoxins and it is opportune that these aspects are also considered in this paper. Although reviews are available (Diekman and Green, 1992; Etienne and Dourmad, 1994; Prelusky et al., 1994a), current evidence questions the validity of some of the original conclusions. New aspects of the aetiology of specific syndromes and the nature of interactions among *Fusarium* mycotoxins have also recently emerged. In addition, the toxicological implications of co-occurrence of *Fusarium* mycotoxins with those from *Aspergillus* fungi deserve attention to enable a proper assessment of risk in animal production. Placinta et al. (1999) examined the evidence of global contamination of grain and animal feed with *Fusarium* mycotoxins but the toxicological aspects were alluded to in a perfunctory manner without critical appraisal of published data. Furthermore, the question of synergism among co-occuring mycotoxins remained largely unexplored. Similarly, D'Mello et al. (1997) presented a superficial account of the toxicology of *Fusarium* mycotoxins focusing on structural diversity and biological activity and on interactions involving fusaric acid. The general reviews of Flannigan (1991) and of D'Mello and Macdonald (1997, 1998) covered wide-ranging aspects including factors affecting mycotoxin production, their role as disease elicitors in plants and risk assessment in human health. Specific updated aspects concerning the effects of *Fusarium* mycotoxins in farm animals were not considered in the aforementioned papers in the detail justified by recent developments. There is thus a compelling case for a critical and comprehensive review of *Fusarium* mycotoxicology that embodies important issues such as metabolism, syndromes, interactions, residues, tolerance limits and amelioration.

2. Metabolism

Metabolism of mycotoxins by animals may affect the manifestation of adverse effects. There may also be additional implications for carcass and milk quality if extensive transformation occurs within the digestive tract or within the tissues of animals.

The microbial transformation of DON in the gut of animals is well documented. A recent investigation attempted to elucidate the site at which this activity was greatest in pigs and the nature and relative toxicity of the metabolites formed (Kollarczik et al., 1994). Using an in vitro system it was determined that, as might be expected, microbial transformation of DON was highest in the caecum, colon and rectum. The only metabolite detected was de-epoxy-DON, a product also formed in the hind gut of other animals including cattle. Furthermore, it was shown that this transformation of DON was accompanied by a significant loss of cytotoxicity to pig kidney cells (Kollarczik et al., 1994).

The ovine metabolism of ZEN has been proposed to include the synthesis of at least five metabolites including zearalanone, α-zearalenol, β-zearalenol, α-zearalanol and β-zearalanol (Miles et al., 1996). High levels of some of these forms may be excreted in the urine as glucuronides by grazing sheep. Passage through the rumen is not necessary to promote these transformations. It should be noted that α-zearalanol has been marketed as a growth promoter with the name zeranol. Its use has been banned in the European Union (EU), but monitoring is continuing in countries where zeranol is permitted in order to comply with requirements for carcasses imported into the EU. However, complications have arisen due to the occurrence of zeranol in the urine of untreated pasture-fed ruminants and equines. The studies of Miles et al. (1996) indicate that urinary zeranol may arise in such animals as a result of metabolism of ZEN and related compounds present naturally in pastures infected with *Fusarium* species.

The adverse effects of ZEN will be partly determined by the processes of elimination. In pigs, as in sheep, ZEN is conjugated with glucuronic acid and in addition may be metabolised to α-zearalenol. However, the studies of Biehl et al. (1993) with sexually immature pigs indicate that biliary excretion and enterohepatic cycling are important processes affecting the fate of ZEN. It was suggested that the glucuronide of ZEN was substantially excreted in bile to be re-absorbed and metabolised further by intestinal mucosal cells, ultimately entering the liver and the systemic circulation via the portal blood supply. It was proposed that this entero-hepatic cycling has the effect of prolonging the retention of ZEN and its derivatives in the circulatory system, retarding elimination and enhancing the duration of adverse effects. It was also suggested that the reduction of ZEN to α-zearalenol occurred most actively in the intestinal mucosa (Biehl et al., 1993).

3. Toxicology and syndromes

In common with other physiologically active compounds, the *Fusarium* mycotoxins are capable of inducing both acute and chronic effects. The effects observed are often related to dose levels and duration of exposure.

Although acute and chronic effects in farm livestock are readily demonstrated under experimental conditions, similar manifestations have been reported in natural outbreaks of *Fusarium* mycotoxicoses in Europe, Asia, New Zealand and South America (Fazekas and Bajmocy, 1996; Prathapkumar et al., 1997; Kramer et al., 1997; Galhardo et al., 1997). Chronic exposure of farm animals to DON is a continuing hazard in Canada, the USA and continental Europe. In Japan, several cases of mycotoxicoses in animals have been attributed to consumption of cereals contaminated with DON and NIV (Yoshizawa, 1991). A number of specific syndromes in farm livestock have now been positively linked with exposure to certain trichothecenes, ZEN, and fumonisins. These include feed refusal, emesis and anorexia; oral and gastro-intestinal lesions; ill-thrift; reproductive dysfunction; equine leukoencephalomalacia; and porcine pulmonary edema. In addition, Duodenitis/proximal jejunitis and acute mortality syndrome have tentatively been linked with particular *Fusarium* mycotoxins.

Table 1
Adverse effects of individual trichothecenes in pigs

Trichothecene	Effects	Data source
DON	Emesis and feed refusal	Review by Diekman and Green (1992)
DON	Decreased feed intake, growth and feed efficiency	Bergsjo et al. (1992)
DON	Reduced feed intake and growth; stomach lesions	Friend et al. (1992)
DON	Depressed feed intake, growth, feed efficiency and carcass weight; reduced blood levels of total protein, albumin, Ca and P	Bergsjo et al. (1993)
DON	Reduced feed intake and growth; recovery in pigs fed pure DON	Prelusky et al. (1994b)
DON	Immediate reductions in feed intake and growth followed by partial dose-dependent recovery	Trenholm et al. (1994)
DON	Reduced feed intake and growth; increased corrugation of mucosa in stomach; transient decrease in serum protein levels	Rotter et al. (1995)
DON	Renal lesions	Harvey et al. (1996)
DON	Dose-dependent reduction in secondary antibody response to tetanus toxoid	Overnes et al. (1997)
NIV	Profound reduction in feed intake at high doses; diets also contaminated with ZEN	Williams and Blaney (1994)
NIV	Increased time to consume feed	Hedman et al. (1997)
T-2 toxin	Reduced feed intake	Friend et al. (1992)
T-2 toxin	Dermatitis of snout, nose and buccal commissures; feed refusal; depressed feed intake, growth, and blood glucose levels; increased blood levels of inorganic P and Mg	Rafai et al. (1995a)
T-2 toxin	Decreased red blood cell count, corpuscular volume and hemoglobin concentration; reduced leucocyte count, proportion of T lymphocytes, antibody formation and blastogenic transformation of lymphocytes	Rafai et al. (1995b)

3.1. Trichothecenes

In acute tests with trichothecenes, type A members such as DAS and T-2 toxin have been found to be more toxic than type B components such as DON and NIV (Leeson et al., 1995). However, the effects and syndromes arising from chronic intake of these mycotoxins are likely to be more important in practical situations (Tables 1 and 2; Fig. 1). In addition, a feature of at least two trichothecenes (DON and T-2 toxin) is their ability to impair immunocompetence, a property which may well be associated with other mycotoxins in this group (Kubena et al., 1997a; Vanyi et al., 1994b). Furthermore, in sows, T-2 toxin may cause infertility, and after parenteral administration during the last trimester of gestation, is able to precipitate abortion within 48 h (Weaver et al., 1986). Its role as an endocrine disrupter is now beginning to emerge (Table 2).

3.1.1. Feed refusal, emesis and anorexia

Earlier studies with pigs indicated that DON was a potent feed intake inhibitor and emetic factor. These effects were appropriately represented by the alternative term for this mycotoxin, namely 'vomitoxin'. However, recent studies (Table 1) have only confirmed the anorectic effects. In quantitative terms marked effects of DON on feed intake

Table 2
Adverse effects of individual trichothecenes in non-porcine livestock

Trichothecene	Animal species/type	Effects	Data source
DON	Broiler chickens	Increased relative weights of gizzard, bursa of Fabricus and heart	Kubena et al. (1997a)
DON	Laying hens	Transmission to eggs following oral administration	Prelusky et al. (1987)
DON	Mink	Preference for non-contaminated feed	Gibson et al. (1993)
3-ADON	Lambs	Depressed feed intake	Brewer et al. (1996)
NIV	Broiler chickens	Reduced feed consumption and weight gain; gizzard erosions; reduced relative liver weight	Hedman et al. (1995)
T-2 toxin	Virgin female rabbits	Impaired ovarian function; low plasma progesterone levels	Fekete et al. (1992)
T-2 toxin	Turkey poults	Reduced bodyweight gain; oral lesions	Kubena et al. (1995)
T-2 toxin	Broiler chickens	Reduced bodyweight gain; oral lesions	Kubena et al. (1997a)
T-2 toxin	Ducklings	Reduced bodyweight and weights of thymus, spleen and bursa of Fabricii; oral and oesophageal ulcerations	Neiger et al. (1994)
T-2 toxin	Geese	Decreased egg yield and hatchability; mortality	Vanyi et al. (1994a)
T-2 toxin	Geese	Dose-dependent cessation of follicle maturation in ovaries; follicle degeneration; involution of oviduct; lymphocyte depletion; lesions in adrenal and thyroid glands	Vanyi et al. (1994b)
DAS	Broiler chickens	Reduced bodyweight; dose-related mouth lesions	Ademoyero and Hamilton (1991)
DAS	Turkey poults	Reduced feed intake, weight gain and feed efficiency; oral lesions	Kubena et al. (1997b)
DAS	Cattle	Anorexia; gastro-intestinal lesions; diarrhoea; reduced milk production	Galhardo et al. (1997)

inhibition have been observed particularly in the range 6–15 mg/kg diet (Fig. 1). At a level of 15 mg/kg, feed intake was only 0.38 of control values. Even at the higher levels of DON, however, emesis was not observed (Trenholm et al., 1994). A particular feature of the appetite depression is that although the effect can be immediate, varying degrees of recovery over time have been reported, without withdrawal of DON from the feed. Thus, Trenholm et al. (1994) noted partial, dose-dependent, adaptation to DON-contaminated diets, the effects being reflected in proportionate reductions in weight gain. On the other hand, Prelusky et al. (1994b) observed feed intake depression for the initial two days of feeding the contaminated diet followed by sufficient compensation thereafter to permit feed intakes and growth rates equivalent to those in control pigs. Despite these differing responses a distinct dose-related effect of dietary DON on feed intake in pigs is still evident (Fig. 1) even in the long-term (Trenholm et al., 1994). Pair-feeding studies with pigs indicate that at the lower dose ranges, the effects of DON on growth may be explained entirely by the effects on voluntary feed intake (Rotter et al., 1995). However, Prelusky (1997) concluded that at relatively high dietary concentrations of DON (above 9 mg/kg), the deleterious effects on weight gain may not be fully explained by the

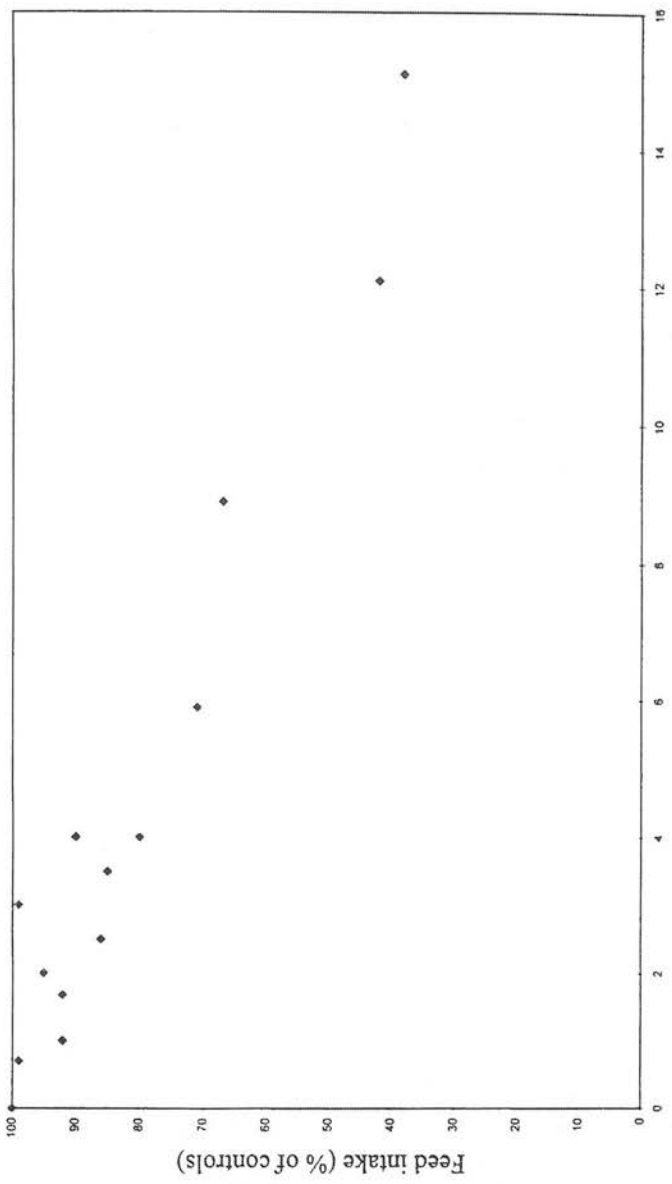

Fig. 1. Effects of dietary deoxynivalenol (DON) levels on voluntary feed intake in pigs. Data selected from Bergsjo et al. (1992), Friend et al. (1992), Bergsjo et al. (1993), Prelusky et al. (1994b), Trenholm et al. (1994), and Rotter et al. (1995).

suppression in feed intake. Under these conditions it is possible that defects in immunocompetence (Table 1) may contribute to the growth depression, perhaps resulting from the incidence of overt or sub-clinical disease. It should be noted that most of the recent studies on the effects of DON on feed intake in pigs (Table 1; Fig. 1) have been conducted with *Fusarium*-contaminated grain and the effects tend to be greater with such diets than with those supplemented with the pure form of the mycotoxin. In at least two instances (Bergsjo et al., 1992, 1993) limited quantities of other trichothecenes such as 3-ADON and NIV were also present together with ZEN. It is may be significant that other trichothecenes including NIV and T-2 toxin have been implicated in both feed refusal and anorexia in pigs (Table 1). Thus, Rafai et al. (1995a) observed dose-related depressions in feed intake within one week of feeding diets contaminated with T-2 toxin. By the end of the 3-week study pigs fed T-2 toxin at 3 mg/kg had feed intakes which were only 0.59 of control values. Furthermore, fusaric acid can enhance brain metabolism in pigs and a potential interaction with co-occurring DON has been proposed in feed refusal and emesis (D'Mello et al., 1997). Thus, additive or even synergistic effects on feed intake cannot be ruled out in studies based on the feeding of *Fusarium*-contaminated grain to pigs. It now also appears that NIV and DAS reduce feed intake in broiler chickens (Table 2) whereas DON has no effect on feed intake or growth (Kubena et al., 1997a). In mink, feed choice is affected by levels of DON as low as 0.28 mg/kg diet (Gibson et al., 1993). Dairy cows are considerably more tolerant to DON, as exemplified by the lack of effect on feed intake and milk production (Charmley et al., 1993; Ingalls, 1996). However, in Brazil poisoning of cattle fed citrus pulp has been attributed to contamination with DAS which has the potential to cause characteristic clinical symptoms including anorexia, weight loss, haemorrhagic lesions in vital organs and even death (Galhardo et al., 1997).

3.1.2. Oral and other gastro-intestinal lesions

Nivalenol, T-2 toxin and DAS induce gizzard erosions and oral lesions in poultry (Table 2). In the case of DAS, lesions are directly related to duration of exposure to the mycotoxin and to its concentration in the diet (D'Mello and Macdonald, 1998). Feeding a high fat diet to broiler chicks increases the growth depression caused by DAS, suggesting that such a diet facilitates lipid micellar absorption of the mycotoxin which is then able to inhibit protein synthesis at the ribosomal level. T-2 toxin also induces lesions in pigs, specifically on the mucosa of the pars oesophageal region, the incidence being dose-related. In addition, T-2 toxin can cause dermatitis of the snout, nose and buccal commissures in the pig (Table 1).

3.1.3. Ill-thrift

Although this condition is not universally recognised or adequately characterised, there is evidence for a distinct if ill-defined syndrome. Ovine ill-thrift occurs despite the presence of abundant and nutritious feeds and has been ascribed to a mixture of toxins present in pasture, some arising from fungal sources. However, as part of a study on ovine ill-thrift in Nova Scotia, Brewer et al. (1996) demonstrated a 44% decline in feed intake of female lambs in the 4 days following a single intraruminal administration of 3-ADON. This was accompanied by a 5% depression in apparent digestibility of the feed. Despite the clarity of these responses, the exact aetiology of ill-thrift remains nebulous,

particularly in view of the occurrence of *F. solani* in soils of Nova Scotia. *F. solani* produces neosolaniol, DAS and T-2 toxin as the principal trichothecenes and judging by the effects observed in other animals (Tables 1 and 2) it is possible that ill-thrift in sheep may be the result of combinations of mycotoxins exerting additive or synergistic effects on feed intake.

3.2. Zearalenone

It is acknowledged that ZEN is of relatively low toxicity, with an LD_{50} value of 2–10 g/kg body weight as determined with mice (Flannigan, 1991). However, its role as a mammalian endocrine disrupter is being recognized, with effects in both males and females of different species, although evidence of its genotoxicity has recently emerged from studies with mice (Pfohl-Leszkowicz et al., 1995).

3.2.1. Reproductive dysfunction

Chronic investigations (Table 3) demonstrate that the estrogenic properties of ZEN towards mammals are an important feature at levels as low as 1.5–3 mg/kg diet. Thus, ZEN induces vulvovaginitis in premature gilts, anestrus in cycling females or delayed return into estrus post-weaning. During pregnancy, ZEN reduces embryonic survival when administered above a threshold level and sometimes decreases fetal weight. ZEN may affect the uterus by decreasing LH and progesterone secretion and by altering the morphology of uterine tissues (Etienne and Dourmad, 1994). In male pigs, ZEN can depress serum testosterone, weights of testes and spermatogenesis, while inducing feminisation and suppressing libido. In cows, infertility, reduced milk production and hyperestrogenism have been associated with ZEN or with *Fusarium* species producing this mycotoxin. When dairy heifers were fed ZEN over three estrous cycles, conception rates declined from 87 to 62% (Weaver et al., 1986). Additionally, ZEN from pastures in New Zealand has been implicated in the development of infertility in cattle and sheep (Towers and Sprosen, 1993). Possible additive or synergistic effects on fertility with T-2 toxin are possible since both often co-occur in *Fusarium*-contaminated feeds. Indeed, Hungarian studies suggest that under certain dietary conditions, ZEN and/or T-2 toxin may cause ovarian dysfunction in cows (Trucksess, 1997).

Fusarium fungi are capable of producing *trans* and *cis* forms of ZEN, α-zearalenol and β-zearalenol on grain and all may contribute to estrogenic effects in mammals to varying extents. It is established that *trans*-α-zearalenol is 3 to 4 times more estrogenic than ZEN (Richardson et al., 1985). The contribution of the different isomers in natural occurrences of reproductive disorders of cattle and sheep, as in New Zealand, needs to be assessed.

3.3. Fumonisins

The adverse effects of fumonisins has emerged as a predominant issue in mycotoxicology and is likely to remain so for some time. There is overwhelming evidence of diverse morphological, cellular and biochemical damage in farm animals fed fumonisin-contaminated diets. Profound effects in terms of lesions in the liver, gastrointestinal tract, brain and lungs have been reported in pigs poultry, calves and

Table 3
Reproductive and other disorders induced by zearalenone

Animal type	Conditions	Effects	Data source
Sows	Natural contamination or addition of pure ZEN	Vulvovaginitis, anestrus; delayed return to estrus post-weaning; reduced embryonic survival; decreased luteinising hormone and progesterone secretion	Review by Etienne and Dourmad (1994)
Sows, gilts and piglets	*Fusarium*-contaminated feed	Reduced conception rates, litter size; stillbirths; enlargement of ovaries and uterus; swelling of vulva in piglets	Vanyi et al. (1994c)
Piglets	Piglets suckling sows fed contaminated grain	Edematous swelling and reddening of vulva; necrosis of tail; congenital lesions of the external genitalia	Dacasto et al. (1995)
Boars	Natural contamination or addition of pure ZEN	Depression of serum testosterone, testes weight and spermatogenesis; feminisation and suppression of libido	Review by Diekman and Green (1992)
Cows	Pure ZEN	Infertility; reduced milk production; hyperestrogensim; depressed conception rates	Weaver et al. (1986)
Cattle and sheep	Pastures in New Zealand	Infertility; increased number of barren ewes and fewer ewes with twins	Towers and Sprosen (1993)
Mink	Pure ZEN	Severe endometrial hyperplasia of uterus; uterine atrophy; endometritis; degeneration and atrophy of ovarian follicles; reduced reproductive performance	Yamini et al. (1997)
Rabbits	Pure ZEN	Reduced bodyweight; histopathological changes in liver, kidneys and uterus; females highly susceptible	Abdelhamid et al. (1992)
Rats	Pure ZEN	Reduced serum testosterone levels and sperm counts	Kaliamurthy et al. (1997)
Mice	Pure ZEN	Persistent estrous; sterility	Ito and Ohtsubo (1994)
Mice	Pure ZEN	Genotoxic; induction of hepatocellular adenomas	Pfohl-Leszkowicz et al. (1995)

Table 4
Adverse effects of fumonisins in farm livestock

Animal species/type	Effects	Data source
Weanling pigs	Nodular hyperplasia of liver; oesophageal lesions; gastric ulceration	Casteel et al. (1993)
Weaned piglets	Dose-dependent induction of pulmonary edema	Zomborszky et al. (1997a)
Weaned piglets	Pulmonary edema; death	Fazekas et al. (1998)
Growing pigs	Reduced weight gain; erratic growth and feed intake patterns; increased serum cholesterol; increased ratios of sphinganine to sphingosine in liver, lung and kidney	Rotter et al. (1996)
Growing pigs	Increased liver and lung weights	Harvey et al. (1996)
Growing–finishing pigs	Increased variability in feed intake and carcass fat content; elevated serum cholesterol	Rotter et al. (1997)
Growing pigs	Reduced pulmonary clearance; predisposition to disease	Smith et al. (1996a)
Growing pigs	Pulmonary hypertension	Smith et al. (1996b)
Chicken embryos	Dose- and time-related mortality (up to 100%); hydrocephalus, enlarged beaks and elongated necks in embryos; pathological lesions in internal organs	Javed et al. (1993a)
Chicken embryos	Sub-cutaneous and hepatic haemorrhages; disruption of sphingoid metabolism	Zacharias et al. (1996)
Broiler chickens	Dose-related clinical manifestations; reductions in weight gain and increase in mortality	Javed et al. (1993b)
Laying hens	Diarrhoea	Prathapkumar et al. (1997)
Calves	Increases in serum enzyme levels; mild microscopic hepatic lesions; impaired lymphocyte blastogenesis	Osweiler et al. (1993)
Horses	Equine leukoencephalomalacia: liquefactive necrosis of cerebral white matter; severity of lesions related to duration of exposure	Schumacher et al. (1995)
Horses	Equine leukoencephalomalacia: reduced appetite, ataxia, edema of lungs and brain, death	Fazekas and Bajmocy (1996)

equine animals (Table 4). There is also evidence of an immunosuppressive dimension in its toxicity to farm animals. For example, Smith et al. (1996a) concluded that, even at sub-lethal doses for the pig, fumonisins can inhibit the action of pulmonary intravascular macrophages in the removal of particulate matter and pathogens from the circulation. Animals may therefore become more susceptible to disease. Dose- and time-related mortality is another feature of fumonisin toxicology (Javed et al., 1993a, b; Fazekas and Bojmocy, 1996). In addition, it is becoming increasingly apparent that a part of the action of the fumonisins hinges on structural analogy. FB_1 bears a remarkable resemblance to sphinganine and sphingosine, intermediates in the biosynthesis and degradation of sphingolipids (D'Mello et al., 1997). Indeed, it has been demonstrated in vitro and in situ that FB_1 blocks sphingolipid biosynthesis by specifically inhibiting sphingosine (sphinganine) N-acyltransferase. A consequence of this inhibition is the accumulation of sphingoid bases in the sera of ponies, pigs and rats fed contaminated corn or culture material from *F. moniliforme* containing known levels of FB_1. Indeed, tissue and serum sphinganine (Sa) to sphingosine (So) ratios can be used as markers to indicate exposure of animals to fumonisins. Over recent years a number of fumonisin-linked syndromes have been established or proposed.

3.3.1. Equine leukoencephalomalacia (ELEM)

Feeding corn contaminated with *F. moniliforme* or culture material derived from this fungus may result in the induction of equine leukoencephalomalacia (ELEM). Cases of ELEM have been confirmed in Hungary, Brazil, South Africa and the USA (Marasas, 1995) and have been attributed specifically to the activity of fumonisins. ELEM is an acutely fatal neurological disorder of horses and donkeys with clinical signs such as ataxia, paresis, hypersensitivity and locomotor derangements. Lesions in the brain comprise liquefactive necrosis in one or both hemispheres. Oedema of both brain and lungs may also occur (Table 4). Serum Sa : So ratios are also markedly increased by feeding culture material of *F. moniliforme* to horses (Goel et al., 1996).

3.3.2. Duodenitis/proximal jejunitis (DPJ)

Duodenitis/proximal jejunitis (DPJ) is a condition characterised by copious, often haemorrhagic gastric reflux, affecting horses over two years of age (Schumacher et al., 1995). Lesions include mucosal and sub-mucosal edema in mild cases and sloughing of villus epithelium, villus atrophy and haemorrhage in severe cases. The cause of DPJ remains to be elucidated. Although Schumacher et al. (1995) were unsuccessful in reproducing the clinical signs of DPJ in horses fed *F. moniliforme* culture material, they were unable to definitively exclude toxins from this fungus in the aetiology of the syndrome.

3.3.3. Porcine pulmonary edema (PPE)

The porcine manifestation of fumonisin toxicity is characterised by pulmonary edema (PPE) (Table 4) as well as pancreatic and liver damage, with cases confirmed in Hungary, Brazil and the USA. A dose-dependent relationship has been observed (Zomborszky et al., 1997a). Elevated serum cholesterol levels appears to be consistent feature of the condition and hepatic enzymes may also be enhanced. Pulmonary hypertension caused by hypoxic vasoconstriction has also been implicated in PPE. Pigs fed fumonisins showed enhanced pulmonary artery pressure, decreased heart rate, cardiac output and mixed venous O_2 tension (Smith et al., 1996b).

In studies on the chronic toxicity of FB_1, weanling pigs fed a diet containing the mycotoxin at 100 mg/kg for 7 days followed by a diet containing 190 mg/kg for 83 days, developed nodular hyperplasia of the liver (Casteel et al., 1993). These nodules, of various diameters, were composed of solid sheets or nests of hepatocytes. In other pigs, the formation of papillary downgrowths of the stratum basale of the distal oesophageal mucosa were observed. Although incidence of PPE has not been reported in other studies (Rotter et al., 1996), increased Sa : So ratios have been observed with fumonisin levels of 10 mg/kg diet. At lower fumonisin levels, erratic feed intake and growth patterns and increased carcass fat content may also occur (Table 4).

3.3.4. Acute mortality syndrome

Field observations have implicated fumonisin-containing maize in an acute mortality syndrome of broiler chickens. The condition is characterised by markedly increased mortality in chicks between 10 and 16 days of age. Javed et al. (1993a,b) observed dose-related increases in mortality of chicken embryos and of broiler chicks following dietary

administration of pure FB_1. MON also enhanced mortality in broiler chicks but the onset was accelerated after feeding the two mycotoxins together. Since maize may be contaminated with both FB_1 and MON, it is possible that the syndrome represents the additive effect of the two mycotoxins. Ledoux et al. (1995) also observed enhanced mortality in broiler chicks following dietary administration of MON but questioned whether it was by itself involved in the syndrome as it occurs under commercial conditions. In contrast, Vesonder and Wu (1998) implicated MON and not FB_1 in acute death of ducklings. The evidence was derived from feeding studies involving *Fusarium* culture materials and pure mycotoxins. An assessment of the effects of MON on mortality in poultry may be compounded by species differences. Vesonder and Wu (1998) noted that ducklings were more sensitive to the lethal effects of MON than turkey poults which in turn were more sensitive than chicks.

3.4. Moniliformin

In addition to inducing mortality and to its putative interaction with fumonisins, MON is endowed with cardiotoxic effects in a wide range of laboratory and domesticated animals including rats, chickens, turkeys and ducks. Primary lesions include myocardial degeneration and necrosis in all these species. Ledoux et al. (1995) reported that just 50 mg/kg diet enhanced heart weights in broiler chickens. Gross lesions included generalised cardiomegaly with dilation of the right ventricle. Histopathological changes included a high incidence of variable-sized cardiomyocyte nuclei, with numerous large round and oval nuclei.

3.5. Embryotoxicity and teratogenic effects

The chick embryo is regularly used in mycotoxicology as a rapid and cost-effective assay model and the prospects are that interest will continue as more emphasis is placed on the elucidation of interactions among co-occurring mycotoxins. However, its limitations must be recognised. For example, the chick embryo cannot excrete xenobiotics such as mycotoxins and metabolic transformations are largely restricted. Nevertheless, recent results are consistent with those observed in conventional experiments. Thus, Javed et al. (1993a) observed embryo mortality on inoculating fertile chicken eggs with FB_1, effects which were dependent upon duration of exposure and dose level and which were replicated in trials with broiler chicks. In addition, however, evidence of embryonic deformities was presented (Table 4). Zacharias et al. (1996) observed dysfunction of sphingoid metabolism in chick embryos exposed to FB_1, as in pigs and horses, and furthermore correlated these changes with gross morphological aberrations.

Placental transfer of ZEN can result in teratogenic effects in piglets following consumption of contaminated feed by sows. Dacasto et al. (1995) reported findings of an outbreak in Italy where sows showed no overt signs of hyperoestrogenism, but both male and female piglets from these dams were affected by various abnormalities of the genitalia (Table 3).

Teratogenic effects have also been observed in sows fed fumonisin-containing diets based on a culture of *F. moniliforme* (Zomborszky et al., 1997b). Preliminary results

indicated that feeding such diets to sows in advanced stages of pregnancy caused fetal damage to the extent that PPE of particular severity was evident in piglets slaughtered immediately after parturition. It is implied that fumonisins contained in the culture elicit adverse effects through placental transfer. In rats, teratogenicity of FB_1 is expressed as a suppression of growth and bone development in the fetus (Lebepe-Mazur et al., 1995).

3.6. Cytotoxicity

Assays based on isolated cells have emerged as useful adjuncts to whole-animal toxicology, yielding supplementary information on physiological and biochemical modes of action. FB_1, for example, causes morphological and functional abnormalities in chicken macrophages in vitro, indicative of an immunosuppressive effect . Chicken macrophage viability may be reduced by exposure to T-2 tetraol, a derivative of T-2 toxin (Kidd et al., 1997). Other studies suggest that the cytotoxicity of MON in an L6 myoblast in vitro model arises partly from oxidative damage and altered pyruvate metabolism (Reams et al., 1996). In pig kidney cells, elevated sphingoid bases and complex sphingolipid depletion were identified as the basis of the cytotoxic action of FB_1 (Yoo et al., 1996). The metabolic changes are not dissimilar to those seen in whole-animal studies (Table 4).

A particular attribute of cytotoxicity models is their potential application in screening procedures with grain or animal feed suspected to be contaminated with mycotoxins. A rapid colorimetric bioassay for screening of *Fusarium* mycotoxins has been developed (Rotter et al., 1993). Such procedures may also initiate a search for as yet unidentified mycotoxins in grain and feed. D'Mello et al. (1993), for example, showed that grain extracts obtained from a particular harvest of barley were lethal to two mammalian cell lines, although the identity of the toxic substances has remained elusive.

3.7. Interactions

Under commercial conditions, livestock are exposed to a complex mixture of mycotoxins derived not only from *Fusarium* fungi but from the *Aspergilli* as well. If the net effect is additive then it might be possible to predict the outcome in terms of productivity. Recent evidence, (Table 5) indicates that most interactions involving *Fusarium* mycotoxins are less than additive or additive for responses ranging from mortality (Javed et al., 1993a) to feed intake and growth (Harvey et al., 1996; Kubena et al., 1997a). However, three reports summarised in Table 5 indicate synergistic effects of DON and fusaric acid; DON and FB_1; and DAS and aflatoxins. In addition, potentiation may occur between co-occurring mycotoxins. Although of minor toxicity at levels detected in nature, fusaric acid can enhance the activity of other *Fusarium* mycotoxins. Thus, a toxic interaction between fusaric acid and FB_1 has been demonstrated in the fertile chicken egg. In combination, high lethality was observed whereas individually the mycotoxins had virtually no effect on mortality (D'Mello et al., 1997). Similarly, Kubena et al. (1997a) observed that serum protein and urea nitrogen in broilers were increased only by the FB_1 and DON combination, while serum Ca levels were increased only by the

Table 5
Interactions involving *Fusarium* mycotoxins

Source of mycotoxins	Combinations	Animal species	Responses	Interaction	Data source
Naturally contaminated grains	DON and fusaric acid	Pigs	Growth	Synergistic	Smith et al. (1997)
Inoculated maize and pure mycotoxin	DON and T-2 toxin	Pigs	Feed intake and growth	Adverse effects of DON reduced at intermediate levels of T-2 toxin	Friend et al. (1992)
Contaminated wheat and culture material	DON and FB_1	Pigs	Weight gain	Synergistic	Harvey et al. (1996)
Pure mycotoxins	T-2 toxin and OA	Pigs	Feed intake and weight gain	Additive	Harvey et al. (1994)
Culture material and pure mycotoxin	DON and MON	Broiler chicks	Feed intake and weight gain	Less than additive	Harvey et al. (1997)
Culture material and contaminated wheat	DON and fumonisins	Broiler chicks	Weight gain	Less than additive	Kubena et al. (1997a)
Pure mycotoxin and culture material	T-2 toxin and fumonisins	Broiler chicks	Weight gain	Additive	Kubena et al. (1997a)
Pure mycotoxins	FB_1 and moniliformin	Broiler chicks	Mortality	Additive	Javed et al. (1993a)
Pure mycotoxin and culture material	T-2 toxin and fumonisins	Turkey poults	Weight gain	Additive	Kubena et al. (1995)
			Certain blood and enzyme values	Synergistic	
Culture material and pure mycotoxins	DAS, fumonisins or OA	Turkey poults	Weight gain	Additive or less than additive	Kubena et al. (1997b)
Inoculated rice and pure mycotoxin	DAS and aflatoxins	Lambs	Weight gain and serum γ-glutamyl transferase	Synergistic	Harvey et al. (1995)

FB_1 and T-2 toxin combination. In turkeys, haematological criteria such as haemoglobin and haematocrit values were increased only by the T-2 toxin and FB_1 combination whereas individually the mycotoxins were without effect (Kubena et al., 1995). It has been concluded that although fumonisin levels in poultry feeds are not intrinsically problematic, the risk is heightened by the synergistic and potentiating interactions with other co-occurring mycotoxins.

4. Residues

Residues may arise through carry-over into eggs, milk, meat and offal and, as such, may represent a potential risk to humans. Prelusky et al. (1987) found relatively low but measureable levels of radio-labelled DON in eggs from hens fed a diet contaminated with the mycotoxin. However, residues declined once the contaminated feed was removed. Of some concern, nevertheless, was the observation that only 0.10 of the radioactivity in egg components could be attributed to DON itself, while the nature of its major metabolites remained obscure. Feeding pigs on ZEN-contaminated diets may also result in detectable amounts of residues. Thus, kidney and liver contained, respectively, up to 4.3 and 4.9 ng ZEN/g (Lusky et al., 1997). In contrast, lactational transfer of fumonisins into milk of dairy cows is considered by Richard et al. (1996) to be minimal, at a daily dosage level of 3 mg FB_1/kg bodyweight for 14 days. Similarly, in lactating sows ingesting non-lethal doses of FB_1, the mycotoxin was absent in the milk, and piglets suckling these sows showed no overt or biochemical evidence of toxicity (Becker et al., 1995).

5. Advisory and tolerance limits

Despite the established carcinogenicity of the fumonisins, statutory regulations do not exist for these or any of the other *Fusarium* mycotoxins. In contrast, stringent directives are in place for the *Aspergillus*-derived aflatoxins. However, a selection of advisory and tolerance limits for the *Fusarium* mycotoxins are available in the literature (Table 6). The data are not designed to be exhaustive, but rather illustrative of global values published in the last three to four years. Compilation of data for a particular mycotoxin is fraught with difficulties arising from expected variation between studies and from the criteria used to develop estimates of tolerance. Dose of in terms of intake is more important in determining toxicity than dietary levels. In addition, rates of detoxification of absorbed mycotoxins will have an impact on the eventual outcome. It should also be recognized that no allowance has been made for additive or synergistic effects arising from co-occurring mycotoxins. Consequently, discrepancies will inevitably arise between estimates based on the feeding of *Fusarium*-contaminated grain and those derived from studies with a single pure mycotoxin (Prelusky et al., 1994b). The use of the pure form, however, may still result in differences in estimates. Thus, Rafai et al. (1995a) commented that feed refusal in pigs fed T-2 toxin at 3 mg/kg was a particular problem whereas other earlier studies had indicated no such effects at

Table 6
Estimates of advisory or tolerance limits for *Fusarium* mycotoxins

Mycotoxins	Class of animals	Levels	Data source
DON	Cattle and chickens	10 mg/kg grain and grain by-products	FDA; in Trucksess et al. (1995)
DON	Pigs	5 mg/kg grain and grain by-products	FDA; in Trucksess et al. (1995)
DON	Growing pigs	0.5–1.0 mg/kg diet	Bergsjo et al. (1992)
DON plus ZEN	Growing chicks; laying hens	DON: 3 mg/kg + ZEN: 0.6 mg/kg diet	Keshavarz (1993)
DON	Ducks	6 mg/kg wheat	Boston et al. (1996)
DON	Dairy cows	15 mg/kg barley or 31 mg/kg bodyweight	Ingalls (1996)
T-2 toxin	Growing pigs	< 0.5 mg/kg diet	Rafai et al. (1995a)
ZEN	Pigs	500 mg/kg feed	Diaz and Cespedes (1997)
ZEN plus NIV	Pregnant sows	ZEN: 1.8 mg/kg + NIV: 6.9 mg/kg diet	Williams and Blaney (1994)
ZEN	Sheep	< 3 mg/kg forage	Kramer et al. (1997)
FB_1	Weaned piglets	< 10 mg/kg feed	Zomborszky et al. (1997a)
FB_1	Weanling gilts	70 mg/kg feed	Guzman et al. (1997)
FB_1	Growing pigs	< 0.1 mg/kg feed	Rotter et al. (1996)
MON	Broiler chickens	< 50 mg/kg feed	Ledoux et al. (1995)

considerably higher levels of the mycotoxin. The choice of performance and biochemical criteria may also influence selection of tolerance values. For example, Rotter et al. (1996) suggested that for FB_1 erratic growth occurred in growing pigs at levels as low as 0.1 mg/kg diet, followed by reduced growth and biochemical abnormalities in blood at 1 mg/kg diet. If sphingolipid aberration in tissues are used as the criterion, then changes would not occur until diets contained 10 mg FB_1/kg. It is difficult to reconcile the value of 70 mg/kg feed (Table 6) for gilts in the study of Guzman et al. (1997) even with suggestions that males are more sensitive than females to the effects of FB_1 (Rotter et al., 1996). The importance of choice of criteria is clearly evident in the case of MON. According to Ledoux et al. (1995), significant increases in mortality of broiler chicks are not observed until dietary levels of MON exceed 200 mg/kg. If feed intake depression is to be avoided, then levels must not exceed 100 mg/kg, and if normal heart weight is the selected criterion, levels of MON should remain below 50 mg/kg diet (Table 6).

6. De-contamination and amelioration

A number of de-contamination procedures have been investigated, broadly divisible into physical and chemical principles (Placinta et al., 1999). Physical methods include milling which has been shown to be highly effective for DON, and density segregation which has resulted in reduced levels of trichothecenes and ZEN. Superactivated charcoal is partially effective at reducing the incidence of oral lesions in broilers fed T-2 toxin, but

mortality remains unaffected (Edrington et al., 1997). Furthermore, amelioration of oral lesions was not consistent between experiments. Chemical methods tested include calcium hydroxide monomethylamine, sodium bisulphite and ammonia. The commercial potential of these de-contamination procedures, however, has yet to be determined.

Anti-oxidants such as vitamin E have been considered as dietary supplements to counteract the effect of T-2 toxin. A partial beneficial effect, in terms of reduced in vivo lipid peroxidation, has been reported in one study with chickens (Hoehler and Marquardt, 1996). Vitamin C was ineffective in this respect.

The general consensus now prevailing is that preventive measures offer greater potential than remedial procedures. With ZEN, a feeding strategy for breeding ewes has been suggested, based on the use of chicory pastures containing inherently low levels of the mycotoxin (Kramer et al., 1997). However, selection of cultivars of cereal and forage plants that are resistant to infection by toxigenic species of *Fusarium* pathogens is likely to be the long-term objective of any effort to control contamination with the associated mycotoxins.

7. Conclusions

The major *Fusarium* mycotoxins occurring in animal feeds and forages include the trichothecenes, zearalenone and its derivatives, and fumonisins. A number of compounds within these three groups have been implicated in spontaneous worldwide cases of mycotoxicoses in livestock. In addition, chronic exposure occurs by virtue of continuing global contamination of cereal grains and forages. Of the trichothecenes, DON and T-2 toxin and DAS are associated with particular syndromes. DON is conventionally linked with emesis, feed refusal and reduced feed intake in pigs, but T-2 toxin and NIV may also exert effects in this respect. In poultry, T-2 toxin and DAS are clearly associated with oral lesions. There is unequivocal evidence implicating ZEN in reproductive disorders of pigs, cattle and sheep, but limited evidence suggests that T-2 toxin may produce similar effects. Fumonisins are definitively linked with porcine pulmonary edema and equine leukoencephalomalacia and with disruption of sphingolipid metabolism in affected animals. In addition, *Fusarium* mycotoxins are tentatively implicated in ill-thrift of sheep, acute mortality syndrome of poultry and, to a lesser extent, in duodenitis/proximal jejunitis of horses. Furthermore, underlying features of immunotoxicity and teratogenicity must be considered in assessing risk from these mycotoxins. Additive interactions in precipitation of adverse effects in pigs and/or poultry have been identified between T-2 toxin and ochratoxin; DON and MON; DON and fumonisins; T-2 toxin and fumonisins; FB_1 and MON; DAS and fumonisins. Of greater concern are synergistic interactions between DON and fusaric acid; and DON and FB_1 observed with pigs and between DAS and aflatoxins in lambs. Equally, potentiation between FB_1 and DON or T-2 toxin for several serum components in poultry is worthy of note. Such interactions also impose difficulties in the provision of reliable values for tolerance and regulatory limits. Finally, it is suggested that the long-term objective should be to reduce natural contamination of cereal grains and forages through the exploitation of disease-resistant cultivars.

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8.1.4 Paper 4 "The influence of carbendazim on mycotoxin production in *Fusarium Sporotrichioides*"

BRIGHTON CROP PROTECTION CONFERENCE - Pests & Diseases - 1996 4D-5

THE INFLUENCE OF CARBENDAZIM ON MYCOTOXIN PRODUCTION IN *FUSARIUM SPOROTRICHIOIDES*

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ABSTRACT

Carbendazim was shown to influence production of T-2 toxin by *Fusarium sporotrichioides* maintained at 25°C, but in isolates transferred from 25°C to 11°C, both T-2 toxin and zearalenone production was reduced as carbendazim concentration rose.

INTRODUCTION

Mycotoxins are possibly the most unfamiliar and least investigated of the natural products that affect man and animals. There is an increasing awareness that this structurally diverse group of naturally occurring fungal toxins is being implicated in toxic syndromes. Such toxins are primarily to be found in agricultural crops such as cereals and oilseeds and products derived from them (Smith *et al.*, 1994).

Fusarium species are widely acknowledged to be phytopathogens of cereals. These fungi are also recognised as producers of mycotoxins including zearalenone (ZEN), T-2 toxin (T-2) and neosolaniol (NEO). Effective fungal disease control strategies require the use of fungicides, but they can influence mycotoxin production, their effects being variable and dose-dependent (D'Mello *et al.*, 1996).

Little attention has been given to the range of environmental factors and fungicides which might allow growth and accumulation of mycotoxins in grains.

The aim of this experiment was to investigate, in factorial combination, the effects of time, temperature and fungicide level on mycotoxin production in pure cultures of *Fusarium sporotrichioides.*

MATERIALS AND METHODS

Fusarium sporotrichioides 309349 from IMI was used. Peripheral plugs from colonies of this culture were used to prepare 5d old cultures. A plug from the 5d old culture was placed under aseptic conditions onto the centre of each petri dish containing PDA with different levels of fungicide. Carbendazim (Bavistin, BASF) was used. The concentration of the active ingredient was 50%. The fungicide was dissolved in ethanol and incorporated in the sterilized medium to provide carbendazim concentrations of 1.0, 2.5, 5.0, 7.5 and 10.0 µg/ml. The solvent without fungicide was added to the control. The cultures were incubated at 25°C for 5d, then half of the replicates were transferred to 11°C. At 6, 14 and 26d, colonies were

extracted with 20 ml chloroform. Filtered extracts were reduced in volume with a rotary evaporator and finally dried under N2 prior to storage at -20°C. For the determination of toxins, the extracts were resuspended in 0.1 ml chloroform and spotted on TLC plates. The plates were then developed in TEF (toluene: ethyl acetate: formic acid, 5:4:1) and examined under UV light. The presence of mycotoxins was evaluated visually by comparing the R_f and colour with the appropriate standards and quantified by densitometry. Analysis of variance for a factorial design was carried out using minitab and significant differences established.

RESULTS AND DISCUSSION

Production of ZEN, T-2 toxin and NEO was significantly influenced by time ($P<0.001$), whereas temperature ($P<0.01$) and concentration of fungicide ($P<0.05$) induced significant effects only for ZEN and T-2 toxin synthesis. However, only the main effects of fungicide concentration and temperature are presented in Table 1. At 5 µg/ml, carbendazim significantly ($P<0.05$) increased T-2 toxin production in cultures maintained at 25°C. On the other hand, in cultures in the 25-11°C regime, T-2 toxin production was significantly ($P<0.05$) reduced by carbendazim at 5 µg/ml, whereas ZEN production was reduced by the fungicide at levels of as low as 1 µg/ml ($P<0.05$).

Table 1. The effect of temperature regime and carbendazim application on mycotoxin production in cultures of *Fusarium sporotrichioides*

Carbendazim level (µg/ml)	Temperature regime					
	25°C			25 - 11°C		
	Mycotoxin (µg/ml of culture extract)					
	ZEN	T-2	NEO	ZEN	T-2	NEO
0	0.31	18.5	6.7	0.65	21.8	10.7
1.0	0.26	13.3	5.8	0.28	15.7	5.0
2.5	0.09	18.0	5.6	0.54	15.9	5.4
5.0	0.26	29.8	13.4	0.31	10.0	4.2
7.5	0.15	18.0	10.9	0.51	9.7	4.7
10.0	0.04	15.9	3.6	0.04	2.9	1.1
Significance	NS	<0.05	NS	<0.01	<0.05	NS
SEM (df=35)	0.091	3.30	2.68	0.091	3.30	2.68

ZEN= zearalenone; T-2 = T-2 toxin. NEO= neosolaniol

Taken together with previous results (D'Mello *et al.*, 1996) it is concluded that T-2 toxin production is increased with carbendazim application. In contrast, ZEN production is reduced with this fungicide

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8.1.5 Paper 5 "Production and control of mycotoxin production in *Fusarium* species pathogenic on cereals"

BRIGHTON CROP PROTECTION CONFERENCE - Pests & Diseases - 1996 5B-4

PRODUCTION AND CONTROL OF MYCOTOXINS FROM *FUSARIUM* SPECIES PATHOGENIC ON CEREALS

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ABSTRACT

Several *Fusarium* spp. that are pathogens of cereals are also potential sources of mycotoxins which may contaminate the grain and prejudice human and animal health. Particular risk emanates from the trichothecenes, including T-2 toxin and deoxynivalenol and from the fumonisins. Deoxynivalenol concentrations in cereal grains have been correlated with the incidence and severity of fusarium ear blight. Recent field trials suggest a limited role for fungicides in the control of deoxynivalenol contamination of wheat grain. With T-2 toxin, there is evidence, from laboratory studies, of enhanced synthesis on exposure of *F. sporotrichioides* to certain fungicides. However, the use of cereal genotypes resistant to fusarium diseases offers good prospects for protection from mycotoxin contamination of grain.

INTRODUCTION

Fusarium spp. have long been associated with diseases of economically important fusarium plants. In particular, fusarium ear blight (scab) of wheat, barley and oats has been linked with over 15 species of this genus (Parry *et al.*, 1995). The most common species include: *Fusarium graminearum, F. culmorum, F. avenaceum, F. poae, Michrodochium nivale (F. nivale), F. sporotrichioides* and *F. oxysporum.* Of these species causing fusarium ear blight, *F. graminearum* and *F. culmorum* are considered to be the most pathogenic (Parry *et al.*, 1995). *F. graminearum* has also been implicated in crown rot of wheat (Wildermuth & McNamara, 1994). In maize, ear rot may occur as a result of infection with a number of *Fusarium* spp., including *F. graminearum* and *F. moniliforme* (Schaafsma *et al.*, 1993).

Although ear blight of cereal plants can result in severe losses in yield, an additional penalty may emanate from contamination of the harvested grain with *Fusarium* mycotoxins. These substances are secondary metabolites that are toxic to animals and humans consuming contaminated grain.

TOXIGENIC *FUSARIUM* PHYTOPATHOGENS

Fusarium spp. of fungi produce a wide range of mycotoxins, of which the most significant from the standpoint of human and animal health are the trichothecenes, zearalenone and its derivatives, fusaric acid and the fumonisins (D'Mello *et al.*, 1996a). In excess of 100 trichothecenes have been isolated, characterised and classified into four types. However, most attention has focused on Type A trichothecenes, including T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS) and on Type B trichothecenes, comprising nivalenol (NIV), deoxynivalenol (DON; vomitoxin) and its 3-acetyl and 15-acetyl derivatives

(3-ADON and 15-ADON, respectively). The structures of the major trichothecenes and other *Fusarium* mycotoxins are presented by Flannigan (1991).

The production of mycotoxins by common phytopathogenic *Fusarium* spp. is summarised in Table 1, compiled from investigations over the past 20 years or so (D'Mello *et al.*, 1996a). The table is not exhaustive but rather illustrative of certain distinguishing features in the production

Table 1. Production of *Fusarium* mycotoxins.

Fusarium species	Mycotoxins
F. sporotrichioides; F. poae	T-2 toxin, HT-2 toxin, neosolaniol, diacetoxyscirpenol
F. poae	Nivalenol
F. graminearum; F. culmorum	Deoxynivalenol, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol
F. avenaceum	Deoxynivalenol, 15-acetyl deoxynivalenol
F. sporotrichioides; F. graminearum; F. culmorum	Zearalenone
F. oxysporum	Zearalenone, fusaric acid
F. moniliforme	Fumonisins

of the commonly occurring mycotoxins of this genus. It is clear, for example, that the production of Type A trichothecenes predominates in *F. sporotrichioides* and *F. poae*, although the evidence is less convincing for the latter species (D'Mello *et al.*, 1996a). Production of Type B tricho-thecenes occurs principally in *F. culmorum* and *F. graminearum*. However, there is much evidence to suggest that *F. poae* is also a Type B trichothecene producer. A notable feature is that many *Fusarium* spp. have the capacity to synthesise other mycotoxins, in addition to the trichothecenes. Thus, zearalenone (ZEN) is produced by three of the species previously mentioned as well as by *F. oxysporum* which also synthesises fusaric acid. *F. moniliforme* is a well-recognised source of the fumonisins which comprise six structurally related metabolites. Of these, fumonisins B_1 and B_2 (FB_1 and FB_2 respectively) have been implicated in human and animal disorders (D'Mello & Macdonald, 1996; D'Mello *et al.*, 1996a).

The classical assessment of toxicity of different deleterious substances centres on the determination of LD_{50} values in experimental animals. Flannigan (1991) lists LD_{50} values of 4.1, 5.2, 9.0, 14.5, 23.0 and 70.0 mg/kg body weight for NIV, T-2 toxin, HT-2 toxin, NEO, DAS and DON, respectively when these mycotoxins were administered intraperitoneally to mice. Although the acute toxicity of DON is relatively low, it is widely recognised as a potent

feed intake inhibitor in pigs which accounts for its alternative name, vomitoxin. Zearalenone is even less toxic in the classical sense, with LD_{50} values for different animals ranging from 2 to 10 g/kg (Flannigan, 1991), but it has been associated with infertility, reduced milk production and hyperoestrogenism in cows (see D'Mello *et al.*, 1996a). Fusaric acid appears to be of minor toxicity at levels detected in nature, but there is increasing evidence that it may act synergistically to enhance the activity of other *Fusarium* mycotoxins. The fumonisins are associated with diverse manifestations of toxicity in farm animals, but these mycotoxins have recently been linked with the incidence of oesophageal cancer in humans (Yoshizawa *et al.*, 1994).

FUSARIUM DISEASES AND MYCOTOXIN PRODUCTION IN CEREAL GRAINS

Owing to the ubiquitous occurrence of DON in cereal grains, considerable work has been conducted to elucidate its relationship with fusarium ear blight. In one such study (Miller *et al.*, 1985), a single isolate of *F. graminearum* was used to infect different cultivars of spring wheat, rye and triticale. Resistant cultivars of the three cereals contained low concentrations of DON (mean 0.6 mg/kg) in the kernels whereas grain from susceptible cultivars had considerably higher concentrations (mean 10.2 mg/kg) despite minimal visual evidence of ear blight in the plants. However, in a subsequent study, pathogenicity in field trials was correlated with mycotoxin contamination of wheat grains (Wong *et al.*, 1995). Thus in that study, *F. culmorum* and *F. graminearum* were found to exhibit the greatest pathogenicity in comparison with *F. sporotrichioides* or *F. avenaceum*. Correspondingly, higher concentrations of DON were detected in grain of susceptible wheat cultivars than in grain from resistant cultivars after inoculation with the two most pathogenic *Fusarium* spp. For example, in susceptible cultivars inoculated with *F. culmorum*, DON values ranged from 17 to 121 mg/kg grain whereas in resistant cultivars concentrations of 0.2 to 9.7 mg/kg grain were recorded. In two of these susceptible cultivars, 15-ADON levels ranged from 0.11 to 0.21 mg/kg, but in all resistant cultivars levels of 15-ADON were below the detection limit. In a more recent investigation, with winter wheat, mean concentrations of DON were positively correlated with both ear blight incidence and severity in natural epidemics (Wiersma *et al.*, 1996). Within *F. culmorum*, strains differing in pathogenicity have been found (Snijders & Perkowski, 1990). In plot trials with experimental inoculations, the most virulent strain induced the highest incidence of ear blight in wheat, particularly in susceptible cultivars, and there was close correlation between ear blight incidence and contamination of kernels with DON. None of the other trichothecenes or ZEN was detected in any of the wheat grain samples.

In maize, ear rot has also been attributed to infection with *Fusarium* spp., particularly *F. graminearum*. Field trials with artificial inoculations indicated that ear rot severity was greater with *F. graminearum* than with *F. moniliforme* or *F. subglutinans* (Schaafsma *et al.*, 1993). Ear rot severity generally correlated well with levels of DON and the authors concluded that severity of ear rot may be a useful indicator of mycotoxin production in grain.

It is important to recognise that in the field and at harvest, grain is likely to be colonised by different species of fungi (D'Mello *et al.*, 1993) and the potential for mycotoxin production may be influenced by fungal interactions. Cuero *et al.* (1988), for example, showed that ZEN production was markedly decreased at 16°C by the presence of *A. flavus* but remained unaffected at 25°C.

CONTROL MEASURES

It is axiomatic that preventive measures are of paramount importance in reducing the risk of mycotoxin contamination of cereal grains. Two obvious strategies may be envisaged, both involving the prevention of fungal proliferation and disease. As might be anticipated, fungicides can influence mycotoxin production but the effects are variable and dose-dependent (D'Mello *et al.*, 1996a). In laboratory experiments with pure cultures (Table 2) dicloran, iprodione and vinclozolin were individually effective as inhibitors of DAS and ZEN synthesis in *F. graminearum* but tridemorph and carbendazim each enhanced T-2 toxin production in *F. sporotrichioides*, while 3-ADON production increased in *F. culmorum* treated with difenoconazole.

Table 2. Effects of fungicides on production of mycotoxins in pure cultures of *F. graminearum*, *F. sporotrichioides* and *F. culmorum*.

Fungicide	Methods	Effects	Ref.
Dicloran, iprodione, vinclozolin	Added separately at levels of up to 500 µg/ml potato-dextrose broth; static culture of *F. graminearum*	Dose-related inhibition of growth and production of DAS and ZEN; total inhibition of DAS and ZEN production at higher levels of each fungicide	1
Tridemorph	Shake-flask cultures of *F. sporotrichioides*; fungicide added at 6 and 36 µg/ml	At 6 µg/ml, growth enhanced but T-2 toxin and DAS production inhibited; at 36 µg/ml, growth inhibited but T-2 toxin production stimulated	2
Carbendazim	Cultures of *F. sporotrichioides* on potato-dextrose agar; fungicide added at 5 µg/ml	Growth unaffected by fungicide; significant increase in T-2 toxin production with fungicide	3
Difenoconazole	Cultures of *F. culmorum* on potato-dextrose agar; fungicide added at levels of up to 100 µg/ml	Growth unaffected by 0.1 µg/ml but 3-ADON production signifi-cantly increased	4

Refs: 1. Hasan (1993); 2. Moss & Frank (1985); 3. Placinta *et al.* (1996); 4. D'Mello *et al.* (1996b).

Field trials with fungicides have yielded somewhat conflicting results. Thus Boyacioglu *et al.* (1992) showed that propiconazole reduced infection of wheat by an artificially applied inoculum of *F. graminearum* by 39-55% and DON levels were reduced by 34-78%. However, thiabendazole had no effect on infection level but DON contamination was reduced

by up to 83%. In a subsequent study, also with wheat inoculated with *F. graminearum* (Milus & Parsons, 1994), ear blight incidence and DON concentrations in grain remained unaffected by propiconazole, thiabendazole or tebuconazole applications. On the other hand, combination of tebuconazole and triadimenol in wheat inoculated with *F. culmorum* reduced ear blight but a 16-fold increase in NIV content of grain was observed (Gareis & Ceynowa, 1994).

It is a consistently held view that exploitation of genetic resistance to diseases such as ear blight offers the most promising method for control of mycotoxin contamination of cereal grains. Two elegant studies reinforce this concept. Thus Snijders & Perkowski (1990) showed that in wheat genotypes resistant to ear blight caused by *F. culmorum*, DON concentrations of grain varied from 3.4 to 4.6 mg/kg. However, in susceptible genotypes DON levels increased to 37 mg/kg. Subsequently, Wong *et al.* (1995) demonstrated that several Chinese cultivars of wheat were resistant to ear blight induced by either *F. culmorum* or *F. graminearum* and DON contamination of grain was low, with a maximum value of 9.7 mg/kg. In contrast, three Canadian cultivars susceptible to ear blight had concentrations of up to 121 mg DON/kg kernel.

CONCLUSIONS

Fusarium spp. are significant not only as phytopathogens but also as potential producers of mycotoxins which may prejudice safety of food, particularly cereal grains. The trichothecenes, including T-2 toxin and deoxynivalenol, and the fumonisins are the most important mycotoxins in this context. Several studies suggest good correlation between fusarium ear blight of cereals and deoxynivalenol contamination of grain. Recent field trials have not provided a consensus regarding the efficacy of fungicides to control mycotoxin production. Indeed, laboratory studies consistently indicate that T-2 toxin may be enhanced with applications of certain fungicides. The exploitation of disease-resistant cereal genotypes, however, represents a promising strategy for reducing mycotoxin contamination of grain. Studies are being undertaken at the Scottish Agricultural College to assess how mycotoxin production may be affected in fungicide-resistant strains of *Fusarium* spp.

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8.1.6 Paper 6 "Disparate effects of temperature and fungicides on mycotoxin production in the phytopathogen, *Fusarium sporotrichioides*"

DISPARATE EFFECTS OF TEMPERATURE AND FUNGICIDES ON MYCOTOXIN PRODUCTION IN THE PHYTOPATHOGEN, *FUSARIUM SPOROTRICHIOIDES*

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ABSTRACT

Toxigenic *Fusarium* species are a major agricultural problem not only because of risks to human and animal health, but also because of losses due to plant diseases.
Fungicides may be used to control these diseases but concern over pesticide residues in food is a critical issue even with current efforts to reduce fungicide dosage. However, it was shown that the use of sublethal doses of fungicide leads to an increase in mycotoxin production. This study presents the disparate effects of carbendazim and difenoconazole on mycotoxin production by *Fusarium sporotrichioides* maintained at two different temperature regimes. Cultures were incubated at a constant temperature of 25° C or initially at 25° C for 5 days and then at 11° C until the completion of experiment. The main effects of temperature on T-2 toxin production depended upon fungicide type. Whereas carbendazim at 5µg/ml increased T-2 toxin production at 25° C, both carbendazim and difenoconazole decreased T-2 toxin production in the 25-11° C temperature regime. Temperature also exerted marked effects on production of NEO and HT-2 toxin.

RESUME

Les espèces de *Fusarium* toxigéniques posent un problème majeur en agriculture parce qu'elles entrainent non seulement des risques sur la santé humaine et animale mais également des pertes de rendement liées aux maladies qu'elles engendrent.
Des fongicides peuvent être utilisés pour contrôler ces maladies, mais des soucis liés aux résidus de pesticide présents dans l'alimentation posent problèmes, même en réduisant les doses de fongicide appliquées. En fait, il a été montré qu'à des doses subléthales, le fongicide engendre une augmentation de la production de mycotoxines. Cette étude présente les effets disparates du carbendazim et de la difénoconazole sur la production de mycotoxines par *Fusarium sporotrichioides* soumis à deux régimes de température différents. Les cultures furent incubées soit à une température constante de 25° C, soit à 25° C pendant 5 jours puis à 11°C jusqu'à la fin de l'expérience. Les principaux effets de la température sur la production de la toxine T-2 dépendaient du type de fongicide appliqué. Alors que le carbendazim, à une concentration de 5 µg/ml, augmentait la production de la toxine T-2 à 25° C, le carbendazim et la difénoconazole, soumis au regime de température 25-11° C, diminuaient tous les deux la production de la toxine T-2.

INTRODUCTION

The great importance of plant diseases and their economic effects have been well documented. On a world wide basis it is estimated that about10-15% of potential crop yield is lost annually due to attack by plant pathogens. Toxigenic *Fusarium* species are a major agricultural problem not only because of human and animal health, but also because of losses due to plant diseases. The intensive characteristic of modern agriculture with the demand for the better standards of crop quality and yield has required a high degree of disease control. Currently, fungicides are applied to almost all cereal crops in the UK and many are sprayed two or more times during the growing season. Fungicides continue to play a major role in maintaining the high standards of disease control evident within the intensive farming systems of the European Union. The intensive use of fungicides to achieve consistent and thorough control of disease is likely to remain a feature of growing systems where quality is of great importance. However, the problem of fungicide resistance is most likely to occur. The use of pesticides has come in for criticism in recent years, with respect to public concern including both the possible accumulation of pesticide residues in soil following repeated applications to crops and presence of residues in the food for consumption. However, imminent economic and legislative pressures, as well as concerns over the long term toxicological and environmental effects of pesticides, may lead to reductions in the quantity of fungicides applied to crops and greater reliance on other methods of disease control. Nevertheless it was shown that the use of sublethal doses of fungicides leads to an increase in mycotoxin production (Placinta *et al.*, 1996). *Fusarium* species and their toxins appear to be most prevalent in corn, wheat and barley but they are also found in fruits, vegetables and non-food items.

Fusarium species of fungi produce a wide range of mycotoxins, of which the most important are the trichothecenes, zearalenone and its derivatives, fumonisins, moniliformin, fusarochromanones, fusaric acid, fusarins etc.(D'Mello *et al.*, 1997) Many of the secondary metabolites may be toxic to micro-organisms (antibiotics), plants (phytotoxins) or animals (mycotoxins). Most attention has focused on type A trichothecenes, including T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS) that predominates in *Fusarium sporotrichioides*. The production of *Fusarium* mycotoxins is affected by different factors, such as temperature, pesticide application, to enumerate just a few. In some years, especially those with wet harvest, ecological conditions in the field and in storage may favor mycotoxin development. Also, irrespective of the rainfall that year, there is an ever-present danger that mycotoxins might develop in moist grain pockets within granaries. Mills (1989) showed that strains of *F. sporotrichioides* isolated from overwintered wheat and corn produced T-2 toxin , a fact which demonstrated that the frequency of seed-borne *Fusarium* species was much higher on overwintered crops. It is also known that interactions among these factors may exert significant effects on mycotoxin synthesis. It is the possibility of long-term chronic toxicity which is of special concern because several of these mould metabolites are known to be teratogenic, carcinogenic, oestrogenic or immunosuppressive and their presence in foods may have more subtle effects on human health.

Many countries have set legislative limits to the concentrations of a number of mycotoxins in foods and these may reflect analytical capability rather than a clear understanding of the toxicological significance or distribution of mycotoxins in foods. Food safety, animal health and productivity, and human health problems identified with fungal-contaminated grains most recently have concentrated research on *Fusarium* species and their toxic metabolites. As the chemical nature, biology and toxicology of trichothecenes has become better understood, it is apparent that these toxins may have been underrated as causal agents of serious food or feed related intoxication in man and animals. The widespread nature of the trichothecenes-producing fungi in foods and feeds suggest that they may come to be recognized as the most important of the acutely toxic mycotoxins. Certainly their potential as inducers of mycotoxicoses appears to be much greater than that of the more widely investigated aflatoxins.

The aim of this factorial experiment was to examine the effects of temperature and difenoconazole on mycotoxin production in *F. sporotrichioides*.

MATERIALS AND METHODS

Fusarium sporotrichioides 309349 from IMI was used. Peripheral plugs from colonies of this culture were used to prepare 5-day old cultures. A plug from the 5-day old culture was placed under aseptic conditions onto the centre of each petri dish containing PDA with different levels of fungicide. Difenoconazole (Plover, Ciba Agriculture) was used. The concentration of the active ingredient was 250g/litre. The fungicide was dissolved in ethanol and incorporated in the sterilized medium at concentrations of 0.1, 1.0, 10.0, 100.0 µg/ml. Ethanol without fungicide was added to the control. The cultures were incubated at 25°C for 5 days, then half of the replicates were transferred to 11°C. At 28 days of age, colonies were extracted with 20ml chloroform. Filtered extracts were reduced in volume with a rotary evaporator and finally dried under N_2 prior to storage at -20°C. For the determination of toxins, the extracts were resuspended in 0.1 ml chloroform and spotted on TLC plates. The plates were then developed in TEF (toluene:ethyl acetate:formic acid, 5:4:1) and examined under UV light. The presence of mycotoxins was evaluated visually by comparing the Rf and colour with the appropriate standards and quantified by densitometry. Analysis of variance for a factorial design according to Mead *et al.* (1993) was carried out using minitab and significant differences established after calculation of LSD values.

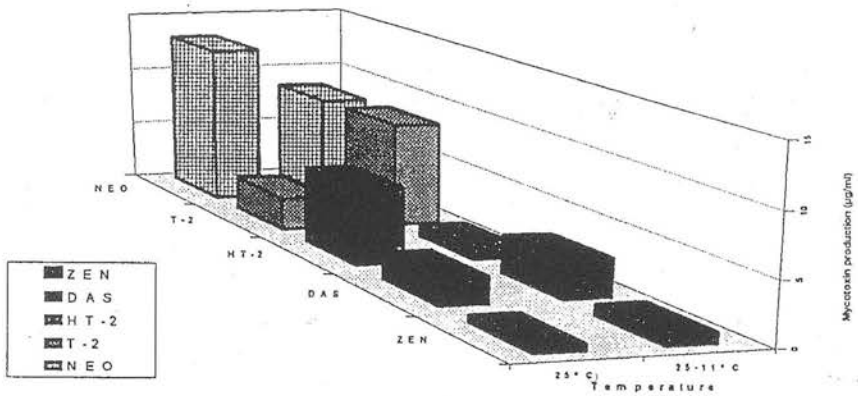
RESULTS AND DISCUSSION

In this study *F. sporotrichioides* produced: zearalenone (ZEN), T-2 toxin (T-2), HT-2, neosolaniol (NEO) and diacetoxyscirpenol (DAS).

As shown in Figure 1, temperature induced significant effects on the production of T-2 toxin ($P<0.001$), HT-2 toxin ($P<0.05$) and NEO ($P<0.01$). However, fungicide application caused significant reductions ($P<0.001$) in the production of ZEN, T-2 toxin and NEO. The interaction between temperature and concentration of fungicide

was significant only for T-2 ($P<0.001$) and HT-2 ($P<0.05$). The effects of difenoconazole concentration and temperature regime on T-2 toxin production will be compared with previous results obtained using carbendazim as fungicide (Placinta *et al.* 1996).

Figure 1: The effect of temperature regime on mycotoxin production by *Fusarium sporotrichioides*



It is clear from Figure 2 that the effect of temperature on T-2 toxin production depends upon fungicide type.

Figure 2: The effects of fungicide type and temperature regime on T-2 toxin production by *Fusarium sporotrichioides.* (Data for carbendazim taken from Placinta *et al.*, 1996)

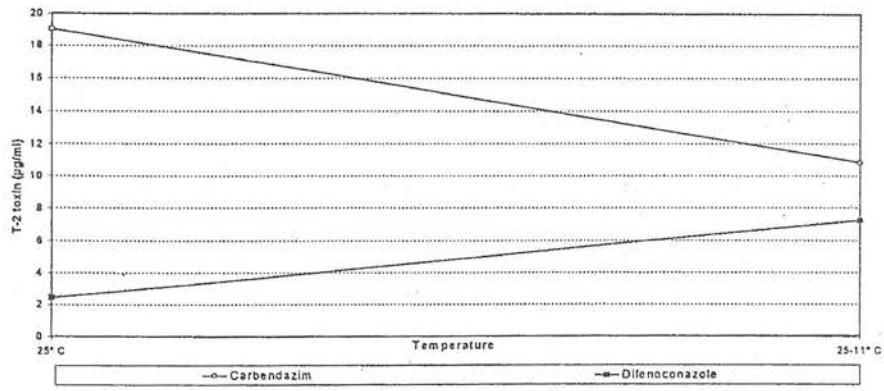
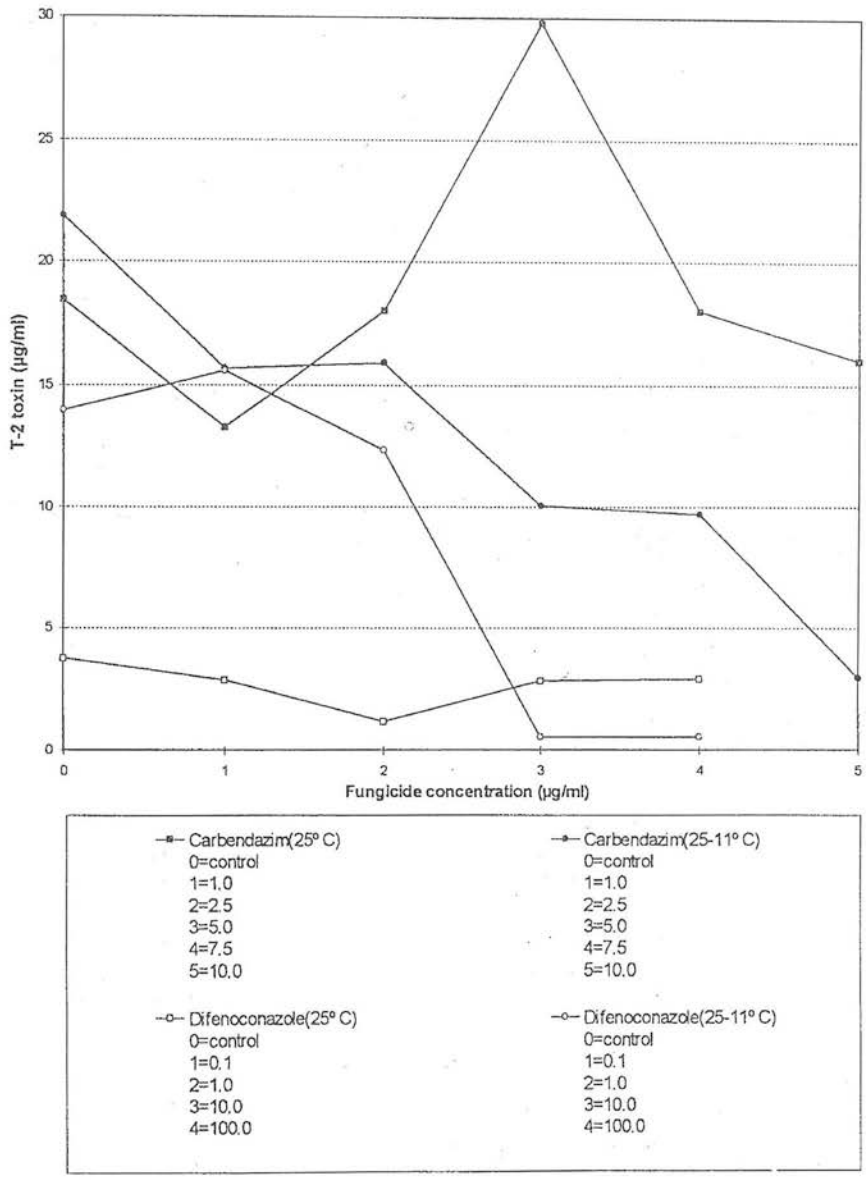


Figure 3: The effects of fungicide type and temperature regime on T-2 toxin production by *Fusarium sporotrichioides*. (Data for carbendazim taken from Placinta *et al.*, 1996)



Whereas carbendazim at 5 µg/ml induced a significant increase in T-2 toxin production at 25°C (Figure 3; Placinta *et al.*, 1996), both carbendazim and difenoconazole caused

significant decrease ($P<0.01$ and $P<0.001$, respectively) in T-2 toxin production in the 25-11°C temperature regime.

CONCLUSION

The use of different types of fungicides (carbendazim and difenoconazole) results in disparate effects on the T-2 toxin production by *F. sporotrichioides* while kept under two different temperature regimes (25° C and 25-11° C).

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8.1.7 Chapter "*Fusarium* mycotoxins" in "Plant and fungal toxicants" - 1997

20 *Fusarium* Mycotoxins

J. P. F. D'Mello, J. K. Porter, A.M.C. Macdonald, and C. M. Placinta

CONTENTS

20.1 INTRODUCTION

Fusarium species of fungi produce a wide range of mycotoxins, of which the most important from the standpoint of animal and human health are the trichothecenes, zearalenone and its derivatives, fumonisins, moniliformin, fusarochromanones, fusaric acid, fusarins, cyclic peptides, and amino acid esters (beauvericin type). In excess of 100 trichothecenes have been isolated, characterized, and classified into four types. However, most attention has focused on Type A trichothecenes, including T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS), and on Type B trichothecenes, comprising deoxynivalenol (DON; vomitoxin) and its 3-acetyl and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV), and fusarenon-X. The structures of the major trichothecenes and other *Fusarium* mycotoxins are presented by Flannigan.[1] In this chapter, we review the production of mycotoxins by the common species of *Fusarium* and the toxicity of a number of these fungal metabolites.

20.2 PRODUCTION OF *FUSARIUM* MYCOTOXINS

The production of mycotoxins by four *Fusarium* species is summarized in Table 20.1, compiled from investigations over the past 20 years or so.[2–28] The table is not designed to be exhaustive

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TABLE 20.1
Production of Mycotoxins by Four Species of *Fusarium* Molds[a]

	(1) Type A trichothecenes				
***Fusarium* species**	**T-2**	**HT-2**	**NEO**	**DAS**	**Ref.**
F. sporotrichioides	+	+	+		2
	+			+	3
	+			+	4
	+	+	+		5
	+	+	+	+	6
	+	+	+	+	7
	+	+	+	+	8
	+				9
	+	+			10
F. culmorum		+			8
F. graminearum	+	+		+	8
F. poae	+	+	+		2
	+	+		–	27
	–	+	–	+	8
	–		–	+	28
	+	+			10

	(2) Type B trichothecenes and ZEN					
	DON	**3-ADON**	**15-ADON**	**NIV**	**ZEN**	**Ref.**
F. sporotrichioides					+	2
	–		–		–	3
					+	5
	+		+	+		8
F. culmorum					+	11
	+				+	6
		+				12
	+	+		–	+	13
	+		+	+		8
	+				+	14
				+		15
	+		+			10
F. graminearum					+	16
	+	+			+	17
	+		+		+	3
	+					18
	+		+			19
	+		+		–	20
					+	11
	+			+	+	6
					+	21
					+	22
	+		+		+	23
	+					24
	+		+	+		8
					+	25
	+				+	14
	+					26
	+		+	+		10
F. poae				+	+	27
	+		+	+		8
	–	–		+	–	28
				+		10

[a] Key: + = confirmed presence; – = confirmed absence (below detection limit); empty cell = no data.

but rather illustrative of certain distinguishing features in the production of the commonly occurring mycotoxins. All four species synthesize a variable mixture of mycotoxins. However, it is clear that the production of Type A trichothecenes predominates in *Fusarium sporotrichioides*. Similar comments apply to *Fusarium poae*, but the evidence is less convincing in view of the confirmed absence of T-2 and NEO in two studies.[8,28] Production of Type B trichothecenes occurs principally in *Fusarium culmorum* and *Fusarium graminearum*, but *F. poae* is also a consistent producer of one of the Type B trichothecenes, namely NIV. A unifying feature is that all four species possess at least some capacity to synthesize zearalenone (ZEN). Similarly, the studies of Abramson et al.[8] indicate that fusarenon-X (FX) production occurs in all four species of *Fusarium* as well as in other species of this genus. Among the latter group, *Fusarium equiseti* is a consistent producer of FX and other Type B trichothecenes[8] as well as ZEN.[6,23] In addition to 3-ADON and 15-ADON, other metabolites of trichothecenes are also produced by various *Fusarium* species. For example, T-2 triol and T-2 tetraol are synthesized by *F. sporotrichioides*[5,7] and 4-acetyl NIV by *F. poae*.[28] A distinctive feature of trichothecene biosynthesis in *F. tricinctum* is the production of a wide array of compounds including T-2 toxin, DAS, NEO, as well as metabolites such as 15-acetoxyscirpenol and acetyl T-2 toxin.[22,29,30]

Other *Fusarium* species are also endowed with the capacity to elaborate trichothecenes and ZEN. Thus, *F. equiseti* produces HT-2, DAS, DON, 15-ADON, and FX,[8] while ZEN is a consistent metabolite.[6,8,23,31] *Fusarium avenaceum* and *Fusarium crookwellense* can synthesize DON and 15-ADON, and in addition, the latter species has been shown to produce FX, a property it shares with *Fusarium acuminatum*.[8] *Fusarium nivale* is capable of synthesizing 3-ADON from DON.[32]

At least seven *Fusarium* isolates producing ZEN may also synthesize closely related derivatives, including cis-ZEN, α- and β-zearalenol and α- and β-zearalanol.[11]

Mycotoxins other than the trichothecenes and ZEN may be synthesized by *Fusarium* species. *Fusarium moniliforme* is capable of producing the fumonisins, moniliformin and fusarin C.[1] The fumonisins comprise 6 structurally related metabolites, of which fumonisins B_1 and B_2 (FB_1 and FB_2 respectively) have been implicated in human and animal disorders.[33] Moniliformin is also produced by *F. avenaceum*,[34] *F. sporotrichioides*, *F. culmorum*,[35] and *Fusarium oxysporum*.[36] In addition, *F. oxysporum* is an established source of the hemorrhagic factor, wortmannin[37] and fusaric acid.[38]

20.3 NATURAL OCCURRENCE OF *FUSARIUM* MYCOTOXINS

The ubiquitous distribution of *Fusarium* species particularly in association with cereal plants predisposes to at least some mycotoxin contamination of grain when appropriate environmental conditions prevail. Scott[39] has provided an exhaustive survey of the global occurrence of the trichothecenes in foods, mainly cereal grains. Since that review in 1989, more evidence has emerged of world-wide contamination of foods and feeds with trichothecenes and other *Fusarium* mycotoxins.

20.3.1 Trichothecenes and Zearalenone

Scott[39] recorded values for DON in the range 0.01 to 20 μg g^{-1} in cereal grains and their by-products in Germany. Values for DAS, NIV, T-2 toxin, and HT-2 toxin varied from 0.05 to 32, 0.01 to 0.94, 0.065 to 14, and 0.1 to 10 μg g^{-1} respectively in positive samples. A more recent study with wheat in Germany[40] yielded similar levels for DON (0.004 to 20.5 μg g^{-1}), but values were markedly lower for NIV (0.003 to 0.032 μg g^{-1}), T-2 (0.003 to 0.25 μg g^{-1}) and HT-2 (0.003 to 0.02 μg g^{-1}). Levels of DAS were below the detection limit, but values

for ZEN ranged from 0.001 to 8.04 μg g^{-1}. As might be expected (Table 20.1), ZEN occurred with DON in a significant number of samples (20%).

In Polish wheats, Scott[39] recorded values of 0.007 to 30 μg g^{-1} for DON but recent data[41] suggest higher levels in samples from central Poland, at 2 to 40 μg g^{-1}, while NIV was lower at 0.01 μg g^{-1}, compared with 0.003 to 0.35 μg g^{-1} previously.[39] In addition, the co-occurrence of 3-ADON with 15-ADON was observed in the recent Polish study[41] while ZEN levels of 0.01 to 2 μg g^{-1}were also reported.

In the survey of Scott,[39] concentrations of DON in feeds and grains in Finland ranged from 0.001 to 0.12 μg g^{-1}, but a recent study in that country[42] indicated higher values in the range 0.007 to 0.3 μg g^{-1}, with 3-ADON at 0.013 to 0.12 μg g^{-1} and ZEN at 0.022 to 0.095 μg g^{-1}. Six lots of oats contained toxic levels of DON (1.3 to 2.6 μg g^{-1}).

Values up to 18 μg g^{-1} for DON in corn samples in Italy were listed by Scott,[39] while ranges for other trichothecenes were: NIV, 0.08 to 0.2 μg g^{-1}; T-2, 0.05 to 0.30 μg g^{-1}; and DAS, 0.15 to 0.30 μg g^{-1}. The occurrence of DON, ZEN, and zearalenols in corn plants infected with stalk rot caused by *Fusarium* species has also been reported recently in southern Italy.[43]

A study in the Netherlands[44] revealed a relatively high frequency of contamination of cereal grains with *Fusarium* mycotoxins, including the natural co-occurrence of DON, NIV, and ZEN. DON concentrations (μg g^{-1}) varied from 0.020 to 0.231 in wheat, 0.004 to 0.152 in barley, 0.056 to 0.147 in oats, and 0.008 to 0.384 in rye. Levels (μg g^{-1}) of NIV were 0.007 to 0.203 for wheat, 0.030 to 0.145 for barley, 0.017 to 0.039 for oats, and 0.010 to 0.034 for rye. For ZEN, data (μg g^{-1}) ranged from 0.002 to 0.174 in wheat, 0.004 to 0.009 in barley, and 0.016 to 0.029 in oats, with a single value for rye at 0.011 μg g^{-1}.

In Japan, there is evidence of consistent and occasionally severe contamination of cereal grains with DON and NIV. Values in positive samples of up to 50 and 37 μg g^{-1} respectively were listed by Scott.[39] More recently, Sugiura et al.[28] reported contamination of wheat in seven locations in Japan, with DON at 0.03 to 1.28 μg g^{-1}, NIV at 0.04 to 1.22 μg g^{-1}, and ZEN at 0.002 to 0.025 μg g^{-1}. In a subsequent study,[45] ZEN levels of 0.010 to 0.658 μg g^{-1} were reported for 14 barley samples.

Data relating to trichothecene contamination of grains and feeds in North America is voluminous, as indicated by the survey of Scott.[39] The impetus has been maintained, particularly in relation to DON,[46] with values ranging from nondetectable to 9.3 μg g^{-1} in wheat grains harvested in the U.S. in 1991. Highest levels were seen in wheat from Missouri, North Dakota and Tennessee. In another survey of the 1991 harvest in the U.S.,[47] values for DON ranged from <0.1 to 4.9 μg g^{-1} in winter wheat and <0.1 to 0.9 μg g^{-1} in spring wheat. In the 1993 harvest, 483 wheat samples had DON concentrations ranging from <0.5 to 18 μg g^{-1}, with 86% of samples from Minnesota and up to 78% of samples from North and South Dakota containing levels in excess of 2 μg g^{-1}. In 147 samples of barley from that harvest,[47] DON concentrations ranged from <0.5 to 26 μg g^{-1}. A new advisory was issued by the FDA, recommending a maximum DON level of 1 μg g^{-1} in finished wheat products destined for human consumption, and 5 to 10 μg g^{-1} in animal feeds. In a study of mycotoxin contamination of grain in Atlantic Canada,[48] DON and ZEN were detected in respectively 53 to 62% and 25 to 29% of samples tested. Most of the values for DON and ZEN were equal to or less than 0.5 and 0.3 μg g^{-1}, respectively. Of 55 samples tested, five had levels of T-2 toxin ranging from 0.16 to 0.31 μg g^{-1}, two contained HT-2 toxin (0.12 and 0.44 μg g^{-1}), and two were contaminated with DAS (both at 0.11 μg g^{-1}).

20.3.2 Fumonisins

World-wide contamination of corn with fumonisins has been reported,[33] with samples from Italy, Portugal, Zambia, and Benin containing FB_1+ FB_2 levels of up to 2.85, 4.45, 1.71, and

3.31 μg g^{-1}, respectively, and with incidence rates of 82 to 100%. In India, FB_1 levels of 300 to 366 μg g^{-1} have been reported in corn infected with *F. moniliforme*.[49]

20.4 FACTORS AFFECTING PRODUCTION OF *FUSARIUM* MYCOTOXINS

The production of *Fusarium* mycotoxins is affected by a diverse array of factors, broadly divisible into biological, physical, and chemical. It is recognized, however, that complex interactions among these factors may exert significant effects on mycotoxin synthesis.

20.4.1 Biological

Wide variations exist in toxigenic potential among different strains of a particular *Fusarium* species.[13] Nevertheless, many of the toxigenic species of *Fusarium* are also pathogenic towards cereal plants. For example, head blight of cereals may be caused by *F. graminearum* and *F. culmorum*, both of which are recognized DON producers (Table 20.1). A direct quantitative assessment of the link between head blight and DON contamination of wheat grains has recently been elucidated in the Netherlands,[13] using genotypes of differing resistance to the disease. A striking correlation was evident between the severity of head blight and DON concentrations in wheat kernels. Work in Canada also supports the view that wheat grains from cultivars susceptible to head blight contain more DON than those from Chinese genotypes which are recognized to be resistant.[10]

At harvest, seeds are likely to be colonized by different species of fungi[50] and mycotoxin production may be affected by fungal interactions, for example during storage.[21]

20.4.2 Physical

A variety of interacting physical factors may affect mycotoxin production under field conditions and during storage.[51] In a number of countries, *Fusarium* head blight has been associated with years of high rainfall. It is also now believed that rain impact may play an important role in the dispersal of *Fusarium* inoculum and in the development of head blight epidemics.[52] It is of interest that the higher than average levels of DON in wheat samples from Missouri, North Dakota, and Tennessee were tentatively attributed to increased rainfall in these states.[46]

Laboratory studies indicate that both time and water activity affect FB_1 production in corn kernels.[53] Maximum synthesis occurred at 21 days of incubation at a water activity of 1, but on lowering water activity to 0.95, maximum production of FB_1 did not occur until 47 days had elapsed. However, both laboratory and field studies[14,18] provide evidence that DON production declines with time. There is some controversy regarding the effects of temperature on ZEN synthesis in laboratory cultures, with Merino et al.[54] maintaining the need for thermic shock while others[14,31,55] have reported ZEN production at constant temperatures.

20.4.3 Chemical

The chemical definition of media which promote mycotoxin production from field isolates of *Fusarium* species has been attempted. Depletion of carbohydrate appears to be an important stimulus for DON production,[3,19] while T-2 formation is enhanced by the presence of sorbic acid.[56]

As might be expected, fungicides can influence mycotoxin production, but the effects are variable and dose-dependent (Table 20.2). Insecticides may inhibit mycotoxin production in *Fusarium* molds,[16,58] but type and dose level of the insecticide are important determinants of efficacy.

TABLE 20.2
Effects of Fungicides on Production of *Fusarium* Mycotoxins

Fungicide	Methods	Effects	Ref.
Dicloran, iprodione vinclozolin	Added separately at levels of up to 500 μg ml^{-1} potato-dextrose broth; static culture of *F. graminearum*	Dose-related inhibition of growth and production of DAS and ZEN; total inhibition of DAS and ZEN production at higher levels of each fungicide	25
Propiconazole, thiabendazole	Field trial with wheat; heads sprayed with *F. graminearum* inoculum 2 days after anthesis; fungicides applied separately prior to, during and after inoculation; propiconazole applied at 120 g ha^{-1}, thiabendazole at 360 g ha^{-1}	Propiconazole reduced *Fusarium* infection by 39–55%; DON levels reduced by 34–78%. Thiabendazole had no effect on infection level, but DON levels reduced by up to 83%	24
Propiconazole, thiabendazole, tebuconazole	Field trial with wheat inoculated with *F. graminearum* at 3 stages from beginning of flowering; fungicide rates: propiconazole, 140; thiabendazole, 280; tebuconazole, 140 g ha^{-1}	Head blight incidence ranged from 83 to 84% irrespective of fungicide (control, 87%); DON levels ranged from 12.9 to 16.7 mg kg^{-1} (control, 12.0 mg kg^{-1})	26
Tebuconazole with triadimenol (Matador)	Field trial with wheat; heads inoculated with *F. culmorum*; Matador applied at 11 ha^{-1}	Fungicide reduced head blight; 16-fold increase in NIV content of grain from fungicide-treated plants	15
Tridemorph	Shake-flask cultures of *F. sporotrichioides* in a defined medium; fungicide added at 6 and 36 μg ml^{-1}	At 6 μg ml^{-1}, growth enhanced but T-2 toxin and DAS production inhibited; at 36 μg ml^{-1}, growth inhibited but T-2 production stimulated	4
Carbendazim	Cultures of *F. sporotrichioides* on potato-dextrose agar; fungicide added at 0.1 and 1.0 μg ml^{-1}	Growth unaffected by fungicide; 6-fold increase in T-2 toxin production with fungicide at 0.1 μg ml^{-1} and small increases in ZEN and NEO production	57

20.5 TOXICOLOGY

20.5.1 Risk Assessment

Food safety, animal health and productivity, and human health problems identified with fungal-contaminated grains most recently have concentrated research on *Fusarium* species and their toxic metabolites. Major world-wide agricultural problems with *Fusarium* have been recognized since: *F. moniliforme* and the fumonisins have been associated with equine leukoencephalomalacia;[59–61] the human oncologic implications with esophageal cancer in certain areas of South Africa, China, northern Italy, and possibly Iran;[62–69] and the routine occurrence of the fumonisins and other *Fusarium* toxins in corn, wheat, barley, rice, and other cereal grains.[70–73] *F. moniliforme* infection of corn can be asymptomatic and presents its greatest concerns since fungal toxins surreptitiously enter animal and human foods.[74] Although *Fusarium* species and their toxins appear to be most prevalent in corn, wheat, and barley, they are also found in nuts, fruits and vegetables, and in non-food items of economic importance (e.g., tobacco, cotton, forage grasses, alfalfa, red clover, and flax).[71,75] Corn, wheat, and barley comprise two thirds of the world cereal production and there are over 24 *Fusarium*

species associated with animal and human health problems.[76] Toxigenic *Fusarium* species, therefore, are a major agricultural problem not only because of human and animal health, but also because of losses due to plant diseases, losses incurred by grain and livestock producers, and effects of contaminated grain on export/import markets.[77]

20.5.2 Structural Diversity and Biological Activity

The spectrum of *Fusarium* metabolites that affect animal and human health are not only the most divergent structurally unrelated, but also are the most divergent biologically acting group of compounds known.[76,78] The fumonisins are pentahydroxyicosanes, structurally similar to sphingosine and dihydrosphingosine, the interference of which in ceramide synthesis is detrimental to cell maintenance.[60,73,79–81] DON is a 12,13-epoxytrichothecene associated with feed refusal and emetic responses primarily in swine.[75,78,82] Zearalenone is a β-resorcylic acid lactone associated with hyperestrogenic activity also in swine,[83] while the fusarins are pyrrolopolyketides of which fusarin C is mutagenic.[84,85] Beauvericin, a cyclic hexadepsipeptide consisting of three N-methylphenylalanyl- and three 2-hydroxy-3-methyl-butyric acid residues in a continuous-alternating sequence, is reported toxic to both mammalian and insect cell lines.[86,87] Fusaproliferin is a new sesterterpene toxic to both brine shrimp larvae and human B-lymphocytes cell line.[88]

Several fumonisins have been identified from *F. moniliforme*-infected grains.[60,72,73] FB_1 is 2-amino-12, 16-dimethyl-3,5,10,14,15-pentahydroxyicosane with a propane-1,2,3-tricarboxylate substituent at C-14 and C-15. FB_2 and FB_3 are the C-10 and the C-5 deoxy analogues, respectively, whereas FB_4 lacks the hydroxyl moiety at both C-5 and C-10. In addition, fumonisin AB_1 and AB_2 have been defined as the corresponding N-acetyl-analogues of FB_1 and FB_2, respectively, and the N-acetyl-C15-keto form of FB_1 has also been reported.[89]

The C-22-aminopentol "backbone" of FB_1, commonly referred to as hydrolyzed FB_1, structurally resembles the free sphingoid bases, sphingosine and sphiganine (dihydrosphingosine).[60,73,79–81] These bases are necessary for ceramide synthesis, which is important to both mammalian and plant cell maintenance and viability.[79,80,90,91] FB_1, FB_2, and hydrolyzed FB_1 (i.e., the C-22-aminopentol backbone) are specific inhibitors of *de novo* sphingolipid biosynthesis, the primary target being sphinganine N-acetyltransferase.[70,79,81] These effects cause an abnormal accumulation of the free sphingoid bases, which are extremely detrimental to cellular function.[80] Analogous inhibitions have been reported with the structurally similar AAL toxins from *Alternaria alternata* f. sp. *lycopersici*.[90,91]

The carcinogenic potential of FB_1 in laboratory animals[62,65] and the statistical correlation of *F. moniliforme*-infected corn and FB_1 with human esophageal cancer has been established.[61–69] Additionally, the possible mechanisms of fumonisin-induced disruption of sphingolipid metabolism and carcinogenesis has been discussed in detail.[61,69] Disruption of *de novo* sphingolipid metabolism also has been correlated with *F. moniliforme* and FB_1-induced hepato- and nephrotoxicity in rats,[92,93] porcine pulmonary edema,[94] and dietary exposure to FB_1 in equidae.[95] These mechanisms are indeed consistent with FB_1-induced chronic hepatotoxicity in primates with an inference to atherosclerosis.[96] The inhibition of sphingolipid synthesis by FB_1 also reduces ganglioside synthesis and affects axonal growth in cultured hippocampal neurons.[97] Whether disruption of sphingolipid metabolism is directly related to liquefactive necrosis in the brain of equines, pulmonary edema in swine, and/or certain syndromes in poultry remains to be determined. However, increases in the sphinganine to sphingosine ratio occur long before any indication of toxicity,[92,98] and the changes in the relative amounts of free sphinganine and sphingosine may be used as a biomarker for animals consuming fumonisins.[94,95,98] Similar results have been reported in plants exposed to both fumonisin(s) and the AAL toxins.[91] Interestingly, FB_1, FB_2, and the AAL toxins have similar effects in cultured mammalian cells.[99]

Although DON is only one of several mycotoxins produced by *Fusarium* species, it is among the most frequent trichothecenes analyzed in cereal crops.[68,78] Animal and public health concerns stem from its occurrence primarily in wheat, corn, and barley and in feed and food commodities derived from these crops. Swine appear to be the most sensitive to the effects of DON; however, several cases of human toxicity have been attributed to the chronic consumption of flour/bread contaminated with DON.[68] The hazard assessments of DON (and other trichothecenes), which are based on toxicology data (i.e., inhibition of protein and DNA synthesis; reproductive, embryotoxic, and possible teratogenic effects), estimate a minimum consumption level at 0.6 mg kg^{-1} body weight (i.e., that which produces no adverse effect).[100] Current guidelines for DON in Canadian wheat are 2 mg kg^{-1} in uncleaned soft wheat used for nonstaple foods, including bran, and 1 mg kg^{-1} in soft wheat destined for infant food.[100]

ZEN and related metabolites are nonsteroidal estrogenic mycotoxins produced by several species of *Fusarium*[78,83] (Table 20.1) and associated with hyperestrogenism in swine, and reproductive problems in cattle and sheep.[83,101] In swine, hyperestrogenism is a well defined syndrome,[83] and although these animals appear to be more sensitive to ZEN and DON, poultry and ruminants seem more tolerant.[78]

20.5.3 INTERACTIONS

Guidelines regulating the amount of mycotoxins found in agricultural commodities used for both animal and human consumption are generally based on toxicologic investigations with pure compounds. Fungal contamination of food and feed, however, rarely involve exposure to a single toxin. *Fusarium* species are common pathogens of a wide variety of crop plants and there is high potential for mycotoxin contamination of many foodstuffs. Routinely, animal toxicity problems occur in which the quantity of the individual mycotoxins found in the suspected feed(s) does not explain the observed syndromes.[68,77,78,100] Currently, the combined effects of mycotoxins on animal and human health have aroused concern because synergistic activities present a unique set of problems in defining both toxicity and food safety guidelines.[77,78]

Dowd[102] has discussed in detail the benefits fungi derive from producing simple molecules that act as synergists with other more complex molecules and the probability that this synergism is widespread in nature. Some of the benefits proposed are energy efficient mechanisms for host invasion, protective mechanisms for survival, and competitive advantage. The endophytic association of *F. moniliforme* and *F. proliferatum* to the corn plant and the external and systemic association to the kernel[71,74] suggest some symbiotic relationships within the *Fusarium*-host associations. Other fungal endophytic-plant interactions that confer insect resistance, drought tolerance, and increased competition with other plants (i.e., allelochemicals) have been described in Chapter 4.

Fusaric acid (5-butylpicolinic acid) is a common metabolite of several *Fusarium* species including *F. moniliforme*, *F. oxysporum*, *F. subglutinans*, and *F. crookwellense*,[75,78,103] and its co-occurrence with other *Fusarium* toxins (e.g., ZEN, fumonisins, DON, and other trichothecenes has been reported.[104] The 10,11-dehydro; 10,11-dihydroxy-; 10-hydroxy-; and the 11-carboxy-analogues of fusaric acid have also been identified from cultures of *F. moniliforme*,[105,106] but fusaric acid appears to be the major analogue occurring in *Fusarium*-contaminated plants and grains. Although of minor toxicity at the levels detected in nature,[104,107,108] there is increasing evidence that fusaric acid may enhance the activity of other *Fusarium* toxins.[71,102–104,107,109,110] This was recognized in tests with certain insects in which DAS, DON, and T-2 toxicity were potentiated by fusaric acid.[102] Furthermore, fusaric acid enhanced the activities of certain insecticides by inhibiting oxidative enzymes responsible for toxin metabolism by insects.[102]

Bacon et al.[103] reported a toxic interaction between fusaric acid and FB_1 in the fertile chicken egg. Each mycotoxin at 5 μg per egg in sterile water produced virtually no effects on the eggs (pip) incubated for 21 days. However, a combination of fusaric acid and FB_1 at 5 μg each per egg was lethal to 46% of eggs. The lethal effects of this combination were dose-dependent and enhanced in a phosphate buffer solution.

Fusaric acid, DON, and ZEN were the major mycotoxins isolated from suspect duck and ostrich feed, and individually were below levels generally considered to cause problems in poultry.[104,108] However, in a 3-week feeding trial using day-old broiler chicks, turkey poults, and ducklings, the feed caused a significant dose-dependent growth depression and mortality, consistent with observed toxicities on both duck and ostrich farms.[104]

The lactational transfer of fusaric acid from the feed of nursing dams to the neonate rat[107] and the potentiation by this mycotoxin of the adverse effects of FB_1 in the fertile chicken egg[103] has raised concern about their synergistic effects. Porter et al.[107] observed fusaric acid concentrations in the stomach colostrum taken from 4-day old rats were directly proportional to the quantity of the mycotoxin in the feed of the nursing dams; these observations were consistent in both F_1 and F_2 generations. Additionally, in both male and female weanlings (21-day old rats), serotonin and tyrosine were decreased in the pineal gland with concurrent increases in serum and pineal melatonin in these animals.[107,111] In adult rats, parental administration of fusaric acid increased tyrosine, serotonin, and dopamine in the brain with concomitant increases of N-acetylserotonin in the pineal gland.[104] Altered neurochemical effects in the brain and pineal gland were partially attributed to fusaric acid inhibiting dopamine-β-hydroxylase and tyrosine-hydroxylase activities and indirectly on its peripheral effects on adrenal catecholamines and serotonin.[104,107] Fusaric acid also elevated serotonin in the brain of swine,[109] and the potential synergistic significance of co-occurring fusaric acid and DON has been proposed in feed refusal and emesis in these animals.[110] Aberrant changes with serotonin and melatonin and/or other neurotransmitters in the brain and pineal gland have been related to adverse effects on seasonal physiological and endocrine changes in both animals and humans.[104,107,112] Therefore, in addition to its synergistic potential, fusaric acid has diverse neurochemical effects that may negatively influence animal productivity, maturation and behavior.

Miller et al.[84] have reported the production of the fumonisins with the fusarins by *F. moniliforme* and suggested that the variable toxicity of fungal extracts may be due to this combination. Although the fusarins A through F have been isolated and characterized, fusarin C has been primarily implicated in the mutagenic activity of *F. moniliforme*.[84,85,113] Following the correlation of FB_1 (and FB_2 and FB_3) with cancer induction and promotion,[114] it is not too inconceivable that the fusarins (e.g., fusarin C) could enhance these activities.

Madahyastha et al.[113] have shown synergistic responses with a combination of T-2 and HT-2; T-2 and T-2-4ol; DON and NIV; or DON and T-2 in a yeast bioassay. Harvey et al.[115] described the synergistic effects of FB_1 and DON on reduced bodyweight gains and serum biochemical indices in growing barrows. To complicate matters even more, varying degrees of antagonism have been observed with other trichothecenes.[113]

Beauvericin is produced by *F. subglutinans, F. proliferatum*, and *F. moniliforme* and fusaproliferin is produced by *F. proliferatum*.[86,88] Although their role in animal and human pathology has yet to be determined, both have been reported to co-occur with FB_1 and moniliformin.[86–88]

20.6 CONCLUSIONS

Fusarium molds may produce a diverse range of mycotoxins. Since these fungi are also pathogenic to cereal plants, it is likely that grain from infected plants may become contaminated with one or more of the *Fusarium* mycotoxins. Although fungicides are generally perceived as effective agents in the control of *Fusarium* diseases, there is limited evidence

to indicate that at sublethal levels some fungicides may enhance mycotoxin production. This effect may be significant in view of proposals to reduce fungicide applications for environmental reasons and also in the context of the development of fungicide resistance in *Fusarium* molds. The co-occurrence of *Fusarium* mycotoxins[116] in cereals should be considered in any assessment of risk for human and animal health since potentiating effects have been observed in mammalian systems. The co-occurrence of the fumonisins, fusarins, moniliformin, the trichothecenes (primarily DON), ZEN, and fusaric acid not only complicates toxicologic investigations but also creates major difficulties in defining tolerance limits and regulatory guidelines.

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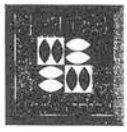
8.1.8 Poster 1 "Studies on the mycology of and potential for mycotoxin production in barley grain" presented at the fifth International Mycological Congress - Vancouver Canada - 1994



Fifth International
Mycological Congress

ABSTRACTS

August 14-21, 1994
Vancouver, British Columbia
Canada

STUDIES ON THE MYCOLOGY OF, AND POTENTIAL FOR MYCOTOXIN PRODUCTION IN BARLEY GRAIN

D'Mello, J.P.F., Macdonald, A.M.C., Parent, B.J.F. and Placinta, C.M. The Scottish Agricultural College, West Mains Road, Edinburgh, EH9 3JG, Scotland.

The purpose of this research was to assess the potential for fungal contamination and mycotoxin production in grains harvested from different cultivars of spring barley grown with or without fungicide applications, and at selected sites in Scotland.

Mycological infection was assessed by surface plate count and by direct spotting of grain. Fungal organisms were identified to genus level. A multi-mycotoxin screening procedure based on thin layer and high performance thin layer chromatography (TLC and HPTLC) was employed to analyse chloroform extracts from barley grains. These extracts were also used in cytotoxicity tests with Hep-2 and BHK mammalian cell lines.

The mycological assessments indicated relatively low but consistent patterns of infection of barley grains at harvest, principally with *Fusarium* and *Alternaria* species, and to a lesser extent with *Penicillium* and *Aspergillus* species. Infection of grain occurred irrespective of cultivar or fungicide treatment.

The multi-mycotoxin screening technique revealed the presence of several metabolites in the extracts of all samples, again irrespective of cultivar or fungicide treatment. Studies are in progress to identify and quantify these substances. Isolates from the grain samples will be used to assess potential for mycotoxin production.

In cytotoxicity tests, a number of extracts were found to be lethal to the mammalian cell lines. The results also confirmed that cytotoxicity tests with cell lines are more sensitive than the TLC screening procedure.

Further work needs to address any link between the compounds isolated on TLC and HPTLC and the incidence of cell lethality.

8.1.9 Poster 2 "The influence of carbendazim on mycotoxin production in *Fusarium sporotrichioides*" presented at Brighton Crop Protection Conference "Pest and Diseases" - 1996

8.1.10 Poster 3 "Disparate effects of temperature and fungicides on mycotoxin production in the phytopathogen, *Fusarium sporotrichioides*" presented at "Crop Protection& Food Quality: Meeting customer needs", University of Kent,Canterbury - 1997

8.2 Mycotoxin standards

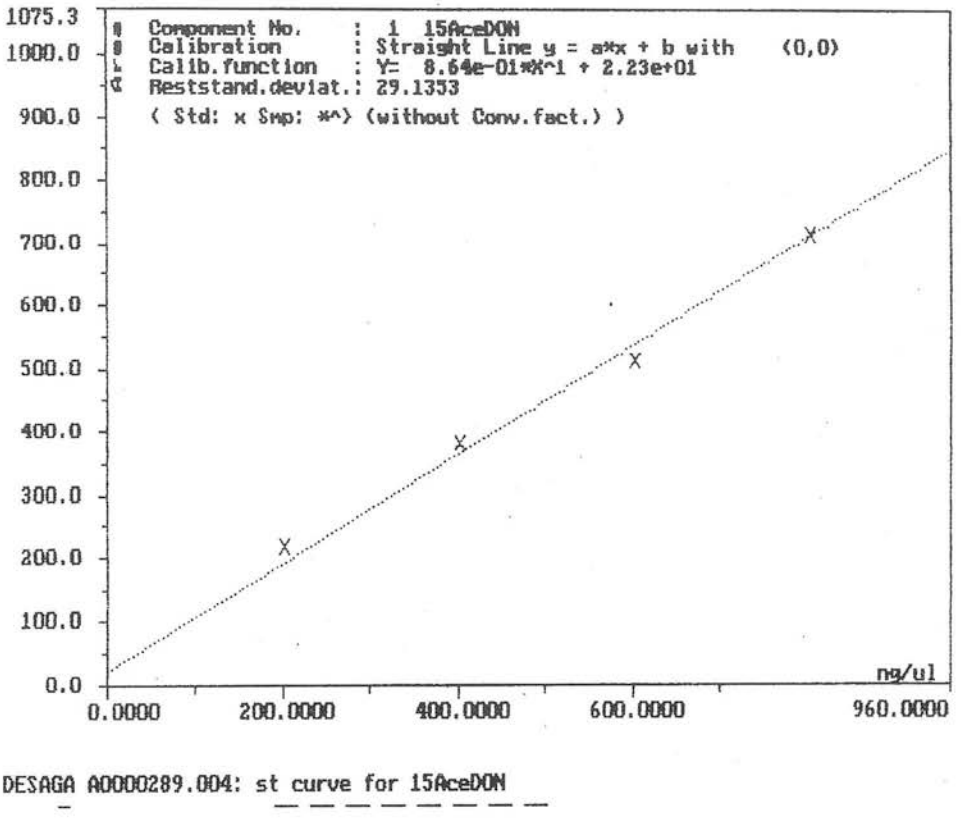


Figure 8.1 Standard curve for 15-ADON

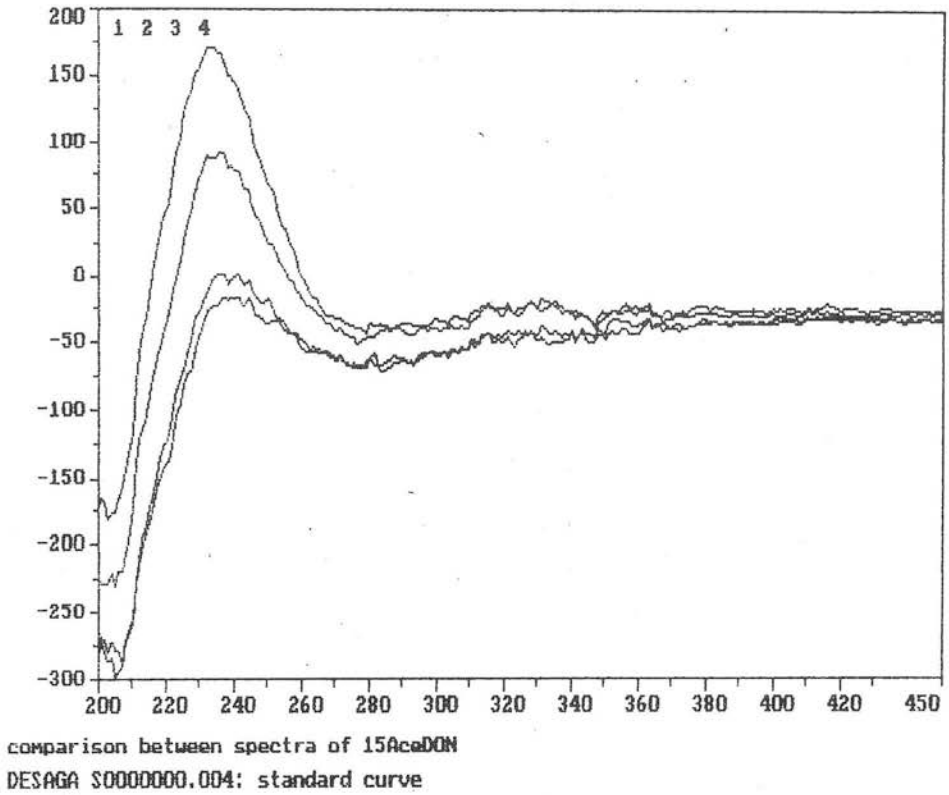


Figure 8.2 Spectral scan of 15-ADON at different wavelengths (nm)

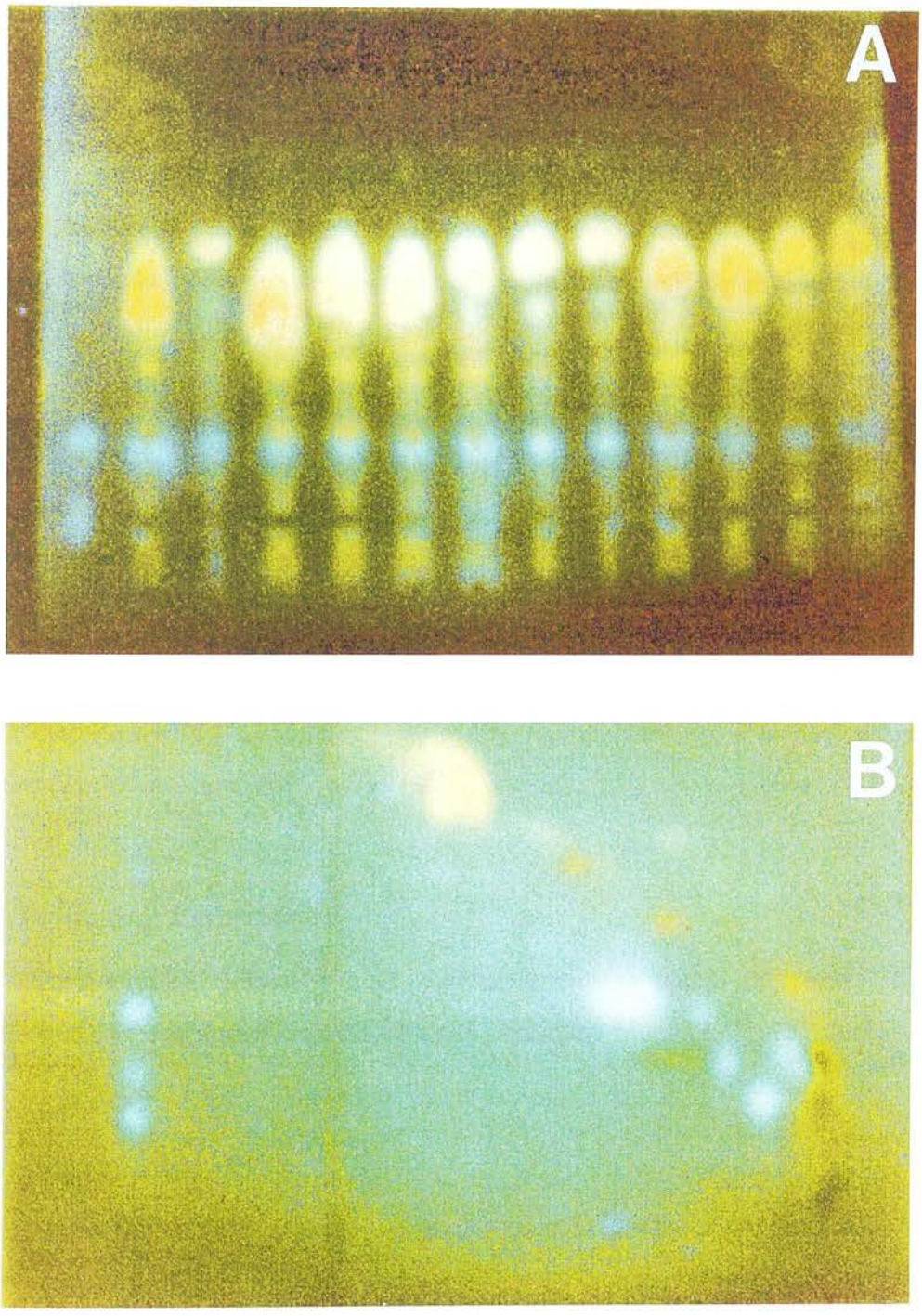


Plate 8.1 TLC plates visualized under UV light. (A) represents a one way TLC and (B) pictures the section of a two dimensional TLC plate developed in TEF.

8.3 Buffers and reagents

8.3.1 DEPC water

To a litre of deionized water, 900 µl DEPC were added using filtered tips. The treated Duran bottle was closed and shaken vigorously by hand until foam formed. The lid was taken off and the bottle left open in a fume hood for at least two hours (overnight), followed by autoclaving two times at 120°C for 15 minutes.

8.3.2 Sodium acetate $C_2H_3NaO_2$, 3 H_2O (3M)

Sodium acetate anhydrous (BDH) was used. The amount of 246.09 g was weighed in a glass beaker (1 l) and deionized water poured up to the 800 ml mark. The beaker was placed on a hot magnetic stirrer in order to help the sodium acetate dissolve. The pH was checked and brought to 5.2 using glacial acetic acid and the content brought up to one litre with DEPC water. The buffer was then divided into treated Duran bottles (2x 500 ml) and DEPC added (450 µl/bottle).

8.3.3 Phenol: Chloroform

To 400 ml saturated phenol, 400 ml of chloroform were added. The treated Duran bottle was wrapped with aluminium foil and kept in the fridge (4°C) at all times.

8.3.4 Guanidine (8M)

The amount of 382.2 g guanidine was measured in a flask/beaker and deionized water added up to 400 ml level. Then 1.953 g MES (20mM) and 3.725 g EDTA (20mM) were added to the guanidine. The solution was stored in the fridge.

Note: Before working with this buffer, the pH was checked and brought to 7.0 using NaOH, the solution filter sterilised, followed by the addition of 175 µl β-mercaptoethanol / 50 ml guanidine, in a fume hood.

8.3.5 NaOH

Sodium hydroxide used for adjusting the pH was prepared using DEPC treated water.

8.3.6 SSC

To 88.2 g Na citrate (0.3M) and 175.4 g NaCl (3M), deionized water was added up to a litre, the pH checked and adjusted to 7.0 with HCl and the solution autoclaved.

8.3.7 SDS

A stock solution of 10% was prepared by adding 100 ml water to 10 g SDS. In order to assist dissolution, it was heated at 68°C.

8.3.8 Acetic acid (1M)

Mwt 60.05

Wt per ml= 1.049 g

1M = 60.05 g per litre = 57.24 ml per litre

Acetic acid solution (1M) was prepared using DEPC treated water (15 ml acid acetic glacial and 235 ml water).

8.3.9 10xMOPS [3-(N-morpholino)-propane-sulphonic acid]

To 800 ml of DEPC treated water, 41.8 g of MOPS were added and the pH adjusted to 7.0. To this, 16.6 ml of 3M DEPC treated sodium acetate and 40.0 ml of 0.25M DEPC treated EDTA (pH 8.0) were added, the solution made up to a litre with DEPC treated water (~143.4 ml) and filter sterilised.

8.3.10 Hybridization buffer-"Gene Images CDP Star detection module"

5xSSC

0.1% (w/v) dextran sulphate

20-fold dilution of liquid block

In order to prepare 100 ml hybridisation buffer, to a measuring cylinder the followings were added:

20 ml 20xSSC

1 ml 10%SDS

5.0 g dextrane sulphate

5.0 ml liquid block

DEPC treated water was added up to the 100 ml mark, the buffer then transferred into a treated Duran bottle and 90 µl DEPC added to it, followed by autoclaving the next day.

8.3.11 Buffer A

100 mM Tris-HCl ($C_4H_{11}NO_3HCl$ 157.6)

300 mM NaCl (pH 9.5) (NaCl 58.44)

To prepare a litre of Buffer A, 15.76 g Tris-HCl and 17.532 g NaCl were measured and added to a treated beaker containing deionized water placed onto a "magnetic stirrer". The pH was checked and brought to 9.5, then the buffer dispensed into treated Duran bottles and autoclaved.

Aliquots of 45 ml Buffer A were dispensed into sterile 50ml plastic tubes (Greiner), 5 ml of liquid block (1:10 dilution) were added to each tube and placed in the freezer.

8.3.12 Loading buffer

Two types of loading buffer were used:

1) Loading buffer for DNA

To prepare 10 ml of loading buffer, the followings were added: 40 µl EDTA (0.25M, pH 8.0 prepared with DEPC treated water), 25 mg bromophenol blue (0.25%), 25 mg xylene cyanol (0.25%), 5 ml glycerol (50%) and DEPC treated water up to 100 ml.

2) Loading buffer for RNA

Sample loading dye

Northern Max^{M} Kit

6 ml (8550G) Ambion

Contains formaldehyde and/or formamide.

8.3.13 TE buffer (pH 8.0)

1 mM EDTA

10 mM TrisCl

TrisCl (0.79 g) was measured and 50ml water added (solution A). EDTA (0.186 g) was measured and 50 ml water added (solution B). Ten ml solution A and 10 ml solution B were added and the mixture made up to 100 ml with ultra pure water.

8.3.14 TAE buffer

A stock solution of TAEx50 was prepared by adding 24.2 g Tris, 5.71 ml acetic acid and 10 ml 0.5M EDTA (pH 8.0), and the mixture brought up to 100 ml with water.

8.3.15 TBE buffer

A stock solution of Tris-borate buffer was prepared. Per litre, the following ingredients were combined: 54 g Tris base, 27.5 boric acid and 20 ml 0.5M EDTA (pH 8.0). The working solution was 0.045M Tris-borate and 0.001M EDTA.

8.3.16 X - Gal

To 200 mg X - Gal 10 ml dimethylformamide were added, then placed at -20°C in the dark.

8.3.17 Ampicillin

A stock solution of ampicillin was prepared by adding 1 ml water to 50 mg ampicillin, sterilized by filtration and stored at -20°C.

8.4 Cloning of probes

1) Production of PCR products

The PCR products were generated using the relevant protocol, making sure that it ended with a final 7-10 minute extension step at 72°C. Each PCR sample (10 µl) was checked/analysed by gel electrophoresis.

2) Cloning into pCR 2.1

Ligation Reaction (10 µl):

Sterile water	2.0 µl
10xLigation buffer	1.0 µl
pCR 2.1 vector (25 ng/µl)	2.0 µl
Fresh PCR product (10 ng)	4.0 µl
T4DNA Ligase	1.0 µl

The ligation reactions were incubated at 14°C for at least 4 hours (preferably overnight). The ligation reaction was centrifuged briefly and placed on ice.

3) Transformation

The appropriate number of vials of One Shot cells (INVαF) were thawed on ice. Two µl of 0.5 M β - mercaptoethanol were pipetted into each vial and mixed by stirring gently with a pipette tip. To this, 1-2 µl of each ligation reaction were added into cells and stirred gently with pipette tip to mix. Vials were incubated on ice for 30 minutes followed by a heat shock for exactly 30 seconds in a 42°C water bath (do not mix). The vials were placed on ice for 2 minutes, then 250 µl of SOC (see 4.3.6)

medium added to each of them. Vials were placed on a shaker (225 rpm) at 37°C for exactly one hour, then vials with transformed cells were transferred on ice.

4) Analysis

Screening for recombinants was done by β-galactosidase presence or absence. This involved 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) which is broken down by β-galactosidase. The break down product was coloured deep blue. Non-recombinant colonies are cells which can synthesise β-galactosidase and present a blue coloration, whereas recombinants with a disrupted β-galactosidase gene are white. Therefore, white colonies were selected. Fifty µl and 200 µl from each transformation vial were plated on a LB plate containing 50 µg/ml ampicillin and X-Gal and incubated at 37°C for at least 18 hours. Plates were moved to 4°C for 2-3 hours for colour development. Ten white transformations were analysed for the presence and orientation of insert by PCR using M13 primers (see 4.7.13.4 -3).

8.5 Preparation of cloned probes

8.5.1.1 Extraction and purification of plasmid DNA

This method involves 3 steps, namely: growth of bacterial culture, harvesting and lysis of bacteria and purification of plasmid DNA.

Growth of bacterial culture

A single bacterial colony was transferred into 2.0 ml of LB media containing ampicillin in a loosely capped 15 ml tube. The culture was incubated overnight at 37°C with vigorous shaking.

Harvesting and lysis of bacterial culture

- **Harvesting**

The next day, 1.5 ml of the culture was transferred into a centrifuge tube, followed by centrifugation at 12,000 rpm, 4°C for 30 seconds in a microfuge. The remainder of the culture was stored at 4°C. The supernatant was removed with a disposable pipette.

- **Alkali lysis**

This protocol is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

The bacterial pellet was resuspended in 100 µl of ice cold Solution I (50 mM glucose, 25 mM Tris- HCl, pH 8.0 and 10 mM EDTA, pH 8.0) by vigorous vortexing. Solution I can be prepared in batches of 100 ml autoclaved for 15 minutes and stored at 4°C.

To this, 200 µl of freshly prepared Solution II (0.2 N NaOH, freshly diluted from a 5N stock and 1% SDS) was added.

(Made up 2.0 ml: 80 µl 5NaOH, 200 µl SDS, 1720 µl water)

The tubes were closed tightly and the contents mixed by inverting the tube rapidly 5 times. It was made sure that the entire surface of the tube came in contact with Solution II, then the tubes were stored on ice. To this, 150 µl of ice cold Solution III (1200 µl of 5M potassium acetate, 230 µl of glacial acetic acid and 570 µl water) were added.

The tubes were closed and vortexed gently in an inverted position for 10 seconds to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 3-5 minutes, followed by centrifugation at 12,000 g, 4°C for 5 minutes in a microcentrifuge.

3) Purification

The supernatant was transferred to a fresh tube and an equal volume of phenol: chloroform (450 µl) added and mixed by vortexing. After centrifuging at 12,000 for 2 minutes at 4°C in a microfuge the supernatant was transferred to a fresh tube. The double stranded DNA was precipitated with 2 volumes of ethanol (900 µl) at room temperature, followed by vortexing. The mixture was allowed to stand for 2 minutes at room temperature, followed by centrifuging at 12,000 rpm, 4°C for 5 minutes in a microfuge. The supernatant was removed by gentle aspiration and the tube left in an inverted position on a paper towel to allow all of the fluid to drain away. Any drops of fluid adhering to the walls were removed, and the pellet of double stranded DNA rinsed with 1.0 ml of 70% ethanol at 4°C. The supernatant was removed as described in the previous step and the pellet of nucleic acid allowed to dry for 10 minutes. Then, the nucleic acids were redissolved in 50 µl of ultra pure water containing DNase free pancreatic RNase (20 µg/ml), vortexed briefly, and stored at -20°C

8.6 Purification of DNA from agarose gels

Several protocols of DNA purification from agarose gel were employed. These include the following kits: WIZARD™ PCR Prep Purification kit, QIAEX II Gel Extraction Kit and DNAce purification system.

8.6.1.1 WIZARD™ PCR Prep Purification kit (PROMEGA)

There were two options when using this kit namely purification without or with vacuum manifold.

Purification without vacuum manifold

The PCR reaction products were separated by electrophoresis in a TAE agarose gel (TBE agarose gels were not recommended when using Wizard™ PCR Preps.). The desired DNA band was visualised on a transilluminator (UV light) and excised quickly (to minimize exposure of the DNA to UV light) with a clean, sterile razor blade or scalpel. The bands were isolated in approximately 300 µl (300 mg) of agarose. The agarose slice was transferred into a 1.5 ml microcentrifuge tube; 1 ml resin added to it and incubated at 65°C in a water bath for 5 minutes or until the agarose was completely melted (resin/DNA mix). For each PCR product a Wizard™ Minicolumn was prepared. The plunger from a 5.0 ml Luer-Lok® disposable syringe was removed and set aside and the syringe barrel was attached to the Luer-Lok® extension of each minicolumn. The resin/DNA mix was pipetted into the syringe barrel. The syringe plunger was inserted slowly and gently the slurry was pushed into the minicolumn.

The syringe was then detached from the minicolumn and the plunger removed. The syringe barrel was then reattached to the minicolumn and 2.0 ml of 80% isopropanol pipetted into the syringe in order to wash the column. The syringe plunger was inserted into the syringe and gently the isopropanol was pushed through the

minicolumn. The syringe was removed and the minicolumn transferred to a 1.5 ml microcentrifuge tube. The minicolumn was centrifuged for 2 minutes at 10,000xg to remove excess wash solution. The minicolumn was then transferred to a new microcentrifuge tube and 30 µl of water added to it and left for 1 minute. The minicolumn was then centrifuged for 20 seconds at 10,000xg in order to elute the bound DNA fragment. The minicolumn was removed and discarded and the purified DNA stored in the microcentrifuge tube at 4 or -20°C.

Purification with vacuum manifold

When the possibility of obtaining a vacuum manifold (Promega VacMan® Laboratory Vacuum Manifold) occurred, the following method was tested. For each PCR product, one Wizard™ Minicolumn was prepared. The provided syringe barrel was attached to the Luer-Lok® extension of each minicolumn. The tip of the minicolumn/syringe barel assembly was inserted into the vacuum manifold. The resin/DNA mix obtained as in the method described above, was pipetted into the syringe barrel. Vacuum was applied in order to draw the resin/DNA mix into the minicolumn. The vacuum was then broke and 2.0 ml of isopropanol 80% were added and the vacuum reapplied in order to draw the solution through the minicolumn. Continuing to draw vacuum for 30 seconds after the solution had been pulled through dried the resin. The syringe barrel was removed and the minicolumn transferred to a 1.5 ml microcentrifuge tube, centrifuging it for 2 minutes at 10,000xg to remove any residual isopropanol. The minicolumn was then transferred to a new microcentrifuge tube and 30 µl of water added, waited for 1 minute and then centrifuged the minicolumn for 20 seconds at 10,000xg to elute the bound DNA fragment. The minicolumn was removed and discarded and the microcentrifuge tube containing the purified DNA stored at 4 or -20°C.

Further purification steps to increase purity

After purification with the minicolumn a total volume of 30 µl was obtained. To this, 300 µl sterile distilled water, 50 µl phenol and 60 µl chloroform were added, mixed and centrifuged. The top layer was removed to a clean microcentrifuge tube and 100 µl H_2O added to the lower layer. The tube was shook, then the top aqueous layer removed and added to the above amount in the clean microcentrifuge tube. To this, 20 µl 3M Sodium Acetate (pH 5.5) and 800 µl 100% ethanol were added, the tube mixed and placed in the freezer at -20°C. After one hour, the tube was spun, the supernatant discarded, 500 µl 70% ethanol added to the tube, mixed and spun again, removed supernatant and the tube inverted to dry the ethanol. It can be stored as a pellet. In order to resuspend the pellet, 50 µl sterile distilled water were added to the tube, then placed into a dry block set at 50°C leave for one hour.

This extra purification step gave better results for the spectrophotometer readings.

1) QIAEX II Agarose Gel Extraction Kit (Qiagen Ltd.)

This protocol is designed for the extraction of 40 bp to 50 kb DNA fragments from 0.3-2% standard or low-melt agarose gels in TAE or TBE buffers.

The DNA band of interest was excised from the agarose gel using a clean, sharp scalpel on a transilluminator and the size of the gel slice minimized by removing the excess agarose. The agarose slice was weighed in a colourless 1.5 ml microcentrifuge tube, then three volumes of Buffer QX1 were added to each volume of gel (i.e. add 300 µl of Buffer QX1to each 100 mg of gel).

The QIAEX II was resuspended by vortexing for 30 seconds, added to the sample according to the details below, and mixed.

<2 µg DNA	Add 10 µl of QIAEX II
2-10 µg DNA	Add 30 µl of QIAEX II
Each additional 10 µg DNA	Add additional 30 µl of QIAEX II

The tube was incubated at 50°C for 10 minutes, to solubilize the agarose and bind the DNA. Every 2 minutes the tube was vortexed in order to keep QIAEX II in suspension, and it was checked that the colour of the mixture was yellow. The tube was kept at room temperature for 10 minutes, flicking it, then centrifuged for 30 seconds. Using a pipette, the supernatant was removed. The pellet obtained was washed with 500 µl of Buffer QX1 (the pellet was resuspended by vortexing, then the sample was centrifuged for 30 seconds and all the traces of supernatant removed using a pipette). This washing step was used to remove residual agarose contaminants.

The pellet was then washed twice with 500 µl of Buffer PE. These washing steps were used to remove residual salt contaminants. The pellet was then air dried for 10-15 minutes or until the pellet became white. To elute the DNA, 20 µl water was added and the pellet resuspended by vortexing and incubated according to the details below.

DNA fragments <4 kb	Incubate at room temperature for 5 min
DNA fragments 4-10 kb	Incubate at 50°C for 5 min
DNA fragments > 10kb	Incubate at 50°C for 10 min

The tube was then centrifuged for 30 seconds and the supernatant containing the purified DNA, pipetted carefully into a clean tube.

2) DNAce purification system (Bioline)

To the slice of agarose gel containing the band of interest, 3 volumes of gel solubilizer were added (6 volumes if it was a TBE gel), then the microcentrifuge tube incubated at 56°C for 5 minutes or until complete gel dissolution. After vortexing the carrier suspension, 7.0 µl were added to each microcentrifuge tube, then placed for 5 minutes on ice. After centrifugation (4°C), at 12000 rpm for 20 s, the supernatant was discarded. One ml of washing buffer (to 15 ml of wash buffer concentrate, 75 ml water and 210 ml 96% ethanol was added and stored at –20°C) was added to each tube, then vortexed, centrifuged and the supernatant removed, this step being

repeated twice. The pellets obtained were dried at 52°C for 5 minutes, resuspended in 20 µl of elution buffer and incubated at 52°C for 5 minutes. After centrifugation (4°C) at 13000 rpm for 5 minutes, the supernatant was collected. The last two steps were repeated and supernatant collected in another microcentrifuge tube, in order to collect everything but at the same time not to dilute the sample. The tubes were stored at -20°C.

8.7 Purification of plasmid DNA using InViSorb™ DNA Extraction Kit (Bioline)

This kit was used for extraction and purification of DNA from TAE agarose gels, TBE agarose gels or aqueous solutions, and the details are presented in APP!!!

For DNA extraction from TAE or TBE agarose gels, the band of interest was excised from the gel using a clean blade, and the volume determined by weight (Note: 1.0 g of gel piece is approximately equal to a volume of 1.0 ml). The gel piece was transferred in a 1.5 ml microfuge tube and 3 volumes of Gel Solubilizer added (volume was increased 2 fold for TBE gels). The tube was then placed in a 56°C water bath for about 5 minutes. A five minute incubation should be sufficient to dissolve the gel piece completely. To the mixed DNA/Gel Solubilizer solution, 5-7 µl of InViSorb 50™ Carrier Suspension (vortexed thoroughly prior to being used) were added and the new mixture vortexed briefly. The microcentrifuge tube was left on ice for 5 minutes, centrifuged at full speed for 20 seconds, then the supernatant carefully discarded. One ml Wash Buffer was added to the tube, vortexed briefly until the pellet was completely resuspended, centrifuged at full speed for 15 seconds, then the Wash Buffer discarded carefully. The last step was repeated twice, then the residual fluid was briefly spun down and removed as completely as possible by aspiration with a pipette. Placing the tube for 5 minutes into a water-bath set at 52°C to completely evaporate the residual ethanol, dried the pellet. Elution Buffer (20 µl) was used to resuspend the pellet by thorough vortexing, then the tube was incubated at 52°C in a water-bath for 5 minutes to elute the DNA. The elution was performed twice with 20 µl of Elution Buffer to increase the yield of DNA. After that, a 5 minutes centrifugation was performed at full speed followed by cautious transfer of the DNA-containing supernatant into a fresh tube (at least 2.0 µl supernatant were left above the pellet in order to avoid carry-over of silica particles). The last step was repeated to minimise the carry- over of silica particles.

Note: regardless of how carefully the final DNA solution was separated from the Silica pellet, some residual particles always remain in the solution which may affect the activity of DNA-modifying enzymes. To avoid associated problems, the DNA solution was centrifuged for 5 minutes at full speed, each time prior to use. A DNA aliquot for further use was taken from the top of the solution immediately after centrifugation.

The yield and purity of the extracted DNA can be verified on an agarose gel using standard control samples or by measuring the UV absorption spectrum.

8.8 QIAquick PCR Purification Kit Protocol (Qiagen Ltd.)

This protocol is designed to purify single- or double-stranded PCR products ranging from 100 bp to 10 kb from primers, nucleotides, polymerases, and salts, using QIAquick spin columns in a microcentrifuge.

Five volumes of Buffer PB were added to one volume of the PCR reaction and mixed. A QIAquick spin column was placed in a 2.0 ml collection tube. In order to bind the DNA, the sample was applied to the QIAquick column and centrifuged for 30-60 seconds at 13,000 rpm. The flow-through was discarded and the column placed back into the same tube. Buffer PE (0.75 ml) was added to the column and centrifuged 30-60 seconds, in order to wash the column, the flow-through discarded and the column placed back in the same tube followed by an additional minute centrifugation. The column was then placed in a clean 1.5 ml microfuge tube.

The DNA was eluted by adding 50 µl 10mM Tris-HCl, pH 8.5 or H_2O to the centre of the QIAquick column and centrifuging for 1 minute. Alternatively, for increased DNA concentration, 30 µl elution buffer were added to the centre of the QIAquick column, allowed to stand for 1 minute, and then centrifuged.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, it was made sure that the pH value was within this range, and DNA stored at -20°C, as DNA may degrade in the absence of a

buffering agent. The purified DNA can also be eluted in TE (10mM Tris-HCl, 1 mM EDTA, and pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

Chapter 9

Bibliography

9 Bibliography

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