F U S A R I A A N D *F U S A R I U M* T O X I N S

I N M A I Z E

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ABSTRACT

Many species of Fusarium are commonly associated with cereals, particularly maize, but in New Zealand, little is known of their significance as mycotoxin producers. These studies have examined the prevalence of fusaria and other fungi in maize and maize fields and have investigated the presence and sources of some major Fusarium toxins in maize.

Fungi in maize, husk, litter and soil from maize fields and in grain at harvest and in storage were assayed. The distribution of fungi was found to be uneven within maize, husk and litter substrates within a field but in soil was more homogeneous. Sampling techniques were therefore developed to ensure representative subsamples were obtained from each source.

Similarly isolation procedures were chosen to ensure adequate recovery of fungi. Dilution and direct platings were used to provide information on total populations and on fungi actually invading kernels, with two media, PDA-D and PCNB. The medium used showed no significant influence on either viable counts or kernel contamination rates nor on the number of different Fusarium spp recovered on the two media, but PDA-D supported a greater overall variety of fungi. The numbers of genera and of Fusarium spp recorded by direct plating were significantly higher than with dilution plating.

The total population and the number of different genera and of Fusarium spp were compared for the four "field" substrates. A total of 25 genera was isolated, most being recovered from soil and litter. Fusarium was present in all samples. Acremonium, Cladosporium, Penicillium and Mucor occurred regularly. The four substrates gave up to ten different Fusarium spp, F. graminearum, F. culmorum and F. acuminatum being the most frequent. Husk and litter samples gave the highest viable counts for both total fungi and Fusarium spp.

Field samples of maize kernels showed 13 genera and ten Fusarium spp. At harvest time total genera increased to 17 but Fusarium spp remained constant. While the total genera remained constant at 17 in stored samples, the number of Fusarium spp dropped to three, only F.

subglutinans, F. graminearum and F. poae being detected. The contamination rate of kernels by fusaria also changed significantly from field samples (75.8%) to harvest samples (58.3%) to only 1.5% in stored maize.

As with Fusarium, Acremonium and Mucor populations decreased from harvest to storage but other genera (e.g. Aspergillus, Beauvaria) were only found in stored maize. The frequency of occurrence of Penicillium remained stable over the whole period.

Three analytical methods, TLC, GC and GC-MS were used for screening maize, poultry ration samples and cultures of Fusarium isolates for five Fusarium toxins. The GC-MS method was the most reliable and sensitive for detection and quantitation of DON, DAS and T-2 toxin, but not for quantitation of ZEA, due to derivatisation problems. TLC and TLC-densitometry were sensitive and reliable enough for detection and quantitation of ZEA and MON respectively. Although the GC results were closer to the GC-MS results, a high percentage of false positives, particularly for T-2 toxin, was noticed.

Of the examined maize samples, 85% were contaminated with fungal toxins. The majority contained ZEA and three samples were each contaminated with four toxins. No MON was detected.

Many isolates, particularly of F. graminearum, were found to be ZEA-producers. Some 63% produced ZEA at >2 ppm. T-2 toxin was produced by 46% of the isolates but at low levels (<1.7 ppm). Low levels of DON and DAS were produced by a few isolates. MON was produced by 30% of isolates, particularly F. subglutinans, and in large amounts (up to 64 ppm).

This thesis is the first report on the natural occurrence of Fusarium toxins in New Zealand maize. T-2 toxin and DAS have not been reported as natural contaminants in this country. MON production has also not been reported in New Zealand.

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF PLATES

ABBREVIATIONS USED IN THIS THESIS

3-ADON	3-acetyl-deoxynivalenol
15-ADON	15-acetyl-deoxynivalenol
BSA	N,O-Bis (trimethylsilyl) acetamide
BSTFA	N,O-Bis (trimethylsilyl) trifluoroacetamide
CFU/g	Colony-forming units per gram
CDA	Czapek-dox solution agar
CLA	Carnation leaf agar
DAN	Diacetylnivalenol
DAS	Diacetoxyscirpenol
2,4-DNPH	2,4-dinitrophenylhydrazine
DON	Deoxynivalenol
ECD	Electron capture detector
EI	Electron impact
FID	Flame ionization detector
FUS-X	Fusarenon-X
GC	Gas liquid chromatography
GC-ECD	Gas liquid chromatography with electron capture detector
GC-FID	Gas liquid chromatography with flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
GYEP	Glucose-yeast-extract-peptone
HFB	Heptofluorobutyryl
HFBI	Heptofluorobutyryl-imidazole
IMC	Initial moisture content
LEM	Leukoencephalomalacia
MAS	Monoacetoxyscirpenol
MC	Moisture content
MID	Multiple ion detection
MON	Moniliformin
MTM	Mycotoxin standards mixture
MS	Mass spectrometry
MS-MS	Mass spectrometry-mass spectrometry
M/Z	Mass/charge ratio of ion fragments in mass spectrometry
NEO	Neosolaniol
NIV	Nivalenol
PCNB	Pentachloronitrobenzene
PDA	Potato dextrose agar
PDA-D	Potato dextrose agar-dichloran
ppb	Part per billion (ng/g)
ppm	Part per million (mg/kg)
PSA	Potato sucrose agar
RIA	Radioimmunoassays
sdw	Sterile distilled water
SIM	Selective ion monitoring
TAS	Triactoxyscirpenol
TLC	Thin layer chromatography
TMS	Trimethylsilyl
TMSI	N-trimethylsilyl-imidazole
TCMS	Trimethylchlorosilane
TIM	Total ion monitoring
ZEA	Zearalenone

PREFACE

Species of the genus Fusarium are usually regarded as soil-borne fungi and are widely distributed throughout the world, particularly in soil associated with plant roots. They are common on all subterranean plant parts as well as plant debris and other organic substrates (Burgess, 1981). From this soil reservoir, they frequently invade the aerial parts of plants. Some Fusarium spp such as F. graminearum (perfect stage Gibberella zeae) can be considered to be among the most destructive of plant pathogens, particularly of maize (Christensen & Wilcoxson, 1966). Several species (F. graminearum, F. culmorum, F. oxysporum etc.) have been found to be very frequently associated with seedling blight and root, stalk and ear rots of many cereal plants in many countries, eg. the U.S.A. (Windels & Kommedahl, 1984), South Africa (Marasas et al., 1979b), Canada (Andrews et al., 1981), New Zealand (Fullerton, 1978). In total, more than half of the Fusarium species are parasites of cultivated plants.

In addition, species of the genus can be agents of mycoses in humans and animals (Austwick, 1984). A few species (eg. F. solani, F. oxysporum) are important in mycotic keratitis and onychomycosis (Rippon, 1982) and more than half of fungal eye infections have been reported to be associated with Fusarium spp (Austwick, 1984).

However, some aspects of the biological and biochemical versatility of the genus can be used to benefit the well-being of man. For example, the pathogenicity of some species to insects (entomogenous fusaria) may be used to control certain insect pests. A number of metabolic products of fusaria (gibberellin, bikaverin etc.) have been found to have specific biological properties which can be turned to human advantage and have therefore been manufactured commercially (Bu'lock, 1984). Primary metabolites of strains of at least one species have been utilised in the production of microbial biomass which can be readily converted into a wide range of foodstuffs (Anderson & Solomons, 1984).

Recently the genus has gained a bad reputation because of the ability of several species to produce toxic secondary metabolites (mycotoxins) on organic substrates, and ingestion of these can result

in illness and even death in both humans and animals (the mycotoxicoses). In particular, during cool and rainy seasons, cereal grains can become grossly spoiled by fusaria and the ingestion of such grain can result in a variety of mycotoxic problems (Marasas et al., 1979a). The apparently harmful consequences of mycotoxin ingestion can also lead to the unnecessary devaluation of many mould-contaminated grain lots (Morita et al., 1984), which itself can have serious consequences on the economic viability of livestock production.

Toxin production by fungi depends on 3 conditions: the actual presence of the fungi, an environment suitable for growth, and suitable substrates for growth (Smith et al., 1984). Determining the toxigenic fungi present in any given food or feed is therefore a useful adjunct for evaluating that food or feed for hazard potential due to mycotoxin contamination (Mislivec, 1977). Thus the study of fusaria and their ability to produce mycotoxins has received wide attention in a number of countries (Snyder, 1986).

Fusaria have been considered to be the most important among all the toxigenic fungi because of the wide range of mycotoxins which they can produce (Hesseltine, 1977; Ghosal et al., 1978). Most field outbreaks of fusariotoxicoses occur in areas with a cooler climate such as that experienced in New Zealand (Richardson et al., 1985), and are frequently associated with contaminated maize which, in turn, is an important grain crop in New Zealand. Maize grain is often considered the most likely source of mycotoxin contamination of animal feeds as reports have indicated that smaller grains (wheat, barley etc.) may be less susceptible to Fusarium mycotoxin contamination, particularly in the field (Smith, 1982).

Maize ranks third among the world's major cereal crops. Total production in New Zealand during the 1983-84 season has been estimated at 200,000 tonnes, making it the third most important cereal crop in this country after barley and wheat (Bansal & Eagles, 1985). Production increased during more recent seasons (1984-85 and 1985-86) to 220,000 and 226,000 tonnes respectively (Agr. Stat. Dept., 1986). The average grain yield of 9.1 tonnes/ha for the 1981-82 season compares with the average of 6.3 tonnes/ha for the U.S.A. for 1979-80 season,

making New Zealand yields among the highest in the world (Bansal & Eagles, 1985).

Approximately 70% of the maize crop in New Zealand is used in the animal feed industry, the remainder being destined for human consumption, pharmaceutical use and for alcohol manufacture. The grain constitutes 63% of the total feed grain used and in the North Island is regarded as the main energy source in feeds for poultry and pigs. Poultry alone consume about 50% of the manufactured feed, while pigs consume about 30% (Chappell, 1985).

In New Zealand there have been extensive studies of two mycotoxin problems, facial eczema and ryegrass staggers, but little is known about the significance of other mycotoxins, particularly those contaminating cereals. In 1982 and 1983, L.G. With at the Poultry Conference held at Massey University brought to the attention of the poultry industry the possibility of economic losses due to the contamination of feedstuff with mycotoxins. He admitted the status of mycotoxins in New Zealand was not known and he emphasised to the poultry industry the need for both monitoring grain samples routinely and maintaining a close watch for any clinical cases. Preliminary studies had shown that poultry feeds can contain high levels of certain mycotoxins and it is strongly suspected that such contaminants regularly affect general health and productivity of poultry flocks (With, pers. comm.) In addition, pig producers should be aware of the problem as swine are the most sensitive animals to the majority of Fusarium toxins (Mirocha, 1984).

The investigations to be reported in this thesis were initiated as no work had been done in this country to determine the toxicity of Fusarium spp associated with maize. It is known that many species of Fusarium are widespread in New Zealand cereals, grasses etc. (Latch et al., 1976; Hampton, 1980; Fowler, 1985). The occurrence of Fusarium mycotoxins could therefore be expected, particularly as the New Zealand climate is often conducive to Fusarium infection and mycotoxin production - the cool, damp conditions that favour fusaria can be prevalent in many districts.

This study has two major components. The first examines the prevalence of Fusarium species in maize fields (on grain, husk and litter and in soil), their standing amongst the general mycoflora of those fields and their changing patterns of occurrence as the crop develops and is placed into storage. The second concerns the screening of Fusarium isolates for their potential toxicity, and examines maize samples collected from the field, at harvest time, and during storage, as well as compounded poultry rations, for some of the important Fusarium mycotoxins. Such mycological and toxicological analyses can give complementary results (Farnworth & Neish, 1980).

CHAPTER 1
INTRODUCTION - LITERATURE REVIEW

1.1. THE GENUS FUSARIUM - TAXONOMY, ECOLOGY AND METHODS OF STUDY

The hyphomycete genus Fusarium is characterised by usually fast growing, pale or bright-coloured colonies (Plates 1-1 to 1-3) with felty aerial mycelium and diffuse or sporodochial sporulation (Domsch et al., 1980). The sporogenous cells arise either directly from the hyphae or from distinct conidiophores. The conidiophores can be either single or complex by branching and terminate in phialides which in turn can be either single (monophialides) or clustered (polyphialides) (Plate 1-4 and Figure 1-1). The phialides taper distally, sometimes have an apical collarette, and produce phialospores (conidia), which may be of two types. The large macroconidia have from one to several septa and are hyaline, elongate, cylindric or curved (frequently boat-shaped) and have a well marked foot cell at the attachment end of the spore. The microspores (microconidia) are smaller, nonseptate or one-septate, ovoid to short-cylindric and are produced on the aerial mycelium, never in sporodochia. They are gathered in short chains or more commonly in spore balls (Barron, 1968).

1.1.1. Taxonomy

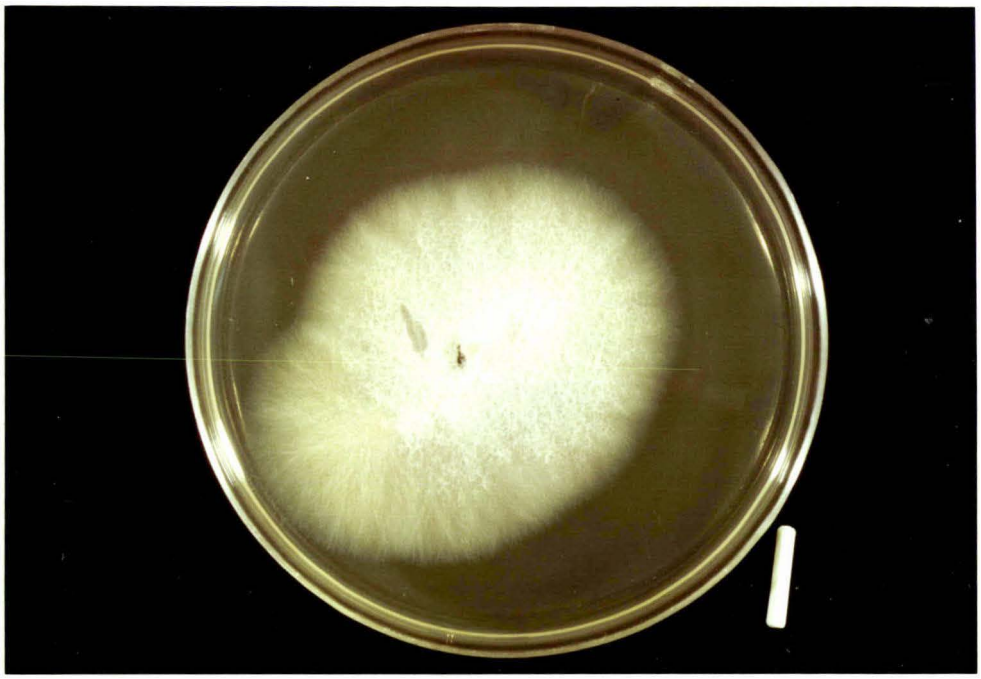
Species of the genus Fusarium are notorious for their variation in morphology on artificial media. Although genetic variability may sometimes be involved, this variation is largely due to cultural conditions (Booth, 1984) and it is, therefore, important to attempt identification of each strain using standardised conditions of culture (Nelson et al., 1983).

The variability displayed within the genus has led to many different opinions being mooted by mycologists regarding its classification. Very often many synonyms can be found in the literature, e.g. the name F. roseum has been used to include F. graminearum, F. equiseti, F. scirpi, F. avenaceum, F. culmorum, while F. tricinctum

Plate 1-1: Five days old *F. subglutinans* culture on PDA

1) Colony with white powdery surface

1a) Reverse with creamy pigmentation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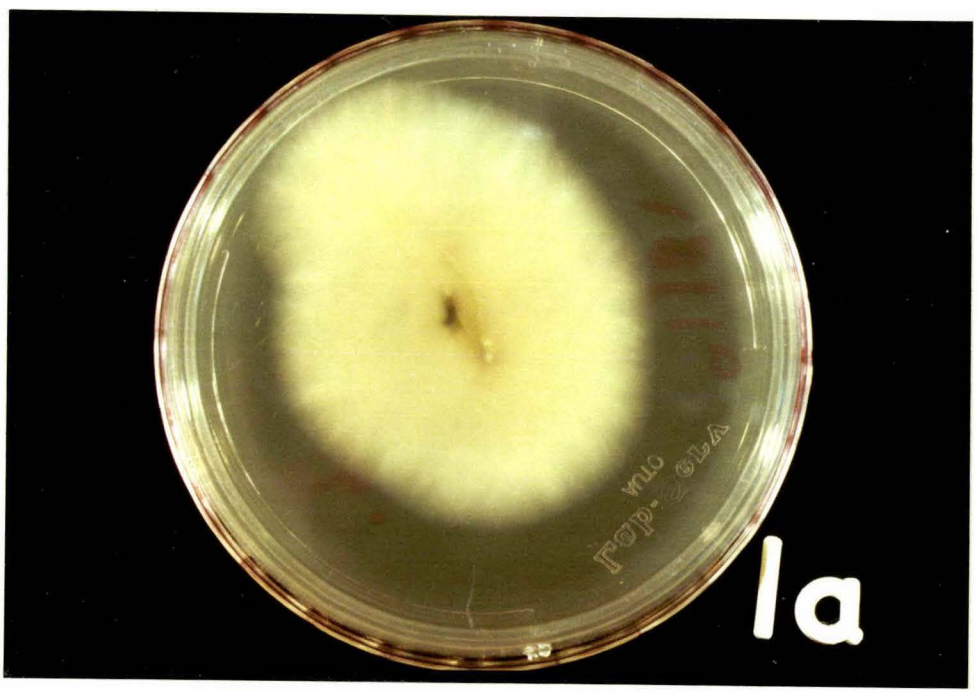
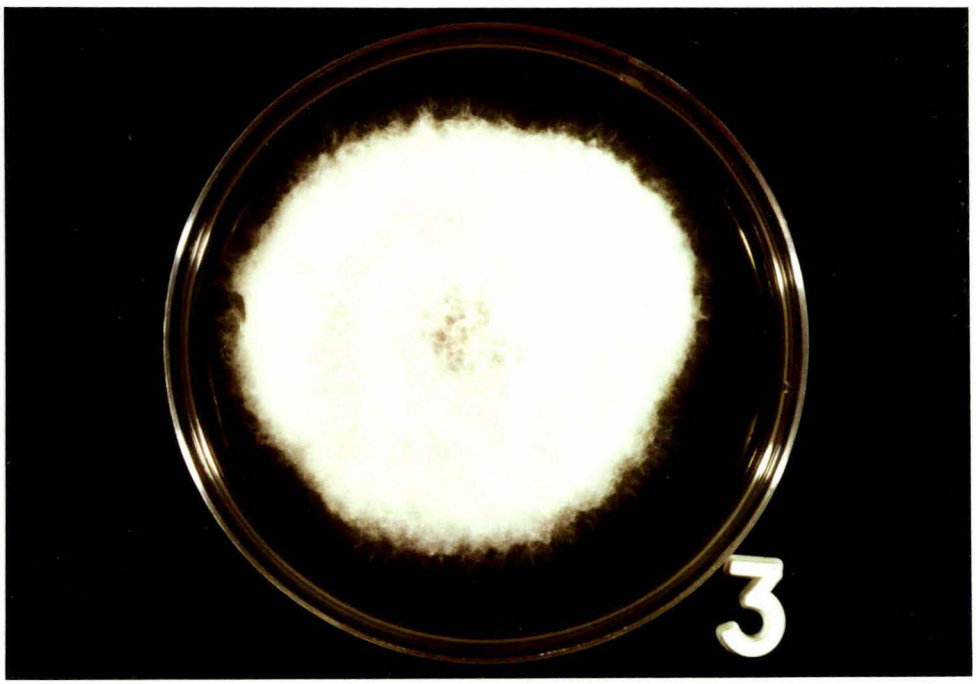

1a

Plate 1-2: Five days old *F. poae* culture on PDA

3) Colony with white cottony surface

3a) Reverse with burgundy pigmentation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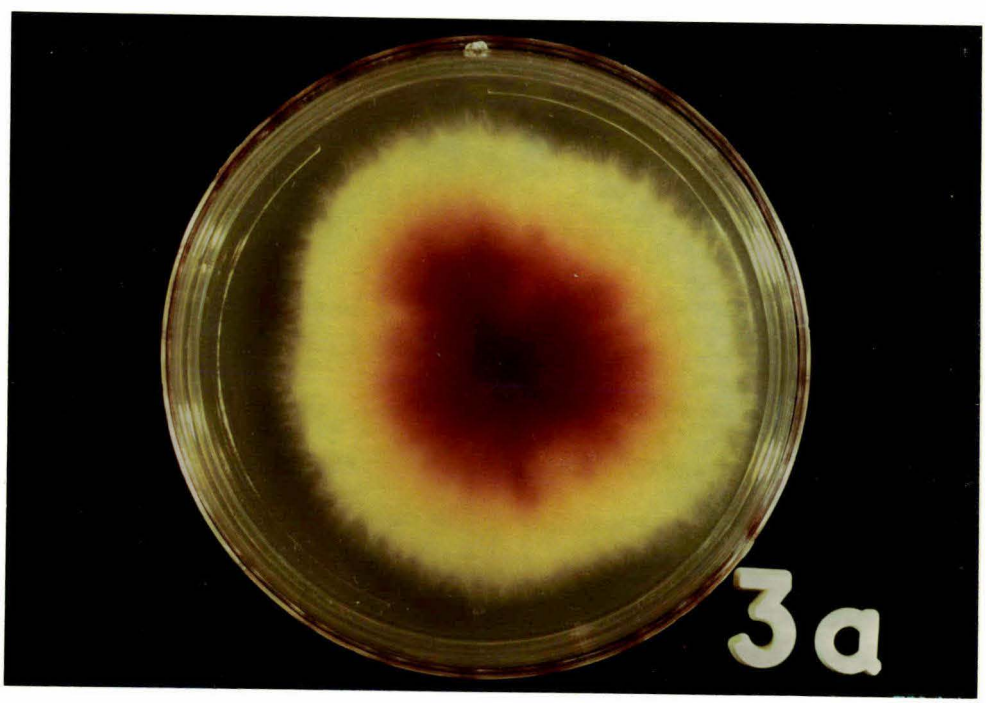
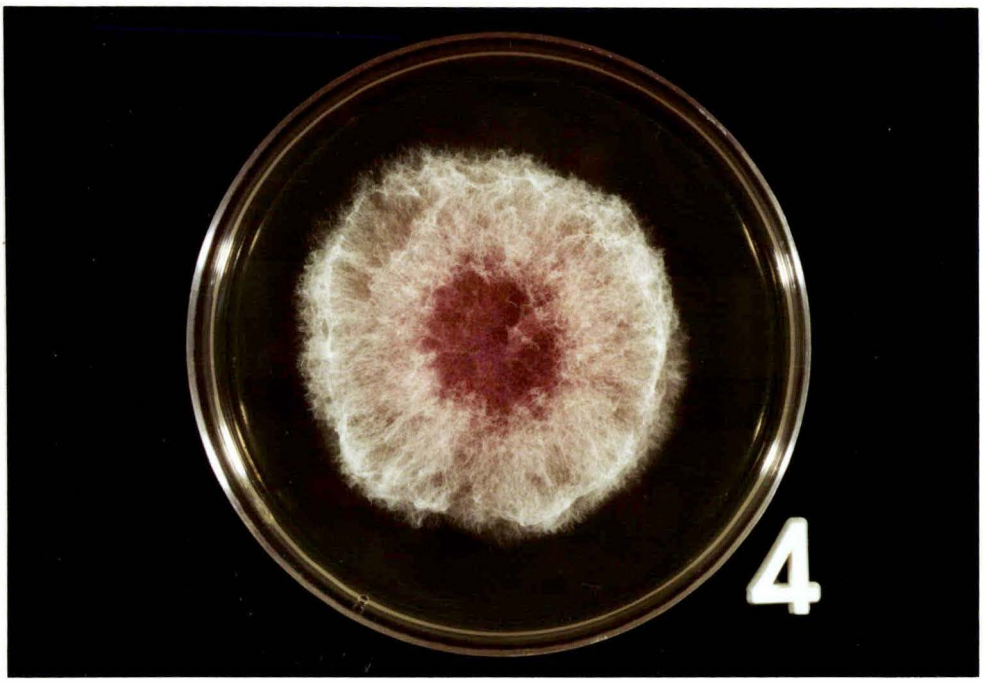

3a

Plate 1-3: Five days old F. graminearum culture on PDA

4) Colony with white floccose surface and showing red pigment at centre

4a) Reverse with red pigmentation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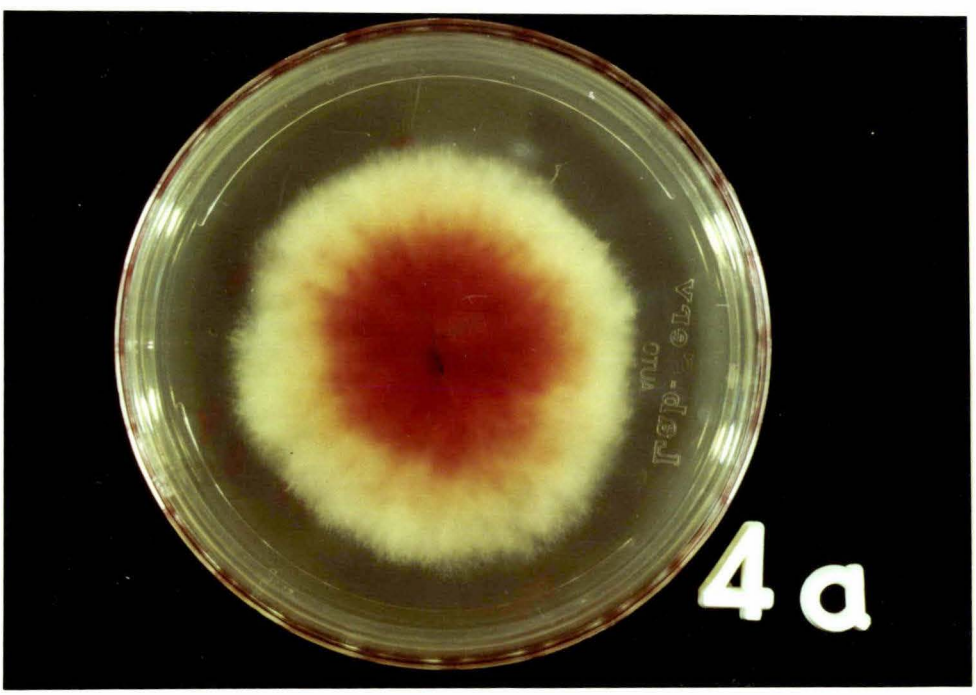

4a

Plate 1-4: Conidiogenous cells and spores of some Fusarium spp

(a)
F. graminearum

Conidiophores with unbranched monophialides
x 400

(b)
F. graminearum

Macroconidia with distinctly foot-shaped basal cell
x 400

(c)
F. poae

Conidiophores with branched monophialides
x 500

(d)
F. poae

Microconidia, globose with distinct papilla
x 500

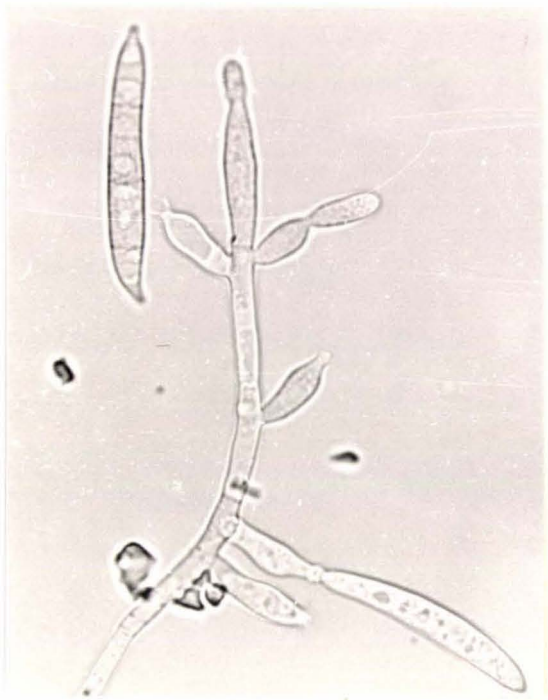
(e)
F. subglutinans

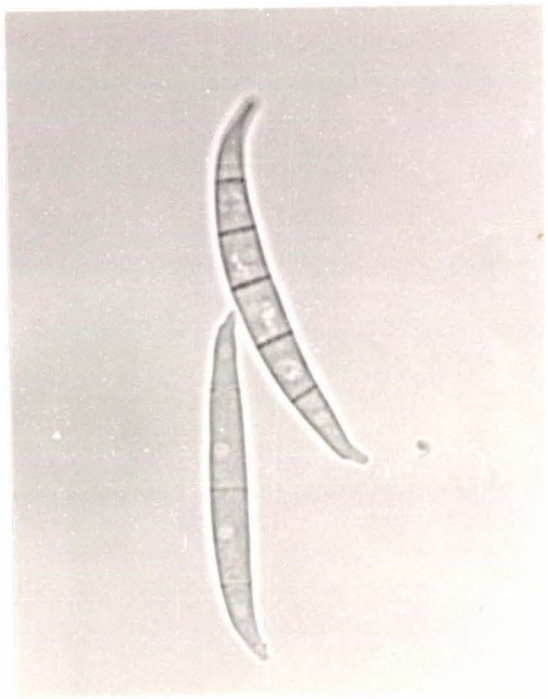
Conidiophores with lateral monophialides
x 400

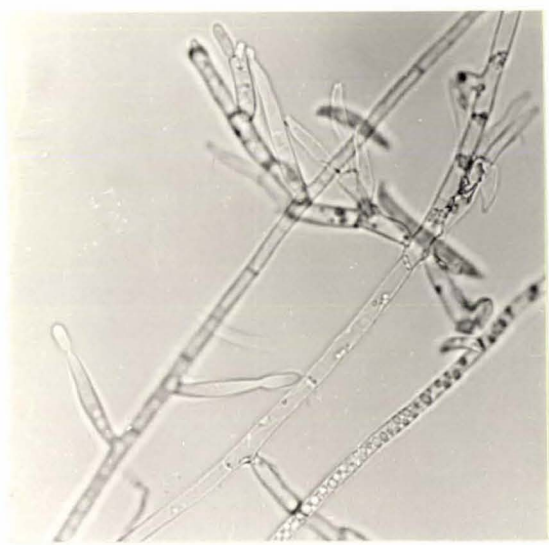
(f)
F. culmorum

Macroconidia

x 400







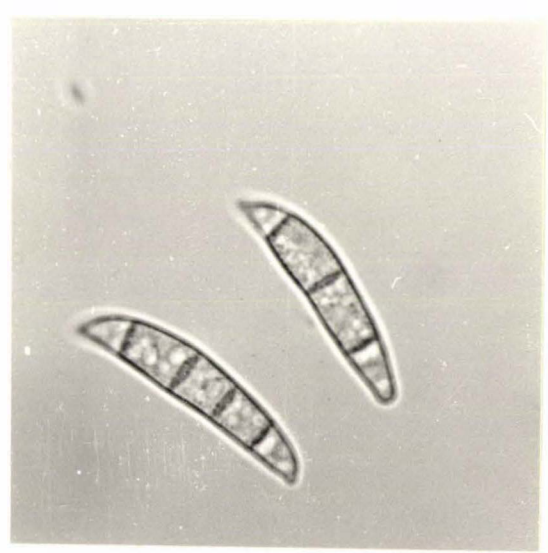
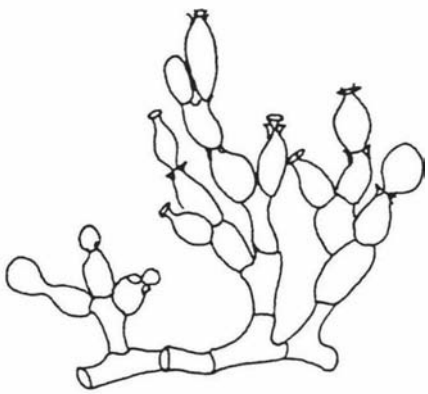
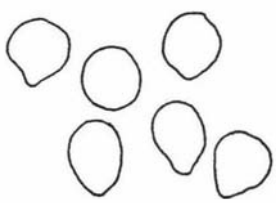


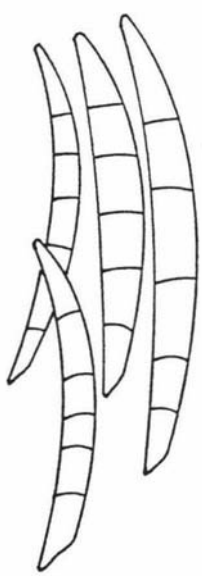
Figure 1-1: Some spores and conidiogenous cells of Fusarium species (from Booth, 1971)



(a) F. poae
Microconidiophores



(b) F. poae
Microconidia



(c) F. graminearum
Macroconidia

has included F. poae, F. sporotrichioides and F. sporotrichiella (Vesonder and Hesseltine, 1981).

That the taxonomy of the genus has been the subject of controversy over the years is partially illustrated in Tables 1-1 and 1-2, which compare just some of the systems which have been proposed. Of the several classifications which have been suggested, some recommended more than 90 species, whilst one consisted of only 9 species. Most have adopted a stand between these two extremes but none, not even the more modern ones, can be used satisfactorily for the identification of all Fusarium spp. (Nelson et al., 1983).

Most of the currently more popular systems are based on the Wollenweber and Reinking scheme first published in 1935. Wollenweber and Reinking grouped the species of the genus into 16 "sections". They included a total of 65 species together with some 55 varieties and 22 "forms". Sections were separated on the presence or absence of microconidia, the shape of the microconidia, the presence or absence of chlamydospores, the location of chlamydospores, the shape of the macroconidia and the shape of the basal (foot) cell on the macroconidia. The division of each section into species, varieties and forms was on the basis of the shape , length, width and number of septations of the macroconidia, in addition to the presence or absence of sclerotia and the colour of the stroma.

In contrast to Wollenweber and Reinking's system, Snyder and Hansen (1940) avoided the creation of large "sections" and used the term "species" to designate the equivalent groupings. This led in some cases to very large collections of quite distinct strains being referred to as the same species. Their species F. roseum was a particularly unfortunate application. About 15 species (in addition to forms) in the sections Arthrosporiella, Discolor, Gibbosum and Roseum of Wollenweber and Reinking were all incorporated into the one complex species F. roseum. To further complicate matters, in 1957 Snyder and Hansen and Oswald (cited by Nelson et al., 1983) proposed the use of the name "Cultivars" for some strains within their species. The cultivar system has been difficult to use because no accurate descriptions of them are available (Nelson et al., 1983).

One commonly used classification is that of Booth (1971) who illustrated 44 species and varieties which were grouped in 12 sections. Booth regarded morphology of the conidium and the sporogenous cells as the major character for the identification of Fusarium spp.

Gerlach and Nirenberg (1982) in their book "The genus Fusarium - a pictorial atlas" used the Wollenweber and Reinking scheme as a basis for arranging fusaria in sections but did not completely accept all Wollenweber and Reinking's concepts. Gerlach and Nirenberg examined all available cultures under various conditions and studied the macroscopic characters (colony growth, aerial mycelium, pigmentation, sclerotial bodies and sporulation) and the microscopic characters (conidiophore, conidia and chlamydospores). Gerlach and Nirenberg used the 16 sections system of Wollenweber and Reinking, but replaced section Discolor with section Fusarium. The sections contained 90 distinct species, in addition to doubtful species and varieties. A comprehensive list of synonyms was included.

Nelson et al (1983) combined several of the systems which have been proposed (eg. Wollenweber and Reinking, 1935; Booth, 1971; Gerlach and Nirenberg, 1982 etc.) in a compromise system. Nelson et al. fully described 30 species and, in less detail, a further 16 species which they designated "questionable species". Sections were arranged in the same order as in Wollenweber and Reinking except that 3 sections, Macroconia, Submicrocera and Pseudomicrocera, were omitted.

Among the more recent publications is the laboratory manual of Burgess and Liddell (1983). It contains practical information on the methods of classification particularly for Fusarium species in Australia. Burgess and Liddell's taxonomic system is essentially based on that of Nelson et al. (1983)

Burgess and Liddell include 28 species in 12 sections (Table 1-3), with no varieties. They listed the most important characters used in their identification scheme as:-

1. Shape of the macroconidium
2. Presence or absence of microconidia

3. Shape and mode of formation of microconidia
4. Nature of the conidiogenous cell bearing microconidia
5. Presence or absence of chlamydospores
6. Colony diameters on PDA after dark incubation for 3 days at 25°C and 30°C
7. Colony morphology on PDA after incubation for 10-14 days at alternating day and night temperature of 25°C/20°C and 12 h photoperiod.

In relation to the importance of conidium morphology, Fisher et al. (1982) found that carnation-leaf agar (CLA) was a good medium to support growth and sporulation and to give a uniform conidium appearance, in contrast to rich media such as potato dextrose agar (PDA) which may cause a delay of spore formation and misshapen conidia.

It is the species concepts of Burgess and Liddell that will be largely followed in the investigations to be reported in this thesis. Most Fusarium isolates were identified according to Burgess and Liddell's Manual but Booth (1971) and Gerlach and Nirenberg (1982) as well as other references were also used for their detailed descriptions.

There is still great disagreement between taxonomists all over the world despite all these efforts. Price (1984) believed, in common with most other authors, that the problem facing work with Fusarium is not the applied mycology but the Fusarium taxonomy. He believed that the array of international taxonomic systems is impressive but, although many are related, there can be pitfalls unless a worker has a degree of familiarity with the area. Booth (1984) caused controversy when he commented that the taxonomic problems with Fusarium were created by people themselves. He stated that there is no mystery about Fusarium taxonomy when a fresh collection from nature is studied and single conidia or single ascospores are cultured on natural media. He considered that no great variation will be found except for F. avenaceum.

Knowledge of the teleomorph state of some species can be of value to the taxonomist (Booth, 1971) although many people, particularly plant pathologists, are concerned only with the anamorph (imperfect)

state. Booth classified the perfect states of Fusarium spp as belonging in the order Hypocreales, of the Ascomycotina (Pyrenomycetes). He includes 3 genera (Nectria, Gibberella and Calonectria), and later suggested two more, Plectosphaerella and Monographella as perfect states for species in the section Arachnites (Booth, 1981).

New technology and advanced knowledge in genetic engineering have recently been used in Fusarium taxonomy. Szecsi and Dobrovolszky (1985) examined genetic distance measured by a comparative computer analysis of DNA thermal denaturation profiles. They found this technique was useful for the classification of Fusarium spp particularly because the thermal denaturation is simple, direct and easily standardised and useful for comparing closely-related DNAs.

Szecsi and Dobrovolszky examined 13 species of Fusarium by this technique. In some cases their findings supported existing groupings, eg. F. graminearum and F. culmorum of the section Discolor were very close. In other cases they found that species presently grouped together, eg. F. sambucinum and F. heterosporum (Booth's Discolor) were, in fact, quite distinct when analysed by average linkage clustering from genetic distance data. Such work may well result in a more accurate taxonomy for Fusarium in the future.

Table 1-1: The principal taxonomic systems for the genus Fusarium

	Wollenweber & Reinking, 1935	Gerlach & Nirenberg, 1982	Nelson, Toussoun & Marasas, 1983*	Booth, 1971	Snyder & Hansen, 1940s
	Sections: 16	Sections: 16	Sections: 12	Sections: 12	Species: 9
1	Eupionnotes	--->	--->	Episphaeria	episphaeria
2	Macroconia	--->	none	"	"
3	Spicarioides	--->	--->	--->	rigidiuscula
4	Submicrocera	--->	none	Arachnites	none
5	Pseudomicrocera	--->	none	Coccophilum	none
6	Arachnites	--->	--->	--->	nivale
7	Sporotrichiella	--->	--->	--->	tricinctum
8	Roseum	--->	--->	Arthrosporiella	roseum
9	Arthrosporiella	--->	--->	--->	"
10	Gibbosum	--->	--->	--->	"
11	Discolor	Fusarium	Discolor	--->	"
12	Lateritium	--->	--->	--->	lateritium
13	Liseola	--->	--->	--->	moniliforme
14	Elegans	--->	--->	--->	oxysporum
15	Martiella	--->	Martiella-	Martiella	solani
16	Ventricosum	--->	Ventricosum	"	"

* Also followed by Burgess and Liddell (1983)

---> Section name of Wollenweber and Reinking retained

Table 1-2: Comparison of the numbers of sections and species in the principal taxonomic systems for Fusarium

	Taxonomic system	Sections	Species	Varieties
1	Wollenweber & Reinking (1935)	16	65	55 + 22 forms
2	Snyder & Hansen (1940s)	-	9	9 cultivars
3	Booth (1971)	12	44	7
4	Gerlach & Nirenberg (1982)	16	90	+ doubtful species and varieties
5	Nelson et al. (1983)	12	30 + 16 questionable	
6	Burgess & Liddell (1983)	12	28	

Table 1-3: Sections and species in the genus Fusarium as listed by Burgess and Liddell (1983)

Section	Species
Arachnites	F. nivale
Spicarioides	F. decemcellulare
Lateritium	F. lateritium
Sporotrichiella	F. poae
	F. tricinctum
	F. sporotrichioides
	F. chlamydosporum
Liseola	F. moniliforme
	F. proliferatum
	F. anthophilum
	F. subglutinans
?	F. 'brevicatenum'*
Elegans	F. oxysporum
Martiella/Ventricosum	F. solani
Discolor	F. culmorum
	F. sambucinum
	F. crookwellense
	F. graminearum
	F. reticulatum
Roseum	F. graminum
	F. avenaceum
Gibbosum	F. acuminatum
	F. 'armeniacum'*
	F. longipes
	F. compactum
	F. equiseti
	F. scirpi
Arthrosporiella	F. semitectum
Eupionnotes	not detected in Australia

* Uncertain status (could be new species)

1.1.2. Ecology of Fusarium Species

1.1.2.1. Factors influencing distribution

The abundance of any fungal species is affected most significantly by the general environmental conditions and the kind and the concentration of available substrates.

A. Environmental factors

The majority of fusaria are widely distributed but it is not uncommon to find that some species appear to be restricted to certain climatic zones (Burgess, 1981). F. rigidiusculum and F. longipes are relatively common in tropical and subtropical zones, for example, while F. sambucinum and F. nivale are most common in cold areas and in alpine soils (Gorden, 1960). Similarly, although F. moniliforme and F. subglutinans can be present in widely varying climates, F. moniliforme tends to be most common in subtropical and temperate areas and F. subglutinans in temperate and cool areas (Kommedahl et al., 1975). Among the many more widely distributed species found in all kinds of climates are F. equiseti and F. acuminatum.

The species F. graminearum was differentiated into 2 groups by Francis and Burgess (1977) who noted that some types were more prevalent in particular climatic regions. Members of Group 1 tended to be more common in drier areas whereas members of Group 2 were more frequent in warm and humid areas. The latter favour the formation of perithecia and ascospore discharge (Tschanz et al., 1976).

B. Type and concentration of substrates

This is the most important part of the fungal environment. States (1981) regarded the fungal habitat as being controlled by the condition, concentration and chemical and structural complexity of the substrate. Fungi have a specific relationship with their substrates as a source of nutrition and energy, and differences in the chemical composition of substrates can act in a selective way for particular groups of fungi (Cooke, 1979). It is not surprising, therefore, that the frequency and range of Fusarium spp isolated from soil or plants

has been found by many ecologists to be affected by the nature and availability of substrate (Burgess, 1981).

When Nash and Snyder (1965) compared the abundance and range of the Fusarium population in cultivated soil and adjacent undisturbed soil, they found that cultivation and cropping had a significant effect on the abundance and range of fusaria in soil. Lim (1974) also found that the distribution of fusaria was influenced by the vegetation and the crop, in addition to the moisture content (MC) of the soil and other inherent characteristics.

Snyder and Nash (1968) reported that F. culmorum was extremely common in long-term cereal plots whereas it was rare in long-term plots planted in broad-leaf crops in the same locality. Similarly Windels and Kommedahl (1974) noted that F. roseum and F. moniliforme were significantly more abundant in a maize field than in non-cultivated soil, but other Fusarium spp were not affected.

The use of resistant cultivars of a particular host crop will also change the population of the relevant Fusarium pathogens (Armstrong and Armstrong, 1975).

The application of fertilizer, particularly nitrogen, can influence the frequency of some species. Warren and Kommedahl (1973) found fertilizer effected more rapid decomposition of residue and thereby reduced the population of F. roseum in the soil, because F. roseum is a poor coloniser and only survives in soil when residue is present. In contrast, F. oxysporum is a true soil inhabitant and aggressive pioneer coloniser of plant tissue.

Damage to the plant may also have an influence on the types of fungi which colonise that plant. Sutton et al. (1980) found that in natural bird injury and simulated bird injury of maize, kernels were colonised rapidly by weakly aggressive fungi. Also the toxin zearalenone was found in injured ears, but not in non-injured ears. They noted that colonised kernels occurred only in portions of ears where husks were loose or shredded. The damaged husks evidently were factors in predisposition of the ear to fungal colonisation. The loosening, opening and shredding of the husks may increase the acces-

sibility of the kernels to fungal propagules and form a favourable environment on the kernels for fungal growth. They suggested that the injured ears are exposed to direct invasion by propagules in the air, in splashed water and on insect, bird and other vectors.

1.1.2.2. Common habitats of fusaria

The majority of Fusarium species are typically soil-borne fungi and they colonise living plant parts or plant residues within the soil or adjacent to the soil surface. Nevertheless, most have both active and passive means of dispersal in the atmosphere and are common colonisers of aerial plant parts (Francis and Burgess, 1977).

A. Soil-borne fusaria

Typical representatives of the soil-borne fusaria are F. oxysporum, F. solani, F. culmorum, F. graminearum group I and F. equiseti (Burgess, 1981). Members of this group often form sporodochia on substrates on or just above the soil surface and the spores can be dispersed by rain drops directly into the soil (Gregory et al., 1959). Air dispersal of this group has also been suggested, particularly in association with dust particles, even for several hundred kilometres (Ooka and Kommedahl, 1977). Propagules of soil-borne fusaria can easily be demonstrated in the atmosphere when soil is cultivated or crops are harvested (Burgess, 1981).

A very common soil isolate is F. oxysporum (Wearing and Burgess, 1978). This species is highly variable and some strains are very pathogenic for plants, while others are typically saprophytic. Because F. oxysporum forms chlamydospores, it is well adapted to long-term survival under diverse conditions (Booth, 1971). It is the predominant fungus isolated from roots and crowns and is active under a wide range of environmental conditions (Burgess et al., 1975).

Another common soil-borne Fusarium in temperate areas is F. solani (Kommedahl et al., 1975). It persists both as a parasite and as a saprophyte. In the absence of suitable substrate it will survive as chlamydospores in soil and crop residue (Nash et al., 1961). It has been isolated occasionally from the air, probably a result of

being present in wind-blown soil or debris (Ooka and Kommedahl, 1977). Burgess (1981) suggested that F. solani is a common air-borne fungus and subsequently it will be deposited on aerial plant parts.

F. equiseti is also frequently found in and on soil (Burgess, 1981). It can survive as chlamydospores or by hyphal fragments either in soil or plant residue. Members of this species are saprophytic but have occasionally been involved in disease problems (Booth, 1971). It has been found to be a common coloniser of senescent wheat and maize tissue (Francis and Burgess, 1975) and forms sporodochia on plant residues from which the conidia are dispersed by rain-splash as well as by wind-blown soil particles.

Variation in the mode of existence can be very marked, even within members of the same species (Burgess, 1981). For example, F. graminearum group 1 and group 2 (Francis and Burgess, 1977) are morphologically similar populations but they are different in their habitats and physiological attributes. Members of group 1 survive as mycelium in host tissue and in the soil, and do not produce chlamydospores. The strains are associated with crown and root rots of cereals and grasses and form abundant sporodochia on the leaf-sheaths (Wearing and Burgess, 1977). It has been suggested that the conidia are dispersed by rain-splash. Members of group 2 are distinguished from group 1 in that they produce perithecia on and in plant residues. Active discharge of ascospores represents a good inoculum source for the infection of aerial plant parts. Members of this group are associated with stalk and ear rots of maize (Francis and Burgess, 1975).

A further ecological group of fusaria, represented by F. moniliforme and F. subglutinans, was designated by Burgess (1981). This group normally is composed of soil-borne fungi which occupy both subterranean and superterranean habitats although they are mainly colonisers of subterranean plant parts and plant residues, in the soil and on the soil surface.

B. Air-borne fusaria

The air-borne fusaria are well adapted to dispersal in the atmosphere and will, therefore, commonly colonise aerial plant parts,

although they are also often isolated from the soil. Active dispersal of ascospores occurs, but appreciable dispersal of spores, including conidia, is achieved by rain-splash, insects, birds etc. (Burgess, 1981).

Typical representatives of this group are F. lateritium and F. stilboides (Burgess, 1981). F. lateritium can cause problems in trees due to its parasitic activity, usually in wet conditions, with ascospores providing the main source of primary inoculum. The fungus is also spread by rain-splash from sporodochia.

F. stilboides is closely related to F. lateritium (Burgess, 1981). This species causes scaly bark and collar rot of coffee (Siddigi and Corbett, 1963). It is mainly spread by rain-splash of conidia and not by ascospores.

1.1.2.3. Incidence and significance of fusaria in maize crops

Kommedahl and Windels (1981) considered that one or more species of Fusarium can be isolated from any part of a maize plant, particularly when the plant is exposed to stress due to biotic, climatic or edaphic factors. However, most of the damage caused to plants (seedling blight, root rot, stalk rot and ear rot) is associated with a complex of opportunistic fusaria and other fungi. The effects of invasion can vary from mild to extensive, depending on the amount and type of stress.

Most of the data available today on the incidence of Fusarium spp in maize has been obtained from plant pathologists and indicate that these fungi, particularly F. graminearum, F. moniliforme and F. subglutinans, are of world-wide occurrence. These species have been found as pathogens of maize in Western Europe (Cassini, 1981), Eastern Australia (Burgess et al., 1981), the U.S.A. (Windels and Kommedahl 1984) and elsewhere. Most of the Fusarium spp which have been found on maize in the U.S.A. belong to the sections Roseum, Gibbosum and Discolor (Vesonder and Hesseltine, 1981).

The majority of Fusarium spp associated with maize are distinctly "field fungi". However, some species (F. culmorum, F. poae and F.

tricinctum) can grow during storage. "Storage fungi" occur particularly when grain with high water activity is stored at a low temperature (Smith et al., 1984). F. moniliforme and F. subglutinans have also been classified as storage fungi of maize (Marasas et al., 1979c; Russell et al., 1982) and even F. graminearum (Jackson et al., 1974) but the separation of field fungi and storage fungi is not absolute and many fungi can show intermediate properties (Lacey et al., 1980).

A. Fusarium spp in "field" maize

F. graminearum is principally associated with head blight, ear rot and stalk rot of maize. Other species, eg. F. avenaceum, F. culmorum, F. subglutinans and F. tricinctum and their teleomorphs have also been frequently recorded in Canada, Eastern Australia, Eastern Europe and the Soviet Union (Burgess et al., 1981; Sutton, 1982; Windels and Kommedahl, 1984).

F. graminearum can appear as a pinkish to reddish mould on the kernels (Sutton, 1982). In severely affected ears, the husks commonly adhere to the kernels and F. graminearum will invade the cob (ear) as well as the shank. The fungus is often found to be more common in the basal halves of the ear when the husks have been shredded by birds (Sutton et al., 1980). Perithecia of Gibberella zeae, the teleomorph of F. graminearum, may develop superficially on infected husks, kernels and stalks particularly in prolonged wet weather. Although the standing plants and seed are sources of inoculum for the spread of the fungus, debris on the soil surface is considered the main reservoir (Sutton, 1982).

During their intensive study of root and stalk rot in maize in the U.S.A. from 1973-1983, Kommedahl and his colleagues found that F. graminearum and F. moniliforme were the main pathogens involved. F. graminearum was isolated more frequently at anthesis (flowering) and extended throughout the growing season. It was the predominant stalk rot organism late in the season (Windels and Kommedahl, 1984).

Kommedahl et al. (1979) isolated several Fusarium spp from roots and stalks of maize. The same species and strains were frequently

found in soil where maize had been grown. F. oxysporum and F. solani were most frequently isolated from roots and F. moniliforme and F. tricinctum most frequently from stalks. F. equiseti, F. acuminatum and F. graminearum apeared more often in the roots than in the stalks.

Moubasher et al. (1984) isolated 3 Fusarium spp, F. moniliforme, F. oxysporum and F. acuminatum from the rhizoplane of healthy and damped-off maize seedlings. F. moniliforme was the most common.

Marasas et al. (1979c) found the most frequent Fusarium spp infecting maize kernels in South Africa were F. subglutinans, F. moniliforme and F. graminearum. They noted that the mean percentage of kernel infection by all the three species increased during storage. Their finding that F. subglutinans was the most prevalent species differed from results available from other countries. They considered that this was due to frequent misidentification and consequent mistaken classification of F. subglutinans as F. moniliforme.

Marasas et al. (1979c) also noted that the three spp were associated with different climatic conditions. F. subglutinans was predominant in temperate areas and F. moniliforme in sub-tropical areas. F. graminearum was common in areas with intermediate climate. Francis and Burgess (1975) also suggested that F. subglutinans is more prevalent than F. moniliforme in cooler climates.

Marasas et al. (1979a) isolated six Fusarium spp from maize and barley grains in West Germany: F. avenaceum, F. culmorum, F. equiseti, F. oxysporum, F. poae and F. tricinctum. Fusarium avenaceum was the most commonly isolated species from bird-damaged maize ears. The predominant species in barley grains was F. poae. Isolates of F. avenaceum, F. culmorum, F. equiseti and F. oxysporum were also shown to be toxic when cultures grown on autoclaved maize were fed to day-old chickens.

Fusarium spp can be frequently isolated from grain left over winter. Neish et al. (1983), examining maize grain over-wintered in a field in Canada, found F. graminearum, F. moniliforme, F. subglutinans and F. sporotrichioides accounted for 84% of the total Fusarium isolates with another five species accounting for the remaining 16%.

B. Fusaria associated with stored maize

The number of fungi found in stored grain depends mainly on the condition of the grain prior to harvest and the conditions of storage (Lacey et al., 1980). The presence and activity of Fusarium spp in stored grain will be controlled by several factors, of which the most important are the water activity of the grain, the temperature, amount of aeration and period of storage, and insect and mite infestation (Lacey et al., 1980). If grain has been heavily infected with Fusarium spp in the field, particularly during any delayed harvesting, the grain may also be contaminated with Fusarium toxins. Thus both Fusarium propagules and toxins will initially be stored with the grain. The majority of fusaria will die after drying or during proper storage but the toxin will stay stable for a long time (Mills, 1982).

Marasas et al. (1979c) found that F. graminearum, F. moniliforme and F. subglutinans were the predominant species on the maize at harvest and the level of infection by these three species increased during an eight-month storage period under commercial conditions, but apparently the grain had not been dried adequately before storage.

Fusarium subglutinans was also found to be the most common isolate in a maize sample stored for 9 months in a grain bin (Farnworth and Neish, 1980). The grain had been left standing in the field during the autumn and winter before harvesting.

Russell et al. (1982) found a high incidence of contamination of maize samples with Aspergillus, Penicillium and Cephalosporium species. After a 35-day period at 40^{o}C and subsequent storage at 6^{o}C, only F. verticillioides (= F. moniliforme or F. subglutinans) was found, the presence of which had not been demonstrated earlier. They assumed development of this species had initially been inhibited by the presence of other seed-associated fungi and it alone had survived the storage successfully.

1.1.2.4. Fusarium species in New Zealand

Some Fusarium spp were known to New Zealand plant pathologists as early as the beginning of the 1930's. The occurrence of members of

this genus, particularly the soil-borne species which were involved in root diseases of cereals and grasses, was investigated extensively (Neil and Brien, 1935; Blair, 1936; Blair and Morrisson, 1949). Neil and Brien (1935) also reported that the dry-rot disease of maize cobs, which had caused severe crop losses in the Bay of Plenty and Gisborne areas, was mainly caused by Fusarium spp. They isolated two Fusarium spp, F. moniliforme and F. subglutinans, and noticed that these species were found on a wide range of cereals and grasses.

Blair (1936) working in the Canterbury region, found that foot-rot of wheat was caused mainly by fusaria, particularly F. culmorum. Other diseases which have been associated with this species are seed-ling blight, spring yellow, whitehead and scab. Fusarium culmorum was also isolated from black root rot in strawberries (Smith, 1953) and from basal rot in carnations (Robinson, 1961).

Blair and Morrisson (1949) listed the wheat diseases in New Zealand as including those involving five Fusarium spp: F. avenaceum, isolated from unthrifty seedlings and foot rot in wheat; Gibberella intricans (perfect stage of F. equiseti), associated with a basal rot in grasses, cereal and Phormium flax; G. zeae (perfect stage of F. graminearum), found as a common cause of basal rot in cereals and grasses as well as causing an ear blight in wheat; G. saubinetii (perfect stage of F. sambucinum), causing a crown rot and foot rot in wheat, and lastly F. sporotrichoides associated with false scab on wheat.

Two other soil-borne fusaria have been commonly found in New Zealand. Fusarium oxysporum has been attributed to several problems, such as destructive wilt of aster (Cunningham, 1931), flax wilt (Baylis, 1940) and vascular wilt in carnations (Robinson, 1961). Fusarium solani causes a dry rot in beans (Brien et al., 1955). There are a few less well-known species such as F. coeruleum, associated with a dry rot in stored potatoes (Brook, 1957), F. redolens causing asparagus seedling blight (Dingley, 1965) and F. semitectum which causes storage rot in fodder beet (Brien and Dingley, 1959).

Matthews (1965) recorded F. nivale for the first time in New Zealand from cereal seed of barley, wheat and ryecorn. F. nivale is

now included within the Hyponectriacae as Microdoctium nivale (Samuels, 1984). It is known to be present on wheat, barley and rye at low levels. Matthews (1965) isolated F. nivale from 7 out of 10 wheat samples, but the percentage of infected seed was very low (0.3-1.0%). In a study of foot-rot problems in New Zealand wheat, Sanderson (1972) found F. nivale together with F. culmorum, F. avenaceum and F. graminearum to be widely spread in New Zealand soils.

Fusaria in pasture soil were investigated by Thornton (1958, 1960 and 1965). He noted that F. oxysporum was the predominant isolate in mild temperatures and in moderately acid soil. It occurred less or was absent in cool, moist conditions and in less acid soils. F. culmorum was isolated from root specimens of ryegrass (Lolium perenne L.) and white clover (Trifolium repens L.) of six soil samples, but at a low level. F. oxysporum was also common from root nodules of the above grass, with F. avenaceum, F. culmorum and F. solani being isolated less frequently.

An ecological study of Fusarium spp associated with grasses was carried out by Keogh (1973). He investigated the influence of grazing animals on the distribution of saprophytic fusaria, using a sample-washing and spore-counting procedure. He classified his findings using the criteria of Colhoun and Park (1964) into 3 groups based on spore characteristics. Group 1 was of F. culmorum, group 2 spores were mostly of F. avenaceum and group 3 contained spores of other Fusarium species.

A number of other researchers have been attracted to Fusarium problems in New Zealand pastures. During a study of root rot of clover, Menzies (1973) found that Fusarium spp formed 19% of a total of 817 fungal isolates. A series of publications by Skipp and Christensen (1981, 1982 and 1983) reported on a study of micro-organisms invading white clover roots, particularly in the Manawatu district. Their results showed that F. oxysporum was the most frequent isolate and was the major root invader among Fusarium spp. Less frequently four other fusaria were isolated: F. avenaceum, F. culmorum, F. nivale and F. equiseti. Loon Lui Lim and Cole (1984) similarly found that F. oxysporum was predominant in white clover seedlings with F. avenaceum being less significant.

Fusarium was one of five genera recorded by Hampton (1977) as seed-borne pathogens of New Zealand wheat. During his study on Drechslera spp in New Zealand cereals, Sheridan (1977) found that Fusarium spp which infected wheat, barley and oat seeds were insignificant and their infection range was between 1% and 8%. However, this result was not supported by Hampton's (1980) work on wheat-seed certification. Hampton found that Fusarium spp were present in over 85% of the infected seeds tested during the two seasons 1977-1978 and 1978-1979. He recorded five Fusarium spp, the most common of which were F. avenaceum, F. culmorum and F. nivale. The remaining two were F. graminearum and F. poae. Chong and Sheridan (1982) found that fusaria were present in 97% of 32 barley seed samples examined with a mean recovery per sample of 16%. Nine species were identified - F. arthrosporioides, F. avenaceum, F. culmorum, F. equiseti, F. fusarioides, F. graminearum, F. lateritium, F. oxysporum and F. poae.

More recently Lee (1986) found that New Zealand barley was contaminated with Fusarium spp at a frequency of 100% (8/8 samples) at harvesting time but this frequency decreased to 71% after 5 months' commercial storage and 33% after 9 months' storage. She isolated 12 species (F. acuminatum, F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. moniliforme, F. nivale, F. oxysporum, F. poae, F. sambucinum, F. semitectum and F. sporotrichioides).

Fullerton (1978) summarised the common fungal problems of New Zealand maize. The pathological problems were stalk rot, cob rot, root rot and sheath blotch, all of which were commonly caused by Fusarium spp. He also found that F. moniliforme, Gibberella zeae, F. oxysporum and F. roseum were present on grasses, debris and in the soil, and discussed the ecosystems of these species. Cob rot occurred when the cobs contacted the soil or other inoculum sources. Maize husks could also be damaged by birds or by caterpillars, thus making the husks more predisposed to infection. Spores situated on debris from the previous crop could be carried by rain or rain-splash into the whorls of seedlings. Fungal spores on leaves could be spread by wind and rain-splash throughout the crop and into adjacent crops.

Falloon (1982) found that F. oxysporum was the most frequently isolated fungus capable of killing maize seedlings. He recorded other

Fusarium spp (F. solani, F. culmorum and F. avenaceum) but these were less common as pathogens in such plants.

In addition to being important pathogens of grass pasture and cereals in New Zealand, fusaria have been linked with another problem, "Ryegrass Staggers". Latch et al. (1976) reported on studies of the fungal population in ryegrass (Lolium spp) pastures in New Zealand over a five-year period. They found that Fusarium spp were the dominant fungi in those paddocks surveyed in which problem staggers occurred. Fusarium conidia were present in over 85% of herbage washing. Fusarium culmorum, F. avenaceum, F. concolor (of Booth, 1971), F. subglutinans, F. nivale, F. oxysporum and F. semitectum were all isolated with F. culmorum and F. avenaceum being the most common. Latch et al. noticed a generally positive correlation between a toxic pasture (one causing ryegrass staggers) and the number of Fusarium conidia present. They maintained a hypothesis that ryegrass staggers may be due to Fusarium mycotoxin production. It has since been found that this may be incorrect (Gallagher et al., 1985).

Recently (Lauren, 1986) has recorded a range of Fusarium spp from North Island pastures, including F. crookwellense, F. culmorum, F. graminum, F. graminearum, F. avenaceum, F. oxysporum and F. acuminatum. Fusarium crookwellense was the most common isolate.

1.2. SAMPLING METHODS FOR THE MYCOLOGICAL STUDY OF FUSARIUM SPP

Fungi in different ecological groups require different sampling techniques for their isolation and enumeration. The failure to recognise fungal communities in the past can be partially attributed to inadequate sampling procedures and inaccurate species identification (States, 1981). Lussenhop (1981) considered that fungal communities have been studied with conservative methods because the relationship between the collected sample and the natural fungal units within it was not clear. Furthermore few reliable statistical procedures have been applied to sampling, either from soil or from grain in the field (Parkinson et al. 1971), although random sampling is a common practice for sampling stored grain (Smith et al., 1984).

Samples are collected for two main reasons - either simply as a source of microorganism (e.g. in investigations of antibiotic production) or for studies of the natural state of the habitat (Parkinson et al., 1971). In the former case very few sampling precautions will be needed and it will not matter whether the sample has been disturbed or altered, even if sieved, air dried and stored for some months before being processed. In contrast, in investigations into relationships, the main aim of any sampling method must be to reveal the general distribution of the fungal units in the field and in the microenvironment. Errors such as sampling errors, selection errors, measurement errors etc. should be reduced as far as practically and economically feasible. Preliminary evaluations of any chosen method must always be performed (Burgess and Liddell, 1983).

Among the factors to be considered in the choice of an isolation technique are the nature of the sample, the number of samples which need to be taken, the range of fungi likely to be present and if it is necessary to isolate all or only some of the fungi (Burgess and Liddell, 1983). If only a few samples are required then a range of techniques can be chosen to maximise the probability of isolation of fungi. But in the case of extensive surveys, the large number of samples which would need to be processed may well preclude the use of more than one procedure. Burgess and Liddell (1983) recommend reducing the number of objectives in extensive surveys wherever possible, to facilitate a reduction in compromises required in the selection of isolation procedures.

There is no single procedure available for the determination of all types of fungi existing within any given habitat, even for the species within one genus (Lussenhop, 1981). In the case of Fusarium, Parkinson et al. (1971) felt that it is impossible to devise a single isolation technique by which all species present in the soil can be isolated, partly because different species are present in different niches within that habitat. McMullen and Stack (1983) pointed out that not only is any isolation technique somewhat selective but also that the fusaria are present in the soil and other substrates in different forms, i.e. conidia, ascospores, chlamydospores and mycelial fragments. They concluded that using a single isolation technique or

medium may be insufficient to fully discover the distribution and abundance of Fusarium spp in the substrate.

1.2.1. Soil

Most of the data available today about sampling procedures has been obtained from studies of the fungal ecology of soil as this natural habitat has been studied more intensively than other habitats (Cooke, 1979). Moreover, soil is an important reservoir of plant-associated fungi and by using appropriate methodology, a strong relationship between the soil fungal community and the plant community can be found.

The basic soil unit can vary greatly from acres of a field to a single horizon. Any sub-sample taken from the soil unit constitutes only a small part of the whole unit but one must assume that such a sample is representative of the whole soil unit (Parkinson et al., 1971).

Nash and Snyder (1962) examined soil to determine whether there was any significant difference between Fusarium (F. solani) viable counts recorded using different sample sizes by comparing composite core samples, single core samples and very small individual samples (43 mg). No significant differences (at the 95% confidence level) were found between the counts of F. solani propagules from the three methods using the dilution-plating technique. Nash and Snyder (1962) also found that propagules of F. solani were evenly distributed in a field when soil recently cropped to beans was examined. However, the viable count dropped markedly and the distribution of the fungus became less uniform where beans had not grown for 1.5 years. The work of McMullen and Stack (1983) also indicated that sampling site did not significantly affect isolation of Fusarium spp but other investigations have found Fusarium to be unevenly distributed. Wearing and Burgess (1977), for example, found that F. graminearum group I was unevenly distributed in soil at sampling sites within wheat fields even where crown rot incidence was uniform.

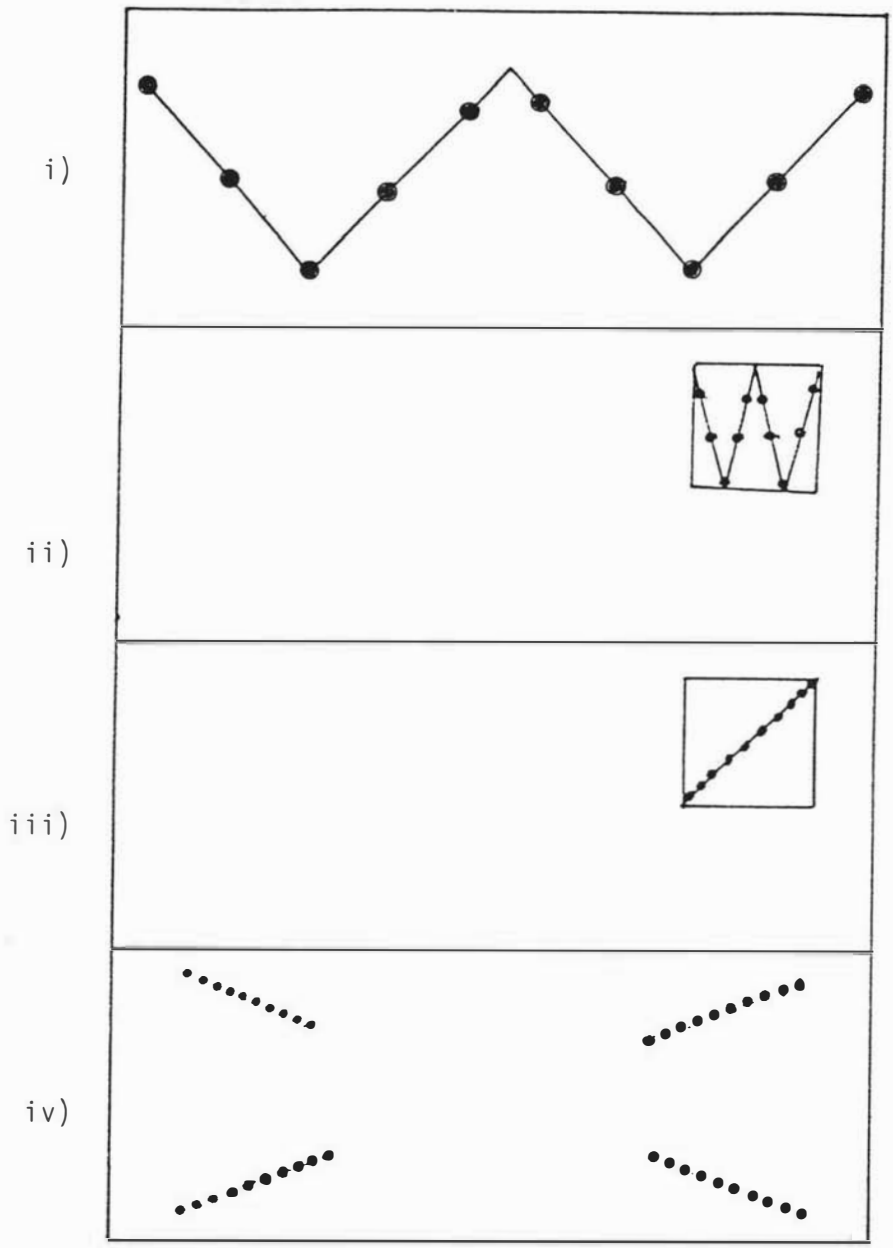
Because Fusarium spp and other fungi can be expected to have uneven distribution in soil, mixing individual soil samples from the

sampling area into a composite sample is a very common practice (Burgess and Liddell, 1983). It must be accepted that if individual fungal species are distributed irregularly, they are likely to be more abundant in some of the individual samples in comparison to their abundance in the composite sample.

Random transection and composite sampling are very common methods of collection. For example, McKenzie and Taylor (1983) collected five composite soil samples on a random transect, each consisting of three cores. Then the composite samples from each transect were bulked, mixed thoroughly and passed through a 200 mm mesh sieve before sub-sampling. Wearing and Burgess (1977) collected 1 kg soil samples from each sample site. Each of these composite samples consisted of sub-samples, each approximately 200 g, from five locations within an area approximately 5 m diameter on a sampling arc 50-75 m from the boundary of the crop (the number of sample sites per field was not given). Wearing and Burgess (1978), using a slightly different method, collected 1 kg composite soil samples per field. Each composite sample consisted of 5 sub-samples of approximately 200 g each, obtained from soil adjacent to five stalk-rot affected plants.

Collecting samples at a constant interval along a path of predetermined shape within the field such as diagonal, V- or W-shaped is common practice (Basu *et al.*, 1977). Basu *et al.* carried out a very useful study to determine the effects of the size of sampling area, the pattern of sampling paths and the spread among sampling sites in relation to the incidence of common leaf spot in alfalfa fields. Basu and his colleagues applied the following methods for sampling (Figure 1-1): i) they sampled 10 approximately equally-spaced sites located on a long W-shaped path covering the entire field; ii) they demarcated a square area of 1 acre in one of 4 quadrants chosen arbitrarily and within the area 10 equally-spaced sampling sites were located along a W-shaped path; iii) they sampled 10 equally-spaced sites located along the 90 m diagonal of the same demarcated area of method (ii) and iv) they sampled 10 equally spaced sites from each of four 90-metre paths located along the field diagonals, each path being halfway between the centre and a corner of the field. After analysis of variance associated with fields, paths within the field and sites within the paths, the authors recommended

Figure 1-2: Diagram showing the relative position (•) of field sampling sites in the 4 sampling methods of Basu et al. (1977)



i) W-shaped path - total 10 site samples

ii) W-shaped path - total 10 site samples from a 1-acre quadrant in the field

iii) 90-m diagonal in a 1-acre area - total 10 site samples

iv) 4 90-m diagonals from each quadrant - total of 40 site samples

the sample should be taken from sites throughout the entire field such as the W-shaped path of 10 site samples (Figure 1-1 (i)).

1.2.2. Soil Organic Matter (Litter)

Satchell (1974) briefly defined the litter layer as dead plant material, or animal manures which may be present on the soil surface. This definition includes all organic materials derived from animals or plants (eg. fallen leaves, dead herbaceous matter, flowers etc.) This organic material on the surface of the soil is sometimes divided into three layers. The "L" (litter) layer is the uppermost, and normally consists of freshly-fallen plant material (not decayed). The "F" (fermentation) layer is beneath the L layer and is where active decomposition is taking place. The lowermost "H" (humification) layer comprises more or less amorphous organic matter (Satchell, 1974).

It is becoming recognised that litter as a specific habitat, with a specific association of fungi is an important part of ecological studies (Cooke, 1979). Such fungal populations are normally derived from the previous generation of litter materials, from soil populations and from the associated areas of plant surfaces which have been colonised (Cooke, 1979).

Dickinson *et al.* (1981) regarded fungi, and in particular the fusaria, as the most important colonisers among the microorganisms of agricultural litter. Litter is the main reservoir of several *Fusarium* spp. Wearing and Burgess (1977) found that *F. graminearum* and *F. avenaceum* survived normally as hyphae in litter, and Kommedahl *et al.* (1979) isolated five *Fusarium* spp from a single fragment of decayed maize stalk.

Because the fungal population persists in different forms in nature and because there is a very wide variety of litter components, a variety of methods for the sampling and processing of litter has been developed. The purpose of the investigation and the nature of the litter (i.e. forest, agricultural etc.) are important factors to consider in the selection of sampling procedures. Some investigators use selective methods while others deal with litter on a broad base. During their long-term study of corn-stalk rot problems in the U.S.A.,

Windels and Kommedahl (1984) selected only rotted corn-stalk debris which was in contact with the soil. In Australia Wearing and Burgess (1977) collected only weathered wheat stubble litter to study F. graminearum (group 1) in a wheat field, and later (1978) weathered maize stubble from a maize field.

Among the more broadly-based studies which have been performed, Goodfellow and Dowson (1978) included the three litter layers (L, F and H) in their sampling of the litter of a spruce forest. Samples (1 kg each) of each layer were aseptically transferred to new polythene bags. The samples were then bulked and mixed before the micro-organisms were assayed. Similarly in his investigation of the interaction of basidiomycete decomposers in the litter of an area of 0.5 ha, Newell (1984) subdivided the litter layer into 4 sublayers - L (1.5 mm in thickness), F1 (5-10 mm), F2 (10-20 mm) and H (10-25 mm) - horizons. Ten random samples of litter (5 g fresh weight) were collected, mixed from the above 4 layers, or 1-2 g separately, every two weeks.

1.2.3. Maize Grains

Sampling of standing crops poses similar problems to those of soil and litter sampling. Samples of maize, for example, may be taken randomly from crops growing in the field, during handling, during storage etc.

Because moulds (and mycotoxins) are rarely uniformly distributed throughout organic materials, their occurrence is rather spasmodic. Sampling technique is possibly the most important factor in any survey of fungi or mycotoxins in natural products such as cereals or animal feeds (Smith et al., 1984).

General recommendations on grain sampling techniques and sample size, particularly from the field, are not easy. Davis et al. (1980) set general guidelines which are discussed below, but these do not completely achieve their purpose as they assumed that large samples will increase accuracy. The cost factor will of necessity have to be considered and reconciled with an acceptable level of accuracy.

1.2.3.1. Sampling maize in the field

Fusarium spp, with few exceptions, can be regarded as field fungi which normally grow on grain before harvest and do not develop further in storage. But sampling from the field is a most difficult task and reliable sampling, representative of the whole field, is not error-free (Davis et al., 1980). The techniques which have been used in the past for maize field sampling have been varied and there has been no standard procedure (Scott and King, 1984). However, Davis and his colleagues found that the coefficients of variation in analyses of aflatoxin could decrease from 133% for a sample of 36 maize ears to 83% for 100 ears and 39% for 800 ears where there was a mean of 100 ppb aflatoxin in the grain. But sub-samples taken after harvesting the whole field gave a coefficient of variation of 29% between all samples. Similarly, Smith et al. (1984) recommended taking a field sample at harvesting time from the harvester or from the loading truck to ensure good random mixing. If it is necessary to collect a sample from growing crops, they recommend collecting large numbers of widely-distributed units or seeds. In practice it is difficult to find investigators following such recommendations. Scott and King (1984) collected the top ear from each of the first ten plants of each plot (a plot was a single 5 m long row) 60 days after the mid-silk stage of development. After the ear had been dried at 40^{o}C for 5-7 days the cobs were shelled and the kernels thoroughly mixed. A random sample was withdrawn and stored at 6^{o}C until used. Vesonder et al. (1978) sampled a maize field 20 rows in from the edges. From each of two diagonally opposite locations 100 cobs were examined visually for Gibberella ear rot. The first 20 to 25 cobs showing the disease were collected, but in the case of no Gibberella rot being found, a random 20 to 25 cobs were collected. The cobs were hand-shelled, put in plastic bags and mixed by shaking. A sample for microbial and mycotoxin assaying was then removed.

1.2.3.2. Sampling stored maize

Again there is no standard method or recommended sample size, and in most cases the stored grain samples are collected by non-mycologists. However, it seems that the stream-cut technique carried out during the unloading of silos or bins is the most reliable method

(Davis et al., 1980). This method involves the collection of small portions of grain from the moving stream during loading or unloading, at periodic intervals, and then combining these portions as a sample. Other methods include that of Lacey et al. (1980), who recommended drawing the stored samples from different depths using sampling probes. Shotwell et al. (1975) collected 8 samples (50-100 g) from different places in a small wooden bin 6.5 x 7 ft. By using a grain probe they ensured inclusion of the top, middle and bottom portions of the same location. Some less popular methods of sampling have been reported, including hand selection of only infected kernels from storage cribs (Marasas et al., 1979c).

Actual sample size has varied from the 70 kg samples of Marasas et al. (1979c) who collected maize samples from a grain elevator, to the 50-100 g samples of Shotwell et al. (1975) described above. Whitaker et al. (1979) handled 400 samples of 1 kg each, collected from commercial lots, which they then combined and subdivided into 10 small lots (40 kg each) called minilots.

1.2.4. Sub-sampling and the Number of Replications

Sub-sampling is necessary as it is impossible to process an entire sample. Sub-sample size is again highly variable, there being no standard method. Indeed, the sub-sampling technique can add another error to the sampling procedure.

Miller et al. (1983) used soil sub-samples of 0.8 g and no replication, whilst McKenzie and Taylor (1983) used 10 g of oven-dried soil and five replicate determinations. Sturz and Johnston (1985) sub-sampled 2.5 g of oven-dried soil from each bulked field sample and eight replicate plates were prepared.

For maize, it is equally important that sub-samples are representative of the whole. This can be achieved in several ways. One popular method is to carefully mix the sample, e.g. by rolling or shaking the grain in a partly-filled jar or closed plastic bag to avoid loss of spores, and subsequently taking small samples from different parts of the bulk sample. Alternatively, bulk samples can

be repeatedly quartered, discarding the two opposite quarters, until a suitable sized sub-sample is obtained (Lacey et al., 1980).

For the actual kernels to be processed the sub-sample size again seems to be arbitrary (Scott and King, 1984). Ikenberry (1961) cited by Scott and King used a sub-sample size of only 4 kernels. Vesonder and Ciegler (1979) used 50 kernels, and Scott and King (1984) 130 kernels. Farnworth and Neish (1980) used a total of 300 kernels which they plated out on 3 different media. The replication of sub-samples also differs widely. Ikenberry (1961) used one, Marasas et al. (1977) two, King and Scott (1981) three and Scott and King (1984) six replications.

Scott and King (1984) carried out an important trial on sub-sample size for maize kernels. Their results showed that a sub-sample size of 13 kernels was too small to give a precise estimation of kernels internally infected with F. moniliforme. A sub-sample size of 65 kernels gave a reasonable estimation, while a sub-sample size of 130 kernels seemed quite satisfactory.

Scott and King (1984) suggested that a 100 kernel sub-sample size and three sub-sample replications are adequate for culturing by direct plating. However, a 100 kernel sub-sample size with no replications is the most common method for the detection of Fusarium spp and other fungal genera (Marasas, 1979b).

In the case of the dilution-plating technique, a sub-sample of 10 g ground maize, with platings of dilutions is often used (Flannigan, 1977), although Miller et al. (1983) used 10 sub-samples of oven-dried ground maize of 0.5 g (total 5 g).

1.2.5. Laboratory Storage of Samples

While most investigators prefer processing their samples as soon as possible after collection, others have stored their samples in a cold place (normally 0-4^{o}C) for varying periods. A few prefer to deep-freeze samples at -18^{o}C. Unfortunately there are very few studies on the effect of low temperature on the death rate of micro-organisms in soils and litters (Parkinson et al., 1971). In addition,

the optimum storage period of samples is not well known, as there is no accurate information on the permissible storage time for different soils. Parkinson et al. (1971) considered most researchers to be rather pragmatic in their work out-look on this question.

Lacey et al.(1980) considered that samples should be processed as soon as possible following collection, but in contrast Windels and Kommedahl (1974) dried soil samples at room temperature for 48 h, then stored them in plastic bags at 8°C for a maximum period of 9 months before processing.

The common storage period is 2-4 weeks. McMullen and Stack (1983) stored their samples at 5°C and processed them within 3 weeks. Wearing and Burgess (1977) stored soil and litter samples in paper bags in the laboratory until processing.

Grain samples should be stored at 0°C or even -18°C to prevent change in the microflora in cases where storage is unavoidable (Lacey et al., 1980) although in general grain with moisture content of 13-13.5% is safe for long-term storage (Davis et al., 1980). Davis and his colleagues presented data about the relationship between moisture content, period of storage, temperature and numbers of fungal propagules/g grain. Maize stored at 0°C for 8 days at 21.3% moisture content showed no change in fungal propagules. But when a duplicate sample was stored at 24°C fungal propagules increased 1500-fold. When another maize sample was stored at a MC of 12.6% at 24°C no change in the numbers of fungal propagules was noticed. Davis and his colleagues pointed out that samples should preferably be stored at 0°C, although economics generally preclude this practice in large surveys.

1.3. ISOLATION TECHNIQUES FOR *FUSARIUM* SPP

1.3.1. Isolation Procedures

The standard dilution-plating technique is one of the oldest and most useful techniques for quantitative studies of fungi, particularly in the soil environment, despite severe and perhaps undeserved criticism (States, 1981). Parkinson et al. (1971) considered that this method has in many cases been inappropriately applied, and they

suggested that other techniques such as the soil-washing technique and direct isolation are adequate, particularly for rhizoplane fungi (fungi associated with roots).

The soil plate technique, normally used for fast-growing fungi which are present as spores, has for some fungi been found to be better than the standard dilution-plating technique (Warcup, 1957). McMullen and Stack (1983) compared the use of the soil plate technique with others because it seemed to be less time consuming. Their conclusions after preliminary studies were that there was no difference between the numbers and types of Fusarium spp which could be isolated by each technique.

Enumerating grain fungi can be carried out either by plating whole grains (direct plating), by serial dilution of washings from the whole grain, or by dilution of a suspension of comminuted grain (Smith et al., 1984).

By using the dilution-plating technique and suitable replication, the fungal viable counts as colony forming units (CFU) can be obtained using media such as Nash and Snyder's (1962) peptone-pentachloronitrobenzene (PCNB) agar for Fusarium or potato dextrose agar (PDA) for fungi in general (see Section 1.3.2).

Price (1984) suggested that the accuracy of measuring the presence of Fusarium in the soil is a function of the technique, whatever selective medium is used and whatever the number of propagules/g. Much depends upon the pretreatment of the soil before dilution and the diluent used. Both the number and uniformity of distribution are much improved with more viscous diluents.

Probably all plant pathologists use the direct plate technique (Marasas et al., 1979c; Farnworth and Neish, 1980; Vesonder et al., 1978). The usual technique for detecting seed-borne pathogens is to first pre-treat the seeds to eliminate surface-borne "saprophytic fungi" and then, after drying, to plate out on solid medium (Flannigan, 1977).

Smith et al. (1984) preferred the use of the direct plate technique over the dilution-plating technique, particularly for soil debris, plant roots, litter and grain kernels. Wearing and Burgess (1978) isolated F. graminearum group 2 in 4 out of 5 samples by using the direct plate technique, but from only 2 out of 5 samples using the dilution-plating technique.

Mislivec and Bruce (1977) found the direct plating technique to be more effective in detecting individual fungal species from soybean and dried bean samples. These authors reported an average of 12.9 species by direct plating but an average of only 4.4 species by dilution plating. Although direct plating can yield a wider range of species than dilution plating, particularly for fungi that do not produce abundant spores, e.g. Alternaria, Epicoccum and Fusarium (Lacey et al., 1980), it is nevertheless very common practice for mycologists to use both techniques and more than one medium to increase the frequency, density and diversity of Fusarium spp obtained (McMullen and Stack, 1983).

There are many other less commonly used techniques which can be applied depending on the type of substrate and the aim of the study. Latch et al. (1976) studied the fungal population in New Zealand ryegrass pasture using three techniques - herbage washing, Brook spore trapping and propagule plating. No one technique gave a complete record of the species present. Burgess and Liddell (1983) isolated perithecium-producing fusaria by washing out small pieces of infected plant tissue bearing perithecia and placing them on the inner side of the lid of an inverted petri dish containing water agar (WA) or carnation-leaf agar (CLA). The tissue was held in place with petroleum jelly. After 24 h incubation at 25^{o}C the ascospores were released on the surface of the agar where they then germinated.

1.3.2. Media for Isolation and Identification

Because fungi vary in their nutritional requirements, the medium used can be a determinant of which species are isolated (Papavizas, 1967). Selective media for several Fusarium spp are available. Martin's rose bengal agar (Martin, 1950) consists of 10 g dextrose, 5 g peptone, 1 g KH_2PO_4, 6.5 g $MgSO_4.7H_2O$, 20 g agar, 1,000 ml tap water

and 1:30,000 rose bengal and 30 µg/ℓ streptomycin. Nash and Snyder's (1962) peptone PCNB agar consists of 1.5% Difco peptone, 2% agar, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 1,000 ml water, 300 ppm streptomycin and 1:100 pentachloronitrobenzene (PCNB). Papavizas' (1967) modified peptone-PCNB has a similar composition to Nash and Snyder's medium except that 0.5 g oxgall and 50 mg of chlorotetracycline HCl are included. Several modifications of Nash-Snyder medium have been suggested (Kerr, 1963; Papavizas, 1967; Stoner and Cook, 1967), using various concentrations of PCNB and different antibiotics, but none add any real advantage.

To evaluate these selective media and their effect on the isolation of Fusarium spp, Papavizas (1967) tested 18 different media for the recovery of fusaria from the soil and concluded that the peptone-PCNB agar in its original form or in a modified form was overwhelmingly advantageous. The selectivity of the medium depends on the PCNB as a good fungal inhibitor for the troublesome fungi (Mucor and Rhizopus). The PCNB also has been added to potato dextrose agar medium by the European Brewing Committee (1981) for the isolation of Fusarium spp from barley.

As an alternative to PCNB (it has been reported as potentially carcinogenic by Andrews and Pitt [1986]), dichloran (2,6-dichloro-4-nitroaniline) has been used. King et al. (1979) added dichloran (2 µg/ml) to rose bengal medium and Andrews and Pitt (1986) replaced the PCNB in Nash-Snyder medium by dichloran for isolating Fusarium spp and dematiaceous hyphomycetes from cereals. King et al. (1979) found dichloran one of the best of 31 antifungal compounds for ability to inhibit fast-growing moulds (Mucor and Rhizopus), while allowing growth of other fungi, particularly the mycotoxigenic fungi.

Lacey and Dutkiewicz (1976) considered that it is extremely critical to choose a specific medium for all fusaria. They found that potato dextrose agar (PDA) was satisfactory for isolating species of this genus. While not being a selective medium, it gave good counts when using inhibitory reagents such as oxgall and PCNB to restrict the growth of fast-growing fungi such as the Zygomycetes. Burgess and Liddell (1983), however, found that PDA was useful only in isolating the slow-growing fusaria, e.g. F. lateritium, F. decemcellulare etc.

They considered that this medium, in common with other rich carbohydrate media, had many disadvantages such as its tendency to encourage the growth of non-target organisms (Zygomycetes etc.) and its apparent promotion of rapid mutation in some Fusarium spp. Homemade PDA is preferred by many mycologists because it stimulates spore production processes adequately (Burgess and Liddell, 1983; Sitton and Cook, 1981).

The carnation-leaf agar (CLA) suggested by Toussoun and Nelson (1976) has been strongly recommended by Fisher et al. (1982) and Burgess and Liddell (1983) as an isolation medium as well as an identification medium. It is not a selective medium, but will encourage the growth and sporulation of a large range of fungi including fusaria. The low nutrient level inhibits bacterial growth and the growth of troublesome fast-growing fungi. Therefore Burgess and Liddell (1983) recommended using CLA and PDA media for the identification of Fusarium spp and did not consider the use of selective media was necessary for identification. CLA is often first choice for identification purposes, as it promotes good growth and sporodochium formation and produces uniform conidia of typical morphology (Fisher et al., 1982; Nelson et al., 1983).

Domsch et al. (1980) found oatmeal agar (OA), potato-sucrose agar (PSA) and the use of black light or daylight were good for inducing sporulation in fusaria. PSA medium was also recommended by Neish et al. (1983).

McMullen and Stack (1983) in their comparison of techniques and media useful for the isolation of fusaria from soil, roots and debris, found that the diversity of fusaria was greatest with Martin's rose bengal medium, although this medium supported the recovery of several other fungi, eg. Trichoderma, Penicillium, Rhizopus and Aspergillus.

It is not uncommon to use more than two media in mycological investigations when more than one fungal genus is being studied. Farworth and Neish (1980) used 3 different selective media and the direct plate technique for maize kernels - ferric citrate medium (Aspergillus differential medium, ADM) for the detection of A. flavus, tomato juice agar - salt medium (TJS) for the isolation of

other storage fungi, and Nash and Snyder's (1962) peptone-PCNB agar for the isolation of Fusarium spp.

1.3.3. Single Spore Culturing

Single spore culturing was first devised by H.N. Hansen and has been recommended by most mycologists particularly for taxonomic purposes (Booth, 1971; Toussoun and Nelson, 1976; Burgess and Liddell, 1983). Fusarium colonies initiated from single spores are uniform and consistent in appearance and this can be useful in identification. The hyphal tip technique was also recommended by Burgess and Liddell (1983) as an alternative to single spore isolation.

1.3.4. Incubation Conditions

Generally most microorganisms including fusaria, grow well at 25°C - 30°C or at room temperature (Smith et al., 1984). Daylight or ultra-violet light is regarded as an important factor for sporulation and for development of pigmentation characteristics (Snyder and Hansen, 1947). Burgess and Liddell (1983) always incubated their cultures in an alternating temperature (25°C day/20°C night) with a 12 h photoperiod. The cultures were incubated 40 cm below a bank of light consisting of 4 white fluorescent tubes (40 w) and 1 tube of black light (40 w). Toussoun and Nelson (1976) incubated Fusarium cultures at 20°-22°C with a 12 h alternating cycle of light and dark. But while fluctuating temperatures are the best, all the Fusarium spp (except F. nivale) grow well at a constant temperature of 20°-22°C (Nelson et al., 1983).

The period of incubation can vary from 1 week to 4 weeks. Windels and Kommedahl (1984) used PCNB agar with a 12 h photoperiod under fluorescent lamps for 1-2 weeks before transferring to home-made PDA medium. The cultures were left for 10-14 days before first examination. Isolates were transferred to CLA and incubated under fluorescent lamps again for 4 weeks, then examined for perithecia and mature ascospores as well as chlamydospores. Both ascospores and chlamydospores are important in the identification of some Fusarium spp

(Burgess and Liddell, 1983). Chlamydospores can also be produced on poor media such as WA and CLA or by using soil extracts or soil plates (Nelson et al., 1983).

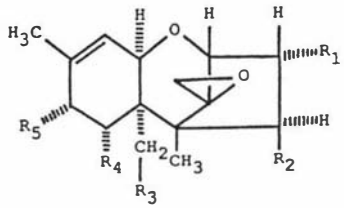
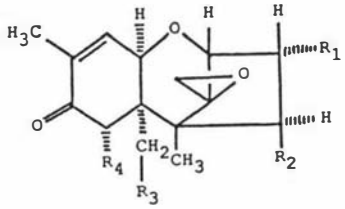
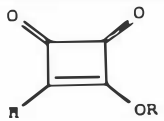
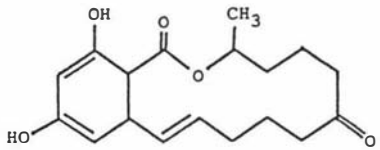
1.4. THE FUSARIUM TOXINS AND FUSARIOTOXICOSES.

1.4.1. The Toxins of Fusarium Species

Mycotoxins are generally considered to be secondary metabolites formed by biochemical pathways which represent branches of normal cellular anabolic and catabolic pathways (Hayes, 1985). Chemically (Table 1-4), Fusarium toxins are relatively low molecular weight, non-antigenic secondary metabolites capable of causing a toxic effect in man and animals. The main Fusarium toxins which have been found to be naturally produced are those of the trichothecenes group and zearalenone (ZEA) and moniliformin (MON). The most important are the trichothecenes, which comprise a large group of about 75 chemically-related and biologically active secondary metabolites. Although possessing common structural features including the tricyclic ring system (trichothecane), the 12,13 epoxide group trichothecenes contain many variations (Gilbert, 1984). Trichothecenes in general are colourless, crystalline, optically active and stable in the solid state and can be stored at room temperature for years without loss of activity (Snyder, 1986).

ZEA is structurally different from the trichothecenes (Table 1-4), being an oestrogen, 6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid-lactone (Mirocha et al., 1977). ZEA is the only Fusarium mycotoxin found useful commercially. Derivatives of ZEA give an enhanced growth rate in cattle when used at the proper time and in proper amounts.

Table 1-4: Structure and some chemical properties of the most common Fusarium toxins (Ueno, 1983; Cole and Cox, 1981)

Mycotoxin structure	Compound	Abbr.	M.W.	Formula	R1	R2	R3	R4	R5
	Type A: *								
	T-2 toxin	-	466.21	$C_{24}H_{34}O_9$	OH	OAC	OAC	H	$OCOCH_2CH(CH_3)_2$
	HT-2	-	424.20	$C_{22}H_{32}O_8$	OH	OH	OAC	H	$OCOCH_2CH(CH_3)_2$
	Diacetoxyscirpenol	DAS	366.16	$C_{19}H_{26}O_7$	OH	OAC	OAC	H	H
	Neosoleniol	NEO	382.16	$C_{19}H_{26}O_8$	OH	OAC	OAC	H	OH
	Monacetoxyscirpenol	MAS	324.15	$C_{17}H_{24}O_6$	OH	OH	OAC	H	H
	Triacetoxyscirpenol	TAS	408.17	$C_{21}H_{28}O_8$	OH	OAC	OAC	OH	OAC
	Type B: *								
	Deoxynivalenol	DON	296.12	$C_{15}H_{20}O_6$	OH	H	OH	OH	
	Nivalenol	NIV	312.12	$C_{15}H_{20}O_7$	OH	OH	OH	OH	
	Fusarenon-X	FUS-X	354.13	$C_{17}H_{22}O_8$	OH	OAC	OH	OH	
	Diacetylnivalenol	DAN	396.14	$C_{19}H_{24}O_9$	OH	OAC	OAC	OH	
	3-acetyldeoxynivalenol (Monoacetyl DON)	3-ADON	338.13	$C_{17}H_{22}O_7$	OAC	H	OH	OH	
	15-acetyldeoxynivalenol (Diacetyl DON)	15-ADON	380.18	$C_{19}H_{24}O_8$	OAC	H	OAC	OH	
 R = Na or K	moniliformin	MON	119.98	C_4HO_3Na	Sodium salt				
			136.07	C_4HO_3K	Potassium salt				
			98.00	$C_4H_2O_3$	Free acid				
	zearalenone	ZEA	318.14	$C_{18}H_{22}O_5$					

* Trichothecenes

MON (1-hydroxy-cyclobut-1-ene-3, 4-dione) is another important Fusarium toxin. It is found as either the sodium or potassium salt and is soluble in water.

Most Fusarium mycotoxins are produced by five main sections of the genus: Sporotrichiella, Liseola, Gibbosum, Roseum and Discolor (Samuels, 1984; Vesonder and Hesseltine, 1981). The majority of the species belonging to these sections have been found to be common on cereal grains. Table 1-5 summarises the most common Fusarium spp that have been found to be toxin producers.

1.4.2. Natural Occurrence

The role of Fusarium toxins as aetiologic agents in mycotoxicoses was first reported by Hsu et al (1972) when they demonstrated the presence of 2 ppm of T-2 toxin in mouldy maize associated with lethal toxicoses in dairy cattle in the U.S.A. In Japan several outbreaks of red mould toxicosis in humans and animals were attributed to ingestion of grain infected by Fusarium spp (Osborne, 1982). Obviously, Fusarium toxins occur naturally in grains, particularly in maize, and it seems to be a serious problem in various countries (Miller et al., 1983).

In practice, only small numbers of mycotoxins have regularly been found naturally, compared to over 200 fungal metabolites which have been shown to be potentially toxic to man and animals. The majority of these toxins have been primarily produced in cultures under laboratory conditions (Smith et al., 1984).

The level of natural mycotoxin contamination varies greatly from one country to another as well as within a country. This has been attributed to several factors and probably the most important of these are environmental factors, agricultural practices and food hygiene (WHO, 1979). Obviously three main conditions must be fulfilled before mycotoxin contamination becomes a problem: toxigenic fungi must be present, the substrate must be suitable for their growth, and environmental conditions must allow adequate growth for toxin production (Hesseltine, 1976).

Table 1-5: The principal Fusarium spp producing mycotoxins.

Fusarium species	Mycotoxins*	References
F. graminearum	ZEA, DON, 15-ADON, NIV T-2 toxin, DAS, TAS, MAS, MON, 3-ADON	Miller et al., 1983; Ichinoe & Kurata, 1983; Chatterjee et al., 1986; Rabie et al., 1986 Scott et al., 1987
F. culmorum	ZEA, DON, 3-ADON (DAS, NEO, T-2 toxin & HT-2)**	Ichinoe & Kurata, 1983; Cole & Cox, 1981; Greenhalgh et al., 1986
F. equiseti	ZEA, MON, T-2 toxin, DAS, NEO, NIV, Fus-X, Butenolide	Cole & Cox, 1981; Rabie et al., 1982; Richardson et al., 1985
F. acuminatum	T-2 toxin, NEO, HT-2, NIV, DAN, DAS, MON, ZEA, FUS-X	Ichinoe & Kurata, 1983; Rabie et al., 1982; Richardson et al., 1985
F. poae	DAS, NEO, T-2 toxin, HT-2 toxin	Ichinoe & Kurata, 1983; Ueno et al., 1973.
F. sporotrichioides	T-2 & HT-2 toxin, NEO, DAS,	Ueno et al., 1973;
F. sulphureum	T-2 & HT-2 toxins, NEO, DAS, TAS, MAS	Harwig et al., 1979; Cole & Cox, 1981.
F. solani	NEO, T-2 toxin, HT-2, DAS	Ueno et al., 1973.
F. avenaceum	NEO, T-2 toxin, DAS, MON	Ueno et al., 1973; Marasas et al., 1979a
F. sambucinum	DAS	Cole & Cox, 1981
F. crookwellense	ZEA, DAN	Lauren, 1986

Table 1-5 (continued)

Fusarium species	Mycotoxins*	References
F. nivale	NIV, FUS-X, DAN	Ichinoe & Kurata, 1983
F. oxysporum	ZEA, MON, T-2 toxin	Steele et al., 1976; Marasas et al. 1977; Rabie et al., 1986.
F. tricinctum	ZEA, T-2 toxin, DAS, butenolide	Steele et al., 1976; Bottalico et al., 1984 Cole & Cox, 1981.
F. moniliforme	MON, ZEA, DAS, T-2 toxin	Kriek et al., 1977; Ghosal et al., 1978.
F. subglutinans	MON	Thiel et al., 1982.
F. semitectum	MON, DAS, T-2 toxin	Rabie et al., 1982, 1986 Cole & Cox, 1981.
F. lateritium	T-2 toxin	Rabie et al., 1986

* Full trivial names are listed in the Abbreviation Table

** According to Greenhalgh et al. (1986), DAS, NEO, T-2 toxin and HT-2 toxin production by F. culmorum was an error, due to an incorrect classification of the isolate.

Toxigenic fusaria normally develop and release their toxin in cool, wet weather, particularly during autumn and winter, in the field, and during storage (Andrews et al., 1981). Despite most Fusarium spp being distinctly field fungi, there are some storage species which appear either before harvesting or more rarely during storage (Burgess, 1981). The identification of toxigenic fungi may not be of diagnostic value in investigations of mycotoxicoses if there is no detection of the mycotoxin itself. But the presence of a known toxigenic fungus in a reasonable quantity does imply some degree of hazard (Smith et al., 1984).

Although Fusarium toxins have mostly been found in kernels on the cobs or in shelled maize, some of the mycotoxins (ZEA, DON and T-2 toxin) have also been found in maize stalks (Mirocha et al., 1979) and in stalks, stems and husks (Young and Miller, 1985).

1.4.2.1. National Surveys

Much of the information now known about the Fusarium mycotoxins and mycotoxicoses has been obtained either from the study of isolated outbreaks of animal disease on the farm or from the assay of selected samples of feeds and feedstuffs (Eppley, 1982). There have been general surveys of the mycotoxin contamination of feeds in only a few countries.

As a result of a national survey in Canada during 1978 and 1979 carried out by a variety of government, university and private agencies (Andrews et al., 1981), it was determined that most mycotoxin contamination of maize and wheat in Western Canada (British Columbia and the prairies) was associated with high moisture cereals, particularly when there had been delayed harvesting or the grain was left overwinter in the field. T-2 toxin and ochratoxin were the most prevalent in these areas but ZEA was common in South Manitoba and deoxynivalenol (DON) was common in the Quebec region. DON was also found in white winter crops in Ontario at the level of 85 ppm during a general survey in 1980 reported by Trenholm et al. (1981) and DON seems to be the most significant mycotoxin in Canada (Scott, 1984).

The results of the 1980 general survey in Canada provoked an immediate surveillance programme in the U.K. for DON in feed and malting barley and in imported maize (Gilbert et al., 1983). These investigations showed that in both feed and malting barley 90% of samples were contaminated at levels of less than 0.02 ppm. Only a few contained DON at levels above 0.02 and up to 0.36 ppm. A total of 36% of imported maize samples were contaminated, 25% at a level of less than 0.02 ppm and 10% from 0.02 to 1.4 ppm.

Further surveys in the U.K. investigating home-grown wheat from three harvests (1980-1982), and imported wheat samples, were reported by Osborne and Willis (1984). A total of 232 samples were analysed for the presence of seven trichothecenes: nivalenol (NIV), fusarenon-X (FUS-X), neoselaniol (NEO), diacetoxyscirpenol (DAS), DON, T-2 toxin and HT-2. Only DON was detected. Of the 199 U.K. home-grown wheat samples, only 32 contained DON at levels ranging from 0.02 to 0.4 ppm. Out of 33 imported wheat samples 23 were found to be contaminated, at levels ranging from 0.02 to 1.32 ppm. Recently, Tanaka et al. (1986), using more sensitive methods, detected 3 Fusarium toxins (DON, NIV and ZEA) in U.K.-grown cereals (wheat and barley) at frequencies of 65, 55 and 13% of samples respectively.

In Australia a survey for several mycotoxins (aflatoxin, ochratoxin A, zearalenone, sterigmatocystin and T-2 toxin) was carried out in eight districts of North Queensland (Blaney et al., 1984). Samples from maize harvested during 1982 were collected from a loading truck. Zearalenone had contaminated 85% of the 293 samples. No other mycotoxins were detected.

Several surveys have been carried out in different states of the U.S.A. to determine the level of mycotoxins occurring in maize. Shotwell (1977) reported that aflatoxin was detected in 31% of 1283 truck-loads of white corn from seven counties in South-Eastern Missouri. Another survey in the southern states (Eppley et al., 1974) showed that 17% of 223 samples analysed were contaminated with ZEA. Hagler et al.(1984) reported three mycotoxins contaminating wheat crops from four states. DON was identified in 31 out of 33 samples and the level of contamination ranged between 0.12 and 5.5 ppm. Also 23 samples were contaminated with aflatoxin B1, at levels ranging from 0.8 to 17.0 ppm.

ZEA was found in 3 of the 33 samples, with the highest level of contamination being 254 ppm.

In Japan, data accumulated between 1976 and 1982 on the natural occurrence of mycotoxins showed that DON and NIV contamination was a major problem in Japan's domestic barley and wheat. Over 200 samples were examined and 74.6% were positive. Wheat and barley grains purchased by the government in 1981 and 1982 were also contaminated by DON and NIV at a level of 80% of samples examined (Yoshizawa, 1984).

National surveys of mycotoxins and mycotoxicoses can thus be very important in establishing potential problems facing livestock producers (Andrews et al., 1981).

1.4.2.2. Naturally contaminated cereals and feedstuffs

A. Zearalenone

ZEA has been well known and well documented as a Fusarium mycotoxin in many countries. It was detected in hay at a level of 14 ppm in England, from maize in France, Yugoslovia, U.S.A. and England at levels ranging from 0.1 to 206 ppm and from animal feeds at levels of 0.01 to 2900 ppm in U.S.A. (Shotwell, 1977). Agnew et al. (1986) found all of 12 New Zealand wheat samples were contaminated with ZEA. ZEA has also been found naturally in pasture. di Menna et al. (1985) found that ZEA was a natural contaminant of New Zealand pasture grass leaves at levels of 0.6-2.6 ppm.

ZEA found in animal feeds originally comes from contaminated maize or barley, according to Smith et al. (1984). This conclusion was reached from data collected by the Veterinary Services Branch, Ontario, Canada on 2,022 feedstuff samples tested between 1972 and 1977, indicating that ZEA was present in 277 samples (14%) and was the most significant mycotoxin associated with maize crops. The contamination level fluctuated from year to year. Aflatoxin, ochratoxin and T-2 toxin were also detected in some samples but not in significant amounts (Funnell, 1979).

Several Fusarium species such as F. graminearum, F. culmorum, F. tricinctum, F. oxysporum, F. sporotrichioides and F. moniliforme have been commonly reported as ZEA producers (WHO, 1979). These species usually attack the developing seeds during a period of heavy rainfall particularly at low temperatures (12-14^{o}C). The general survey of maize harvested during 1982 in North Queensland, Australia, reported by Blaney et al. (1984), showed that 85% of the samples examined (293 samples) were contaminated with ZEA. The mean concentration in all samples was 0.17 ppm, but there were four samples in which the concentration exceeded 1 ppm. There was a positive correlation between the amount of rainfall during the period of maize growth, Gibberella zeae cob rot and the ZEA contamination (Blaney et al., 1984).

B. Trichothecenes

These toxins are produced mostly by Fusarium spp but also by some species of other fungal genera such as Myrothecium, Stachybotrys and Trichoderma (Eppley, 1975). Ueno (1977) reported over 18 species of the genus Fusarium as being trichothecene producers under laboratory conditions and one isolate could produce more than one Fusarium toxin. Over 75 trichothecenes have been isolated and characterised (Gilbert, 1984), and over 47 of them are naturally-occurring (Snyder, 1986), but only about 15 are biologically active (Bottalico et al., 1984). The principal naturally-occurring trichothecenes are NIV, FUS-X, DON, 3-ADON, DAS, T-2 toxin and HT-2 toxin (Rosen and Rosen, 1984) and 15-ADON (Abbas et al., 1986). Eppley (1982) listed other trichothecenes as occurring naturally, such as diacetylnivalenol, diacetyldeoxynivalenol, deoxynivelenol acetate, acetoxyscirpendiol, 4-acetoxyscirpendiol and neosolaniol. These trichothecenes are less frequently reported because they are not commercially available, it is difficult to screen for them and there has been a lack of extensive examination (Smith et al., 1984).

Only four of the trichothecenes (DON, T-2 toxin, DAS and NIV) are frequently reported from cereals and mixed feed (Ueno, 1977). The predominant contaminant among these four toxins is DON, particularly in maize (Pathre and Mirocha, 1979). DON and ZEA most frequently occur together in maize (Shotwell et al., 1980; Vesonder and Ciegler, 1979). DON, ZEA and NIV have commonly been found in wheat

samples in New Zealand (Agnew et al., 1986) and in barley and wheat samples from Japan, Taiwan and China (Ueno et al., 1986). DON, 15-ADON and ZEA were found together in two U.S.A. maize samples which had been associated with feed refusal (Abbas et al., 1986).

The natural occurrence of DON in Canadian grains has been well documented by several authors. National surveys at different times indicated that the level of contamination with DON in Ontario soft winter wheat ranged from 0.22-0.74 ppm, from Quebec hard spring wheat 0.33-3.0 ppm and in wheat from the Maritime Provinces 0.13-0.47 ppm. Maize was also contaminated with DON to a level of 0.2-0.62 ppm (Scott, 1984).

DON was the main mycotoxin contaminating both domestic and imported grains in the U.K. (Gilbert et al., 1984).

Vesonder et al. (1978) reported 24 out of 52 preharvest maize samples from Northwest Ohio, U.S.A. were contaminated with DON. These samples came from a delayed harvest under wet weather and the crop was extensively contaminated with Fusarium spp. Yoshizawa (1984) found DON and NIV to be natural contaminants of Japanese grain (wheat and barley). Their data, which were collected between 1976 and 1982, indicated that 74.6% (of 205 samples) of domestic grains were contaminated at a level ranging from 0.3 ppm to over 2 ppm. A similar level had been found in grain imported during 1981-1982 with an overall frequency of 80% of samples contaminated.

On very rare occasions DON and T-2 toxin have been found naturally together in cereals. In Canada, the two toxins have occasionally been linked with livestock toxicoses and refusal of feed (Neish et al., 1982). Recently, DON has been found contaminating about 60% of breakfast cereals (60 samples) at an average level of 100 ppb (Trucksess et al., 1986).

There are several reports which indicate that T-2 toxin has been involved in animal and human mycotoxicoses. The first major outbreak, known as alimentary toxic aleukia (ATA), occurred in the Soviet Union between 1941-1947 (Joffe, 1978). Hsu et al. (1972) reported that mouldy maize associated with a lethal toxicosis in dairy cattle was contaminated with 2 ppm of T-2 toxin. Cases of mouldy grain toxicosis

which involved ducks, geese, horses and swine were attributed to the presence of 25 ppm of T-2 toxin in the grain (Puls and Greenway, 1976).

Mirocha et al. (1976b) found that in addition to T-2 toxin, DAS and DON contaminated mixed feed samples from different states of the U.S.A. T-2 toxin, at levels of 0.076 ppm was found in one mixed feed associated with bloody stools in cattle. DAS was found in two mixed feed samples associated with haemorrhagic bowel syndrome in swine. The level of contamination was 0.380-0.500 ppm. From India, Ghosal et al. (1978) reported T-2 toxin, DAS and ZEA in sweet corn infected with F. moniliforme growing in the field. The levels of contamination were 4 µg/g, 14 µg/g and 16 µg/g for T-2 toxin, DAS and ZEA respectively. Jemmali et al. (1978) reported that T-2 toxin as well as three other Fusarium toxins (NIV, DON and ZEA) occurred naturally in maize samples associated with infertility and refusal symptoms in swine in France. The level of contamination with T-2 toxin was relatively small at 0.02 ppm.

Thus, the natural occurrence of trichothecenes in foodstuffs and animal feed has been of concern to health authorities in various parts of the world (Ichinoe et al., 1983).

C. Moniliformin (MON)

This mycotoxin was found for the first time to occur naturally in two maize samples in a South African crop by Thiel et al. (1982). The levels of contamination were 16 and 25 ppm. Two other Fusarium toxins, DON and ZEA, were also isolated from the above samples. A mycological study of the two samples revealed that F. graminearum, F. moniliforme and F. subglutinans were the main isolates. The latter two species are among the most prevalent fungi in maize world-wide (Marasas et al., 1979c). Under laboratory conditions a large amount of moniliformin (11.3 g/kg maize) was produced by F. subglutinans isolates by Kriek et al. (1977), and Steyn et al. (1978) reported 2 to 16 g of moniliformin/kg maize. Other Fusarium spp have also been found to be good moniliformin producers, namely F. avenaceum, F. acuminatum, F. oxysporum, F. semitectum, F. equiseti and F. concolor (Rabie et al., 1982).

Recently Thiel *et al.* (1986) found that maize samples from the U.S.A. were naturally contaminated with MON (2.87 ppm) and fusarin C (0.39 ppm).

1.4.3. Effects of Fusarium Toxins on Human and Animal Health

Fusarium spp have for many years been recognised as being widely present in cereal grain, and consumption of mouldy grains by either humans or animals has resulted in a variety of health problems. During the second half of the eighteenth century Woronin (1891), cited by Mirocha (1984), described outbreaks of human mycotoxicosis in both Sweden and Eastern U.S.S.R. The main symptoms were dizziness or staggering after consumption of bread made from mouldy grain. The cereal disease associated with these symptoms was 'scab' or blight caused by *Fusarium*. The mycotoxicosis can still be a problem today, and it has been demonstrated that ingestion of bread made from mouldy rye can result in headache, dizziness, shivering, vomiting, disturbance of vision and general malaise. Animals such as dogs, horses, pigs and chickens are also susceptible (Mirocha, 1984).

Several other outbreaks of mycotoxicoses all around the world have been associated with *Fusarium* toxins, and in particular the trichothecenes, as summarised by Pathre and Mirocha (1979) and Osborne (1982). These include taumelgetreide toxicosis and alimentary toxic aleukia (ATA) in man, horse and pig (U.S.S.R.); stachybotrotoxicosis and dendrodochiatoxicosis in horses (Europe); bean-hull toxicosis in horses and red mould toxicosis in horses, swine and cows (Japan); mouldy corn toxicosis in swine and cows (U.S.A.); oesophageal cancer in man (Transkei, South Africa); degrala disease in cows (India) and Fusariotoxicosis in ducks, geese, horses and swine (Canada) (Table 1-6).

Table 1-6: Some Fusariotoxicoses associated with cereal grain and animal feedstuffs. *

Fusariotoxicosis	Country	Toxin(s) implicated	Type of cereals	Fungi isolated	Animals affected	Symptoms	References
Taumelgetreide toxicosis	Siberia	Trichothecenes	Rye	G. saubinetti	Man, horse, pig, fowl	Headache, chills nausea, vomiting	Woronin, 1891
Alimentary toxic aleukia (ATA)	USSR	T-2 toxin	Wheat, oats barley, rye	F. sporotrichioides	Man, horse, pig	Vomiting, diarrhoea, skin inflammation, leukopenia, angina	Joffe, 1978
Bean-hull toxicosis	Japan	T-2 toxin, NEO	Bean hulls	F. solani	Horse	Convulsions, cyclic movement	Ueno et al., 1972
Red mould toxicosis	Japan	DON, NIV	Wheat	F. graminearum	Man, horse, swine, cow	Vomiting, diarrhoea, emesis, abortion	Saito & Tatsuno, 1971
Mouldy corn toxicosis	U.S.A.	DON, DAS, T-2 toxin	Maize	F. roseum, F. tricinctum	Cow, swine	Emesis, haemorrhage, refusal of feed	Hsu et al., 1972
Fusariotoxicosis	Canada	Trichothecene	Barley	G. zeae	Ducks, geese horse, swine	Emesis, refusal of feed, excessive salivation, thirst	Greenway & Puls, 1976
Osophageal cancer	Transkei	DON, ZEA, MON	Maize	F. graminearum F. moniliforme F. subglutinans	Man	Oesophageal tumours	Thiel et al., 1982
Leukoencephalo-malacia	U.S.A., Egypt, China, S. Africa U.S.A.	Culture of F. moniliforme	Maize	F. moniliforme	Horse, mule, donkey	Nervous depression, incoordination liquefactive necrosis of white matter of cerebral hemisphere	Pienaar et al., 1981 Wilson et al., 1985.

* Largely presumptive

Of the several Fusarium toxins which have been firmly established as causing intoxication in humans and animals after ingestion, T-2 toxin, DAS, DON and ZEA are the most common (Mirocha, 1983). However, as the fungi produce an array of toxins in nature, some known and some as yet undescribed, causality can be multiple.

Mycotoxicoses in general represent a diagnostically difficult problem for veterinarians since many of the symptoms are slight and easily confused with other diseases. The insidious effects on animals will cause reduced growth rate, reduced feed conversion and increased disease susceptibility. However, on the basis of information on cause and effect relationships available today, the usual concentration of naturally-occurring toxins in contaminated foodstuffs so far reported does not appear to be high enough to explain the natural or experimental symptomatology which has been apparent in animals. This discrepency can in part be attributed to problems associated with analysis and can also be due to the synergistic effects of more than one toxin (Pathre and Mirocha, 1979).

1.4.3.1. Humans

The relationships between mycotoxins and human health are difficult to ascertain as there is no direct evidence of such involvement in terms of controlled experiments with man. Smith (1981) thought that the susceptibility of different animal species to many mycotoxins must be considered as strong evidence that man could be affected if submitted to similar exposure levels. Good epidemiological evidence has been collected which indicates that several fusariotoxins affecting human health can be confirmed. ATA, also called "septic angina", "panmyelotoxicosis" and several other names, was described in dramatic outbreaks occurring in the U.S.S.R., particularly in the Orenburg district, between 1941 and 1947. The disease was associated with the consumption of breads prepared from mouldy grains (millet, wheat, rye and oats) left under the winter snow. The typical symptoms were described as spots on the skin, leukopenia, agranulocytosis, necrotic angina, haemorrhagic rash, sepsis, exhaustion of the bone marrow, bleeding from the nose, throat and gums. Patients with ATA showed impaired nervous reflexes, general depression, hyperaesthesia, encephalitis etc., and reported burning sensations in the mouth,

oesophagus and stomach (Joffe, 1978). The victim developed a severe emesis, diarrhoea and abdominal pain; 60% of the victims died. It was found that the moulds associated with ATA were mainly F. tricinctum and F. poae and their mycotoxins, particularly T-2 toxin, were most likely responsible (Yagen et al., 1977).

Food poisoning was also reported in Japan in the 1940's and 1950's due to consumption of rice contaminated with G. zeae and F. nivale. Nausea, vomiting,headaches and convulsions were the main clinical signs (Vesonder and Hesseltine, 1981). Mouldy rice has also been found to cause yellow rice disease in Japan, a disease with similar symptoms to beriberi (Osborne, 1982).

There are many other records of nausea, vomiting and diarrhoea associated with the contamination of wheat and rice by fusaria. In 1956 and 1963 the harvested wheat of Western Japan was severely attacked by Fusarium and the resulting human mycotoxicoses were attributed to Fusarium toxins, particularly trichothecenes (Yoshizawa, 1983).

In Africa there has been a clear association between maize cultivation and the occurrence of oesophageal cancer (Cook, 1971). Maize samples consumed by people developing oesophageal cancer in the Transkei area in South Africa were found to be contaminated with Fusarium spp (Marasas et al., 1977). DON and other trichothecenes were suspected to be the main cause. Similarly Lin and Tang (1980) (cited by Schoental, 1981) reported that oesophageal tumours in man and animals in Henan Province in North China could be due to toxic metabolites produced by fungi such as fusaria. Pure T-2 toxin has also been reported to be carcinogenic to rats (Pathre and Mirocha, 1979).

Recently Schoental (1985) presented epidemiological evidence of the relationship between Fusarium mycotoxins and psychiatric disorders in humans. He believed that perinatal exposure to the mycotoxins could cause damage to many human organs, including the central nervous system (CNS). Psychiatrists had observed that some ATA patients sometimes showed opposite signs and symptoms, eg. either a tendency to somnolence or asomnia, and either psychomotor excitement or anergia,

apathy etc. Some of the patients experienced vivid visual and aural hallucinations.

According to Schoental (1985), there is other evidence from epidemiological studies which indicates that the risk of serious neonatal abnormalities of the CNS such as encephaly, Down's, Turner's and Klinefltov's Syndromes as well as chromosome aberration in the newborn and schizophrenia is related to the season of birth and is higher among those born during the winter or spring months. Outbreaks of precocious development of sex organs which affected thousands of very young children in the early 1980's in Puerto Rico also appear related to excessive intake of ZEA and its derivatives which were detected in the blood of some children (Saenz de Rodriguez, 1984).

There is an endemic osteoarthritic disease of man known in China, Eastern U.S.S.R. and North Korea as Kaschin-Beck's Disease. It, too, seems to be associated with maize and wheat contaminated with fusaria (Smith and Moss, 1985).

1.4.3.2. Horses

Equine mycotoxicoses have been recognised for some time. On occasions these mycotoxicoses have caused high mortality. Stachybotryotoxicosis is believed to be caused by trichothecenes produced by Stachybotrys alternans. It shows a similarity to mycotoxicoses caused by Fusarium spp (Hintikka, 1977).

There have been several sporadic disease outbreaks for which Fusarium mycotoxins, particularly trichothecenes, were believed to be responsible. For example, in the U.K. in 1927 "grass disease" or "grass sickness" in horses was associated with unusual seasonal weather. Although the aetiological agent of the disease was not established the condition appears to be similar to that caused by trichothecenes (Schoental, 1981).

On Hokkaido Island, Japanese scientists reported sporadic fatal outbreaks of Akakabi byo (Red mould disease) in humans and horses, for which Fusarium mycotoxins were later found to be the main cause. Konishi and Ichijo (1970) (cited by Ueno et al., 1972) reported that

horses fed bean hulls during winter showed convulsion, disturbed respiration and decreased heart rate. The mortality rate was about 15%. No definitive aetiology was established but microbiological examination of the hulls found heavy contamination with F. solani. This finding led most Japanese scientists to believe that Fusarium toxins may have been the cause.

The most serious mycotoxicosis of horses which is most certainly associated with feed contamination by fusaria is leukoencephalomalacia (LEM), also referred to as "corn stalk disease" and "mouldy corn disease". Pienaar et al. (1981) reviewed most of the available literature about the LEM cases. They consider that the problem can be traced back to the 18th and 19th centuries when high mortality in horses in the U.S.A. was preceded by nervous symptoms after consumption of mouldy maize. The disease was later found to have a world-wide distribution, and was reported from South America, China, Egypt, Greece and South Africa (Pienaar et al., 1981). Recently the disease has been reported from France and Brazil (Wilson et al., 1985).

Despite the early indications that this disease was caused by a mycotoxin, the responsible fungus, F. moniliforme was not identified until 1971 (Wilson and Maronpot, 1971). LEM was found to cause liquefication and necrotic lesions in the white matter of one or both cerebral hemispheres. Similar symptoms have been reproduced experimentally in both donkeys and horses by feeding F. moniliforme (Badiali et al., 1968; Marasas et al., 1976; Wilson et al., 1985).

Recently Wilson et al. (1985) reported numerous LEM cases in the U.S.A. These authors reproduced similar clinical signs, disease course, gross and histopathological lesions in horses fed mouldy maize. Although F. moniliforme has been found to be the main cause of the disease, the actual mycotoxins responsible for producing the disorder are still unknown (Wilson et al., 1985).

1.4.3.3. Cattle

Several outbreaks of mycotoxicosis in cattle that are apparently related to ingestion of feedstuff contaminated by fungal toxins have been recorded in different parts of the world (Petrie et al., 1977).

What appears to be the first clear report was described by Hsu et al. (1972) in the U.S.A. The lethal toxicosis was called mouldy corn toxicosis and was characterised by bloody diarrhoea, massive haemorrhagic lesions in the stomach, heart, intestine, lung, bladder and kidney as well as frequent abortions. T-2 toxin at a level of 2 ppm was detected in the mouldy maize that had been blamed for the outbreak.

Similar symptoms were described by Petrie et al. (1977) following outbreaks in which 9 cows out of 115 died after eating mouldy feedstuff. T-2 toxin, NEO, FUS-X and DAS were detected (but without quantification). Petrie and his colleagues assumed that this outbreak was one of T-2 toxicosis although there is a possibility that other trichothecenes might have been involved.

Experimentally, Kosuri et al. (1970) administered T-2 toxin intramuscularly to cattle and reported massive haemorrhage in the large intestine as well as weight loss. Weaver et al. (1980) did not find haemorrhagic lesions when they experimentally studied the effect of T-2 toxin in pregnant cows, but observed refusal of rations containing 50 ppm T-2 toxin. Congestion of mucosa and the blood vessels in the lamina propria of the omasum, rumen and jejunum in both cows and calves were also noticed.

Mirocha (1983) reviewed most of the reports on this subject and his conclusion was that T-2 toxin when ingested by dairy cows at a concentration of less than 1.0 ppm does not cause haemorrhage. Therefore, any haemorrhaging associated with contaminated feed is likely to be caused by some toxin as yet undiscovered.

Some research has been done on the effects of Fusarium toxins on the bovine immunosystem. Buening et al. (1982) experimentally administered T-2 toxin to calves at the rate of 0.6 ppm and found that this resulted in a significant decrease in chemotaxis and migration of neutrophils. Several changes were observed by Mann et al. (1982) in the bovine immune system. They fed 43 day-old calves T-2 toxin at the rate of 0.6 ppm and found a significant reduction in the levels of alpha, beta 1, beta 2 and gamma globulin. Serum protein levels and

the C_3 level were decreased and the levels of IgM and IgA were significantly lowered, but not the IgG.

1.4.3.4. Swine

Feed refusal and hyperoestrogenic symptoms appear to be the most dramatic effects of Fusarium mycotoxins on swine (Mirocha, 1984).

A. Feed refusal and emesis

Vesonder and Hesseltine (1981) noted that outbreaks of barley scab caused by Gibberella zeae in the U.S.A. were reported in 1919, 1928 and the 1930's, and this barley was rejected by swine. One of the causative agents of emesis and feed refusal in swine has been found to be DON (vomitoxin) (Pathre and Mirocha, 1979). Vesonder et al. (1973) found it to be the major agent. Later Forsyth et al. (1977) demonstrated a dose-response relationship between the level of DON administered and the feed refusal factor and vomiting in swine. The authors found that the swine completely refused the mouldy maize containing 13 ppm DON. Although DON accounts for a major part of the refusal symptom, Mirocha (1983) experimentally found that a concentration of pure DON equal to that in maize which swine totally refused, did not cause total refusal and suggested that refusal might be due to other metabolites produced by Fusarium and as yet unidentified.

Other trichothecenes such as T-2 toxin and DAS (Vesonder et al., 1979) as well as 15-ADON (Abbas et al., 1986) were isolated from maize that had been naturally refused, in addition to DON.

T-2 toxin and DAS have also been found to cause feed refusal as well as other symptoms in swine. Weaver et al. (1978b) fed swine a diet containing different doses of T-2 toxin (12, 16, 24 and 32 ppm). The diet with 24 and 32 ppm caused complete refusal. Animals fed the 16 ppm diet ate scantily while those fed 12 ppm ate 16% less than the control group. So the authors concluded that swine will accept 10-12 ppm of dietary T-2 toxin and can continue such a diet for up to seven months without any apparent clinical sign of intoxication. At

autopsy, however, severe congestion and haemorrhage of the liver were found.

Weaver et al. (1981) studied the activity of DAS in young swine. A diet contaminated with 2, 4, 8 and 9 ppm DAS was fed to young swine for nine weeks. All the above doses caused lingual and buccal mucosal lesions and some of the swine developed multiple focal mucosal erosion. No haemorrhage or congestion was noticed in the intestine except that a few animals showed diffuse congestion in the spleen. All doses caused a decrease in food consumption and in weight gain. The authors concluded that 10 ppm DAS could cause complete refusal.

B. Oestrogenic effect of ZEA

ZEA and its isomers can cause serious reproductive problems when consumed by swine or dairy cattle, but have little effect on poultry (Mirocha, 1984). Pigs are considered to be the most sensitive animals to ZEA, developing an oestrogenic syndrome and infertility. The most common symptoms found include swelling of the genital system. The vulva becomes swollen and oedematous and there may be vaginal or anal prolapse, while the uterus becomes enlarged and oedematous and ovaries become atrophied. The testes and mammary glands are also affected (Chang et al., 1979; Long et al., 1982).

Chang et al. (1979) stated that infertility in swine is commonly attributed to the ingestion of maize infected with Fusarium spp capable of producing ZEA. The main symptoms found by Chang and his colleagues was that ZEA caused nymphomania, pseudopregnancy, ovarian atrophy and changes in the endometrium in mature female swine. Long and Diekman (1986) demonstrated that three gilts out of four which ingested 108 ppm ZEA on postmating days seven to ten, were not pregnant and showed regression of corpora lutea.

The effect of Fusarium toxins on fertility may not be solely due to ZEA, however. Mirocha (1984) suggested that trichothecenes are also involved. T-2 toxin and its effect on fertility and abortion in sows were investigated by Weaver et al. (1978a). These authors reported no effect on pregnancy when the T-2 toxin was fed with the normal

diet. But when T-2 toxin was administered parenterally at a dose of 0.21-0.41 ppm body weight, abortion occurred after 48-80 hours.

1.4.3.5. Poultry

There are few reports of natural outbreaks of T-2 toxicosis in poultry. During one outbreak the morbidity ranged between 10 to 25%, with about 10% mortality (Bryden, 1986). Chickens appear less sensitive than other livestock to the toxic effects of most Fusarium toxins.

The effect of pure T-2 toxin on chickens has been studied by several researchers but there is still no clear understanding of the amount of T-2 toxin which can be tolerated by chickens. Chi et al. (1977a) observed no changes in chickens dosed less than 4.0 ppm, but those fed a level higher than 4.0 ppm showed decreased body weight, reduced feed consumption and oral lesions. The oral lesions, described as circumscribed, proliferative caseous-like plaques, were regarded as an excellent indicator of T-2 intoxication. However, other studies have given different results. In Italy, Cirilli (1983) found that a small amount of T-2 toxin (40-30 ppb) in maize caused serious symptoms such as hepatic necrosis, nephrosis and death due to avitaminosis and metmyoglobinaemia in broiler chickens. Chi and Mirocha (1978) tested other trichothecenes to see if oral lesions could be produced. Only DAS at a level of 5 ppm caused severe lesions. DAS has also been reported to cause oral lesions and affect food intake and growth rate of chickens (Bryden, 1986).

T-2 toxin has also been reported as causing neural disturbance in chickens (Wyatt et al., 1973; Chi et al., 1981). Wyatt and his coworkers observed that chickens fed 4, 6 and 8 ppm toxin showed hysterical wing movement, seizures and an impaired righting reflex.

The effect of Fusarium toxins on egg-production of poultry has also been studied. Wyatt et al. (1975) fed laying hens 20 ppm of T-2 toxin and observer oral lesions, reduction in feed consumption, and reduced egg-production. Egg-shells were also thinned. Chi et al. (1977b) obtained similar results by feeding 8.0 ppm T-2 toxin which

also affected both hatchability of eggs and the blood chemistry of the hens.

Poultry have been regarded as being relatively insensitive to DON (Huff et al., 1981; Kubena et al., 1985). Kubena et al (1985) fed DON at a level of 0, 9 or 18 ppm to chicks from one day-old to 35 days old. They demonstrated that the body weight was not affected, but liver weights were significantly lower and gizzard weights increased. The haemoglobin and haemocrit were decreased significantly. Allen et al. (1981) investigated the effects of DON, either purified (0, 8 and 16 mg/kg of diet) or from Fusarium culture (8, 16 and 64 mg/kg of diet), on broiler chickens. They found that 8 and 16 mg/kg were without effect but a dose of 64 mg/kg of diet was found to reduce weight gain and feed consumption. Three birds out of ten died but no lesions were noticed upon necropsy.

Chickens also seem highly resistant to ZEA up to 800 mg/kg of diet (Chi et al., 1980; Allen et al., 1981). Chi et al. (1980) found that chickens are highly tolerant to ZEA compared to swine and turkey poults. The oestrogenic effects of the ZEA were increased with increasing toxin levels (eg. 400 and 800 mg/kg); also the effect was greater when it was administered in multiple doses rather than in a single dose. The authors also noticed that serum calcium increased and serum phosphorus decreased.

Moniliformin was found to be highly toxic to poultry experimentally by Cole et al., 1973. There are a limited number of Fusarium spp capable of producing this toxin, but these spp showed an ability to release a large amount (up to 33.7 g/kg of substrate) under laboratory conditions (Rabie et al., 1982). Cole et al. (1973) reported that the oral LD_{50} of purified MON was 4.0 mg/kg in one day-old cockerels. Birds which lived more than two hours showed ascites with oedema of mesenteries and haemorrhage in the proventriculus, gizzard, intestine and skin. Ducklings showed even higher susceptibility to MON than chickens (Kriek et al., 1977).

Chickens have been reported to be highly susceptible to crude Fusarium cultures, possibly because of the mixture of toxins present in such material (Mirocha, 1984). Adams and Tuite (1976) found, when

feeding laying hens with maize contaminated with G. zeae, that egg production and feed consumption were significantly reduced. Reduction of weight gain, chemical and gross pathological changes and severe haematopoietic damage were observed when one day-old chickens were fed mixed crude extracts of F. poae and F. sporotrichioides by Joffe and Yagen (1977). Walser et al. (1982) reported a high mortality rate when broiler chickens were fed rations containing 5% and 10% grain contaminated with F. graminearum. The mortality rate was 75% and 100% respectively, within one to three weeks.

Probably the best example of these unknown or perhaps synergistic effects of mixtures of Fusarium toxins was observed by Allen et al. (1982) who found that although DAS could be responsible for reduced hatchability, an unknown Fusarium toxin produced by F. graminearum cultures had a greater effect. Two groups of laying hens were fed either 0.5 ppm purified DAS or 3% of Fusarium culture. The hatchability of fertile eggs was reduced by 24% with DAS and 99% with the Fusarium culture.

Another example of such unknown toxin(s) is that which causes tibial dyschondroplasia (TDP) in chickens. This disease (bone deformation) is commonly seen in chickens, turkeys, swine, horses, cattle and dogs. In the past the causal role had been assigned to rapid weight gain, nutrition and genetic factors, and toxin in cereals was not considered (Mirocha, 1984). Walser et al. (1982) found a group of water-soluble toxins to be associated with TDP. Chickens fed 2% F. graminearum cultures grown on rice, or partially purified toxin, developed the disease. The lesion was characterised by the presence of a cartilage which extended distally from the proximal tibiotarsi. More recently Lee et al. (1985) isolated six major water-soluble components and one of these (assigned TDP-1) was apparently the main cause of TDP. The toxin also reduced egg hatchability to nearly zero.

There are few reports on the association of Fusarium with feed refusal in poultry, although Burditt et al. (1983a) found evidence linking mycotoxins directly to feed refusal. Hagler et al. (1981) demonstrated that culture filtrates of F. roseum containing high levels of DAS produced dramatic feed refusal when added to feed or substituted for drinking water. Burditt et al. (1983a, b) regarded

contamination with T-2 toxin and DAS as one of the main factors in feed refusal in poultry.

Trichothecenes have been demonstrated to be immunosuppressants in laboratory animals (Rosenstein et al., 1981; Friend et al., 1983), and some research has been carried out on such effects in poultry. Hoerr and Carlton (1979) studied the toxicity of T-2 toxin to 7 day-old male broiler chickens. The surviving birds developed atrophy in the cortex of the bursa of Fabricus and thymus. Birds administered 7 consecutive daily doses of 1.25 mg/kg showed yellow bone marrow. Boonchuvit et al. (1975) reported the effects of T-2 toxin on Salmonella infection of chickens. The results indicated that neither the Salmonella infection nor T-2 toxin alone caused mortality but the two in combination enhanced mortality. T-2 toxin was found to exert a thymolytic effect in turkeys and both T-2 toxin and DAS have been found by Pier et al. (1979) to depress bone marrow activity and cause leukopaenia and thymic involution .

Richard et al. (1978) compared T-2 toxin intoxication in both turkeys and chickens. Their results indicated that turkeys were more susceptible to T-2 toxin than chickens at dose levels of 10 ppm. Thymus glands in turkeys were markedly decreased in size and histological examination of these glands revealed a depletion of cortical lymphocytes.

1.4.4. Fusarium Mycotoxins in New Zealand

The potential danger of Fusarium mycotoxins in countries with a temperate climate such as New Zealand has been pointed out repeatedly (Marasas et al., 1979a; Pier, 1981 etc.).

In New Zealand Fusarium populations have been found in the majority of fields on which ryegrass staggers develop, and Latch et al. (1976) have suggested that fusaria might be partially responsible for such outbreaks.

It has also been suggested that the natural contamination of pasture with ZEA can be partially responsible for low lambing percentages in some parts of New Zealand (di Menna et al., 1985). A series

of trials was carried out to establish the incidence of Fusarium spp in the pasture, particularly in the North Island and to analyse their ability to produce toxins and their impact on livestock (Lauren, 1986). Three districts, Gisborne, Pukekohe and Wanganui, were covered by this study. Mycological data obtained showed that F. crookwellense was the most frequent isolate, with F. culmorum, F. graminum, F. graminearum, F. avenaceum, F. oxysporum and F. acuminatum also found in several sites (Lauren, 1986). Mycotoxin analysis of the grass samples for ZEA contamination revealed that ZEA was present in six samples out of seven from the low-land sample of the Gisborne district, but in only one out of five samples from high land in the same district. The contamination level ranged between 0.6 and 2.6 ppm. ZEA was found less frequently in the Pukekohe and Wanganui districts (2/24 and 1/24 samples respectively).

Smith et al. (1986) found experimentally that the reproductive performance of ewes was markedly reduced by oral dosing of 25 ppm ZEA daily from Day 7 to Day 17 of the oestrus cycle. Smith et al. (1986) carried out a further trial which showed that ZEA could cause 70% reduction in the reproductive performance of sheep when given at a level of 16 ppm of dietary intake. Lauren (1986) predicted that chronic exposure to low levels of Fusarium toxin could reduce stock live-weight and predispose the animals to infectious diseases.

Fusarium isolates from pasture have been tested for their toxigenic activity on a number of occasions. Gallagher (1985) reported that F. culmorum isolates produced ZEA at levels of >200 ppm on oats medium. Lauren (1986) screened 25 strains belonging to F. crookwellense, F. culmorum, F. equiseti, F. sambucinum, F. avenaceum, F. graminum and F. semitectum and several Fusarium mycotoxins were isolated. ZEA was produced on rice medium by isolates of F. crookwellense and F. culmorum at levels of 1-29 ppm. In addition to ZEA, F. culmorum produced 69 ppm of β-zearalenol and 3 ppm of α-zearalenol. DON (vomitoxin) and acetyl-vomitoxin were produced by F. semitectum and DAS by F. sambucinum. One F. crookwellense isolate was found to produce various trichothecenes such as 4, 15 diacetylnivalenol, other species were found to produce DON, 15 ADON and 4,15 diacetoxyscirpenol (Lauren, 1986).

In terms of New Zealand cereals contaminated with Fusarium mycotoxins, Agnew et al. (1986) assayed 12 wheat samples and found two significantly contaminated with 0.02 and 0.77 ppm of NIV. A further two samples were contaminated with DON at levels of 1.5 and 10.8 ppm. A significant level of a third, unidentifiable, compound was present in four samples. All samples were contaminated with ZEA at levels ranging from 0.04 to 0.35 ppm.

Recently (Anon, 1986) a warning was issued about the potential seriousness of Fusarium mycotoxin contamination not only of New Zealand cereals but also of products and feeds. It was said "the presence of high levels of trichothecenes could have grave health implications for farmers, harvesters, millers and consumers as well as for animal feed producers and animals."

1.4.5. Laboratory Methods for the Production of Fusarium Mycotoxins

Among the factors which influence the production of toxins by Fusarium spp in the laboratory are the substrate and its initial moisture content, the temperature, the period of incubation and the strain of the fungus used.

1.4.5.1. Substrate

Both natural substrates and synthetic substrates normally in a liquid state, have been used for toxin production (Greenhalgh et al., 1983).

A. Natural substrates

Natural media used for the study of mycotoxin production by Fusarium have been mostly maize or rice, either alone or with added glucose (Mirocha et al., 1971) and have led to somewhat conflicting results.

Eugenio et al. (1970) studied the substrate factor extensively by using different natural and synthetic media to observe their effect on ZEA production. They found the amount of ZEA varied from excellent production on polished rice, somewhat less on maize, and none on

soybean and peas. Only trace amounts of ZEA were produced in liquid media (Czapek-Dox, Sabouraud's and potato-dextrose broths). Early work done by Burmeister (1971) showed that white maize grits were superior to pearled wheat and polished rice for producing T-2 toxin at 15°C. Greenhalgh et al. (1983) produced two Fusarium toxins, DON and ZEA, on both rice and maize but rice was found to be the better substrate for DON and maize the better for ZEA.

Bottalico et al. (1984) reported that in general maize kernels supported the production of different Fusarium mycotoxins (DON, T-2 toxin and ZEA) better than rice kernels, while rice was superior for moniliformin production. To produce moniliformin under laboratory conditions, Kriek et al. (1977) and Rabie et al. (1978) used maize-in-water cultures incubated at 25°C for 21 days and found maize to be the best substrate for MON.

A comparative study investigating the production of ZEA, zearalenol, T-2 toxin and DON was carried out by Richardson et al. (1984) on three different media: moist autoclaved rice, vermiculite moistened with nutrient broth and a liquid starch-glutamate-sodium citrate medium. White rice and vermiculite cultures supported the production of all of the toxins but the liquid medium supported only poor production of T-2 toxin and DON. Vermiculite was superior in that it yielded a clear extract compared to the rice culture which yielded an oily extract which reduced the sensitivity of the detection technique.

B. Synthetic media

Liquid or solid synthetic media have been less popular for investigating Fusarium toxin production in spite of their advantage in reducing interfering substances and subsequently shortening the extraction and purification processes (Richardson et al., 1984). The information available indicates that a synthetic medium is suitable for screening and production of ZEA and its derivatives and it has been used extensively for this purpose. About 32 g/ℓ of ZEA were obtained in a surface fermentation of a defined medium containing glucose, urea, casein hydrolysate (N-Z-Amine A), K_2HPO_4, $MgSO_4.7H_2O$ KCl and $ZnSO_4.7H_2O$ (Hidy et al., 1977). Richardson et al (1984)

considered the synthetic media also adequate for the detection of trichothecenes.

Recently, Pestka et al. (1985) used various synthetic media to produce DON and 15-ADON from F. graminearum isolates. They obtained the highest amount of DON (16.5 mg/ℓ) by adding 4% corn steep liquor to a modified Fries medium. The highest amount of 15-ADON (14.0 mg/ℓ) was produced in glucose-yeast extract peptone (GYEP). Greenhalgh et al. (1984) used 80ℓ of GYEP for the large-scale production of 3-ADON by F. graminearum and F. culmorum.

Several other synthetic media have also been routinely used for mycotoxin production. Czapek-Dox medium supplemented with 0.5% peptone was used by Morooka et al. (1972) and again by Yoshizawa and Morooka (1977). Modified Gregory medium and Vogel synthetic medium were used by Cullen et al. (1982).

1.4.5.2. Initial Moisture Content (IMC)

IMC and the relative humidity greatly influence toxin production on natural substrates. Richardson et al. (1984) studying the accumulation of ZEA in both rice and vermiculate media, found the optimum IMC to be 70% water on a dry-weight basis, and Eugenio et al. (1970) found that ZEA was produced better on rice at MC 60-65% or on maize at MC 45% w/w basis. Caldwell et al. (1970) and Naik et al. (1978) produced significant amounts of ZEA on maize at IMC of approximately 40%. In the case of DON, Greenhalgh et al. (1983) found optimal production at 40% IMC after a 24-day incubation period. Similarly Neish et al. (1983) found that optimum DON production was at 35% IMC.

1.4.5.3. Temperature and period of incubation

As well as the substrate used and its initial moisture content, incubation temperature and period of incubation can greatly influence toxin production in the laboratory (Greenhalgh et al., 1983; Bottalico et al., 1984). Burmeister (1971) emphasised temperature and the nature of substrate in his studies of T-2 toxin production. He obtained the highest amount of T-2 toxin on white maize grits at

15°C. He also noted that the amount of T-2 toxin declined when the temperature increased. The T-2 toxin was 50% less at 20°C and 85% less at 25°C and no toxin was detected at 37°C. Commonly the culture is first incubated at 27°C one week, followed by three weeks at 12°C to maximise T-2 toxin production (Bottalico et al., 1984). Greenhalgh et al. (1983) found that a strain of F. graminearum known to be a ZEA and DON producer, produced mainly ZEA at 19.5°C. The amount of ZEA increased according to both IMC and the time of incubation, reaching a maximum (333 ppm) after 40 days at 48% IMC. But at the higher temperature of 28°C DON production was optimal, 515 ppm being produced after 24 days at 40% IMC. Greenhalgh et al. considered that the incubation period should exceed three weeks to allow maximum production of DON.

The effects of incubation temperature and the length of incubation on moniliformin production have been studied by Rabie et al. (1978). Using three different incubation temperatures (20°C, 25°C and 31°C) and incubation periods of 7, 14 and 21 days, they reported that optimal production was after 21 days' incubation at 25°C.

Probably the longest period of incubation used was that of Caldwell et al. (1970) for ZEA production. Cultures were grown on 150 g maize either for ten weeks at 16°C or at 24°C for two weeks followed by 2 weeks at 12°C.

1.4.5.4. Fungal strain (the biological factor)

Jarvis (1971) concluded that not all strains of a particular fungal species are able to produce mycotoxins, but no explanation is so far available for this fact. A number of investigators have noticed that variation between strains is influenced in part by physical and nutritional factors. Greenhalgh et al. (1983), for example, noted that some Fusarium spp which appear to be weak producers of toxin, and even those which are apparently non-toxin producers, could produce toxin (or more toxin) under specific physical and nutritional conditions. These authors found three out of three strains of F. graminearum produced DON and ZEA on maize, whereas on rice only two of the strains produced significant amounts.

The interaction between temperature and strain was demonstrated by Naik et al. (1978). These authors found only one strain out of five of F. graminearum produced greater amounts of ZEA at 10°C compared to those produced at 25°C, and only one strain out of the five produced greater amounts at 35°C. Similar results were obtained by Bottalico et al. (1984) concerning the interaction of strain and temperature. The majority of their strains of F. graminearum produced maximum ZEA at a constant incubation temperature of 27°C but a few produced ZEA only at a low temperature (12°C). One of the strains also produced DON only at 12°C.

1.4.5.5. Miscellaneous factors

There are other less important factors such as pH, O_2, CO_2 concentration, culture flask size etc. The pH factor has normally been adjusted in synthetic media only. Hidy et al. (1977) used a starting pH of 6.8-7.2 for ZEA production, but to reduce the risk of contamination the pH was allowed to drop to about 4. Similarly El-Kady and El-Maraghy (1982) found that ZEA was produced optimally at an initial pH of 7. The optimum pH for DON production ranges between 5.2 and 6.5, according to Greenhalgh et al. (1983).

Unfortunately there is inadequate information available about the other factors and their influence on Fusarium toxin production. Greenhalgh et al. (1983) experimented with different sizes of culture flask containing different amounts of rice. No mycotoxin was detected in 2,000 and 2,800 ml flasks but production was good in 500 ml flasks. They interpreted such phenomena as being due to the larger flasks requiring longer incubation periods for toxin production or alternatively due to the gaseous environment affecting the biosynthesis of the toxin. Hidy et al. (1977) stated that aeration for ZEA production in liquid media was not an important factor, although a minimal amount was essential.

1.5. PROCEDURES FOR THE ANALYSIS OF *FUSARIUM* MYCOTOXINS

1.5.1. Sample Extraction

There are several steps to be completed before a sample is ready for mycotoxin analysis (Gorst-Allman and Steyn, 1984).

1.5.1.1. Sample and subsample size

The size of sample used for toxin analysis has varied greatly and it is difficult to make a general recommendation (Davis *et al.*, 1980; Smith *et al.*, 1984). Smith *et al.* suggested that a larger sample size (not specified) would increase the accuracy but analysis would be costly. Because it is difficult to analyse the whole (large) sample, subsampling is necessary.

Davis *et al.* (1980) recommended grinding the original sample to pass through a No. 14 sieve then thoroughly blending and subdividing into 1 kg samples. The 1 kg sample should then be ground to pass through a No. 20 sieve before blending and subdividing into 500 g analytical samples. They also suggested that it is necessary to use a larger subsample for coarsely-ground material than for finely-ground material.

In practice the subsample size has ranged from the 10 g of maize used by Cohen and Lapointe (1980) to the 300 g of feedstuff analysed by Mirocha *et al.* (1976b). Trenholm *et al.* (1985) considered the size of the final sample (subsample) used for mycotoxin analysis to be very important. They found that the precision of the analysis improved markedly when the subsample size ranged between 50 to 200 g.

1.5.1.2. Extraction solvents and methods

Pathre and Mirocha (1977) divided the *Fusarium* toxins (trichothecenes) into two groups based on solubility properties. Group A included T-2 toxin, HT-2 toxin, NEO, DAS, monoacetoxy scirpenol, verrucarin, vatridin and verrucol. Group B included scirpentriol, T-2 tetraol, DON and NIV. Group A trichothecenes are all about the same order of polarity and so normally can be extracted from contaminated

commodities with an aprotic (non-polar) solvent such as ethyl acetate, acetone, chloroform, methylene chloride or diethyl ether. Group B, which are highly hydroxylated and relatively polar, can usually be extracted with protic (polar) solvents such as methanol and ethanol or even by aqueous methanol, aqueous acetonitrile or water. Forsyth et al. (1977) suggested that ethyl acetate as well as acetonitrile are the choice of solvents for the extraction of toxins in Group A. Forsyth and his colleagues recovered 80% of T-2 toxin and 97% of DAS from mixed feed samples using acetonitrile. Ethyl acetate was even better (87% and 99% recovery). Good recovery (85%) of DAS and FUS-X was achieved by Nakano et al. (1974) using a mixture containing 100 ml hexane and 200 ml methanol - 1% NaCl (55:45).

For most practical purposes in choosing a solvent capable of extracting all Fusarium toxins simultaneously, aqueous methanol solvents ranging between 50% and 95% methanol have been used extensively (Kamimura et al., 1981; Gilbert, 1984; Cohen and Lapointe, 1984). For example, Cohen and Lapointe applied methanol-water (50:50) to extract group A trichothecenes (T-2 toxin, HT-2 toxin and DAS) from cereal grain, while Scott et al. (1981) successfully extracted DON (group B) from wheat samples with 50% methanol. Kamimura et al. (1981) extracted ZEA, MON, butenolid and several trichothecenes from both groups with methanol-water (95:5). But Trenholm et al. (1985) found that acetonitrile-water was better than methanol-water, in particular by giving less interfering contamination.

Generally there are two main methods for the extraction of contaminated samples or fungal cultures. They may either be extracted continuously with an appropriate solvent mixture for 24 h or more in a Soxhlet apparatus or, where a faster extraction is wanted or the mycotoxin is heat-sensitive, the sample can be extracted with an appropriate solvent in a Waring blender for several minutes (Gorst-Allman and Steyn, 1984).

Considerable variations in results have been found between laboratories or procedures or both (Trenholm et al., 1985). These variations are partially due to the diverse extraction and clean-up procedures used by various laboratories (Eppley, 1979). Such differences can be particularly obvious in the extraction of DON.

However, when DON was extracted from cereal grains with methanol-water (1:1) and blended for 5 min in a high-speed blender, the recoveries from wheat averaged 72% and 80% from two different laboratories and this was regarded as a good result (Scott et al., 1981). Quite similar results were reported by Bennett et al. (1983) using the same solvent but with a wrist-action shaker for 30 min. The recovery ranged from 77.3% to 86.3% for DON spiked in wheat samples. Acetonitrile-water (84:16) solvent was used by Chang et al. (1984) in the extraction of wheat samples. Extraction was completed with 3 min of high-speed blending, with nearly 100% recovery. But Trenholm et al. (1985) found 3 min blending released only 53% DON in naturally-contaminated samples. They recommended a minimum of 16 min for complete DON extraction from naturally-contaminated samples.

Soxhlet extraction has been commonly used for extracting MON from feed and foodstuff. Rabie et al. (1978, 1982) extracted mouldy meal with aqueous methanol (80%) in a Soxhlet extractor for 48 h, whereas Steyn et al. (1978) used methanol as the solvent and a Soxhlet-type automatic extractor for 3 h only.

A few studies have evaluated the efficiency of the actual extraction technique. Trenholm et al. (1985) assessed 3 mixing apparatus (high-speed blender, wrist-action shaker and mechanical stirrer) and their effect on extraction of DON from naturally-contaminated grains. Two solvent systems, acetonitrile-water (21:4) and methanol-water (1:1) were used as extraction solvents. The high-speed blender was the best extraction device, requiring a minimum of 16 min blending. They found that naturally-contaminated samples needed a longer time (about 2 h) on a wrist-action shaker; nevertheless this shaker was useful for large-scale sample analysis.

1.5.1.3. Clean-up procedures

Any extract from grain, foodstuff or fungal cultures usually contains numerous carbohydrates, lipids, pigments and other contaminants, so it is necessary to introduce a clean-up step to remove as many interfering compounds as possible before analysis (Gorst-Allman and Steyn, 1984). Purifying the extract will improve the sensitivity, detection, selectivity and quantification. Two techniques are com-

monly employed for purification, liquid-liquid and solid-liquid partition (Pathre and Mirocha, 1977).

A. Liquid-liquid (solvent-solvent) partition

In their review, Pathre and Mirocha (1977) noted that partitioning of the extract in organic solvents as well as in a heterogeneous solvent system has been reported to be without any significant loss of trichothecenes. The main problem with this procedure is that many trichothecenes from group A (T-2 toxin, DAS etc.) have some finite solubility in hydrocarbon solvents (such as hexane etc.). The partitioned hydrocarbon solution (lipid-hydrocarbon) might, then, act as a solvent for dissolving some of the trichothecenes and lowering their recovery.

There are several systems of liquid-liquid clean-up. Pathre et al. (1976) used acetonitrile/petroleum (60-70) 50:50 for purifying MAS, DAS and T-2 toxin. Bennett et al. (1981) extracted DON from maize grains with aqueous methanol 80% and the aqueous solution of DON was partitioned in ethyl acetate solution. Defatting is very often accomplished by partitioning the extract solvent with either n-hexane or iso-octane (Takitani and Asabe, 1983).

B. Solid-liquid partition

This technique has been extensively used in the isolation and analysis of trichothecenes and uses absorbent silica gel (Hagen and Tietjen, 1975; Cohen and Lapointe, 1980), charcoal (Ueno et al., 1973), florisil (Kamimura et al., 1981), Amberlite XAD-2 (Visconti and Mirocha, 1985) and XAD-4 (Kamimura et al., 1981).

TLC on silica gel is a very common cleaning-up technique. The TLC plate may be spotted with crude extract and then developed in benzene-hexane (3:1) etc. In this system the mycotoxin remains on the base line whereas many of the contaminants move with the solvent front (Hagen and Tietjen 1975).

Amberlite XAD-2 and XAD-4, both highly porous synthetic adsorbents on polystyrene beads, have been found to be promising agents for

clean-up purposes. For example, Visconti and Mirocha (1985) applied XAD-2 to clean-up chicken organ tissue. The T-2 toxin and its metabolites were eluted with 90% aqueous methanol.

It is not uncommon to find many researchers including more than one cleana-up step and occasionally both techniques (solid-liquid and liquid-liquid) are involved in the purification process (Abbas et al., 1986). Kamimura et al. (1981) applied two steps, namely XAD-4 column chromatography and florisil column chromatography to purify mixtures of Fusarium mycotoxins. The sample extract was applied first to AMberlite XAD-4 resin in a chromatographic column. Moniliformin was first eluted with 50 ml of water and then ZEA and five trichothecenes with 100 ml of methanol. The methanol extract was evaporated and the residue redissolved in a 10 ml mixture of chloroform-methanol (9:1). This was then subjected to florisil column chromatography as a second step clean-up, the trichothecenes and ZEA being eluted with 100 ml chloroform-methanol (9:1). A two-step clean-up was used by Cohen and Lapointe (1980) following extraction of ZEA from aimal feeds with chloroform-methanol. Clean-up was initially with a Sep-Pak silica gel cartridge followed by column chromatography on Sephadex LH-20.

A three-step clean-up method can also be used. Rosen and Rosen (1984) used methanol-hexane partitioning, reversed-phase and normal-phase Sep-Pak C18 cartridges to clean-up T-2 toxin, HT-2 toxin, ZEA and DAS from maize samples.

1.5.2 Physico-chemical Assays

A considerable number of analytical methods for Fusarium mycotoxins have been proposed, but there have been few overall comparisons of their application. The methods in use for detecting and quantifying mycotoxins can be classified into physico-chemical or biological assays (Pathre and Mirocha, 1977).

Several physico-chemical assay methods have been developed and used during the past decade, including thin-layer chromatography (TLC), gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), mass spectrometry-mass spectrometry (MS-MS) and high performance liquid chromatography (HPLC). Scott (1982) evaluated accuracy,

precision and limits of detection of the above methods and his conclusion was that GC with an electron capture detector (ECD) or GC-MS were the best techniques for detection and quantification of trichothecenes but the methods involve expensive instrumentation. Gilbert et al. (1984) ranked the most common chemical analytical methods for the separation and detection of Fusarium mycotoxins in order of increasing specificity as follows: HPLC (with UV detector), TLC after spraying with P-nitrobenzyl-pyridine reagent, GC with ECD, GC-MS and MS-MS. As the specificity and sensitivity of the technique increases, the instrumentation becomes more expensive and less available for routine laboratory assays.

1.5.2.1 Thin-layer chromatography

Thin-layer chromatography is the technique which is the most widely used for the detection and analysis of mycotoxins, including Fusarium toxins. It depends on three variables, namely the type of absorbent, the solvent system and the method of detection (Gorst-Allman and Steyn, 1984). TLC is frequently the method of choice for the semi-quantitation of trichothecenes (Scott, 1982). The analysis is most frequently performed on a silica gel phase. Fusarium toxins, excepting ZEA, have no fluorescent or ultra-violet absorbing properties, so their detection and quantitation on TLC can be difficult. The chromatogram should be sprayed with a chromogenic reagent and the toxin detected by fluorescence or by colour (Takitani and Asabe, 1983). However, some disadvantages accompany this detection method. The reagents are not specific and for natural extracts the chromatograms are difficult to interpret and the technique is, at best, semi-quantitative. There is also no single reagent which has enough sensitivity, selectivity and stability for universal use.

Pathre and Mirocha (1977) considered that the TLC method is not quantitative because of the difficulty encountered in uniformly spraying the plate with the reagent. Detection and quantitation also become very critical when the extract contains many interfering components. But Gilbert et al. (1984) concluded that TLC (preferably in two dimensions) can offer adequate separation when a combination of spray reagents is used, although the sensitivity for certain

trichothecenes (T-2 toxin in particular) is poor and quantitation is at best only approximate.

Pathre and Mirocha (1977), and Takitani and Asabe (1983) conducted general reviews of the main reagents. Any of the following could be chosen depending on the purpose of the assays and the type of toxin.

a) Sulphuric acid (H_2SO_4) at a ratio of 10-50% in aqueous or ethanolic solution. After being sprayed, the TLC plate is heated for about 20 min at 100-130°C (Sorenson et al., 1975). Trichothecenes type A will give a greyish-black colour and type B a brown colour.

   The detection limit according to Takitani and Asabe (1983) was 0.25 µg/spot. Under UV light (360 nm) only type A will give a blue fluorescence, but the detection limit was improved by about 0.05 µg/spot. Hofman (1980) (cited by Scott, 1982) reported improved sensitivity using two-dimensional TLC and spraying with 20% H_2SO_4. He detected 15 µg/kg T-2 toxin in the muscle and liver tissue of chicken.

b) Aluminium chloride ($AlCl_3$). The use of this reagent is based on resultant fluorescence. Naoi (1983) detected FUS-X after plates had been sprayed with 50% $AlCl_3$ and heated for 10 min at 130°C. The toxin was detected by its blue fluorescence under 360 nm UV light and the detection limit was 0.05 µg/spot. Kamimura et al. (1981) detected type B trichothecenes by spraying plates with 20% $AlCl_3$ and heating for 10 min at 110°C; the detection limit was between 20-50 µg/kg for DON, NIV and FUS-X.

   Recently Eppley et al. (1986) reported on an extensive collaborative study (18 collaborator laboratories) for the determination of DON using a rapid screening method in which TLC plates were sprayed with $AlCl_3$. The lower limit of detection was actually 50-100 ppb but reliable determinations of DON in wheat were achieved at levels of 300 ppb or more.

c) 2,4 dinitrophenyl hydrazine (2,4-DNPH). Sorenson et al. (1975) reported a semi-quantitative determination of trichothecenes on TLC after derivatives were obtained from reaction with 2,4-DNPH. The detection limit was 6 µg/ml. Gorst-Allman and Steyn (1979) reported an orange colour from the reaction of 2,4-DNPH with T-2 toxin and dark orange-yellow with ZEA. But the 2,4-DNPH reagent was not sensitive enough compared to other reagents such as H_2SO_4, $AlCl_3$ etc.

Kamimura et al. (1981) developed a densitometric quantitation and confirmation of moniliformin by using 2,4-DNPH derivatives. The detection limit of this technique was 50 µg/kg. Steyn et al. (1978) applied two reagents, 1% of 2,4-DNPH and 1% of methanolic ninhydrin solution sprayed on the TLC plate (0.3 mm Camag D-5 silica gel).

d) Other reagents such as P-anisaldehyde (Scott et al., 1970), 4-(P-nitrobenzyl) pyridine (Takitani et al., 1979), Nicotinamide-2-acetyl pyridine (Sano et al., 1982) also have been used successfully for detection and quantification of some Fusarium toxins.

Quantitation of mycotoxins by TLC can be carried out by evaluation either on the plate or after extraction from the plate. Both need a precise application of a fixed amount of standard mycotoxins (Gorst-Allman & Steyn, 1984).

There are two main methods applicable for quantifying the Fusarium mycotoxins on TLC: a) visual determination which is, according to Schuller et al (1976), at best semiquantitative. A comparison of spot areas of the sample is made to the area of known concentration of standard run on the same plate; b) instrumental measurement. Schuller et al. (1976) found both accuracy and precision of analysis can be improved by using a densitometer to determine the transmission or reflection properties of the mycotoxin spot on the TLC plate. The densitometer can be used on spots which are coloured, either naturally or with chromatogenic reagent, are charred, or absorb UV light or fluoresce (Gorst-Allman & Steyn, 1984). UV spectroscopy is also commonly used as a quantitative method for the mycotoxin extracted

from the adsorbent layer of TLC. TLC absorbence obtained from unknown samples is used in conjunction with the molar absorptivity (E) of pure toxin to calculate the concentration (Burmeister et al., 1979).

A large variety of solvent systems has been developed to detect and characterise various toxins. The most frequently used system consists of different percentages of methanol (2-7%) in chloroform, although this system is very sensitive to environmental factors such as humidity.

Different developing solvent systems for the detection and identification of trichothecenes on silica gel TLC plates have been developed. Pathre and Mirocha (1977) reviewed some of these systems, eg. chloroform-methanol (98:2, 97:3 and 95:5), toluene-ethyl acetate (1:3) and benzene-acetone (3:2). The Rf values for different trichothecenes are affected by the polarity of the solvent. Acetone has been effectively substituted for methanol and the optimum ratio of chloroform to acetone was reported as 90:10 by Gorst-Allman and Steyn (1979) or 85:15 by Genest and Smith (1963) (cited by Gorst-Allman and Steyn, 1984). Eppley et al. (1986) used chloroform-acetone-isopropanol (8:1:1) for DON detection. Neish et al. (1982) developed TLC plates in toluene-ethyl acetate-formic acid (6:3:1) or benzene-methanol-acetic acid (24:2:1) to detect T-2 toxin and DAS.

1.5.2.2. High performance liquid chromatography

While high performance liquid chromatography has successfully been used for detection and quantitation of ZEA (Gilbert, 1984), it was found too difficult to apply for most trichothecenes (except for some group B trichothecenes, e.g. DON, NIV etc.) because of the lack of UV absorption. Furthermore this method was earlier considered unsuitable for grain analysis, because of poor sensitivity (Scott, 1982). However, sensitivity has now been increased considerably particularly after improvements in the sensitivity of modern UV detectors (Lauren and Greenhalgh, 1987). Lauren and Greenhalgh detected 15 and 50 ng/g of NIV and DON respectively from spiked wheat and maize samples.

1.5.2.3. Gas chromatographic and associated techniques

Scott (1982) concluded that gas chromatography alone or in combination with other techniques was the most satisfactory method of analysis for Fusarium mycotoxins, being better than TLC in terms of detection and sensitivity. However, GC has been said to have only limited application in the investigation of unknown mycotoxins (Gorst-Allman and Steyn, 1984) although when coupled to mass-spectrometry a considerable amount of qualitative data concerning the identify of the compound being analysed can be obtained.

A. Derivatisation

As Fusarium mycotoxins are insufficiently volatile themselves they must be transformed into volatile derivatives, typically trimethylsilyl (TMS) ethers (Kamimura et al., 1981) or heptafluorobutyryl (HFB) esters (Scott et al., 1981). The derivatisation step is of primary importance in analytical procedures, but the conditions that favour the reaction are complicated (Kientz and Verweij, 1986).

A large number of derivatisation reagents are commercially available (Table 1-7) and the use of some reagents seems arbitrary. Some authors have also used incomplete derivatisation of mycotoxin according to Gilbert et al. (1985).

Various Fusarium toxins have been silylated with TRI-SIL "TBT" which is a standard mixture of TMSI, BSA and TMCS (3:3:2). It is a powerful silylating reagent with high boiling point, but it is difficult to remove excess (Gilbert, 1984; Mirocha et al. 1976a; Visconti and Mirocha, 1985). Rosen and Rosen (1984) heated the mixture of BSTFA reagent with mycotoxins for 1 h at 90°C for derivatising several trichothecenes as well as ZEA.

Heptafluorobutyrylimidazole (HFBI) has been used by several authors, providing good sensitivity particularly by using GC with ECD for analysis (Gilbert, 1984; Cohen and Lapointe, 1984; Scott et al., 1981).

Mixtures of TMSI and TMCS in ethyl acetate were successfully used by Kamimura et al. (1981) for silylation of T-2 toxin, HT-2 toxin, NIV, DON, FUS-X and ZEA. One ml of mycotoxin standard or 0.5 ml of extract sample was transferred into a 20 ml pear-shaped flask and after evaporation 0.5 ml of derivatising reagent was added. The reaction was complete within 15 min at room temperature.

Table 1-7: Names of derivatising reagents *

Reagent	Abbreviation
N,O-Bis(trimethylsilyl)acetamide	BSA
N,O-Bis(trimethylsilyl)trifluoroacetamide	BSTFA
Trimethylchlorosilane	TMCS
Hexamethyldisilazane	HMDS
N-Trimethyldilylimidazole	TMSI
BSA-TMCS(5:1)Tri-Sil BT **	BT
TMSI-BSA-TMCS(3:3:2)Tri-Sil TBT **	TBT
Trifluoroacetic anhydride	TFAA
N-Trifluoroacetylimidazole	TFAI
N-Methyl-bis(trifluoroacemamide)	MBTFA
Heptafluorobutyrylimidazole	HFBI

* From Kientz and Verweij, 1986, and Gilbert et al., 1985.

** Trade name of Pierce

Some studies have attempted to deduce the optimum conditions required for a universal derivatisation procedure for the quantitative analysis of samples contaminated with Fusarium toxins. Gilbert et al. (1985) examined the optimum condition for trimethyldilylation of DON. They used different derivatising reagents (BSTFA and TMSI) and 3 different temperatures (room temperature, 60°C and 100°C) for completing the reaction. The authors found that DON could be derivatised completely only in the presence of TMSI whatever the thermal treatment, while using BSTFA alaone, despite prolonged heat treatment and the presence of an acidic catalyst such as TMCS, the derivatisation of DON was not completed. The authors concluded that TMSI was

also a powerful reagent for all members of type B trichothecenes (eg. NIV, FUS-X etc.). The main disadvantage of TMSI is that it is not easily evaporated and the excess has been found to cause damage to the stationary phase of the column.

A further and more extensive study was carried out by Kientz and Verweij (1986) using seven different derivatisation reagents for DAS, NEO, DON, NIV, T-2 toxin, HT-2 toxin and ZEA. Their conclusion was that reagents containing TMSI in the presence of BSA or BSTFA seemed to be essential to obtain a high response for both type A and type B trichothecenes. However, ZEA derivatives are highly unstable even with BSTFA reagent (Rosen and Rosen, 1984).

Kientz and Verweij (1986) suggested that the unsatisfactory results obtained in many cases were most likely due either to incomplete derivatisations, mostly happening with DON, or to decomposition of the derivative during gas chromatographic analysis, particularly with ZEA. It was hard to distinguish between these two factors.

B. Gas-liquid chromatography

Gas-liquid chromatography can obtain good resolution and often good sensitivity of detection if the toxin is sufficiently volatile at the column temperature or can be converted into a volatile derivative (Mirocha et al., 1976a). Increasing the temperature of the column either linearly or non-linearly will allow the sequential elution of metabolites of greatly varying (although not overlong) retention times without peak broadening (Gorst-Allman and Steyn, 1984).

The main disadvantage of GC is the possibility of false positive results, particularly with flame ionization detection (FID), because there are many components which co-chromatograph with trichothecenes (i.e. have an identical retention time) (Mirocha et al., 1976b). By using GC with an electron capture detector (ECD), this problem can be partially overcome and this combination (GC-ECD) can be regarded as the best compromise between expensive instrumentation and reliable quantitation (Scott, 1982).

Most of the Fusarium toxin derivatives (TMS in particular) have been analysed on a short non-polar packed column. Several stationary phases are available such as OV-1 (Steele et al., 1976; Chaytor and Saxby, 1982) or OV-17 (Kamimura et al., 1981). SE-52 was used by Thouvenot and Morfin (1979) on capillary glass for detection of ZEA and fused silica capillary columns coated with SE-54 by Cohen and Lapointe (1982) for the detection of DON. According to Pathre and Mirocha (1979), the most suitable phases for trichothecenes are SE-30 and OV-17. Recently capillary columns (unpacked) have been found superior to the packed columns and are now used more often (Rosen and Rosen, 1984). Cohen and Lapointe (1984) used fused silica DB-5 (30 m x 0.32 mm id) for the detection of T-2 toxin, HT-2 toxin and DAS.

Sensitivity of the GC method is largely dependent upon the separation of the mycotoxin under investigation from other interfering components in the extract. The detection limit depends to a large extent on the type of detector, normally either a FID or ECD.

Scott (1982) concluded that GC-ECD is more sensitive than GC-FID for determining TMS ethers of both type A and type B trichothecenes, particularly for group B members because of their conjugated carbonyl group. Kamimura et al. (1981) compared the detection efficiency of GC-FID and GC-ECD for several trichothecenes and ZEA. They found that the detectable amount of trichothecenes of type A (T-2 toxin and DAS) was about 200 µg/kg and about 100 µg/kg for type B (NIV, DON and FUS-X) when GC-FID was used, compared to about 80 µg/kg for type A and about 2 µg/kg for type B when GC-ECD was used. This means that more than a 2-fold improvement in the sensitivity for type A and a 50-fold improvement for type B was noted with the use of GC-ECD. Kuroda et al. (1979), cited by Naoi (1983) have reported an even greater increase in sensitivity with ECD, dependent on the presence of C=O at the 8th position of the trichothecene structure for type B compounds.

Although gas chromatography with FID is less sensitive for Fusarium toxins compared to ECD, it is still commonly used, but most confirmation is done by a combination of GC-FID with mass spectrometry (Steele et al., 1976; Cullen et al., 1982; Kientz and Verweij, 1986).

C. Gas chromatography - mass spectometry

The GC-MS technique is extremely selective and sensitive and can achieve most of the requirements of screening processes (Chaytor and Saxby, 1982). However, the instrumentation is expensive and not freely available to the routine laboratory (Gilbert et al., 1984). Pathre and Mirocha (1979) considered GC-MS to be the best analytical tool, giving precise identification of compounds even when there is substantial interference.

As the components of an injected sample are eluted from a GC column, the mass spectrum of each component is recorded, each toxin and its derivatives having a characteristic mass spectrum (MS). A computer can be interfaced with the GC-MS system and the MS data stored and analysed.

There are two modes for recording the mass spectra - either full-scan (total ion detection) or selected ion monitoring (SIM). The full-scan mode is useful for the identification and confirmation of the presence of the toxin as it is eluted off the column, but the sensitivity is limited and sometimes hampered by an overwhelming concentration of an interfering compound (Pathre and Mirocha, 1977). SIM is used in a continuous-scanning mode during which MS are obtained at regular intervals (Mirocha et al., 1976b). The specificity and sensitivity of SIM provide a definite additional advantage over other electronic methods of detection (Rosen and Rosen, 1984). Self (1979) demonstrated that SIM with a multi-channel computer controlled at high resolution should give a high sensitivity.

Rosen and Rosen (1982) described the SIM procedure for the detection of several Fusarium toxins from "yellow rain" samples. Three characteristic molecular ions for each toxin were chosen. The same technique was applied later by the authors to extracts of maize samples to identify and quantify T-2 toxin, HT-2 toxin, DAS and ZEA (Rosen and Rosen, 1984). They demonstrated that the identification of these mycotoxins as well as their reliable quantitation could be achieved down to levels of abut 20-50 ppb. However, they noticed that the ZEA derivatives began to deteriorate after one hour.

The GC-MS (SIM) technique is the recommended routine screening method for DON in MAFF Food Science laboratories in the U.K. (Gilbert et al., 1984). The procedure uses TMS derivatives. Monitoring is carried out for two characteristic molecular ions (mass/charge ratio [m/z] of ion fragments 512 and 422). In the case of multi-toxin assays the SIM technique can be applied with capillary column multiple ion detection (MID) for switching between the two groups of eight characteristic molecular ions. Up to eight trichothecenes in a single chromatographic run can be monitored (Gilbert et al., 1984).

The number of molecular ions used for monitoring varies greatly among researchers. Scott et al. (1981) monitored a single characteristic ion (m/z 884) for DON-HFB derivatives with the detection limit $\leq$ 100 ppb in samples and Cohen and Lapointe (1984) confirmed the presence of T-2 toxin, HT-2 toxin and DAS-HBFI derivatives by using Electron Impact (EI) and SIM mode (one ion). Yoshizawa (1984) monitored at m/z 512 and 497 for DON-TMS and at m/z 482 and 413 for NIV. The detection limit for the toxins was 100 ppb. Chaytor and Saxby (1982) detected and quantitated T-2 toxin from maize samples by monitoring on two ions, m/z 350 and m/z 436. The detection limit was extremely low (5 ppb) with an average recovery of 80%.

Using three selected ions is a not uncommon technique which has been employed for trichothecenes and ZEA detection. Mirocha et al. (1974) described the SIM method for the isolation, detection and quantitation of ZEA-TMS derivatives. The detection limit was 10 ng/g in maize by using m/z 447, 462 or 429 and 333. Rosen and Rosen (1982) selected three ions for most toxins. T-2-TMS was monitored at m/z 436, 350 and 290, DON-TMS at 513, 512 and 497, DAS-TMS at 378, 350 and 290, ZEA-TMS at 462, 445 and 350. For HT-2-TMS only two ions were used (466 and 347). In addition, deuterated TMS derivatives were used as internal standards and two selected ions chosen for monitoring each standard.

The same technique, when it was applied to maize samples, showed a detection limit of about 20-50 ppb.

The largest number of selected ions was used by Mirocha et al. (1976b). Nine characteristic fragment ions were chosen to detect several Fusarium toxins contaminating a mixed feed sample.

Several modes of ionization can be used for the GC-MS assay method, i.e. Electron Impact (EI), Negative Ion Chemical Ionization (NCI), Positive Chemical Ionization (PCI), Oxygen Negative Chemical Ionization (ONCI) etc. (Miles and Gurprasad, 1985). Most of the applications of MS for Fusarium toxin identification have utilised the EI-MS mode and detection limits range between 25 pg and 100 pg (Mirocha et al., 1976b; Scott et al., 1981; Cohen and Lapointe, 1984).

Other modes of ionization may give better sensitivity than ET. Rothberg et al. (1983) obtained better sensitivity and selectivity by using NCI with the SIM technique for the detection of six trichothecenes. Rothberg and his colleagues compared three MS modes, EI, PCI and NCI. The limits of detection for the three modes were 100, 500 and 0.1 pg respectively. Miles and Gurprasad (1985) applied ONCI-MS for the detection of nine trichothecenes as well as ZEA from different agricultural commodities. By selecting two ions to be monitored for each toxin, they were able to screen for ten toxins in a single analytical run. No derivatisation was needed, so the trichothecenes were stable and the analysis could be carried out at a longer time after sampling. Recovery ranged between 77% and 106%.

D. Mass spectrometry - mass spectrometry

MS-MS is the most sophisticated instrumental technique which has been used for both the separation and detection of mycotoxins. MS-MS as described by Plattner and Bennett (1983) uses one stage of mass separation to select the compound of interest from the matrix and a second stage for analysis after collisionally activated dissociation (CAD) by collision with the target gas. Potential multi-toxin screening is possible by direct analysis of a crude solvent extract without clean-up or derivatisation (Plattner and Bennett, 1983). These authors successfully used this method to detect ZEA and DON, and the sensitivity was 100 ug/kg in cereal samples. But this technique is severely limited by the availability of equipment due to the high cost of the instrument (Gilbert et al., 1984).

1.5.2.4. Multi-mycotoxin assay

No principles exist for predicting the presence of a particular mycotoxin in foodstuffs other than those drawn from our limited knowledge of fungal ecology (Gorst-Allman and Steyn, 1979). It is well known that natural samples are often contaminated with more than one mycotoxin (Lee et al., 1980) and that some Fusarium spp can produce more than one toxin. This will make it difficult to predict which Fusarium toxins are significant (Gilbert et al., 1984). As analysis for each individual toxin can be tedious and time-consuming, multi-mycotoxin screening methods have been introduced and strongly recommended (Lee et al., 1980).

Any method for multi-mycotoxin analysis should fulfil four main criteria (Gilbert et al., 1984). These criteria are: a) a minimum of four Fusarium mycotoxins should be monitored simultaneously; b) the method should achieve a limit of detection below 100 µg/kg for animal feed and probably below 50 µg/kg for human; c) rapid procedures are desirable, to allow the analysis of a large number of samples; d) the method should provide reasonably high specificity. This is particularly important for trichothecenes, which do not show native fluorescence nor electron-capturing properties without derivatisation.

A variety of multi-mycotoxin screening methods have been proposed. TLC has been a frequent analytical tool. Gorst-Allman and Steyn (1979) used TLC to separate 13 important mycotoxins from a contaminated maize sample into chemically neutral and acidic components. The analysis was achieved by using several solvent systems and spraying plates with appropriate chromogenic reagents. The limit of detection from the pure sample ranged between 1.0 and 0.01 ppb. It is unlikely that these values could be achieved in the analysis of naturally-contaminated samples.

TLC systems are often slow, of relatively low sensitivity and require the use of several solvents (Lee et al., 1980). TLC in combination with GC was applied for screening a large number of Fusarium toxins by Kamimura et al. (1981) and Richardson et al. (1984). Kamimura et al. described how several trichothecenes as well as ZEA,

MON and butenolide can be simultaneously determined in a spiked maize sample by TLC and GC either with FID or with ECD. The authors also applied this method to 43 samples of wheat and barley and 30 were positive for DON and NIV.

GC or GC-MS or both have also been commonly used for the detection and confirmation of the presence of more than one Fusarium toxin. Mirocha et al. (1976b) screened feedstuff for 6 Fusarium toxins by using GC-MS. The results indicated that DON, DAS and ZEA naturally contaminated the samples. In the course of a survey of food and fodder cereals in the U.K., Osborne and Willis (1984) applied only GC with occasional MS confirmation to assay wheat samples for seven common trichothecenes but only DON was detected.

Gilbert et al. (1984) considered that the GC-MS method is the most promising and achieves the desired criteria for a multiple-mycotoxin assay. Miles and Gurprasad (1985) used GC-MS in the ONCI mode to successfully analyse nine underivatised trichothecenes.

1.5.2.5. Biological detection methods

In cases where there is no established physico-chemical assay technique or if the toxigenic fungus produces an uncommon or unknown mycotoxin, toxicity can be monitored by a bioassay method (Cole, 1984). Fusarium toxins (particularly trichothecenes) possess antibiotic, phytotoxic, cytotoxic and dermatitic activities which allow such characteristics to be employed for bioassay (Pathre and Mirocha, 1977). Skin tests and immunoassays are probably the most reliable and sensitive tests (Scott, 1982) but some methods are not specific to Fusarium toxins (Ueno, 1983).

A. Skin bioassay

Several investigators have successfully used skin bioassay for trichothecenes, particularly T-2 toxin and HT-2 toxin. The method provides a highly selective bioassay but not all trichothecenes showed dermal toxicity (Cole, 1984). The test is carried out by topical, intradermal or subcutaneous administration (Wehner et al., 1978), using mice, rats, guinea pigs or rabbits. Ueno (1983) considered the

guinea pig to be the most sensitive to trichothecenes. The prominent features of the reaction (skin irritation, erythema, oedema and necrosis) are read after 24, 48 and 72 h (Chung et al., 1974). Hayes and Schiefer (1979) could estimate 5 to 60 ug/ml of T-2 toxin and DAS quantitatively by cutaneous injection of rats and rabbits. However, skin bioassay is at best semi-quantitative and it cannot distinguish between members of the trichothecene family (Eppley, 1975).

B. Immunoassay

There are now available specific sensitive and relatively inexpensive immunoassay methods for detection and quantitation of some Fusarium toxins and other mycotoxins (Chu et al., 1979; Gilbert et al., 1984).

Because Fusarium toxins have no natural antigenic characters, it is necessary to produce specific antibodies to use in either radioimmunoassays (RIA) or enzyme-linked immunosorbent assays (ELISA) (Ueno, 1983). The RIA method has been used as a technique for the detection and quantitation of Fusarium mycotoxins. The instrumentation is relatively inexpensive and the method is rapid and can be carried out on crude sample extracts, or even sometimes without extraction, as in the case of biological fluids (Fontelo et al., 1983). The main disadvantages are that it requires specialised skill and experience initially to produce anti-sera and it is not suitable for monitoring for more than a single toxin at a time (Gilbert et al., 1984). A significant cross-reaction could occur between any chemically-related mycotoxins such as T-2 toxin and HT-2 toxin, or zearalenone and its derivatives (Fontelo et al., 1983; Thouvenot and Morfin, 1983).

Lee and Chu (1981a, b) developed a RIA for determining T-2 toxin in maize, wheat and biological fluid. No cross-reaction was noticed between the antibody and other trichothecenes which were tested except for HT-2 toxin. The detection limit was 2.5 ng/g in maize, 1 ng/g in wheat and 0.5 ppb for serum, urine and milk. Similar results were reported by Fontelo et al. (1983) by a RIA method in which no extraction step was used for the biological fluids. A sensitivity of 1 ng per assay or 10 ng/ml was achieved. Antibodies for RIA of zearalenone

and zearalenol were developed in pigs by Thouvenot and Morfin (1983) and were applied to human serum. It was found that this method was able to detect as low as 5 ppb of ZEA or zearalenol in human and animal sera. But it cannot differentiate zearalenone from its metabolites or zearalenol from its transformation products (Thouvenot and Morfin, 1983).

ELISA assay methods were successfully employed by Peters et al. (1982) to detect T-2 toxin down to 2 pg per assay of purified mycotoxin. The ELISA method has advantages over the RIA method as the cross reaction of T-2 toxin antiserum with other trichothecenes was weak. The method is also expensive. ELISA techniques have most commonly been used for aflatoxin (Ram et al., 1986).

CHAPTER 2
MATERIALS AND METHODS

2.1. MYCOLOGICAL TECHNIQUES

2.1.1. Principal Media

A. Potato Dextrose Agar (PDA):-

Potato broth	500 ml

(prepared by boiling 200 g peeled and diced old-crop potatoes for 1 h in 500 ml tap water followed by filtering through double-folded cheesecloth, to a final volume of 500 ml); add

Dextrose	20 g
Agar	20 g
Water	500 ml
Autoclaved	15 min at 120°C

B. Potato Dextrose Agar + Dichloran (PDA-D):-

To molten PDA at 45°C the appropriate quantities of antibiotic stock solutions (see below) were added to give final concentrations of:

Benzyl penicillin	50 units/ml
Streptomycin sulphate	100 units/ml
Dichloran*	1 ml

* 2,6-dichloro-4-nitroaniline

C. Modified Nash and Snyder's (1962) Peptone-PCNB agar (PCNB):-

Difco peptone	15 g
Agar	20 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
PCNB (stock)*	5 ml
Water	1 ℓ

Autoclaved for 15 min at 120°C.

* Pentachloronitrobenzene

(continued)

To the molten medium cooled to 45°C the appropriate quantities of antibiotic stock solutions (see below) were added to give final concentrations of:

Streptomycin sulphate	100 mg/ℓ
Chlorotetracycline HCl	50 mg/ℓ

Antibiotic Stock Solutions for Media B and C

Benzyl penicillin	5000 units/ml in water
Streptomycin sulphate	10000 units/ml in water
Chlorotetracycline HCl	5 mg/ml in water
Dichloran	0.2% in ethanol (1 ml equivalent to 2 mg/ℓ)
Pentachloronitrobenzene	10% in acetone

D. Potato Sucrose Agar (PSA):-

Potato broth	500 ml
Sucrose	20 g
Agar	20 g
Water	500 ml

Autoclaved for 15 min at 120°C.

E. Carnation-leaf Agar (CLA):-

Fresh carnation leaves, free from fungicide or insecticide, were cut into about 5 x 5 mm pieces and dried at 55-60°C for 1.5 h (until brittle). Approximately 5 g quantities were placed in plastic bags, sealed, and sterilized by γ-irradiation (ICI New Zealand Ltd, Wellington). Sterilized leaves were stored at 4°C until used.

Several (approximately 10) pieces of the carnation leaves were placed in empty petri dishes and covered with molten, cooled, tap-water agar. Prior to use the plates were left at room temperature for 4 days to check for any contamination.

F. Czapek-Dox Agar (CDA):-

Na_2NO_3	3.0 g
KH_2PO_4	1.0 g
$MgSO_4.7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4.7H_2O$	0.01 g
Glucose	30.0 g
Agar	15.0 g
Distilled water	1.0 ℓ

Autoclaved for 15 min at 120°C.

2.1.2. Source of Samples

2.1.2.1. Maize (see Table 2-1)

A. Field samples

Samples were collected from maize fields at Flyger's Line near Palmerston North (J1), near Levin (J2) and from four fields from Kairanga County (T1, T2, T3 and T4). Locations are indicated in Figure 2-1 and Table 2-1.

B. Harvest samples

These were collected from the same fields as the samples at harvest time with the exception of field J1, which was not sampled. A further two harvest samples, RH from a maize field at Settlers Line in Bunnythorpe County and KH from a field at Flyger's Line near Palmerston North, were collected during the unloading of a truck at Rowe & Collis Ltd. Drying Complex.

C. Stored samples

a) Three samples from Silo A from Kairanga County were collected between 10.8.84 and 10.10.84. The grain was a mixture of that from fields T2, T3 and T4.

b) Three samples from Silo B from Levin were collected between 10.9.84 and 29.11.84; the grain originally came from field J2 (Levin).

Table 2-1: Source of samples.

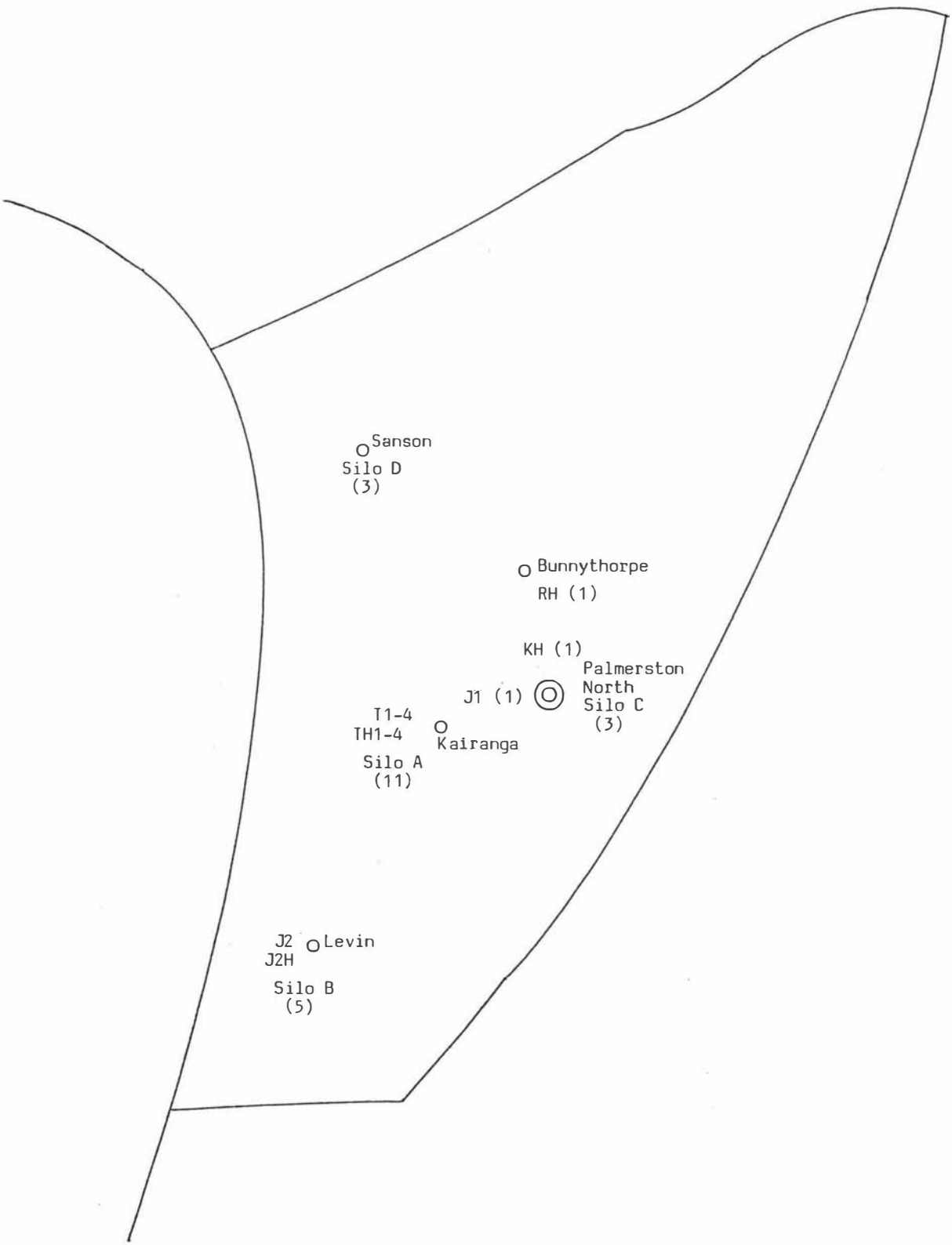
a) Field and harvest samples.

Field samples	Date of sampling	Harvest sample	Date of sampling	Breed of maize	Location
T1	19.06.84	T1H	26.06.84	Pioneer 3901	Kairanga
T2	04.07.84	T2H	05.08.84	De Kalb 54 + XL45	"
T3	01.05.84	T3H	12.06.84	PX 442 +	
			05.08.84	XL45	"
T4	11.06.84	T4H	05.08.84	XL45	"
J2	11.06.84	J2H	11.07.84	Pioneer 3901	Levin
J1	20.04.84	-	-	Pioneer 3901	P.N., Flygers Line
		KH	17.08.84	De Kalb 54	" "
		RH	12.08.84	Pioneer 3709	Bunnythorpe

b) Stored samples.

Sample code		Date of sampling	Breed of maize	Location
Silo A:	1	10.08.84	De Kalb 54 XL45 and PX 442	Kairanga, ex fields T2, T3, T4
	2	03.09.84		
	3	10.10.84		
Silo B:	1	10.09.84	Pioneer 3901	Levin, ex field J2
	2	17.11.84		
	3	29.11.84		
Silo C:	1	30.09.85	Not known	Poultry Research Centre, Massey University
	2	02.12.85		
	3	24.01.86		
Silo D:	1	17.10.85	Not known	Sanson
	2	16.11.85		
	3	27.12.85		

Figure 2-1: Sources of field, harvest and stored maize samples collected in the Manawatu District. Location of samples collected is coded KH, RH etc.; number in parentheses indicate number of samples.



c) Three samples from Silo C from the Poultry Research Centre, Massey University, were collected between 30.9.85 and 24.1.86.

d) Three samples from Silo D in Sanson County were collected between 17.10.85 and 27.12.85.

2.1.2.2. Soil, husk and litter samples

These materials were collected from fields J1, J2, T3 and T4 at the time of collection of field maize samples.

2.1.3. Sampling Technique

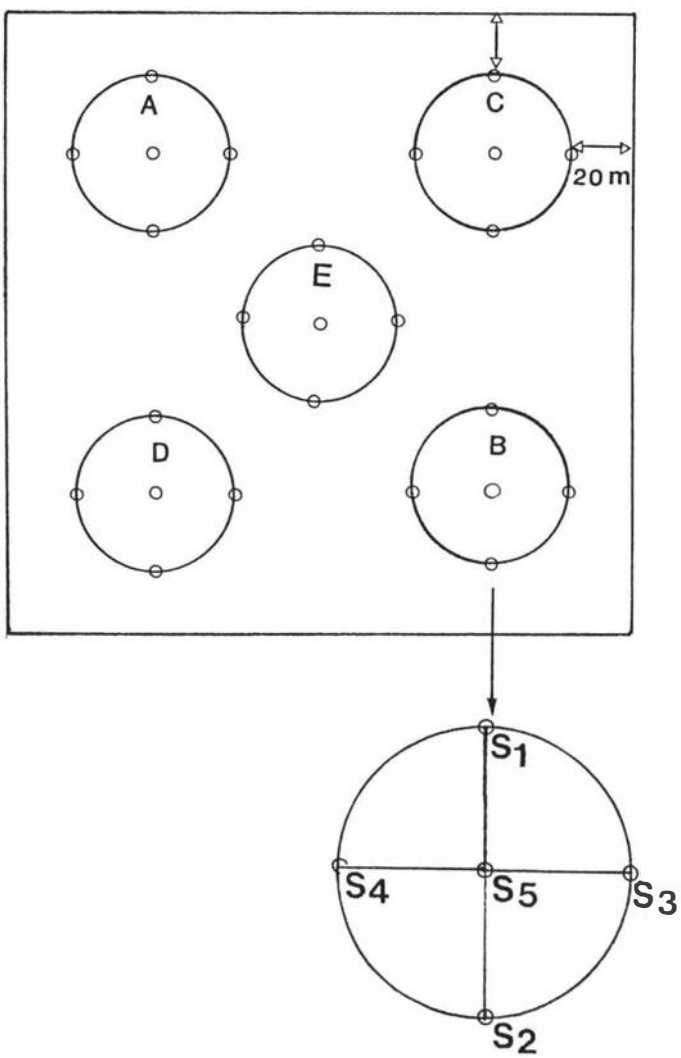
2.1.3.1. Field samples

Samples were collected between 1-8 weeks before harvesting. Each field was divided arbitrarily into five circular plots approximately 75-100 m in diameter and 20 m into the crop from the margin of the field. These plots were coded A, B, C, D and E (Figure 2-2). In each plot four sampling sites (S1-4) were selected at the corners of each quadrant (i.e. on radial transects). A fifth sampling site (S5) was established in the centre of the plot.

The following samples were collected from within an area of 2 m^2 at each sampling site:-

A. Four maize cobs complete with husks (each one from a separate plant);
B. 2 to 3 g of litter (dead maize leaves, dead grass leaves etc. in contact with the ground);
   Disposable plastic gloves were used to avoid cross-contamination. The maize and litter samples were collected into sterile heavy-duty polythene bags.
C. Approximately 30 g soil to a depth of 10-12 cm, using a soil sampler (a stainless steel tube 20 cm long and 3 cm internal diameter). The samples were placed in brown paper bags. The soil sampler was washed and disinfected with 70% alcohol between each sampling.

Figure 2-2: Field sampling method used for maize, husks, litter and soil, showing the relative positions of sampling plots A, B, C, D and E and sampling sites S1, S2, S3, S4 and S5



Distribution of plot samples within the field

Distribution of site samples within the plot

The samples from the five sites were pooled (Figure 2-3) to form plot samples (composite samples) for each sample type - i.e. 20 cobs, about 10-15 g of litter and about 150 g soil.

D. Husks were detached from all the 100 cobs before leaving the field and placed in sterile polythene bags. After mixing well about 50 whole husk leaves were randomly chosen and stored in fresh bags.

Following preliminary studies (see Section 3.1.1), the plot samples were pooled together to form composite field samples (Figure 2-3) which consisted of 100 cobs, approx. 750 g of soil and 50-75 g litter. Upon return to the laboratory (within 2 h of collection), all the maize cobs were shelled aseptically by hand to obtain between 4-5 kg kernels. Husks as well as litter were cut into small pieces approx. 3 x 5 mm with sterile scissors. All samples were stored in a cold room at 0-4^{o}C until processing (maximum storage period two months).

Field samples of maize, husk, litter and soil from four fields (J1, J2, T3 and T4) were subjected to full mycological assay and comparison, while only maize samples from fields T1 and T2 were examined.

2.1.3.2. Samples of maize at harvest

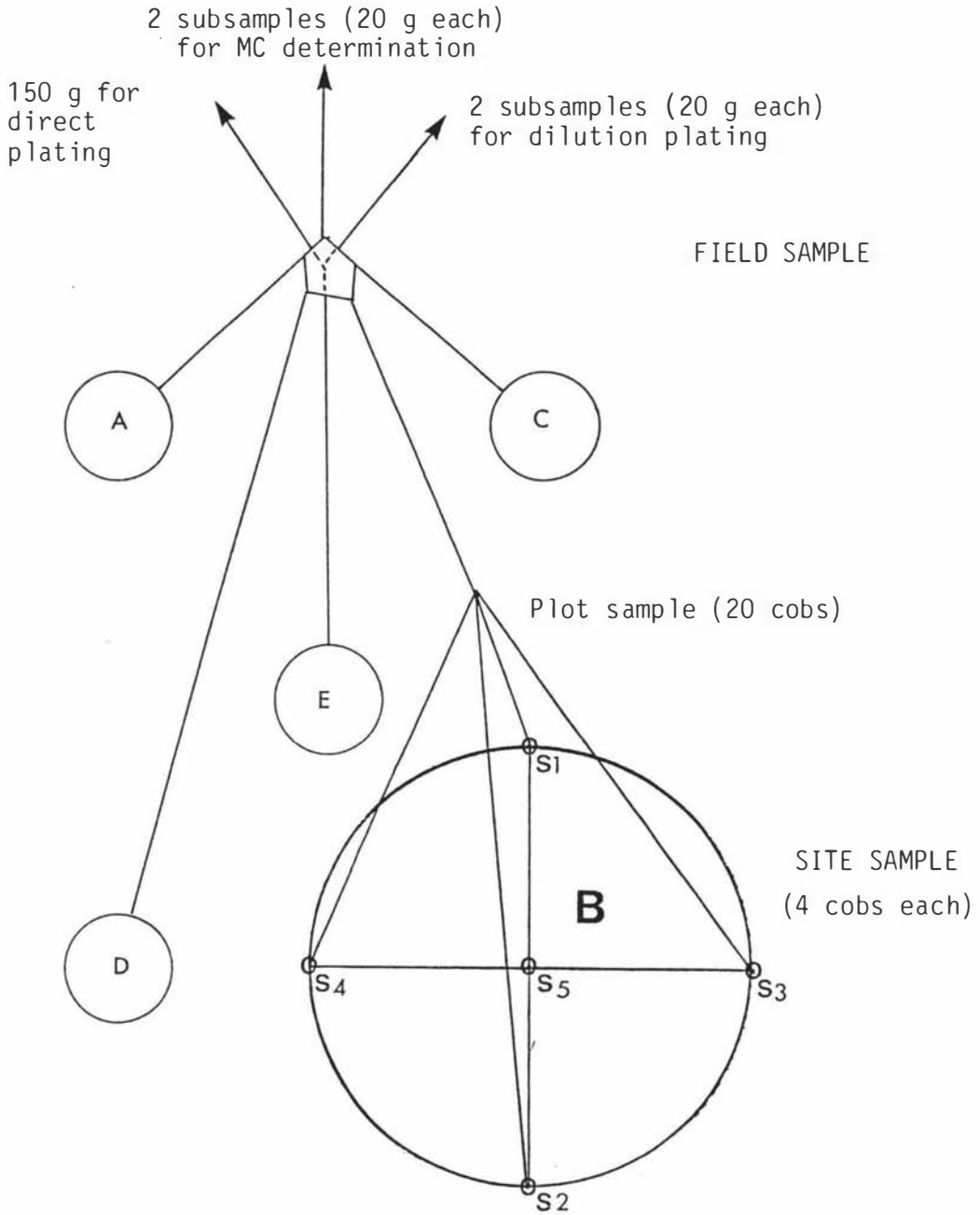
Sampling of maize was performed either during the loading of a truck from the combine harvester or during unloading into the silo. A 2000 ml plastic jug was used to cut the grain-stream at periodic intervals until a total sample of 5 kg/field was collected.

2.1.3.3. Samples from stored maize

Approximately 3-5 kg of maize were collected from silos by using one of the following methods according to the structure of the silo:-

A. From the top of the silo, made up as a composite of 5 portion samples, each portion being drawn from approx. 1 m^{2} x 30 cm depth of the maize bulk after thorough mixing.

Figure 2-3: Scheme for pooling of site and plot samples of maize to the field sample and subsequent subsampling



B. By using a grain probe, the sample being collected from both top and side doors at different depths and then bulked to form a composite sample.
C. From the grain stream during the unloading of the silo.

Details of sampling intervals are included in Table 2-1.

2.1.4. Sub-sampling Technique

2.1.4.1. Maize

A small tumbler mixer of variable speed (Design Electronics Ltd., Palmerston North, New Zealand) and with a PVC barrel was used for the sub-sampling procedure. To investigate the effect of number of rotations (no. of rolls/min) on the release of fungi, a preliminary investigation was made as follows on maize samples derived from fields J2 and T1.

The polythene bag containing the whole original sample (approx. 4-5 kg) was placed in the mixing barrel. Three sub-samples were taken at zero time (i.e. with no shaking), each one of about 20 g, by hand but wearing disposable gloves. Each sub-sample was drawn independently from three different areas of the composite sample. The bag was filled with air, tied firmly and the mixer spun for 1 min (35 rotations) after which a further three sub-samples were withdrawn (20 g each). The above procedure was repeated at 2 min (70 rotations), 3 min (105 rotations) and 4 min (140 rotations).

The preliminary investigation of the above sampling method indicated that 70 rotations gave the maximum release of propagules, so all further composite samples were sub-sampled after two minutes' rolling (see Section 3.1.1).

Using the above technique the following sub-samples were drawn routinely:-

A. Two sub-samples (about 20 g) for moisture content measurement
B. Two sub-samples (about 20 g) for examination by the dilution-plating technique

C. One sub-sample (about 150 g) for the direct plating technique.

Sub-samples were placed in small sterile polythene bags and kept in a cold room at 0-4^{o}C before processing.

2.1.4.2. Husk and litter

Samples were sub-sampled by filling the polythene bags with air, shaking vigorously by hand several times in different directions, then withdrawing two sub-samples (about 5 g each) with sterile forceps. Sub-samples were stored in a cold room.

2.1.4.3. Soil samples

Large soil particles were crushed by hand and the soil mixed well. Approximately 50 g were then withdrawn. This sample was dried in an incubator for about 12 h at 38^{o}C following which it was mixed thoroughly and then two sub-samples of 10 g each were withdrawn with a spatula, placed in individual universal bottles and stored in the cold room.

2.1.5. Processing of Samples

2.1.5.1. Moisture content (MC) determination (for maize only)

This was tested according to I.S.T.A. Rules and Annexes 1976, Seed Science and Technology, using tin dishes with foil lids. The test was carried out in duplicate on two independently drawn samples, each of which was about 20 g. All field and harvest samples, (MC over 20%) were pre-dried for 3-5 h at 70^{o}C. The maize was then ground into final form.

About 10 g of sample was ground in a coffee grinder and dried at 130^{o} $\pm$ 3^{o}C for 4 h, followed by a cooling period of about 30 min in a desiccator containing silica gel. The MC was calculated as a percentage of the weight loss using the following formulae:

1. $$m_2 - m_3 \times \frac{100}{m_2 - m_1}$$

where m_1 = the weight of the dish and its cover
m_2 = the weight of the dish, maize and cover before drying
m_3 = the weight of the dish, maize and cover after drying.

If the material was pre-dried, the MC was calculated from the results obtained in the first and second stages as follows:

$$2.\quad S_1 + S_2 - \frac{S_1 \times S_2}{100}$$

where S_1 = moisture lost in the first stage
S_2 = moisture lost in the second stage.

2.1.5.2. Assaying of fungal flora

Investigations were carried out on two media, PDA-D agar for general purposes and PCNB agar selective medium for fusaria. All field and harvest samples were processed by both dilution plating and direct plating except for soil samples, which were examined by dilution plating only. Samples of stored maize were examined by direct plating only.

A. Isolation and counting techniques

i) Dilution plating

a) Maize samples: All field sub-samples were dried for about 20 h, and harvest samples for about 8 h, at 38°C in an incubator to reduce the MC below 17% to facilitate grinding. The whole sub-sample (about 20 g) was then ground finely in a coffee grinder and 10 g weighed out accurately and added to 100 ml of sterile soft agar (0.08% agar (Oxoid No. 3) in water) in the sterile stainless steel jug of a Waring blender (Dynamics Corporation of America - New Hartford, U.S.A). The suspension was blended for 5 min at a speed of 17,000 rpm, and 10 ml of the homogenised sample transferred to 150 ml bottles containing 90 ml of soft agar. The bottle was shaken vigorously for about 30 sec. Two or three further ten-fold dilutions were prepared. Samples of 1 ml of each of the final three dilutions were pipetted in duplicate into sterile disposable petri dishes to which PDA-D or PCNB medium was then added (i.e. a total of 12 plates/sub-

sample for both media). About 12-15 ml molten agar medium at 45°C was poured into each petri dish and the inoculum mixed well with the medium.

b) Husk and litter samples: Each 5 g sub-sample was thoroughly mixed and 1 g removed to a 200 ml screw-cap bottle. Sterile distilled water (sdw, 100 ml) was added, and the preparation shaken for 15 min at 250 rpm on a shaker (New Brunswick Scientific). The supernatant was decanted immediately and serial dilutions (up to 10^{-6})in sdw prepared. Cultures on PDA-D and PCNB were established as for the maize samples. The husk and litter segments were reserved for the direct plating technique.

c) Soil samples: The two sub-samples of 10 g each were treated similarly to the ground maize samples, except that the soil dilution factors were higher than those for the maize (to 10^{-5}), and the last three dilutions used for plating.

All plates were incubated at 25°C in the dark. Colonies were counted after 3, 5 and 7 days of incubation.

Slow-growing and unidentified colonies and all Fusarium colonies were subcultured onto PDA and other media for further examination. If more than 30 colonies of any particular morphological type were isolated on the dilution plates, only a random selection of about 20 colonies was subcultured.

ii) Direct plating technique

a) Maize kernels: Maize kernels (about 100 g) from the sub-sample (Section 2.1.4.1) were placed in a 400 ml screw-cap bottle. The kernels were disinfected by handshaking with 1% sodium hypochlorite (NaOCl) for 1 min. After discarding the NaOCl, the kernels were rinsed with sdw three times, each time being shaken for about 20 sec. After drying on a sterile filter paper, 50 kernels were cultured on PDA-D medium, and another 50 kernels were cultured on PCNB medium (preferably 5 days old) at a density of 5 kernels per plate. All plates were incubated in the dark at 25°C. The plates were first examined after 5 days' incubation and slow-growing colonies

subcultured. They were re-examined after 10 days. All Fusarium spp and unidentified fungal genera were subcultured onto identification media.

b) Husks and litter segments: The segments which were reserved from the dilution plating procedure were handshaken with 1% NaOCl continuously for 1 min. After the NaOCl was discarded the segments were washed three times with sdw, each time being shaken for about 20 sec. The segments were then dried on sterile filter paper. A total of 30 segments per sub-sample was plated out on both PDA-D and PCNB (15 segments each) at a density of 5 segments/plate. Both incubation conditions and examination of the plates was as for kernels.

B. Identification and classification

i) Fusarium spp. growing on plates from both techniques were identified after culturing on PSA, PDA and CLA media.

The single spore technique (Booth, 1977) was applied when necessary, particularly for those isolates to be later used for mycotoxin screening. The cultures were incubated in the dark for 3-4 days at 25^{o}C and growth rate (colony diameter) measured. The plates were re-incubated under illumination (two 40 w cool white fluorescent tubes and one 40 w black light tube, 360-370 nm [Philips TL 40/08], positioned 40 cm above the cultures) with a 12 h photoperiod, and alternating temperature of 25^{o}C day/20^{o}C night. The cultures were examined after 10-14 days and again after 4 weeks to check for perithecia and chlamydospore production. The cultures on PDA and PSA were used to study colony morphology i.e. growth rate, pigmentation, sporodochia production and other variables but identifications were largely based on morphology of conidia, conidiophore, type of sporing head and chlamydospore production etc. on CLA medium. The above characters were studied macroscopically, with a stereo-microscope and by preparing wet-mounts in sdw for light microscopy. All Fusarium spp were identified using the criteria of Burgess and Liddell (1983), Gerlach and Nirenberg (1982) and Booth (1971). Other available references were also used as necessary.

ii) Other fungal genera were identified into genus only except for some aspergilli. Most colonies were identified on the isolation media but colonies with uncertain affinity were transferred to other appropriate media such as PDA, CDA and CLA. Genera with poor sporulation were cultured on CLA and incubated under black light.

C. Viable counts

i) Dilution plating: Colonies were counted on both media after 3, 5 and 7 days' incubation at 25^{o}C. Plates carrying between 10-100 colonies were used. The mean of colony numbers from two plates of the same dilution on the same medium were expressed as colony-forming units per gram sample (CFU/g). In the case of Fusarium spp, colonies growing on all 6 plates for each medium were counted and the mean of these counts were used to obtain the Fusarium viable count as CFU/g.

ii) Direct plating: The number of maize kernels or segments of litter or husk that yielded Fusarium spp or other fungal genera were counted after 5 and 10 days' incubation in the dark at 25^{o}C. The results were expressed as a percentage of contaminated kernels or segments of the total yielding fungi.

D. Statistical analysis

The data were subjected to a one-way analysis of variance (Steel and Torrie, 1980) to test for significant differences between the means of a) the fungal populations in field plot samples, b) the fungal populations and numbers of fungal genera and Fusarium spp isolated on different media or by different techniques, and c) the numbers of fungal genera and Fusarium spp isolated from field and harvest samples. Duncan's test was used to assess plot differences in Tables 3-1 to 3-4.

2.1.5.3. Preservation of fungal cultures

A. Fusarium isolates

Two methods were used for maintaining the Fusarium cultures:-

i) CLA slants: The CLA slant was recommended by Fisher et al. (1982) for short-term storage. In the present studies, Fusarium

cultures stored on CLA slants were found to remain viable for over two years without mutation. The cultures were stored at room temperature.

ii) Lyophilisation: Isolates of Fusarium spp for lyophilisation were grown on CLA plates for 7-10 days under a 12 h alternating cycle under the light bank at 20°C night/24°C day. The cultures were checked for adequate growth and purity. Five vials for each isolate were prepared for lyophilisation. Sterile skim-milk 10% (0.2 ml) (skim milk powder - Anchor New Zealand Co-operative Dairy Co. Ltd., Hamilton) was introduced into each sterile freeze-dry vial labelled with the isolate number. Two colonised carnation leaf pieces were transferred to the vials under sterile conditions. The vials were then stoppered with a sterile cotton plug and held in stainless steel bowls containing either crushed dry ice or liquid N_2 and propan-2-ol (isopropyl alcohol) for 30 min. Lyophilisation was performed using standard procedures. The lyophilised samples were randomly checked for viability immediately and after each 6 months. Cultures were stored at -20°C.

B. Other fungal genera

These isolates were maintained on both PDA and CLA slants and subcultured every 6-8 months.

Representatives of all Fusarium species and other fungal genera were deposited at the herbarium and culture collection of the Plant Diseases Division, D.S.I.R., Auckland (Appendix A). The author specifically acknowledges the help of Dr G. Samuels in the identification of some of the isolates as well as confirming all the others, and for his general guidance in Fusarium taxonomy.

Representatives of the isolates of Fusarium spp were also deposited with the American Type Culture Collection (ATCC), U.S.A. (Appendix B).

2.2. TECHNIQUES FOR MYCOTOXIN EXTRACTION AND ANALYSIS

2.2.1. Source of Samples

Twenty samples of maize and four samples of poultry rations were assayed for the presence of mycotoxins:

A. A total of 18 maize samples destined for use in animal rations, including field, harvest and stored samples, collected as described in Section 2.1.2 (Table 2-1)

B. Two maize samples from a commercial supplier producing human foodstuffs.

C. Four compounded poultry ration samples provided by a large chicken production unit.

Also:

D. A total of 40 isolates of Fusarium spp. (Table 2-2) which had been obtained from maize, husk, litter and soil were tested for their ability to produce mycotoxins in culture.

Table 2-2: Fusarium isolates tested for their potential toxigenicity.

Fusarium spp.	No. tested
graminearum	11
subglutinans	15
moniliforme	1
equiseti	2
avenaceum	2
crookwellense	1
acuminatum	1
stilboides	1
culmorum	2
oxysporum	2
merismoides	1
sambucinum	1

2.2.2. Reagents

2.2.2.1. Mycotoxin reference standards

A. Original stocks

Zearalenone (ZEA) and T-2 toxin were obtained from Sigma Chemical Company, St Louis, MO, U.S.A., Diactoxyscirpenol (DAS) and Deoxynivalenol (DON) were obtained from Myco Lab. Company, Chesterfield, MO, U.S.A. Moniliformin was generously provided by Dr P.G. Thiel, National Research Institute for Nutritional Disease, Tygerberg 7505, South Africa and Dr. J.L. Richard, National Animal Disease Center, Ames, Iowa, U.S.A.

B. Standard mycotoxin mixtures

i) The standard mycotoxin mixture for TLC (referred to as MTM1) consisted of DON (15 µg/ml) and T-2 toxin, DAS, ZEA and MON (each 30 µg/ml in chloroform/methanol (1:1).

ii) The standard mycotoxin for GC and GC-MS (MTM2) consisted of DON (40 µg/ml) and DAS, T-2 toxin and ZEA (each 80 µg/ml) in chloroform/methanol (1:1).

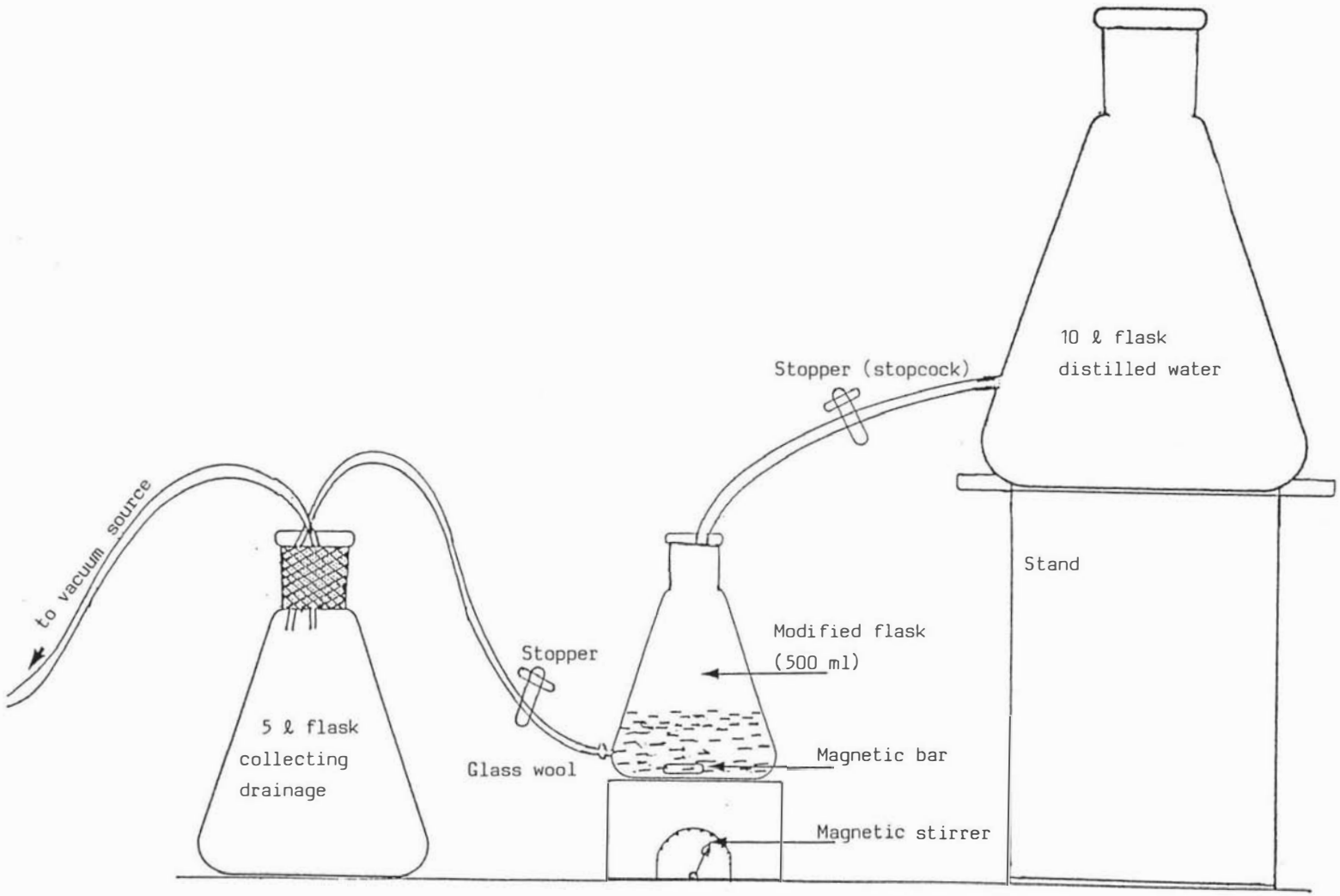
All mycotoxin stocks were stored in a -18^{o}C freezer. When required for analyses they were thawed to room temperature.

2.2.2.2. Column chromatography materials (for clean-up of samples)

A. Amberlite XAD-4

Amberlite XAD-4 (Rohm and Haas Co., Philadelphia, PA 19105, U.S.A.) was generously provided by Rohm and Haas New Zealand Ltd., Otahuhu, Auckland. Conditioning of the Amberlite ion exchange resin was performed as described by Naoi (1983) with slight modification. About 250 g of the resin was placed in a 500 ml modified Erlenmeyer flask, with a magnetic bar for stirring. The flask had a side arm fitted and hose attached for drainage, as shown in Figure 2-4. Distilled water for flushing was added while stirring. After draining the water sufficient 1N NaOH was added to cover the Amberlite beads and

Figure 2-4: Scheme showing the apparatus used for conditioning the Amberlite XAD-4 resin.



the resultant mixture was stirred for 1 h. Then the NaOH was drained off and another similar amount of 1N NaOH was added, stirred for another hour, then drained. The NaOH and the XAD-4 were then flushed continuously with distilled water whilst stirring, and continuously drained until the effluent water was at neutral pH (approx. 10-15 ℓ rinsing water required). Sufficient methanol to cover the Amberlite was then added and the mixture stirred for 5 min before the methanol layer was drained off. This was followed by three applications of hot methanol, with stirring at each application. Finally, sufficient water was flushed through the resin to remove the methanol. The prepared XAD-4 resin was then stored in distilled water at room temperature.

B. Florisil

Florisil (BDH Chemicals Ltd., Poole, England), about 100-200 US mesh, was activated by drying 1½ h at 105^{o}C and stored in a desiccator with silica gel adsorbent.

2.2.2.3. TLC reagents

A. TLC solvent systems (developing solvents)

All solvents were analytical reagent grade. Chloroform and methanol were redistilled prior to use. Toluene and acetone were stored over molecular sieves (4A) (Type 4A-4-8 mesh beads - Ajax Chemicals Ltd, Sydney, Australia) to remove water.

The following solvent systems were prepared fresh each day for use on that day:

Solvent #1. chloroform-methanol (93:7)
Solvent #2. Toluene-acetone-methanol (5:3:2)
Solvent #3. Toluene-acetone (97:3)
Solvent #4. Chloroform-methanol (3:2).

B. Spray reagents

1. Aluminium chloride solution:-
   20 g reagent grade $AlCl_3.6H_2O$ was dissolved in 100 ml 50% ethanol-water.
2. Sulphuric acid solution:-
   H_2SO_4 (20% v/v in water).
3. 2,4-Dinitrophenylhydrazine (2,4 DNPH) solution:-
   0.32 g reagent grade 2,4-dinitrophenylhydrazine $(NO_2)_2.C_6H_3.NH\text{-}NH_2$ (Ajax Chemicals Ltd., Sydney, Australia) was dissolved in 100 ml 2N HCl. The same reagent was used for derivatisation of moniliformin (Section 2-2-6-5).
4. Diazonium salt reagent:-
   Fast violet B salt (Sigma Chemical Company, St Louis, MO, U.S.A.) as 0.7% solution in distilled water.

2.2.2.4. Reagents for gas chromatography (GC and GC-MS)

A. Derivatising reagents

Two trimethylsilyl (TMS) derivatising reagent mixtures were employed:
Derivatising Reagent [a] (N-Trimethylsilyl-imidazole) [TMSI], research grade (Serva, Feirbiochemica, Heidelberg, New York) + Trimethylchlorosilane [TMCS] (dispersed into 1 ml ampoules under nitrogen) + Ethyl acetate (glass distilled and stored over molecular sieve). These 3 reagents were mixed under oxygen-free nitrogen in the ratio 1:0.2:9 in 20 ml bottles for use on the same day, or dispersed in 1 ml ampoules and sealed under nitrogen for storage at 0-4^{o}C until used within 2 weeks.

Derivatising reagent [b] (a commercial preparation): TRI-SIL TBT (1 ml ampoules) - this is a special formulation of TMS-imidazole (TMSI), Bis-TMS-acetamide (BSA) and trimethyl chlorosilane, 3:3:2 (v/v) (Pierce Chemical Company, Rockford, IL 61105, U.S.A.)

B. Internal standard

C_{24}, C_{26}, C_{28}, C_{30} n-alkanes mixed in hexane each at a concentration of 50 mg/5 ml (Alltech Association, Takapuna, Auckland, New Zealand).

C. Phosphorus pentoxide

P_2O_5 (E-Merk Darmstadt, F.R. Germany) for absorbing moisture before derivatisation.

2.2.3. Apparatus

2.2.3.1. Gas chromatography

A. For GC-FID, a Pye Unicam series 304 gas chromatograph (Philips Ltd., Cambridge, England) was used. This was equipped with flame ionization detector (GC-FID) and a Pye Unicam CPP4 computing integrator. The instrument was located at the New Zealand Dairy Research Institute, Palmerston North.

Two columns were employed:

1. 12 m x 0.25 mm i.d. vitreous silica capillary column with bonded phase BP1 (dimethyl siloxane equivalent, non-porous bonded phase) (Scientific Glass Engineering Pty Ltd, Ringwood, Victoria 3134, Australia).

2. 50 m x 0.25 mm i.d., otherwise identical to column #1.

Operating conditions: Gas carrier: Helium (He). Linear gas velocity at 150°C = 55 cm/sec, chart speed 30 cm/h. The temperature was programmed starting at 180°C for 5 min isothermal, then rising to 220°C at a rate of 5°C/min, followed by a rise to 280°C at a rate of either 3°C/min for the short column or 8°C/min for the long column. The On-column injector temperature was 60°C and the detector temperature 320°C. The attenuation of the electrometer was initially set at 64, then decreased to 32 or 16 with some samples, depending on the observed peak height and signal-to-noise ratios.

B. For GC-MS a Hewlett Packard (model 5985 B) system was used coupled to a HP 1000 data system (Hewlett-Packard Co., Palo Alto, CA, U.S.A.). The instrument was located at the Forest Research Institute, Rotorua.

Electron Impact (EI) mode ionization was used, with ion source temperature 200°C and electron energy 70 eV. The instrument was fitted with a BPI bonded phase vitreous silica capillary column (12 m x 0.25 mm i.d.) interfaced to the mass spectrometer by an open-split interface heated at 250°C. The column temperature was programmed from 180°C to 250°C, with an initial hold of 5 min at 180°C and final hold of 10 min at 250°C. The first programme rate was 8°C/min until 220°C, then 3°C/min until 250°C. The injector temperature was 65°C (on-column system). The carrier gas was helium at a mean linear velocity of 50 cm/sec.

The mass spectrometer was programmed to monitor 5 characteristic ions each for DON, DAS, T-2 toxin and ZEA (Table 2-3). The scanning for Group 1 (DON and DAS) proceeded from 9 min after injection until 16 min, then scanning for Group 2 (T-2 toxin and ZEA) proceeded from 16 min until 25 min. The chromatograms were obtained as hard-copy from the computer system.

Table 2-3: Mass-to-charge ratios (M/Z) of ions used for selective ion monitoring during GC-MS.

Group 1		Group 2	
DON:	512	T-2 toxin:	436
	422		350
	407		290
	259		173
	235		122
DAS:	378	ZEA:	462
	350		444
	290		429
	175		333
	106		151

2.2.3.2. Thin-layer chromatography (TLC)

A. TLC plates:

1. 20 x 20 cm aluminium sheets, pre-coated with 0.2 mm silica gel 60 without fluorescent indicator (Art-5553 from E. Merck, Darmstadt, F. R. Germany).
2. 20 x 20 cm aluminium sheets pre-coated with 0.2 mm silica gel 60 with fluorescent indicator (Art-5554 from E. Merck, Darmstadt, F. R. Germany).

B. Viewer

Chromato-Vue cabinet Model CC-60, ultraviolet illumination system (UUVP Inc., San Gabriel, CA, U.S.A.). The unit has two U-V tubes 254 and 365 nm wavelength.

C. TLC densitometer

A Shimadzu model CS-90 dual wavelength TLC scanner (Shimadzu Ltd., Kyoto, Japan) was used with the following operating conditions:-

Slit width 0.3 x 10 mm; linear scanning mode; scan speed 10 mm/min; wavelength: reference 700 nm, sample 480 nm. For confirmation, 3 wavelengths, 400, 480 and 540 nm were used.

2.2.3.3. U.V. spectrophotometry

SP8-400 UV/Vis spectrophotometer (Pye Unicam Ltd., Cambridge, England). Operating conditions were scan 300 nm to 200 nm, record range 2-A (2 absorbance units full scale), band width (nm scan speed) 4 nm/s.

2.2.3.4. Glassware

All glassware was soaked in 1% NaOCl for about 3 h then rinsed several times with distilled water, soaked in chromic acid overnight, then rinsed again several times with tap water, several times with deionized water and finally oven-dried before use.

2.2.4. Mycotoxin Production under Laboratory Conditions

Quantities of 100 g of maize, free of mycotoxins, were placed in 75 ml distilled water in 500 ml Erlenmeyer flasks stoppered with cotton wool plugs. The flasks were left with occasional shaking for 1 h at room temperature and then stoppered with foil and autoclaved twice at 121°C for 45 min on two consecutive days. One ml amounts of fungal inoculum (consisting of spores and hyphal segments in sdw) from a 7-day old culture on CLA was used. The cultures had been incubated under a black light bank with a 12 h photoperiod to stimulate sporulation. After inoculation, the maize in the flask was shaken several times to distribute the inoculum. The flasks were then incubated at 25°C for four weeks, with the exception of F. subglutinans which was incubated for three weeks only. After the incubation period, cultures were dried in the oven at 50°C for 24 h, then ground to a semifine meal with a Waring blender and stored at 0-4°C until use.

2.2.5. Extraction of Samples and Cultures

Extraction and clean-up steps were based on the method of Kamimura et al. (1981) with minor modifications.

Maize sample (100 g) or maize culture (100 g) was finely ground in a coffee grinder or Waring blender, then transferred to a 500-ml Quickfit glass-stoppered flask; 200 ml aqueous methanol (95 methanol:5 water) were added and the flask shaken for 1 h, using a wrist-action shaker at 800 shakes/min. The contents of the flask were then filtered through Whatman No. 1 filter paper. The approximately 100 ml of filtrate obtained was transferred to a 300-ml round-bottomed flask and evaporated to about 10 ml under reduced pressure at 50°C in a Buchi rotary evaporator.

2.2.5.1. Column chromatographic fractionation and clean-up

The Amberlite XAD-4 column was prepared as described by Kamimura et al. (1981).

The slurry of Amberlite XAD-4 resin was placed into a glass chromatography column 20 cm x 1.4 cm id, plugged with glass wool,

until the height of the settled column was about 10 cm. The sample extract was added gently to the top of the column. The flask was washed with distilled water followed by small portions of methanol totalling 5 ml. A first fraction (F1), containing any moniliformin, was obtained by eluting the column with water into a 300-ml round-bottomed flask at a rate of 1.5 ml/min for the first 30 ml and then 3 ml/min for a further 50 ml and the two eluates combined. (Preliminary investigation had indicated that part of the moniliformin content was eluted with the first 30 ml water and the rest with the remaining 50 ml water.)

DON, DAS, T-2 toxin and ZEA were then eluted with 100 ml methanol at a rate of 6 ml/min. The eluate was collected in a 300-ml round-bottomed flask to form fraction 2 (F2).

2.2.5.2. Clean-up of F1

Ethanol (20 ml) was added to the eluate and the mixture then evaporated to dryness under reduced pressure on a water bath at 70^{o}C. The residue was dissolved in 5 ml methanol, then mixed with 15 ml acetone.

The resulting solution was filtered through Whatman No. 1 filter paper into a 300-ml round-bottomed flask. The filtrate was evaporated to dryness under reduced pressure on a water bath (50^{o}C). The residue was then dissolved in 1 ml methanol and stored at -18^{o}C until required for assay of moniliformin.

2.2.5.3. Florisil chromatographic column for clean-up of F2

The florisil column was prepared as described by Kamimura *et al.* (1981). A glass column (20 cm x 1.5 cm i.d.) plugged with glass wool was used. About 10 ml chloroform and 5 g anhydrous sodium sulphate (Na_2SO_4) were added to the column, followed by a slurry of 10 g florisil in chloroform/methanol (9:1). When the gel settled it was topped with about 5g Na_2SO_4. The solvent was then drained to the top of the Na_2SO_4.

F2 was evaporated to dryness under reduced pressure on a water bath at 50°C. The residue was dissolved in 10 ml chloroform/methanol (9:1) then transferred onto the column. Mycotoxin was eluted into a 300-ml round-bottomed flask with 100 ml chloroform/methanol (9:1) at a rate of 2 ml/min. After collection, the eluate was evaporated to about 10 ml and then transferred to a 30-ml pear-shaped flask and evaporated to dryness. The residue was dissolved in 1 ml methanol and then stored at -18°C until required. It was used to screen for DON, DAS, T-2 toxin and ZEA.

2.2.6. Screening Methods

2.2.6.1. Thin-layer chromatography

The TLC plate was first developed in either solvent #1, chloroform-methanol, or solvent #2, toluene-acetone-methanol to wash out any impurity. Then it was reconditioned in an oven at 110°C for 90 min.

A light pencil line (spotting line) was marked 3 cm from the bottom edge of the plate. 20 µl aliquots of F1 and F2 and 20 µl mycotoxin reference (MTM1) were spotted at 1.5 cm intervals with micropipettes. After drying the spots with a hairdryer, the TLC plates were developed in unlined and unequilibriated tanks using the following solvent system:

A. TLC plates without fluorescent indicator were developed in solvent #1 until 18 cm from the bottom edge. After development the plate was dried for about 10 min and then observed under 365 and 254 nm U.V. light for fluorescent spots. ZEA appeared as a blue fluorescent spot particularly at 254 nm. The plate was sprayed with $AlCl_3$ solution (20% w/v) and heated at 110°C for 10 min, then observed again under 365 nm U.V. light. DON and ZEA showed bright blue fluorescent spots. The plate was then sprayed with H_2SO_4 solution (20% v/v in water), heated at 110°C for 10 min and observed under 365 nm U.V. light. The T-2 toxin and DAS exhibited blue-green fluorescent spots. The third spray reagent was 2,4 DNPH solution 0.32% in 2N HCl. After spraying, the plate was heated at 110°C for 10 min. MON appeared as a red-brown spot on the spotting line.

B. TLC plates with fluorescent indicator were developed in solvent #2 until the solvent front was 18 cm from the bottom edge. The plate was dried for about 10 min and then observed under 254 nm U.V. light. ZEA and MON appeared as absorption (dark) spots. When the plate was sprayed with 0.32% 2,4 DNPH solution and heated at 110^{o}C for 10 min, MON appeared as red-brown (brick red) spots.

2.2.6.2. Gas Chromatography

A. Derivatisation: 0.1 ml of mycotoxin reference (MTM2) and 0.2 ml of sample extract (F2) were placed in separate 1 ml reaction-vials fitted with teflon septum stoppers. The vials (opened) were put in a desiccator with phosphorus pentoxide to absorb moisture. The desiccator was then connected to a water vacuum pump for 3-4 h to evaporate the solvent, then to a high vacuum pump (Email Industries Ltd., New Zealand) for 4 h to remove residual solvent and water. The desiccator was then flushed with nitrogen, and the vials immediately sealed. The following amounts of TMS reagent were added through the septum: 100 µℓ for the mycotoxin reference vial, 200 µℓ for the sample extract vials. The vials were then left at room temperature for 30 min. Derivatisation was performed fresh daily.

B. Preparation of samples for injection into the GC system: Samples were normally diluted 50% with pyridine or with ethyl acetate before injecting onto the column. Derivatised samples or mycotoxin standards (0.4-0.6 µℓ) were co-injected with 0.2 µℓ internal standard mixture (C_{24}-C_{30}, 0.5 µg/µℓ).

C. Detection and quantitation of mycotoxins: Calculation of the concentration of mycotoxins was performed using peak areas calculated by an integrator appropriately programmed for the purpose (on some occasions peak height was used). The internal standard was used to monitor any variation in the retention time between assays. Extracts of all samples and twelve Fusarium cultures were assayed by the GC method.

2.2.6.3. Gas chromatography - mass spectrometry

TMS-derivatives of mycotoxin standards (MTM2) and of sample extracts were prepared fresh each day as for the GC method. Dilution of the sample extract was necessary for those with high mycotoxin concentration or if they contained materials which could interfere with the analysis, eg. carbohydrates, lipids etc.

One µl aliquots of derivatised MTM2 or sample extract were injected directly into the capillary column (on-column injector). The mass spectrometer was programmed to monitor the DON and DAS ions for retention times between 9 and 16 min, and the ions for T-2 toxin and ZEA were monitored between 16 and 25 min. The mass spectrum of each mycotoxin was analysed individually by choosing a narrow window time, eg:

DON between 9 and 13 min with five ions, DAS between 12 and 16 min, T-2 toxin between 18-22 min, and ZEA between 21-25 min. The ions' mass spectrum as well as the ion chromatograms were checked for the above toxins.

Quantitation of the derivatised mycotoxins in samples was performed by comparing their computer-integrated peak areas (mean of 5 ions) with those of the corresponding mycotoxin standard. Each mycotoxin had been analysed for intensities of selected ions as a function of retention time as well as the ratio between these ions. This information was used to identify the mycotoxin. Extracts of all samples and 25 Fusarium cultures were analysed and confirmed by the GC-MS method.

2.2.6.4. Quantitation of zearalenone

Because the ZEA-TMS derivative is unstable a TLC method was employed for quantitation. Each TLC plate (Section 2.2.3.2,A 1) was spotted at 1.5 cm intervals with 3 spots, (2, 5 and 10 µl respectively) of ZEA standard (40 µg ZEA per ml chloroform/methanol) and three spots (5, 5, and 15 µl respectively), of sample extract (F2). Then 5 µl of ZEA standard was applied to one of the 5 µl sample spots as internal standard for co-chromatography. The plate was developed as described in Section 2.2.6.1, A. After development the plate was left to dry

for 10 min at room temperature and then viewed under both short-wave U.V. (254 nm) and long-wave U.V. (365 nm). ZEA appeared as a blue fluorescent spot, more intense under short-wave. By visually matching the intensity of fluorescence of the sample spots with those of the ZEA standards, a semi-quantitative assay of the amount of ZEA was made. Any samples that showed more intense fluorescence than those of the mycotoxin standard spots were subjected to further dilution and rechromatographed. After visual assessment, the plate was sprayed with 20% $AlCl_3$ for confirmation as already described. Occasionally, further confirmation was obtained by spraying a second plate with fast violet B, as described by Scott et al. (1978).

2.2.6.5. Densitometric analysis for quantitation and confirmation of moniliformin

The method of Kamimura et al. (1981) was used with a slight modification:

Aliquots of 0.3 ml each of sample extract F1 and moniliformin standard were transferred to separate small screw-cap vials and evaporated to dryness under a stream of nitrogen. The residue was then dissolved in 2 ml 2,4 DNPH solution (Section 2.2.2.3, B). The vial was then sealed, mixed gently by swirling and placed in a 50°C water bath for 2 h to allow formation of the MON-2,4 DNPH derivatives. The derivatives were transferred into 100 ml separatory funnels with 10 ml water, and the vial rinsed with additional small portions of acetone and several portions of water to give a total volume in the separatory funnel of 20 ml.

The aqueous layer was extracted with 10 ml chloroform by shaking for 5 min. This was repeated with an additional 10 ml chloroform. Approximately 10 g of anhydrous sodium sulphate was added to the chloroform phase and this was stirred before filtration into a 30-ml pear-shaped flask. The filtrate was then evaporated to dryness under reduced pressure on a steam bath. The residue was dissolved in 0.5 ml acetone. A TLC plate with fluorescent indicator was spotted at 1.5 cm intervals with 10 and 20 µl of sample derivative and 5, 10 and 20 µl standard MON-derivative. The plate was then developed in solvent #3

(toluene-acetone 97:3) until the solvent front was 18 cm from the bottom edge.

After development, the plate was dried for 10 min at room temperature and then scanned on a TLC densitometer at 480 nm, for quantitation by estimating the peak area with the recorder speed set at 2.5 cm/min. A second scan was performed with the recorder speed at 0.5 cm/min for measuring the peak height. The plate was also scanned at 400 nm and 540 nm to confirm the identity of the MON peak, by comparing the ratios of peak heights at the different wavelengths for each sample spot with those of the standards at the same wavelengths.

MON-derivatives gave a brick-red colour on the TLC plate at approx. Rf 0.3. All F1 samples and Fusarium culture extracts were assayed and confirmed by this method.

2.2.6.6. U.V. spectroscopy for confirmation of MON

50 µℓ of sample extract (F1) and MON standard were spotted on a TLC plate with fluorescent indicator, developed in solvent #4 (chloroform-methanol 3:2) for 18 cm then dried and viewed under short-wave U.V. light (254 nm). MON was detected as a dark absorbent band which was then scraped off and eluted with chloroform-methanol (1:1, about 20 ml). The silica gel extract was then filtered into a 30 ml pear-shaped flask and evaporated to dryness, redissolved in 0.5 ml methanol and the solution diluted or concentrated as required. The absorption spectrum was obtained by scanning between 300 and 200 nm on a recording spectrophotometer, as described in Section 2.2.3.3.

2.2.7 Safety Measures

Because mycotoxins are extremely dangerous substances, all samples and Fusarium cultures were handled with extreme care, particularly when handling the pure mycotoxins. In all cases a face mask with filter, and disposable gloves, were worn. All processing was carried out in a laminar flow cabinet. Any spill of mycotoxin-containing materials was treated with 5% sodium hypochlorite. This solution was also used for glassware detoxification (see Section 2.2.3.4).

CHAPTER 3
RESULTS

3.1. THE MYCOFLORA OF MAIZE AND ASSOCIATED SUBSTRATES FROM FIELD TO STORAGE

3.1.1. Preliminary Investigations

3.1.1.1. Field sampling techniques

This investigation included a comparison of the total fungal propagules, and of the Fusarium propagules in particular, from the five individual plot samples of maize, husk, litter and soil collected as described in Section 2.1.3.1 from fields J1 and T3. Samples were analysed by the dilution-plating technique, using the two media PDA-D and PCNB agar (Section 2.1.1). Fungal viable counts from the maize samples were expressed as the mean of eight plate replications and those from husk, litter and soil samples were calculated as the mean of four plate replications.

A summary of the total fungal viable counts (CFU/g) for the four sample types from field J1 are presented in Table 3.1. There was a significant difference between the fungal viable counts for maize from the five separate plot samples ($P<0.01$), and for husks and litters ($P<0.001$), but no significant difference was found between the soil samples ($P>0.05$).

A comparison between the total fungal viable counts recovered from the four substrates as a mean of the five plot samples indicated that litter gave the highest counts of 3.7×10^7 CFU/g, although the counts of husks (1.7×10^7 CFU/g) were close to those of litter. The maize kernels consistently gave the lowest counts for all five plot samples (mean 4.7×10^3 CFU/g).

The propagules of Fusarium spp recorded from the five plot samples are listed in Table 3.2. Counts of fusaria showed significant differences in the five separate plot samples for maize ($P<0.01$) and litter ($P<0.05$), but no significant differences were noted for husk and soil samples ($P>0.05$).

Table 3-1: Total fungal viable counts (CFU/g) of maize, husk, litter and soil from the five plot samples in field J1.

Plot sample	Total viable counts (CFU/g)*			
	Maize	Husk	Litter	Soil
A	8.6×10^{3a}	1.1×10^{7a}	8.0×10^{7a}	1.0×10^{4a}
B	8.4×10^{3a}	5.8×10^{6b}	3.7×10^{6b}	2.0×10^{4a}
C	3.1×10^{3b}	6.8×10^{6c}	3.0×10^{7c}	1.6×10^{4a}
D	2.2×10^{3c}	2.6×10^{7d}	4.0×10^{7d}	1.6×10^{4a}
E	1.2×10^{3d}	3.4×10^{7c}	2.5×10^{7c}	1.8×10^{4a}
Mean	4.7×10^{3}	1.7×10^{7}	3.7×10^{7}	1.6×10^{4}

* Counts from maize as mean of eight plate replications from the two media (PDA-D and PCNB); from the other substrates as mean of four plate replications from the two media.

a,b,c,d overall means in the same column with no common superscripts differ. For maize $P<0.01$ and for husk and litter $P<0.001$.

Table 3-2: Viable counts of Fusarium spp (CFU/g) of maize, husk, litter and soil from the five plot samples in field J1.

Plot sample	Fusarium viable counts (CFU/g)			
	Maize	Husk	Litter	Soil
A	12.5^{a}	1.3×10^{5a}	6.5×10^{6a}	3.2×10^{3a}
B	4.9×10^{2a}	1.5×10^{5a}	6.0×10^{5b}	1.8×10^{3a}
C	$o.0^{b}$	7.5×10^{5a}	2.6×10^{6bc}	1.2×10^{3a}
D	1.7×10^{2a}	7.5×10^{4a}	1.4×10^{6bd}	3.2×10^{3a}
E	20.0^{a}	4.8×10^{5a}	1.3×10^{5be}	2.5×10^{3a}
Mean	1.4×10^{2}	3.2×10^{5}	2.3×10^{6}	2.3×10^{3}

a,b,c,d,e overall means in the same column with no common superscripts differ. For maize $P<0.01$ and for litter $P<0.05$.

Comparing the mean viable counts of fusaria in the five plot samples for the four substrates gave a similar pattern of occurrence to those of total fungi. Litter gave the highest counts of both Fusarium and total fungi.

The total fungal viable counts obtained from the three substrates (maize, husk and litter) examined from field T3 are listed in Table 3-3. Again the fungal viable counts of the individual maize, husk and litter samples for the five plot samples were significantly different ($P<0.001$).

The fungal populations of both husk and litter samples were very high and were similar to those of field J1. However, two of the maize plot samples (B and D) gave no fungi. These two samples consisted of a different maize variety (XL45) within the same field; samples from plots A, C and E were of maize variety PX442. The maize samples again gave the lowest fungal viable counts (mean 3.8×10^2 CFU/g compared to 3.5×10^7 mean for the husk samples).

Fusarium counts from the five plot samples of field T3 are summarised in Table 3.4. The maize samples showed heterogeneous distribution of Fusarium and the viable counts ranged between 20 CFU/g from plot C to zero from plots B, D and E ($P<0.01$). Thus, as with field J1, fusaria propagules on maize were distributed unevenly and were present in very small numbers. Husk and litter samples showed no significant differences ($P>0.05$).

The results of this preliminary study, and the considerations regarding the time factor for processing and the economic cost, emphasise the importance of combining plot samples to obtain a representative field sample for use in the studies to be reported.

Table 3-3: Total fungal viable counts (CFU/g) of maize, husk and litter from the five plot samples in field T3.

Plot sample	Total viable counts (CFU/g)		
	Maize	Husk	Litter
A	1.3×10^{2a}	3.5×10^{7a}	3.2×10^{7a}
B	0.0^{b} *	1.0×10^{7b}	6.0×10^{7b}
C	1.3×10^{3c}	7.3×10^{7c}	1.7×10^{7c}
D	0.0^{b} *	2.6×10^{7a}	2.3×10^{7cd}
E	4.8×10^{2a}	3.0×10^{7a}	3.4×10^{7a}
Mean	3.8×10^{2}	3.5×10^{7}	3.3×10^{7}

* Maize in plots B and D was of a different variety (see text)

a, b, c, d Overall means in the same column with no common superscripts differ ($P<0.001$).

Table 3-4: Viable counts of Fusarium (CFU/g) of maize, husk and litter from the five plot samples in field T3.

Plot sample	Fusarium viable counts (CFU/g)		
	Maize	Husk	Litter
A	12.5^{a}	2.5×10^{5a}	1.1×10^{6a}
B	0.0^{b}	4.0×10^{4a}	2.5×10^{6a}
C	20.0^{a}	3.5×10^{5a}	1.9×10^{6a}
D	0.0^{b}	5.3×10^{4a}	6.0×10^{6a}
E	0.0^{b}	9.0×10^{5a}	1.4×10^{6a}
Mean	6.5	3.2×10^{5}	1.5×10^{6}

a, b Overall means in the same column with no common superscripts differ ($P<0.001$).

3.1.1.2. Laboratory subsampling technique

Two samples derived from fields J2 (Sample 1) and T1 (Sample 2) were used to investigate the validity of the subsampling procedures for maize grains and to determine the relationship between the number of rotations (rolls) during sample mixing and the fungal viable counts as described in Section 2.1.4.2.

The results from sample 1 were expressed as a mean of three subsamples on one medium PDA-D (six plate replications). For sample 2, the results were calculated as a mean of two subsamples on two media, PDA-D and PCNB agar (eight plate replications). Table 3-5 and Figure 3.1 summarise the results from both samples. Table 3-5 shows the mean CFU/g and the mean log 10 of CFU/g with confidence limits of 95%. Using an analysis of variance test it was shown that there was no significant difference ($P>0.05$) in terms of the total fungal viable counts between the five rotations. At zero time (no spin) sample 1 showed the lowest viable counts (data from Sample 2 is not available). Two minutes of rolling gave the highest viable counts from both samples.

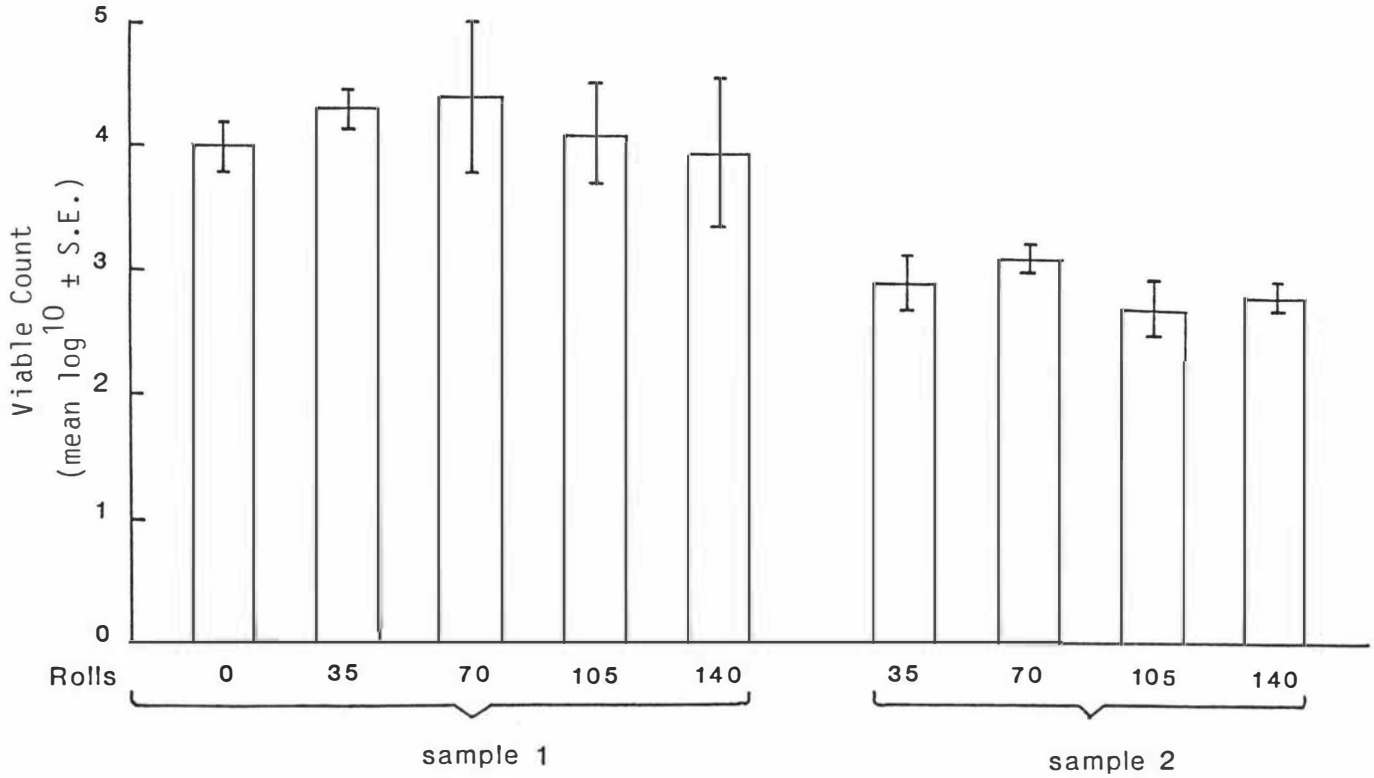
Table 3-5: Relationship between numbers of rotations of sample mixer and the subsequent total fungal viable counts (CFU/g) as determined by dilution plating.

Rotation time (min)	Number of rotations	Viable counts (CFU/g)* Sample 1 Count	Sample 1 log 10 ± S.E.	Sample 2 Count	Sample 2 log 10 ± S.E.
0	-	1.2×10^3	4.02 ± 0.209	N.A.	-
1	35	2.6×10^3	4.29 ± 0.1	1.0×10^3	3.97 ± 0.11
2	70	7.3×10^3	4.40 ± 0.675	1.2×10^3	4.04 ± 0.07
3	105	1.7×10^3	4.09 ± 0.44	6.2×10^2	3.74 ± 0.17
4	140	2.1×10^3	3.94 ± 0.68	8.4×10^2	3.83 ± 0.07

* Counts for Sample 1 as mean of six plate replications, for Sample 2 mean of eight plate replications

NA.: not assayed

Figure 3-1: Relationship between number of rotations (rolls) of sample mixer and the total fungal viable counts (CFU/g) of maize



3.1.1.3. Evaluation of media and isolation techniques for the recovery of *Fusarium* spp

As the *Fusarium* population was given priority in this study, isolation methods were initially tested for their efficiency in recovering fusaria from maize samples. The two media PDA-D and PCNB and the dilution plating and direct plating techniques were compared using grain from fields J1, T3 and J2 (Table 3-6). For each technique, no significant difference ($P>0.05$) was noticed between the total *Fusarium* populations recovered on the two media.

It was difficult to make a comparison between the two techniques themselves in relation to the estimated total population of *Fusarium* spp because the amount of maize assayed by dilution-plating was measured by weight (10 g) while that for direct plating was measured by number of kernels (100). However, use of the number of different *Fusarium* spp recovered could be considered valid as a criterion.

A comparison of the efficiency of the two media and the two techniques for the recovery of different *Fusarium* spp is summarised in Table 3-7. In these preliminary tests there was no significant difference ($P>0.05$) between the two media when number of species on each medium was compared in the separate techniques. However, the difference between the two techniques was highly significant ($P<0.001$). No *Fusarium* spp were recovered by dilution plating from four samples out of ten, while by direct plating *Fusarium* spp were recovered from all samples. The mean numbers of species from all ten samples were 0.8 and 2.5 by dilution-plating and direct plating respectively.

The media and technique factors will be examined further later in this chapter after further data have been accumulated, including those for total fungal genera.

Table 3-6: Preliminary evaluation of the two media (PDA-D and PCNB agars) and their efficiency for recovery of Fusarium spp from maize kernels by the direct plating and dilution-plating techniques

Field code	Plot Sample	Direct plating: Contamination rate (%) PDA-D	Direct plating: Contamination rate (%) PCNB	Dilution plating: Viable counts (CFU/g) PDA-D	Dilution plating: Viable counts (CFU/g) PCNB
J1	A	78	50	0.3×10^2	-
	B	72	68	3.7×10^2	2.7×10^2
	C	76	18	-	-
	D	68	90	3.3×10^2	0.05×10^2
	E	92	94	-	0.4×10^2
T3	A	20	94	0.2×10^2	0.3×10^2
	B	8	-	-	-
	C	100	94	0.3×10^2	0.1×10^2
	D	2	-	-	-
	E	80	98	-	-
J2	A	98	96	*	*
	B	100	100	|	|
	C	94	98	v	v
	D	92	100		
	E	92	96		
Mean		71.5	73.0	0.78×10^2	0.36×10^2
Mean log 10		1.45	1.6	1.9	1.6
		NS P>0.05		NS P>0.05	

- = sample negative for Fusarium

↓ = not assayed

NS: not significant

Table 3-7: The numbers of different Fusarium spp recovered on PDA-D and PCNB agars by the direct plating and dilution plating techniques.

Sample		No. of Fusarium spp					
		Direct plating		Dilution plating		Total, both media	
		PDA-D	PCNB	PDA-D	PCNB	Direct plating	Dilution plating
J1	A	2	2	1	-	2	1
	B	3	4	2	1	4	2
	C	2	3	-	-	3	-
	D	3	2	1	1	3	1
	E	2	2	-	1	2	1
T3	A	2	2	1	1	2	1
	B	1	-	-	-	1	-
	C	4	3	2	1	4	2
	D	1	1	-	-	1	-
	E	3	1	-	-	3	-
Mean		2.3	2.0	0.7	0.5	2.5	0.8
		NS P>0.05		NS P>0.05		*** P<0.001	

3.1.1.4 Moisture content of maize grains

Moisture contents (MCs) of the grain were estimated as described in Section 2.1.5.1 and the results are listed in Table 3-8 for maize samples from the field, at harvest and from storage. At the time of collection of field samples the grain's MC was moderately high, with a mean of 32.3% (range 36.1% [J2] to 25.7% [T2]). At harvest time the MCs dropped to within the range 20.8% (sample T4) to 25.8% (sample RH), mean 22.8%. Because field T3 was planted with two different maize varieties (PX442 matures earlier than does XL45), two different MCs are listed.

The mean MC of stored samples was 13.7%. The MC for samples from each individual silo was close, and means were 14.4, 14.5, 13.2 and 12.8% for silos A, B, C and D respectively (Table 3-8).

Table 3-8: Moisture contents (MC) of maize grain from field, harvest and stored samples

Field samples	MC (%)	Harvest samples	MC (%)	Stored samples	MC (%)
T1	27.3	T1H	23.2	A1	14.7
T2	25.7	T2H	20.9	2	14.5
T3 (1)	34.0)	T3H (1)	22.0)	3	14.0
(2)	31.6) 32.8	(2)	21.1) 21.6	mean	14.4
T4	29.5	T4H	20.8	B1	15.0
J1	32.3		*	2	13.5
J2	36.1	J2H	25.1	3	15.0
KH	*	KH	22.0	mean	14.5
RH	*	RH	25.8	C1	13.3
				2	13.1
mean	32.3		22.8	3	13.2
				mean	13.2
				D1	13.3
				2	12.2
				3	13.0
				mean	12.8
				mean of 4 silos	13.7

* Not sampled

3.1.2. The Mycoflora of Growing Maize and Associated Substrates in the Field

Maize from fields T1, T2, T3, T4, J1 and J2 was fully examined as described in Section 2.1.5.2 and, for comparison, husk, litter and soil samples were also included for fields J1, J2, T3 and T4. Table 3-9 summarises the total fungal counts from these substrates as determined by the dilution plating technique. The highest fungal population was from husk (mean 5 x 10^7 CFU/g) while the lowest was from soil (mean 2.4 x 10^4 CFU/g).

Samples of maize, husk and litter were also assayed by the direct plating technique and results expressed as percentage contamination rate of maize kernels or segments of husk or litter (Section 2.1.5.2). Debris from soil was not assayed in this way. Table 3-10 compares the results from these three substrates. It was found that 100% of husk segments from all the fields were contaminated by fungi. All kernel samples except that from field T3 were also contaminated at this level. Litter segments showed a lower mean contamination rate (86.5%), due to the low result (50% contamination) from sample T3.

In Table 3-11 all fungal genera isolated from the field samples by both techniques used are listed and the incidence of these genera in maize, husk, litter and soil substrates is compared. A total of 25 genera was detected from the total of 18 samples examined. The frequency of occurrence of each genus among all the samples varied greatly from 18/18 (100%) for the genus Fusarium to very rare with such genera as Chrysosporium and Myrothecium 1/18 (5.5%).

Four fungal genera occurred regularly. These genera were Fusarium, Acremonium, Cladosporium and Penicillium, at levels of 100%, 94%, 88% and 88% of all samples respectively. A comparison between the four substrates from all fields indicated that the larger number of fungal genera was isolated from soil and litter (19 each) while maize and husk had 13 and 11 genera respectively. Whilst most genera were found in all four substrates, a few were strictly associated with certain substrates only. Coniothyrium, Paecilomyces, Preussia, Pythium and Sependonium were isolated from soil samples only. Rhizopus was isolated from maize only.

Table 3-9: Total fungal viable counts of field samples of maize, husk, litter and soil (mean of two media).

Field code	Viable Counts (CFU/g)			
	Maize	Husk	Litter	Soil
T1	3.0×10^3	NA *	NA	NA
T2	3.4×10^3	NA	NA	NA
T3	3.8×10^2	3.5×10^7	3.3×10^7	2.9×10^4
T4	1.5×10^5	8.0×10^6	3.6×10^6	3.0×10^4
J1	4.7×10^3	1.7×10^7	3.7×10^7	1.5×10^4
J2	5.0×10^4	1.4×10^8	3.4×10^6	2.3×10^4
Mean	3.5×10^4	5.0×10^7	1.9×10^7	2.4×10^4

* NA: not assayed

Table 3-10: Contamination rates (%) of field samples of maize, husk and litter by fungi (direct plating technique, mean of two media).

Field code	Contamination rate (%)		
	Maize	Husk	Litter
T1	98	NA *	NA
T2	98	NA	NA
T3	88	100	50
T4	100	100	96
J1	100	100	100
J2	100	100	100
Mean	97	100	86.5

* NA: not assayed

Table 3-11: Fungal genera isolated from field samples of maize, husk, litter and soil (direct and dilution plating techniques, both media).

Genus	Field Samples: Maize T1	Maize T2	Maize T3	Maize T4	Maize J1	Maize J2	Husk T3	Husk T4	Husk J1	Husk J2	Litter T3	Litter T4	Litter J1	Litter J2	Soil T3	Soil T4	Soil J1	Soil J2	Total occurrence in 18 samples
Acremoniella	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	3
Acremonium	+	+	+	+	+	+	+	+↑*	+	+↑	+↑	+↑	-	+↑	+	+	+↑	+↑	17
Alternaria	+	-	+	-	-	-	+	+	+	+	+	-	+	-	+	-	-	-	9
Arthrinium	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	3
Ascochyta	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	2
Chaetomium	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	2
Chrysosporium	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	1
Cladosporium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	16
Coniothyrium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	2
Epicoccum	-	+	-	-	+	+	+	+	+	-	+	+	-	+	-	-	-	-	9
Fusarium	+	+↑	+↑	+↑	+	+↑	+	+	+	+	+	+	+	+	+	+	+	+	18
Gliocladium	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	+	4
Mucor	+	+	+	-	+↑	+	+	+	+	+	-	-	+	-	-	-	+		11
Myrothecium	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	1
Nigrospora	-	+	-	+	-	-	+	+	-	+	-	+	-	+	-	-	-	-	7
Paecilomyces	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	1
Penicillium	+↑	+	+	+	+	+	+↑	+	+↑	+	-	+	-	+	+↑	+↑	+	+	16
Phoma	+	-	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	-	8
Preussia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	1
Pythium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	2
Rhizopus	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	3
Scopulariopsis	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	+	4
Sepedonium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	1
Trichoderma	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	+	4
Verticillium	-	-	-	-	-	+	-	-	+	-	-	+	+↑	-	+	+	+	+	8
Total genera, each field	9	6	6	5	8	8	8	9	8	8	11	9	6	10	13	10	10	9	
Total genera, all fields			13					11				19					19		

*↑: the predominant genus

The predominant genus (the most frequently isolated genus from a sample, as estimated from all plates of both techniques) in four of the six maize samples was Fusarium. In samples from fields T1 and J1, Penicillium and Mucor were the predominant isolates respectively. Acremonium and Penicillium were common in husk, litter and soil samples.

The frequency of occurrence of each genus could be estimated more precisely in terms of the actual contamination rates by the fungus (% contamination of maize kernels and segments of husk and litter) as recorded by the direct plating technique. Table 3-12 shows that a total of 15 genera was detected from the six field samples. There were 13 genera isolated from the maize samples, and 11 genera from each of the husk and litter samples. Fusarium was the most common contaminant from all substrates. It was found at a level of contamination of 75.6, 69.0 and 43.3% of kernels, husk segments and litter segments respectively. Other genera commonly isolated were Mucor in kernels (mean contamination rate 27.6%), Penicillium in husk segments (mean 32.8%) and Acremonium in litter segments (mean 12.5%).

The population of Fusarium spp as enumerated by the dilution plating and direct plating techniques is summarised in Tables 3-13 and 3-14. Viable count determinations of fusaria in maize, husk, litter

Table 3-12: Contamination rate (%) of field maize and segments of husk and litter by fungi (direct plating technique, mean of two media).

	Contamination rate (%) in Field Samples																
	Maize							Husk					Litter				
Genus	T1	T2	T3	T4	J1	J2	Mean	T3	T4	J1	J2	Mean	T3	T4	J1	J2	Mean
Acremoniella	1	-	-	-	-	-	0.2	-	-	-	6.7	1.7	-	-	-	-	0
Acremonium	6	4	7	19	15	5	9.3	-	3.3	3.3	10.0	4.2	-	20	26.6	3.3	12.5
Alternaria	1	-	-	-	-	-	0.2	25	-	10	6.7	10.4	3	-	-	-	0.8
Arthrinium	-	-	-	-	4	-	0.7	-	-	-	-	0	6.6	-	-	-	1.7
Cladosporium	-	-	2	-	-	-	0.3	-	10	-	-	2.5	-	6.7	10	-	4.2
Epicoccum	-	1	-	-	6	3	1.7	35	6.7	-	-	10.4	-	-	-	-	0
Fusarium	45	72	63	100	77	97	75.6	76	43.3	67	90	69.0	6.6	23.5	46.6	96.7	43.3
Mucor	-	4	61	-	99	3	27.6	26	3.3	50	-	19.8	-	-	-	-	0
Nigrospora	-	2	-	4	-	-	1.0	8	20	-	16.7	11.2	-	36	3.3	10	12.3
Penicillium	72	34	9	3	2	2	20.3	31	46.7	36.7	16.7	32.8	-	-	-	3.3	0.8
Phoma	3	-	-	-	-	-	0.5	-	3.3	-	-	0.8	-	13	3.3	-	4.0
Rhizopus	2	-	-	-	-	-	0.7	-	-	-	-	0	-	-	-	-	0
Scopulariopsis	-	-	-	-	-	-	0	-	-	-	-	0	20	-	-	-	5.0
Trichoderma	-	-	-	-	-	-	0	-	-	-	-	0	3	-	-	-	0.8
Verticillium	-	-	-	-	-	4	0.7	-	-	3.3	-	0.8	-	-	43.3	-	10.8
Total each field	7	6	5	4	6	7		6	8	6	6		5	5	6	4	
Total genera, all fields				13						11					11		

Table 3-13: Total viable counts (CFU/g substrate) of fusaria recorded from field samples of maize, husk, litter and soil (mean of two media).

Field code	Viable counts (CFU/g)			
	Maize	Husk	Litter	Soil
T1	2.2×10^2	NA *	NA	NA
T2	1.1×10^2	NA	NA	NA
T3	0.11×10^2	3.0×10^5	2.4×10^5	4.0×10^3
T4	1.6×10^3	2.3×10^5	9.7×10^4	3.5×10^3
J1	3.4×10^2	3.0×10^5	7.8×10^5	2.5×10^3
J2	4.7×10^3	1.4×10^5	1.0×10^5	7.6×10^2
Mean	1.2×10^3	2.4×10^5	3.0×10^5	2.7×10^3

Table 3-14: Contamination rates (%) of field samples of maize, husk and litter by fusaria (direct plating technique, mean of two media).

Field code	Contamination rate (%)			
	Maize	Husk	Litter	Mean %
T1	45	NA *	NA	
T2	72	NA	NA	
T3	63	76	6.6	48.7
T4	100	43.3	23.5	55.7
J1	77	67	46.6	72.0
J2	97	90	96.7	93.7
Mean %	75.6	69.0	43.3	

* NA: not assayed

and soil indicated that the highest populations were shown by husk and litter samples (Table 3-13). The two substrates showed similar numbers when averaged for all four fields (2.4×10^5 and 3.0×10^5 respectively). Maize samples gave the lowest population from all fields (mean 1.2×10^3 CFU/g).

However, using the direct plating technique, Fusarium spp were found to occur in maize kernels at higher actual contamination levels than in segments of husks and litters (Table 3-14). The mean % of kernels or segments contaminated with fusaria in the fields were 75.6, 69.0 and 43.3% for maize, husk and litter respectively. Field J2 gave the highest overall % contamination (93.7%) compared to the other three fields.

A variety of individual Fusarium spp was detected in all four substrates from field samples as is summarised in Table 3-15. The results showed that overall, F. culmorum was the most frequently isolated (14/15 samples). Three other species, F. graminearum, F. acuminatum and F. oxysporum were also commonly found (13/15, 11/15 and 9/15 samples respectively). Three species, F. merismoides, F. equiseti and F. moniliforme were recorded only once each. A comparison between the number of Fusarium spp isolated from each substrate indicated that the litter samples had the greatest variety (11 species), with soil and maize having ten each, and husks eight species. The predominant species in five of the six maize samples was F. graminearum, while F. subglutinans was the predominant isolate in the remaining sample (T3). The three husk samples had different predominant species, F.graminearum, F. subglutinans and F.oxysporum. Two species (F. culmorum and F. acuminatum) were the predominant isolates from the litter samples. All three soil samples showed F. oxysporum to be the predominant species.

Five species, F. graminearum, F. culmorum, F. subglutinans, F. oxysporum and F. acuminatum were found at high levels in the four substrates when viable counts were determined by dilution plating (Table 3-16 and Figure 3-2). No F. oxysporum was found in maize samples and no F. acuminatum was detected from the soil samples using this technique.

Table 3-15: Fusarium spp isolated from field samples of maize, husk, litter and soil (direct and dilution plating techniques, both media).

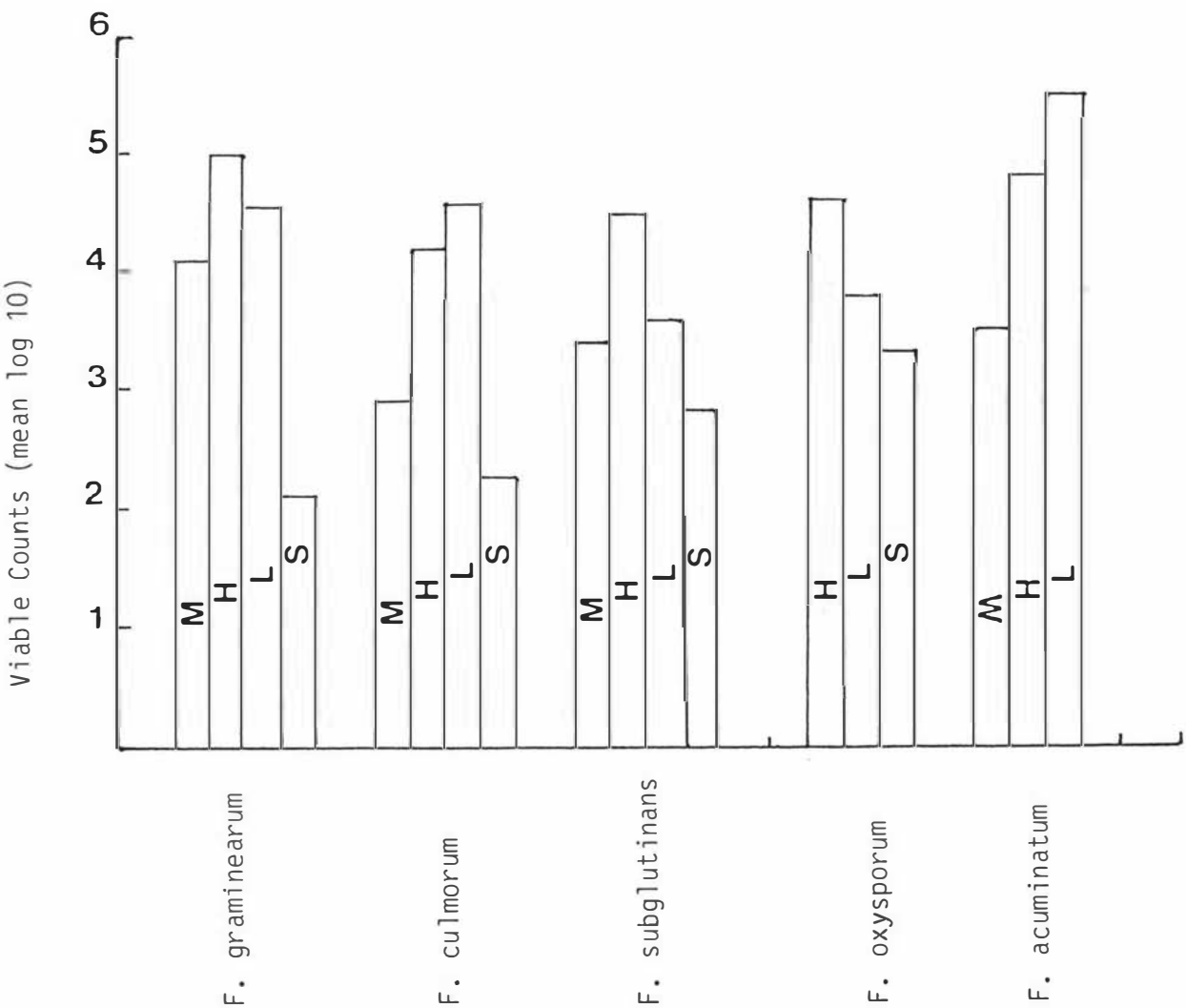
	Field Samples															
	Maize						Husk			Litter			Soil			Occurrence in
Fusarium spp.	T1	T2	T3	T4	J1	J2	T3	T4	J2	T3	T4	J2	T3	T4	J2	15 samples
F. graminearum	+↑*	+↑	+	+↑	+↑	+↑	+↑	+	+	+	+	+	+	-	-	13
F. culmorum	+	+	-	+	+	+	+	+	+	+	+↑	+↑	+	+	+	14
F. subglutinans	-	-	+↑	+	+	-	-	+↑	+	-	+	-	-	+	+	8
F. oxysporum	+	-	+	+	-	-	-	-	+↑	-	+	+	+↑	+↑	+↑	9
F. acuminatum	+	+	+	+	+	+	+	-	+	+↑	+	+	-	-	-	11
F. avenaceum	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	3
F. poae	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	7
F. solani	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	3
F. merismoides	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	1
F. lateritium	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	3
F. equiseti	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	1
F. crookwellense	-	-	-	+	-	-	-	-	+	-	+	+	-	-	+	5
F. sambucinum	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	2
F. stilboides	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	5
F. moniliforme	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	1
Total spp, each field	5	3	4	8	5	6	5	4	7	3	11	8	6	7	5	
Total spp all fields			10					8			11			10		

*↑: the predominant species
(J1 samples of husk, litter and soil not assayed to species level.)

Table 3-16: Viable counts (CFU/g) of the most commonly-occurring Fusarium spp in field samples (mean of three fields T3, T4 and J2).

	Viable counts (CFU/g)				
Substrate	F. graminearum	F. culmorum	F. subglutinans	F. oxysporum	F. acuminatum
Maize	1.3×10^4	8.0×10^2	2.6×10^3	0	3.2×10^3
Husk	1.0×10^5	1.7×10^4	3.0×10^4	4.6×10^4	6.8×10^4
Litter	3.8×10^4	4.3×10^4	4.3×10^3	5.9×10^3	3.7×10^5
Soil	1.3×10^2	1.8×10^2	6.8×10^2	2.2×10^3	0

Figure 3-2: Comparison between the viable counts of the most commonly-occurring Fusarium spp in field samples (maize = M, husk = H, litter = L and soil = S) (counts as CFU/g).



The frequency of occurrence of the individual Fusarium spp in kernels and in segments of husk and litter is shown in Table 3-17. A total of 12 Fusarium spp was detected from all substrates by the direct plating technique. Nine species occurred in the six samples of maize and seven species each in the three samples of husk and litter. F. graminearum was the most frequent isolate from maize and husk samples (mean 44 and 37.7% respectively) while F. culmorum was the most frequent isolate from the litter samples. Three species (F. culmorum, F. subglutinans and F. acuminatum) were also common but at much lower frequencies than F. graminearum.

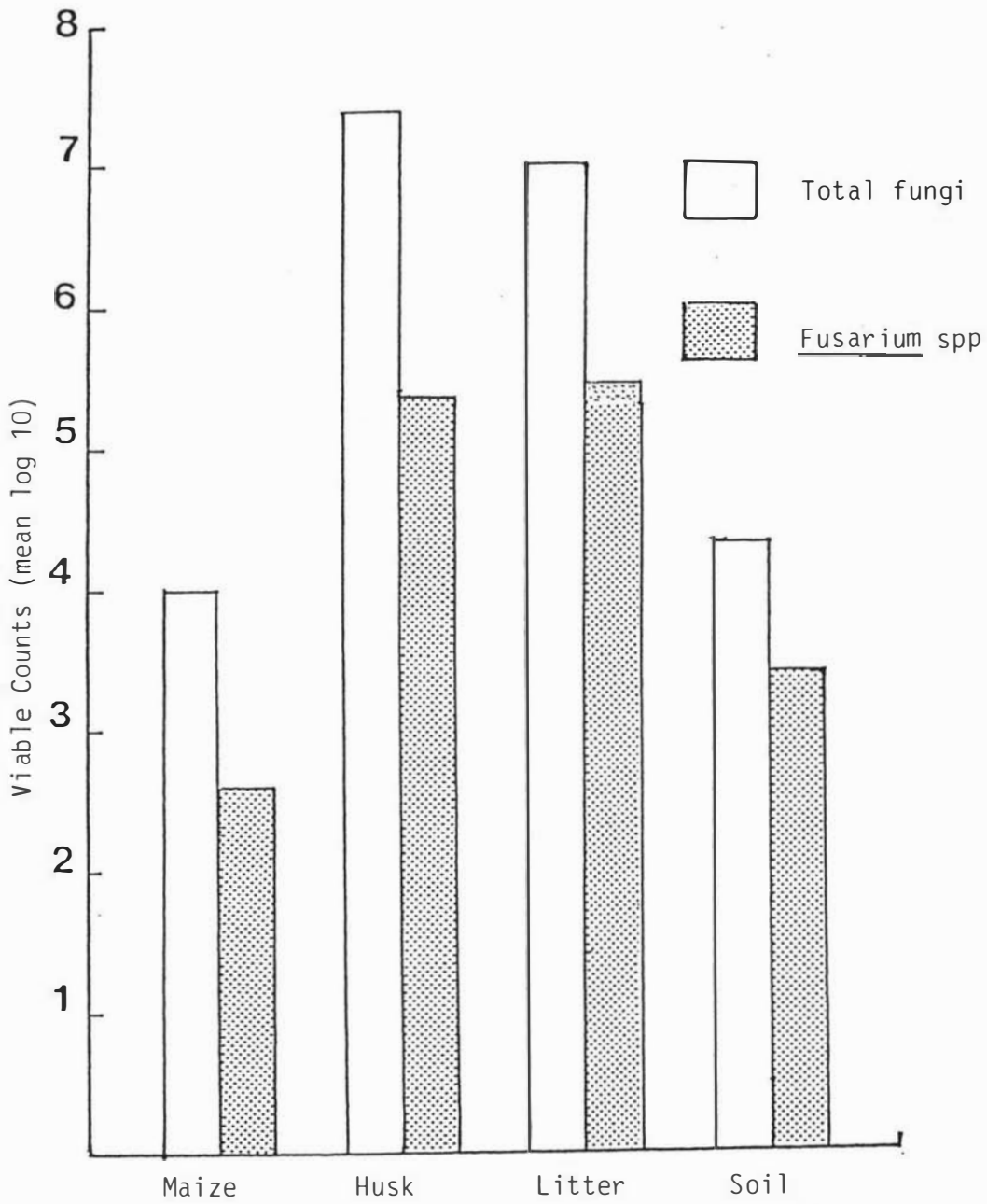
Both total fungal viable counts and counts of Fusarium spp from all substrates are summarised in Figure 3-3. The results are expressed as mean log 10 of counts from all samples obtained from the four fields T3, T4, J1 and J2 for which direct comparisons could be made. The highest counts for both total fungi and Fusarium spp were obtained from husk samples. The figure allows comparison of the total fungal population in each of the four substrates examined and of the relative proportion of Fusarium species in the total fungal population of each of the substrates.

Table 3-17: Contamination rates (%) of field maize and segments of husk and litter by individual Fusarium spp, (direct plating technique, mean of two media).

	Contamination rate (%) in field samples																
	Maize							Husk					Litter				
Fusarium spp.	T1	T2	T3	T4	J1	J2	Mean	T3	T4	J1	J2	Mean	T3	T4	J1	J2	Mean
F. graminearum	23	51	6	62	47	77	44.0	43	20	NA*	50	37.7	7	3	NA	20	10
F. culmorum	10	19	-	34	3	13	13.0	3	10		10	7.7	-	-		40	13.3
F. subglutinans	-	-	28	11	25	-	10.7	-	20		3	7.7	-	-		3	3.3
F. oxysporum	3	-	5	1	-	-	1.5	-	-		-	-	-	3		3	2.0
F. acuminatum	8	4	26	1	2	12	8.8	23	-		20	14.3	-	7		30	12.3
F. poae	3	-	-	1	1	-	0.8	10	3		13	8.7	-	-		-	-
F. crookwellense	-	-	-	3	-	-	0.5	-	-		7	2.3	-	-		-	-
F. stilboides	-	-	-	-	-	1	0.17	-	-		-	-	-	-		-	-
F. moniliforme	-	-	-	1	-	-	0.17	-	-		-	-	-	-		-	-
F. avenaceum	-	-	-	-	-	-	-	13	-		-	4.3	-	-		-	-
F. equiseti	-	-	-	-	-	-	-	-	-		-	-	-	3		-	1.0
F. lateritium	-	-	-	-	-	-	-	-	-		-	-	-	-		10	3.3
Total spp, each field	5	3	4	8	5	4		5	4	0	6		1	5	0	6	
Total spp, all fields				9						7					7		

*NA: not assayed

Figure 3-3: Comparison of the mean viable counts (CFU/g) of total fungi and Fusarium in samples of maize, husk, litter and soil from fields T3, T4, J1 and J2.



3.1.3. The Mycoflora of Maize at Harvest

The maize samples collected at harvest time (Section 2.1.3.2) were assayed by both the dilution plating and direct plating techniques. The total fungal population of these harvest samples is summarised in Table 3-18, which lists the results from both isolation techniques. The overall level of contaminated kernels was high at 94.3% (mean of seven fields sampled). The lowest level of contaminated kernels from an individual field was recorded from field T2H (82%). Four fields (T1H, J2H, KH and RH) were contaminated at 100%. Total fungal propagules as detected by dilution plating were similar for six of the fields (1.0-2.0 x 10^3) but sample KH showed a low population of 70 CFU/g. The mean from all fields was 1000 CFU/g.

The individual occurrence of different fungal genera is presented in Tables 3-19 and 3-20. In Table 3-19 the proportion of maize kernels contaminated by individual genera is given as an average for the two media in the direct plating technique. A total of 16 genera was detected from the seven fields by this technique. The highest number of different genera was detected in field KH (11 genera) while the lowest number was from field T2H (5). The genera Fusarium and Penicillium occurred in all samples. Three genera (Arthrinium, Fusicoccum and Scopulariopsis) occurred rarely (1/7 each). The highest mean contamination levels of kernels were 58.3% for Fusarium, followed by 27.7% for Penicillium. Three fields, J2H, KH and RH were contaminated with high levels of Acremoniella (43, 34 and 31% respectively, mean 15.4%), but no Acremoniella was detected in the remaining fields. In contrast, Epicoccum and Nigrospora occurred commonly in the fields (6/7) but at low kernel contamination rates (1.6 and 5.1 respectively).

Table 3-20 summarises the total number of genera detected by both direct and dilution plating. A total of 17 genera was isolated from the seven harvest samples. Four genera (Acremonium, Epicoccum, Fusarium and Penicillium) were found in all samples, while some genera such as Arthrinium, Fusicoccum etc. were found in only one sample each. Fusarium was the predominant isolate from five samples, with Penicillium predominant in the remainder. Verticillium was detected only by dilution plating.

Table 3-18: Total fungal population of harvest samples of maize (direct and dilution plating techniques, mean of two media).

Harvest sample code	Dilution plating Viable count (CFU/g maize)	Direct plating Contamination rate (%)
T1H	1.0×10^3	100
T2H	1.1×10^3	82
T3H	1.1×10^3	94
T4H	1.3×10^3	84
J2H	1.3×10^3	100
KH	0.7×10^2	100
RH	2.0×10^3	100
Mean	1.0×10^3	94.3

Table 3-19: Contamination rates (%) by different genera isolated from harvest samples of maize (direct plating technique, mean of two media).

Genus	Contamination rate (%) in harvest samples							Mean %	Occurrence in 7 fields
	T1H	T2H	T3H	T4H	J2H	KH	RH		
Acremoniella	-	-	-	-	43	34	31	15.4	3
Acremonium	12	1	1	-	-	2	8	3.4	5
Alternaria	-	-	2	-	4	-	-	0.9	2
Arthrinium	-	-	-	1	-	-	-	0.14	1
Chaetomium	-	-	-	2	-	6	-	1.14	2
Cladosporium	2	-	2	-	-	-	-	0.6	2
Epicoccum	3	1	3	1	1	2	-	1.6	6
Fusarium	87	40	37	39	74	51	80	58.3	7
Fusicoccum	-	-	-	-	1	-	-	0.14	1
Mucor	6	-	1	-	-	3	18	4.0	4
Nigrospora	10	5	4	6	-	3	8	5.1	6
Penicillium	33	44	49	36	22	4	6	27.7	7
Phoma	-	-	-	-	-	26	-	3.7	1
Schizophyllum	-	-	-	-	2	-	-	0.3	1
Scopulariopsis	-	-	-	-	-	1	-	0.14	1
Trichoderma	-	-	1	-	1	8	-	1.4	3
Total genera, each sample	7	5	9	7	8	11	6		

Table 3-20: Fungal genera isolated from harvest samples of maize (direct and dilution plating techniques, both media).

Genus	Harvest samples							Occurrence in 7 fields
	T1H	T2H	T3H	T4H	J2H	KH	RH	
Acremoniella	-	-	-	-	+	+	+	3
Acremonium	+	+	+	+	+	+	+	7
Alternaria	-	-	+	-	+	-	-	2
Arthrinium	-	-	-	+	-	-	-	1
Chaetomium	-	-	-	+	-	+	-	2
Cladosporium	+	+	+	+	+	-	-	5
Epicoccum	+	+	+	+	+	+	+	7
Fusarium	+↑*	+	+	+↑	+↑	+↑	+↑	7
Fusicoccum	-	-	-	-	+	-	-	1
Mucor	+	-	+	-	-	+	+	4
Nigrospora	+	+	+	+	-	+	+	6
Penicillium	+	+↑	+↑	+	+	+	+	7
Phoma	-	-	-	-	+	+	-	2
Schizophyllum	-	-	-	-	+	-	-	1
Scopulariopsis	-	-	-	-	-	+	-	1
Trichoderma	-	-	+	-	+	+	-	3
Verticillium	+	+	-	-	-	-	+	3
Total genera, each sample	8	7	9	8	11	11	8	

*↑: the predominant genus

The total population of Fusarium detected by both techniques is presented in Table 3-21. The dilution plating technique gave low viable counts for all the fields. Field T2H showed no Fusarium by dilution plating while 40% of the kernels were contaminated with Fusarium according to the direct plating technique.

Among the seven fields examined, field RH had the highest viable count of fusaria (680 CFU/g), while KH gave only 5 CFU/g. The mean for all the fields was 150 CFU/g. The direct plating technique indicated a level of contamination of kernels ranging between 37% (field T3H) to 87% (field T1H) with a mean of 58.3%.

A total of nine Fusarium spp was isolated from all harvest samples by direct plating. The number of different Fusarium spp per field ranged between three species from field RH to six species from field KH. (Table 3-22) This table shows that some species were present in all fields (F. graminearum and F. culmorum) whilst others (F. oxysporum and F. crookwellense) were isolated from only one field. F. graminearum was the most common contaminant of kernels (mean 33.4% contamination rate).

The dilution plating technique yielded only seven species from the seven fields (Table 3-23). F. acuminatum, F. crookwellense and F. stilboides were not detected by this technique but F. lateritium was found in one field. The maximum number of Fusarium spp recorded by this method from one field was four species from sample KH, but the majority had two species. F. graminearum was again the most frequent.

Table 3-24 summarises the occurrence of all Fusarium spp as detected by both direct and dilution plating. A total of ten was detected, with F. graminearum, F. culmorum and F. subglutinans being the most frequently occurring (7/7, 7/7 and 5/7 samples respectively). F. graminearum was the predominant isolate in five of the seven samples, with F. culmorum and F. subglutinans being predominant in one sample each.

Table 3-21: Total population of Fusarium spp contaminating harvest samples of maize (mean of two media).

Harvest sample	Dilution plating Viable count (CFU/g maize)	Direct plating Contamination rate (%)
T1H	0.9×10^2	87
T2H	-	40
T3H	0.2×10^2	37
T4H	0.4×10^2	39
J2H	2.0×10^2	74
KH	0.05×10^2	51
RH	6.8×10^2	80
Mean	1.5×10^2	58.3

Table 3-22: Contamination rates (%) of harvest samples of maize by individual Fusarium spp. (direct plating technique, mean of two media).

Fusarium spp	Contamination rate (%) in harvest samples T1H	T2H	T3H	T4H	J2H	KH	RH	Mean %
F. graminearum	24	18	23	29	49	30	61	33.4
F. culmorum	5	19	4	8	12	6	16	10.0
F. subglutinans	34	-	3	1	-	11	2	7.3
F. oxysporum	-	-	-	-	-	1	-	0.14
F. acuminatum	-	3	-	2	-	-	-	0.7
F. avenaceum	-	-	-	-	12	-	-	1.7
F. poae	20	-	7	-	3	2	-	4.6
F. crookwellense	-	1	-	-	-	-	-	0.14
F. stilboides	5	-	-	-	-	1	-	0.9
Total spp, each sample	5	4	4	4	4	6	3	

Table 3-23: Fusarium viable counts (CFU/g) in harvest samples of maize (dilution plating technique, mean of two media).

Fusarium spp	Viable counts (CFU/g) in harvest samples							Occurrence
	T3H	T4H	J2H	T1H	T2H	RH	KH	in 7 fields
F. graminearum	0.2×10^2	-	2.0×10^2	0.7×10^2	-	0.05×10^2	1.6×10^2	5
F. culmorum	-	0.2×10^2	-	-	-	-	0.4×10^2	2
F. subglutinans	-	-	-	-	-	-	3.1×10^2	1
F. oxysporum	-	-	-	-	-	-	1.7×10^2	1
F. avenaceum			0.03×10^2	0.1×10^2	-	-	-	2
F. poae	0.02×10^2	-	-	-	-	-	-	1
F. lateritium	-	0.2×10^2	-	-	-	-	-	1
No. spp, each sample	2	2	2	2	0	1	4	

Table 3-24: Fusarium spp isolated from harvest samples of maize (direct and dilution plating techniques, both media).

Fusarium spp	Harvest samples							Occurrence
	T1H	T2H	T3H	T4H	J2H	KH	RH	in 7 fields
F. graminearum	+	+	+↑	+↑	+↑	+↑	+↑	7
F. culmorum	+	+↑	+	+	+	+	+	7
F. subglutinans	+↑*	-	+	+	-	+	+	5
F. oxysporum	-	-	-	-	-	+	-	1
F. acuminatum	-	+	-	+	-	-	-	2
F. avenaceum	-	-	-	-	+	-	-	1
F. poae	+	-	+	-	+	+	-	4
F. crookwellense	-	+	-	-	-	-	-	1
F. stilboides	+	-	-	-	-	+	-	2
F. lateritium	-	-	-	+	-	-	-	1
No. species, each sample	5	4	4	5	4	6	3	

*↑: the predominant species

3.1.4. Mycoflora of Stored Maize

Samples of maize from storage silos were examined by direct plating only. Table 3-25 summarises the results for both total fungi and *Fusarium* spp from 12 samples of stored maize taken from four silos. A comparison of the total fungal population between the four silos shows that silo C was heavily contaminated (81% kernel contamination rate), whereas there were few contaminated kernels in silo D. Overall the 12 samples were contaminated at a level of 42.4%. Generally there was no increase in the fungal population during the sampling period for most silos except silo A.

The level of *Fusarium* contamination in stored maize was very low (mean 1.5% for 12 samples). The highest level of contamination was from silo B (3% kernel contamination rate).

A total of 17 fungal genera was isolated by the direct plating technique on both media. Table 3-26 summarises the contamination rates by each individual genus. A comparison between the total number of genera isolated from each silo showed that silo B was contaminated with the highest number (11). Some genera were common in the samples. *Penicillium*, *Fusarium* and *Nigrospora* were detected in 11, 9 and 6 samples respectively. Five genera (*Acremoniella*, *Arthrinium*, an unidentified Ascomycete, *Chrysosporium* and *Epicoccum*) were detected in only one sample each. Each silo showed different levels of contamination. *Penicillium* was the most common contaminant in samples from silos A and C. In silo B *Nigrospora* predominated and silo D showed a very low level of contamination, the predominant genus being *Alternaria*.

Table 3-27 shows the rate of contamination of kernels with each individual *Fusarium* spp. Only three species (*F. subglutinans*, *F. poae* and *F. graminearum*) were detected from the 12 samples. *F. subglutinans* occurred most frequently (8/12 samples). *F. poae* and *F. graminearum* were detected only in silo B and the contamination rates were very low.

Table 3-25: Contamination rates (%) of stored maize (direct plating technique, mean of two media).

Silo	Sample no.	Storage period (weeks)	Contamination rate (%) All fungi	Fusarium spp
A	1	4	14	2
	2	7	18	2
	3	12	60	1
	Mean		30.7	1.7
B	1	8	51	3
	2	13	45	4
	3	18	57	2
	Mean		51	3
C	1	54	80	1
	2	56	82	-
	3	63	81	-
	Mean		81	0.3
D	1	12	2	-
	2	16	13	2
	3	21	6	1
	Mean		7	1
Mean			42.4	1.5

Table 3-26: Contamination rates (%) of maize from four silos by different fungal genera (direct plating technique, mean of two media).

Genus	Contamination rate (%)																Occurrence in 12 samples
	Silo A				Silo B				Silo C				Silo D				
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	
Acremoniella	1	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	1
Alternaria	-	-	-	-	-	-	-	-	-	1	2	1	-	7	-	2.3	3
Arthrinium	-	-	-	-	-	-	2	0.7	-	-	-	-	-	-	-	-	1
Ascomycete	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	0.3	1
Aspergillus	-	-	-	-	-	-	-	-	13	31	63	35.6	-	-	2	0.7	4
Beauvaria	-	-	-	-	1	-	-	0.3	1	-	1	0.7	1	-	-	0.3	4
Chaetomium	-	-	2	0.7	2	-	-	0.7	-	-	-	-	-	-	-	-	2
Chrysosporium	-	-	-	-	-	1	-	0.3	-	-	-	-	-	-	-	-	1
Cladosporium	-	-	1	0.3	-	-	-	-	-	1	-	0.3	-	1	1	0.7	4
Epicoccum	-	-	-	-	1	-	-	0.3	-	-	-	-	-	-	-	-	1
Fusarium	2	2	1	1.7	3	4	2	3.0	1	-	-	0.3	-	2	1	1.0	9
Mucor	-	-	-	-	1	-	-	0.3	-	-	-	-	-	2	1	1.0	3
Nigrospora	2	2	6	3.3	33	31	30	31.0	-	-	-	-	-	-	-	-	6
Penicillium	7	13	44	23.0	10	8	14	12.3	79	27	23	43.0	2	1	-	1.0	11
Rhizopus	-	1	1	0.7	-	-	1	0.3	11	-	-	3.7	-	-	-	-	4
Scopulariopsis	1	-	1	0.7	1	-	1	0.7	-	-	2	0.7	-	-	-	-	5
Ulocladium	-	-	-	-	-	-	-	-	-	1	-	0.3	-	-	1	0.3	2
No. genera, each sample	5	4	7		8	4	6		5	5	5		2	6	5		
No. genera, each silo				8				11				9				9	

Table 3-27: Contamination rates (%) of stored maize from four silos by individual Fusarium spp (direct plating technique, mean of two media).

Silo	Sample No.	Contamination rate (%) F. subglutinans	F. poae	F. graminearum
A	1	2	-	-
	2	2	-	-
	3	1	-	-
B	1	-	2	1
	2	1	1	2
	3	1	-	1
C	1	1	-	-
	2	-	-	-
	3	-	-	-
D	1	-	-	-
	2	2	-	-
	3	1	-	-
Mean		0.9	0.25	0.33

3.1.5 Analysis of the Mycological Assays

3.1.5.1. Media and techniques and their effect on the isolation of fungi

The preliminary study carried out at the beginning of this investigation considered effects of media and techniques on the isolation of *Fusarium* spp only (Section 3.1.1.3). After completing the assays it seemed worthwhile to examine the effects of those factors on the isolation of other fungal genera in comparison with *Fusarium* spp.

The overall results from all maize samples examined indicated that the two different media (PDA-D and PCNB) gave no significant differences ($P>0.05$) for either the total fungal population or the *Fusarium* population (Tables 3-28 and 3-29, and Figures 3-4 and 3-5). However, the total fungal population was slightly higher on PDA-D medium in both techniques. *Fusarium* counts were slightly higher on PDA-D using dilution plating and vice versa with direct plating.

The influence of the media on the total number of genera isolated and the number of *Fusarium* spp are summarised in Table 3-30. No significant difference ($P>0.05$) was found between the actual number of *Fusarium* spp obtained from the two media although the mean number of species was higher on PCNB medium (4.2) compared to that on the PDA-D medium (3.5). This overall result agrees with the preliminary results. However, the media factor significantly ($P<0.01$) affected the numbers of other genera which were recovered (Table 3-30 and Figure 3-6). The total number of genera was significantly higher on PDA-D (5.9) than on the PCNB (4.3) ($P<0.01$).

Only one parameter was used to evaluate the influence of the two techniques which were used (i.e. the number of genera and the number of *Fusarium* spp recorded by either technique). Table 3-31 and Figure 3-7 show that both these, when recorded by direct plating, were significantly higher than when recorded by dilution plating, particularly for *Fusarium* spp. The mean number of *Fusarium* spp recorded by direct plating was 4.9 compared to 2.4 by dilution plating ($P<0.001$). Similarly the total number of genera isolated was 6.9 (mean) by direct plating compared to 5.4 (mean) by dilution plating ($P<0.025$).

The interaction between the media and techniques, and their effect on the number of fungal genera and Fusarium spp isolated is summarised in Table 3-32. This table indicates that the number of Fusarium spp isolated was significantly higher ($P<0.001$) by using direct plating, regardless of the medium used. In the case of numbers of fungal genera, direct plating results were significantly higher on PDA-D, but there was no significant difference between the techniques when PCNB was used.

Table 3-28: Evaluation of the two media, PDA-D and PCNB for their effect on the estimated populations of total fungi and Fusarium spp from maize as determined by the dilution plating technique.

	Viable counts (CFU/g)			
Sample	Fusarium		Total fungi	
code	PDA-D	PCNB	PDA-D	PCNB
T1	2.0×10^2	2.4×10^2	3.6×10^3	2.4×10^3
T1H	1.3×10^2	0.4×10^2	1.0×10^3	9.3×10^2
T2	1.3×10^2	0.9×10^2	2.8×10^3	4.0×10^3
T2H	-	-	1.4×10^3	8.7×10^2
T3	0.1×10^2	0.08×10^2	4.4×10^2	3.3×10^2
T3H	0.3×10^2	0.03×10^2	1.1×10^3	1.2×10^3
T4	1.7×10^3	1.6×10^3	2.2×10^5	7.4×10^4
T4H	0.3×10^2	0.3×10^2	2.0×10^3	6.7×10^2
J1	1.5×10^2	5.4×10^2	5.0×10^3	4.0×10^3
J2	6.0×10^3	3.3×10^3	4.5×10^4	5.5×10^4
J2H	2.3×10^2	1.7×10^2	2.0×10^3	5.1×10^2
RH	6.2×10^2	7.3×10^2	2.7×10^3	1.5×10^3
KH	-	0.1×10^2	0.7×10^2	0.4×10^2
Mean log 10	2.0	1.9	3.4	3.2
	NS ($P>0.05$)		NS ($P>0.05$)	

NS: not significant

Table 3-29: Evaluation of the two media PDA-D and PCNB for their effect on the recovery rate (% contamination of kernels) of total fungi and Fusarium spp from maize as determined by the direct plating technique.

Sample code	Contamination rate (%)			
	Fusarium		Total fungi	
	PDA-D	PCNB	PDA-D	PCNB
T1	50	40	100	96
T1H	74	100	100	100
T2	76	70	100	96
T2H	38	42	96	68
T3	58	68	88	83
T3H	28	46	96	92
T4	100	100	100	100
T4H	26	52	94	74
J1	88	66	100	100
J2	96	98	100	100
J2H	68	80	100	100
RH	66	94	100	100
KH	26	76	100	100
Mean	61	71.7	98	93.4
		NS (P>0.05)		NS (P>0.05)

NS: not significant

Figure 3-4: Comparison of the two media PDA-D and PCNB for their effect on the estimated populations of total fungi and Fusarium spp from maize as determined by the dilution plating technique.(CFU/g).

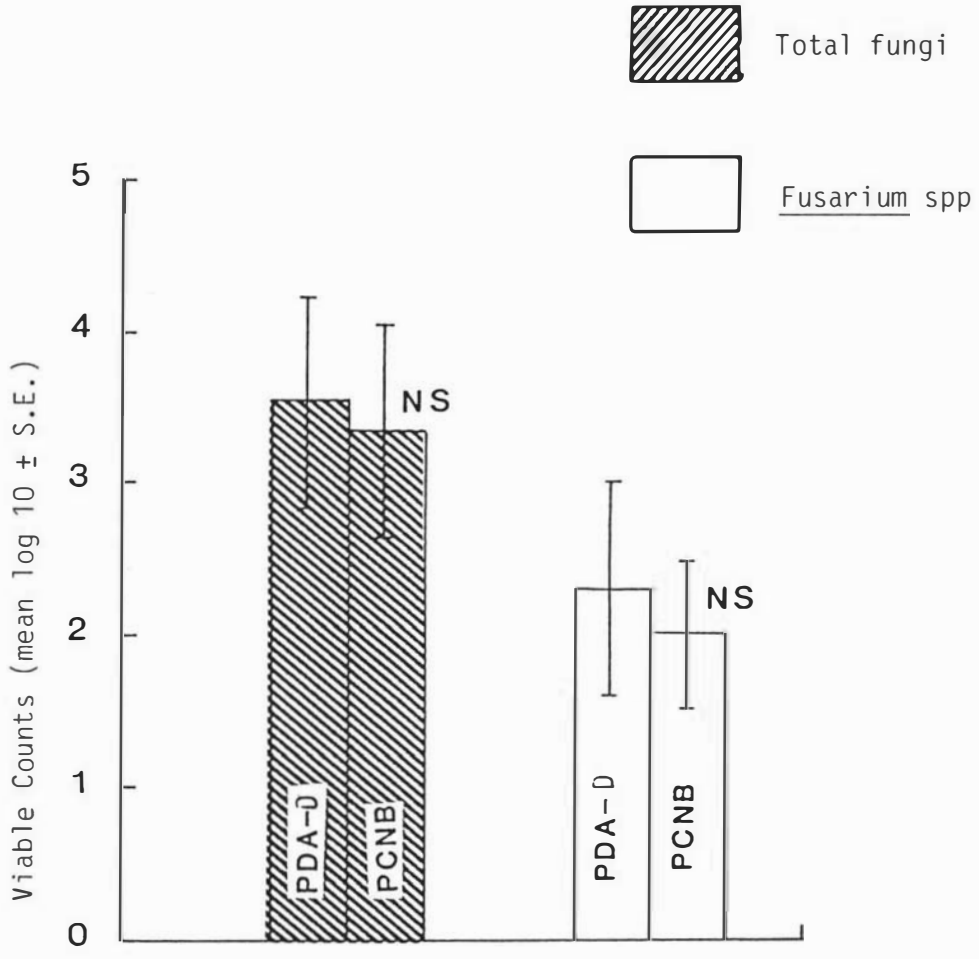


Figure 3-5: Comparison of the two media PDA-D and PCNB for their effect on the recovery rate (% contamination of kernels) of Fusarium spp and other genera, as determined by the direct plating technique.

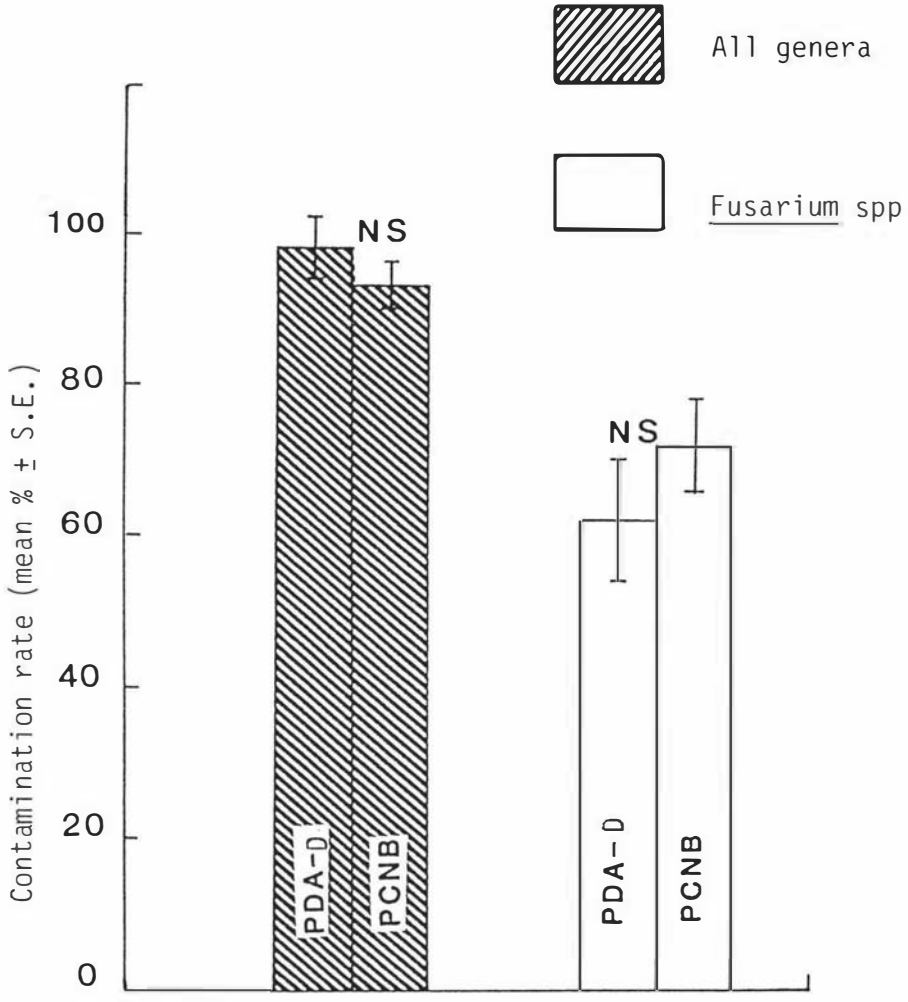
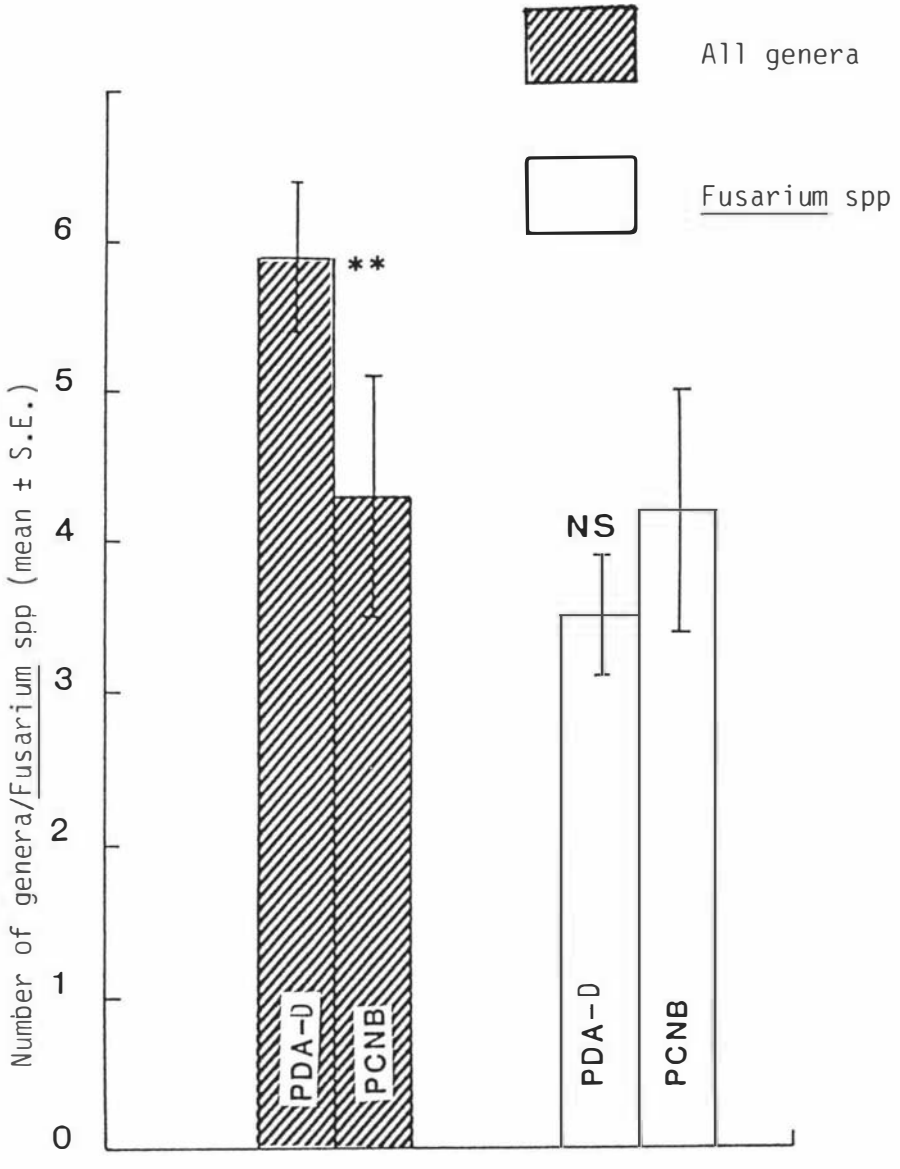


Table 3-30: Evaluation of the two media PDA-D and PCNB for their effect on the number of fungal genera and of Fusarium spp recovered from maize by the direct plating technique.

Sample code	No. Fusarium spp		Total no. fungal genera	
	PDA-D	PCNB	PDA-D	PCNB
T1	5	5	6	3
T1H	3	5	7	3
T2	3	3	5	4
T2H	4	3	5	2
T3	4	4	5	4
T3H	3	4	7	7
T4	4	8	4	3
T4H	3	3	5	5
J1	3	4	6	4
J2	4	3	5	5
J2H	4	4	7	4
RH	2	3	6	6
KH	4	6	9	6
Mean	3.5	4.2	5.9	4.3
	NS (P>0.05)		(P<0.01)	

NS: not significant

Figure 3-6: Comparison of the two media PDA-D and PCNB for their effect on the number of fungal genera recovered from maize samples as determined by the direct plating technique.

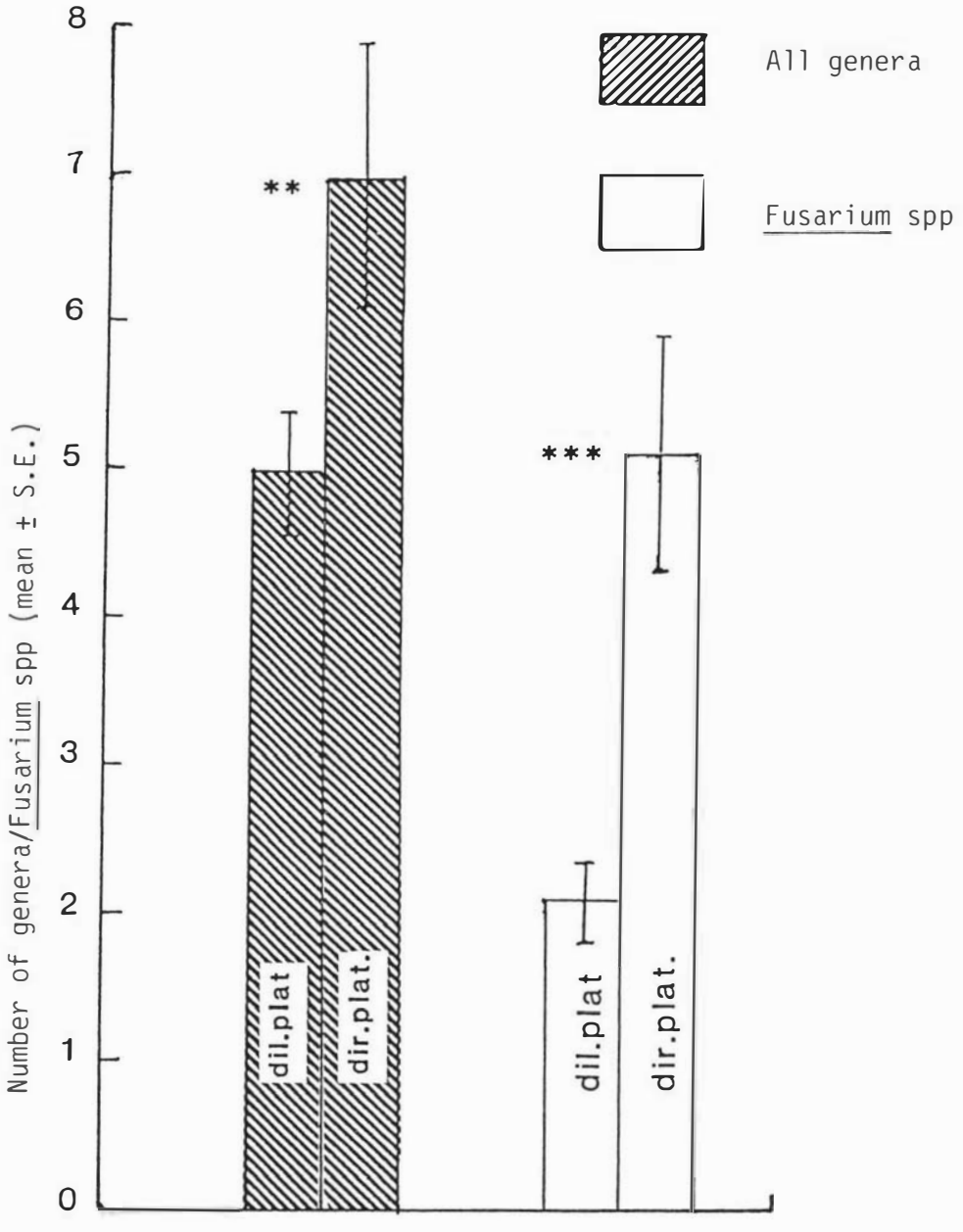


** P<0.01

Table 3-31: Evaluation of the direct plating and dilution plating techniques for their effect on the numbers of fungal genera and of *Fusarium* spp recovered from maize (both media).

Sample code	No. Fusarium spp		Total no. fungal genera	
	Dilution pl.	Direct pl.	Dilution pl.	Direct pl.
T1	4	5	8	7
T1H	3	5	6	9
T2	3	3	5	6
T2H	1	4	5	6
T3	2	4	6	5
T3H	2	4	5	9
T4	2	8	4	4
T4H	2	8	4	6
J1	3	5	7	6
J2	3	4	5	7
J2H	2	4	6	8
RH	2	3	4	6
KH	2	6	6	10
Mean	2.4	4.9	5.4	6.9
	$P<0.001$		$P<0.025$	

Figure 3-7: Evaluation of the direct plating and dilution plating techniques for their effect on the total number of fungal genera and the number of Fusarium spp recovered from maize (both media).



** P<0.01

*** P<0.001

Table 3-32: Evaluation of the two media PDA-D and PCNB and the two techniques dilution and direct plating for their effects on the total numbers of fungal genera and of Fusarium spp recovered from maize.

Sample code	PDA-D				PCNB			
	Fusarium spp		Total genera		Fusarium spp		Total genera	
	Dilution	Direct	Dilution	Direct	Dilution	Direct	Dilution	Direct
T1	2	5	5	6	2	5	4	3
T1H	1	3	5	7	2	5	5	3
T2	2	3	4	5	1	3	4	4
T2H	-	4	2	5	-	3	4	2
T3	2	4	5	5	2	4	6	4
T3H	1	3	4	7	1	4	5	6
T4	2	4	4	4	2	8	4	3
T4H	1	3	4	5	1	3	5	5
J1	2	3	6	6	2	4	6	4
J2	3	4	5	5	2	3	3	5
J2H	2	4	6	7	1	4	6	4
RH	3	2	4	6	3	3	4	6
KH	-	4	3	7	1	6	5	7
Mean	1.6	3.5	4.3	5.8	1.5	4.2	4.7	4.3
	P<0.001		P<0.001		P<0.001		NS (P>0.05)	

3.1.5.2. Frequency of occurrence of individual genera and *Fusarium* spp from all samples

Table 3-33 summarises the overall frequency of isolation of the individual genera from all samples (37) of maize, husk, litter and soil. A total of 30 genera was recorded. Two genera, *Fusarium* and *Penicillium* were recovered from 34 of the 37 samples (92%). *Cladosporium*, *Acremonium* and *Nigrospora* were also common (70-54%), whilst seven genera were each recorded from a single sample only. *Aspergillus* and *Beauvaria* were isolated from stored maize only, while *Coniothyrium*, *Preussia* and *Pythium* were isolated from soil samples only.

Table 3-33: Occurrence of fungi in the 37 samples of maize, husk, litter and soil examined (both techniques, both media).

Genus	No. of samples positive						
	Maize			Husk	Litter	Soil	Occurrence in 37 samples
	field	harvest	stored				
Acremoniella	1	3	1	1	1	-	7
Acremonium	6	7	-	4	3	4	24
Alternaria	2	2	3	4	2	1	14
Arthrinium	1	1	1	-	1	1	5
Ascochyta	-	-	1	-	1	1	3
Aspergillus	-	-	4	-	-	-	4
Beauvaria	-	-	4	-	-	-	4
Chaetomium	1	2	2	-	-	2	7
Chrysosporium	-	-	1	-	1	-	2
Cladosporium	6	5	4	4	4	3	26
Coniothyrium	-	-	-	-	-	2	2
Epicoccum	3	7	1	3	3	-	17
Fusarium	6	7	9	4	4	4	34
Fusicoccum	-	1	-	-	-	-	1
Gliocladium	-	-	-	-	1	3	4
Mucor	5	4	3	4	1	1	18
Myrothecium	-	-	-	-	1	-	1
Nigrospora	2	7	6	3	2	-	20
Paecilomyces	-	-	-	-	-	1	1
Penicillium	6	7	11	4	2	4	34
Phoma	1	2	-	1	4	2	10
Preussia	-	-	-	-	-	1	1
Pythium	-	-	-	-	-	2	2
Rhizopus	3	-	4	-	-	-	7
Schizophyllum	-	1	-	-	-	-	1
Scopulariopsis	-	1	5	-	1	3	10
Sependonium	-	-	-	-	-	1	1
Trichoderma	-	3	-	-	1	3	7
Ulocladium	-	-	2	-	-	-	2
Verticillium	1	3	-	1	2	4	10
Total genera	13	17	17	11	18	19	
No. samples examined	6	7	12	4	4	4	37

Two criteria, the mean contamination rate (% of kernels infected in all samples) and the frequency of occurrence (% presence in all samples examined) as assessed from the results of the direct plating assays can be used to evaluate the changing significance of various fungal genera associated with maize samples from field, harvest and storage.

The seven most frequently encountered genera, together with Aspergillus, are listed in Table 3-34 and their changing patterns of occurrence illustrated in Figures 3-8 and 3-9. Acremonium, Fusarium and Mucor were common in field samples but decreased in occurrence from harvest to storage. Other genera such as Nigrospora and Epicoccum showed greater occurrence at harvest time. Penicillium maintained its level from the field to storage. In contrast, Aspergillus was only recorded from stored maize.

Table 3-34: Comparison of the mean contamination rates (%) and the frequencies of occurrence (%) of eight fungal genera in all field, harvest and stored maize samples.

	Contamination rate (%) in maize from:			Frequency of occurrence (%) in maize from:		
	Field	Harvest	Storage	Field	Harvest	Storage
Acremoniella	0.3	15.4	0.3	16.6	42.8	8.2
Acremonium	9.3	3.4	-	100	71.4	-
Aspergillus	-	-	9.1	-	-	33.3
Epicoccum	1.7	1.6	0.3	50.0	85.7	8.3
Fusarium	75.6	58.3	1.5	100	100	75.0
Mucor	27.6	4.0	0.3	66.7	57.0	25.0
Nigrospora	1.0	5.1	8.7	33.0	85.7	50.0
Penicillium	20.3	27.7	20.0	100	100	91.7
No. samples examined	6	7	12	6	7	12

Figure 3-8: Contamination rates (mean %) of eight fungal genera from field, harvest and stored samples of maize (6, 7 and 12 samples respectively).

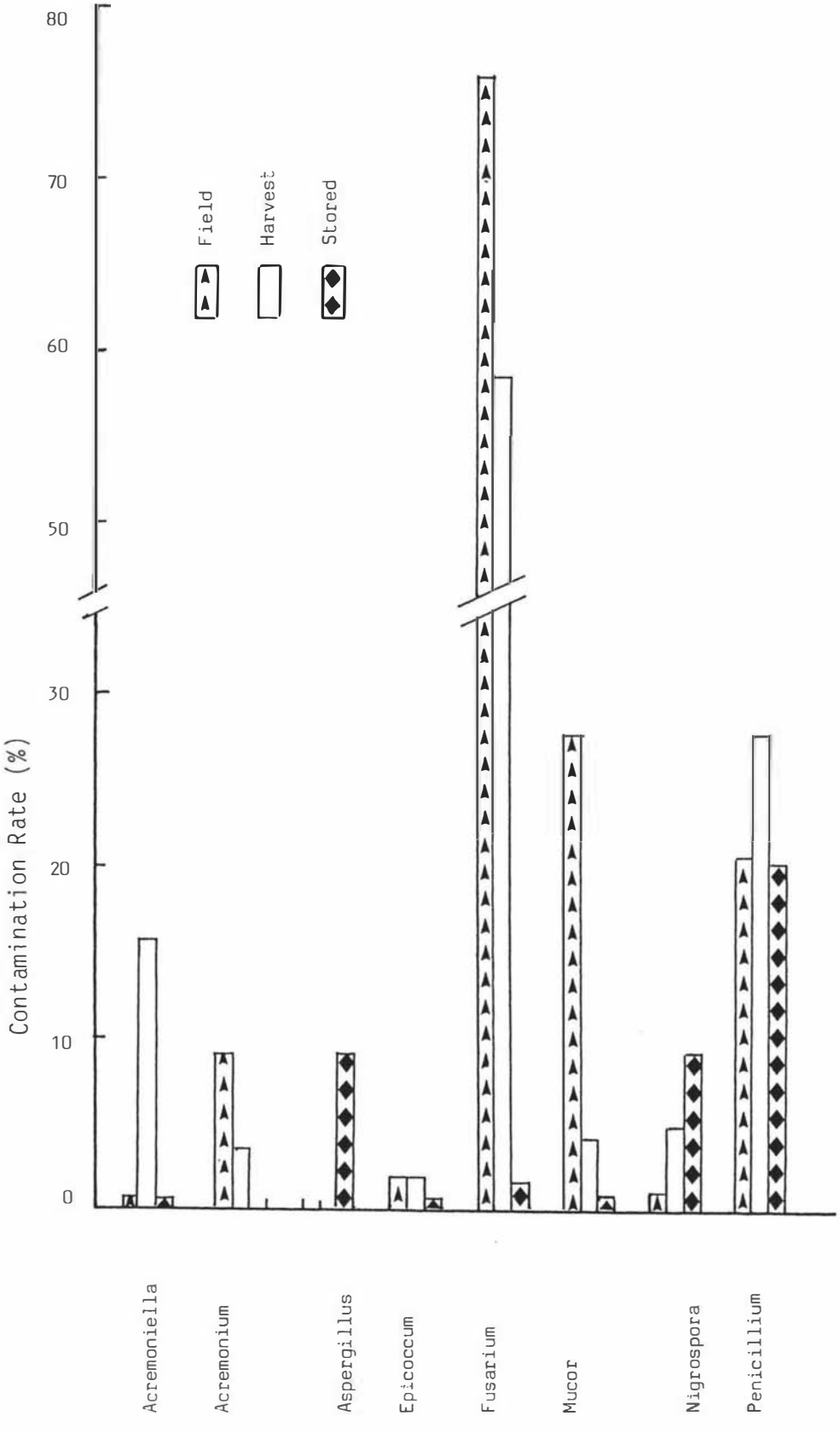
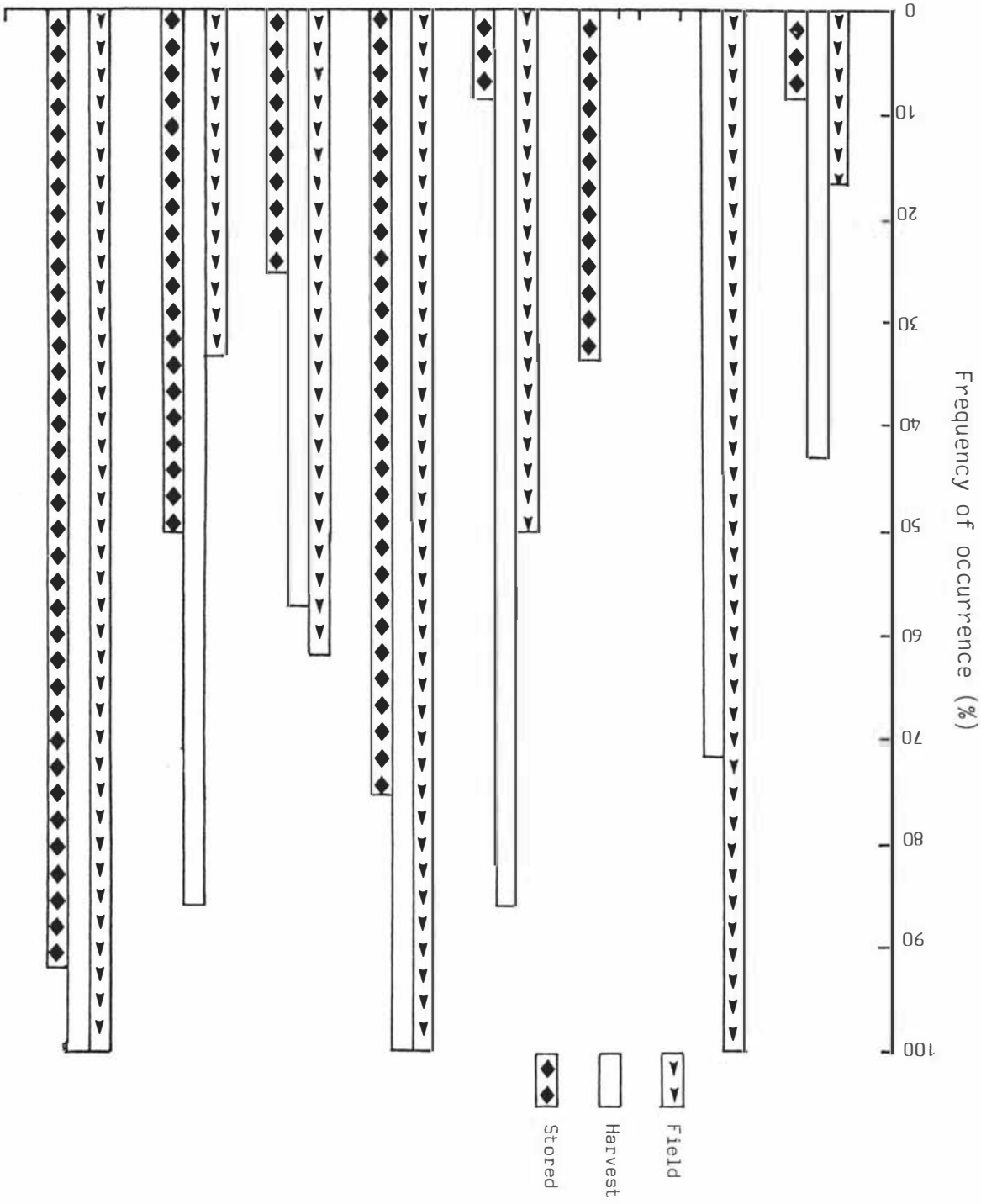


Figure 3-9: Frequency of occurrence of eight fungal genera in field, harvest and stored samples of maize (6, 7 and 12 samples respectively).



A total of 15 Fusarium spp was recorded from the 34 samples examined in which speciation of Fusarium isolates was undertaken (Table 3-35). Stored maize contained the fewest species (F. subglutinans, F. poae and F. graminearum only), whilst soil and litter samples yielded ten and 11 species respectively. Field and harvest maize samples contained ten species each. F. graminearum occurred at the highest frequency (67.6% of all samples), F. culmorum and F. subglutinans were also common at 61.7% each. The lowest frequencies of occurrence (one out of 34 samples each) was recorded for F. merismoides, F. equiseti and F. moniliforme.

When the overall levels of Fusarium spp contaminating maize in the field and at harvest time were compared, a highly significant difference ($P<0.001$) was indicated between the two populations (Table 3-36 and Figure 3-10). Maize samples from the field were found to be contaminated by Fusarium spp at significantly higher levels than harvest samples in enumerations done by direct plating (mean contamination rates 75.8% in field samples, 58.3% in harvest samples). But the actual numbers of different Fusarium spp isolated from field and harvest samples gave no significant difference ($P>0.05$) (mean 4.8 from field and 4.9 from harvest, Table 3-36). In contrast, total fungal population levels from field and harvest samples showed no significant differences ($P>0.05$) although the total number of genera at harvest time slightly increased (mean 7.6 compared to 5.8 from preharvest, Table 3-36).

Table 3-35: Occurrence of Fusarium spp in 34 samples examined (both techniques, both media).

Fusarium spp	No. of samples positive						
	Maize			Husk	Litter	Soil	Occurrence in 34 samples
	field	harvest	stored				
F. graminearum	6	7	3	3	3	1	23
F. culmorum	5	7	-	3	3	3	21
F. subglutinans	3	5	8	2	1	2	21
F. oxysporum	3	1	-	1	2	3	10
F. acuminatum	6	2	-	2	3	-	13
F. avenaceum	1	1	-	1	1	-	4
F. poae	4	4	2	3	-	-	13
F. solani	-	-	-	-	-	3	3
F. merismoides	-	-	-	-	-	1	1
F. lateritium	-	1	-	-	2	1	4
F. equiseti	-	-	-	-	1	-	1
F. crookwellense	1	1	-	1	2	1	6
F. sambucinum	-	-	-	-	1	1	2
F. stilboides	1	2	-	-	2	2	7
F. moniliforme	1	-	-	-	-	-	1
Total spp	10	10	3	8	11	10	
No. samples examined	6	7	12	3	3	3	34

Table 3-36: Contamination rates (%) of maize kernels by all fungi and by Fusarium spp in field and harvest samples (direct plating technique, mean of two media).

	All fungi				Fusarium spp			
	Field		Harvest		Field		Harvest	
Sample code	% contamin.	No. genera	% contamin.	No. genera	% contamin.	No. species	% contamin.	No. species
T1	98	7	100	7	45	5	87	5
T2	98	6	82	5	73	3	40	4
T3	88	5	94	9	63	4	37	4
T4	100	4	84	6	100	8	39	4
J1	100	6	*	*	77	5	*	*
J2	100	7	100	8	97	4	74	4
KH	*	*	100	7	*	*	51	3
RH	*	*	100	11	*	*	80	6
Mean	97.3	5.8	94.3	7.6	75.8#	4.8	58.3#	4.9

*: not assayed
#: P<0.001

Figure 3-10: Comparison of contamination by Fusarium spp of maize in the field and at harvest (direct plating technique).

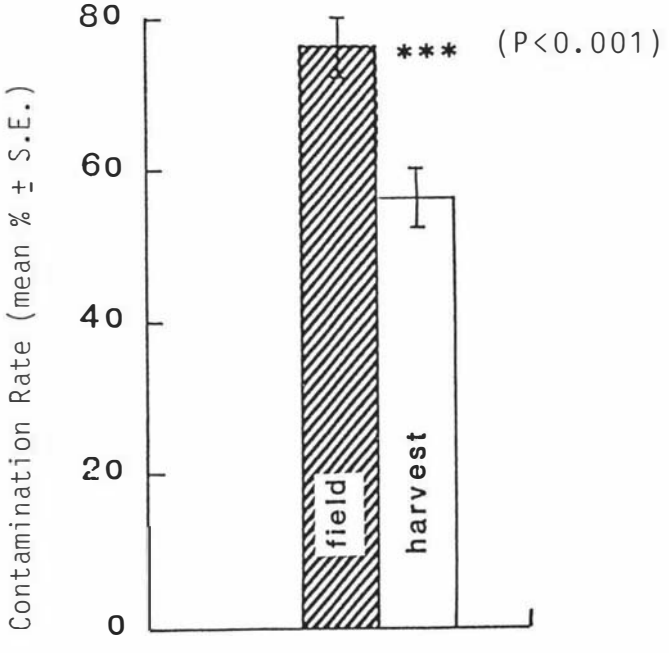


Table 3-37 shows the level of contamination by the individual fusaria in field and harvest samples (five fields). Three Fusarium spp, F. graminearum, F. culmorum and F. acuminatum, showed a significant decrease ($P<0.001$) in harvest samples, whilst F. subglutinans gave the same level. The remaining species were present in low numbers only. Overall, the Fusarium population dropped significantly ($P<0.001$) at harvest time (Figure 3-10).

Table 3-37: Contamination rates (%) of maize kernels by individual Fusarium spp, field and harvest samples (direct plating technique, mean of two media).

	Contamination rate											
	Field samples						Harvest samples					
Fusarium spp	T1	T2	T3	T4	J2	Mean	T1H	T2H	T3H	T4H	J2H	Mean
F. graminearum	23	51	6	62	77	43.8	24	18	23	29	49	28.6
F. culmorum	10	19		34	13	15.2	5	19	4	8	12	9.6
F. subglutinans			28	11		7.8	34		3	1		7.6
F. oxysporum	3		5	1		1.8						0
F. acuminatum	8	4	26	1	12	10.2		3		2		1.0
F. avenaceum						0					12	2.4
F. poae	3			1		0.8	20		7		3	6.0
F. crookwellense				3		0.6		1				0.2
F. stilboides					1	0.2	5					1.0
F. moniliforme				1		0.2						0
No. species each sample	5	3	4	8	4		5	4	4	4	4	

3.2. OCCURRENCE OF FUSARIUM MYCOTOXINS IN MAIZE

Twenty samples of maize obtained from the field, at harvest and during storage were extracted and analysed for the mycotoxins - DON, DAS, T2-toxin, ZEA and MON as described in Section 2.2. In addition, four samples of compounded poultry rations containing maize as their major ingredient were included for comparison.

3.2.1. Thin Layer Chromatography

In Table 3-38, the results from TLC assays of DON, DAS and T-2 toxin are expressed as mycotoxin present (+) or absent (-) after comparison of the fluorescence characteristics of positive spots from samples with those of authentic standards described in Section 2.2.6.1. The Rf values of the Fusarium mycotoxins in the solvent chloroform-methanol (93:7) were approx 0.23, 0.79 and 0.84 (Figure 3-11) for DON, DAS and T-2 toxin respectively. Assays of ZEA (Rf value 0.73) could be presented semiquantitatively as ppm (Section 2.2.6.4).

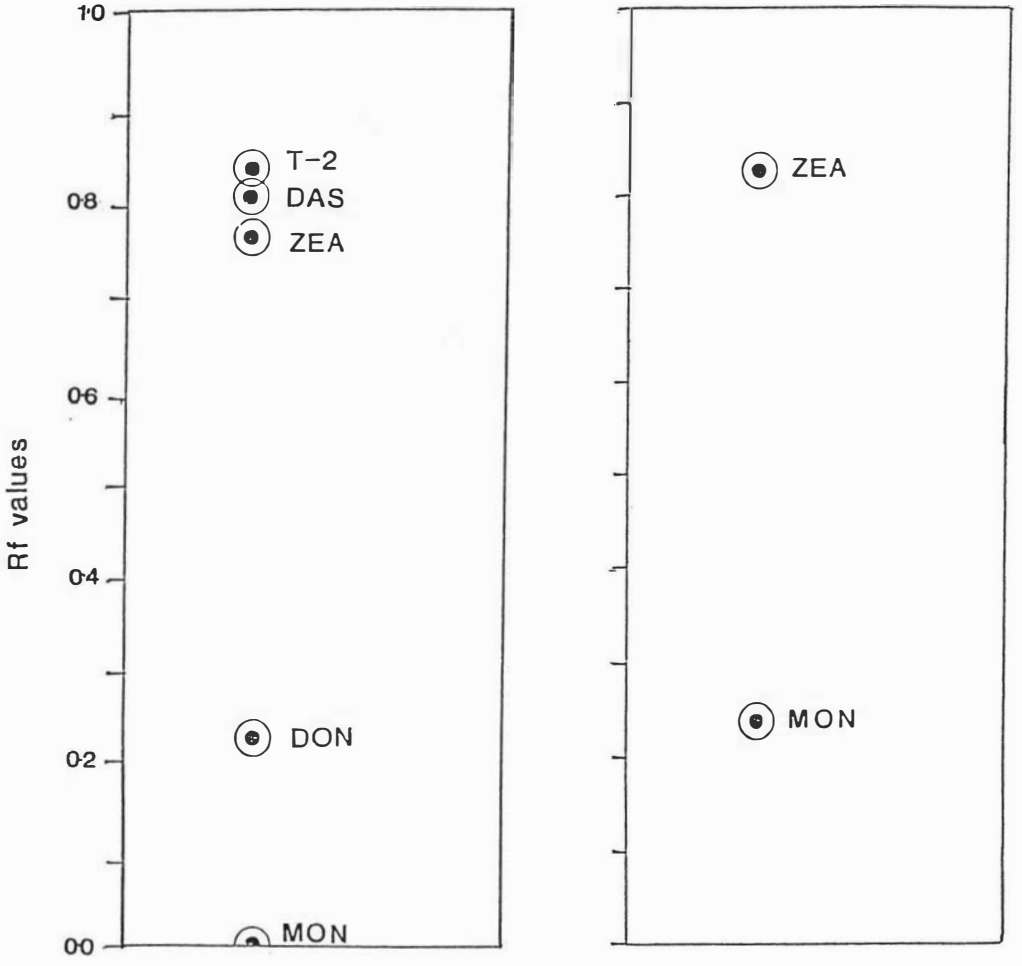
The frequencies of T-2 toxin, DON and ZEA were 25%, 29% and 54.2% respectively in the 24 samples examined. No DAS was detected by TLC in any of the samples. The maximum number of mycotoxins detected by the TLC method in any one sample was three (sample A3) while six samples showed two mycotoxins each. No Fusarium toxins were detected in the poultry rations (GC1-4) by TLC.

Table 3-38: Thin layer chromatography analysis of maize and poultry rations sampled for DON, DAS, T-2 toxin and ZEA.

Sample code	Mycotoxin			
	DON	DAS	T-2 toxin	ZEA (ppm)
J2	-	-	+	0.32
T3	-	-	-	0.5
T4	-	-	-	2.2
T1	-	-	-	0.4
J1	-	-	-	0.4
T2	-	-	-	1.9
T1H	-	-	+*	4.6
KH	-	-	-	-
T2H	-	-	+*	-
RH	+	-	-	2.9
T4H	-	-	+	0.2
J2H	-	-	-	16.0
T3H	-	-	+	-
C2	-	-	-	-
D1	+	-	-	-
B3	+	-	-	-
A1	+	-	-	0.1
A3	+	-	+*	0.1
AN-1	+	-	-	0.8
AN-2	+	-	-	-
GC1	-	-	-	-
GC2	-	-	-	-
GC3	-	-	-	-
GC4	-	-	-	-
Total +ve	7	0	6	13
% +ve	29	0	25	54.2

*: doubtful

Figure 3-11: Diagrammatic representation of the analysis of mycotoxin standards according to their Rf values on developed TLC plates.



TLC without fluorescent indicator, developing solvent chloroform-methanol (93:7).

TLC with fluorescent indicator, developing solvent toluene-acetone-methanol (5:3:2).

3.2.2. Gas Chromatography with Flame Ionisation Detection

Figure 3-12 shows a gas chromatogram of the mycotoxin standards after conversion to the trimethyl silyl (TMS) ethers (as described in Section 2.2.6.2). The amounts of toxin injected were 48 ng for DON and 96 ng each for DAS, T-2 toxin and ZEA. The amount of internal standard (C_{24}, C_{26}, C_{28} and C_{30} as a mixture) was 80 ng. The identities of the peaks are shown on Figure 3-12. Peak f contained unresolved C_{28} and ZEA-TMS. Thus ZEA could not be easily quantitated on the column if the internal standard mix was present. Quantitation of ZEA was unreliable even in the absence of overlapping C_{28} because of the instability of ZEA-TMS. Where C_{28} interfered with estimation of the ZEA-TMS peak area/height, an estimate could sometimes be made by direct comparison with another chromatogram containing internal standards only.

The majority of the maize samples contained large amounts of interfering compounds, particularly those with similar retention times to T-2-TMS. For example, sample B3 (Figure 3-13) was contaminated with small amounts of DON and DAS (peaks a and b) but large amounts of material coinciding with the T-2 toxin peak (peak e). Calculation of the concentrations of these peaks gave values of 0.009, 0.4 and 37 ppm for DON, DAS and T-2 toxin respectively (the value for T-2 toxin was later found to be false and was in fact very small according to the GC-MS method described later).

There were a few samples which did not show too much impurity. Sample J2 (Figure 3-14) was contaminated with T-2 toxin (peak e); the amount of T-2 toxin in this sample was similar by both GC and GC-MS (0.18 and 0.2 ppm respectively). Sample J2 was also contaminated with DON (peak a), DAS (peak b) and ZEA + C_{28} (peak f).

The problems of interference with the assay of T-2 toxin and the overlapping of ZEA with C_{28} were overcome by using a very long capillary column (50 m) with an altered temperature programme (Section 2.2.3.1) as shown in Figure 3-15. All of the mycotoxin standards as well as the internal standards were well separated, with the exception of the DAS-TMS peak b and C_{24} peak c, which came closer owing to the

change in temperature programming, but they still showed near baseline resolution. The long column had the advantage of giving sharp peaks with better resolution.

Figure 3-16 is a gas chromatogram of the TMS derivatives from a naturally-contaminated maize sample (AN-2) with ZEA present (peak d). The ZEA-TMS peak was well separated from the C_{28} peak (peak c).

Figure 3-12: Gas chromatogram of TMS-derivatives of mycotoxin standards on 12 m capillary column. Peak identification: a = DON-TMS, b = DAS-TMS, c = C_{24}, d = C_{26} (internal standard) e = T-2-TMS, f = ZEA-TMS + C_{28} (internal standard), g= C_{30} (internal standard). (Attenuation was changed from 64 to 16.)

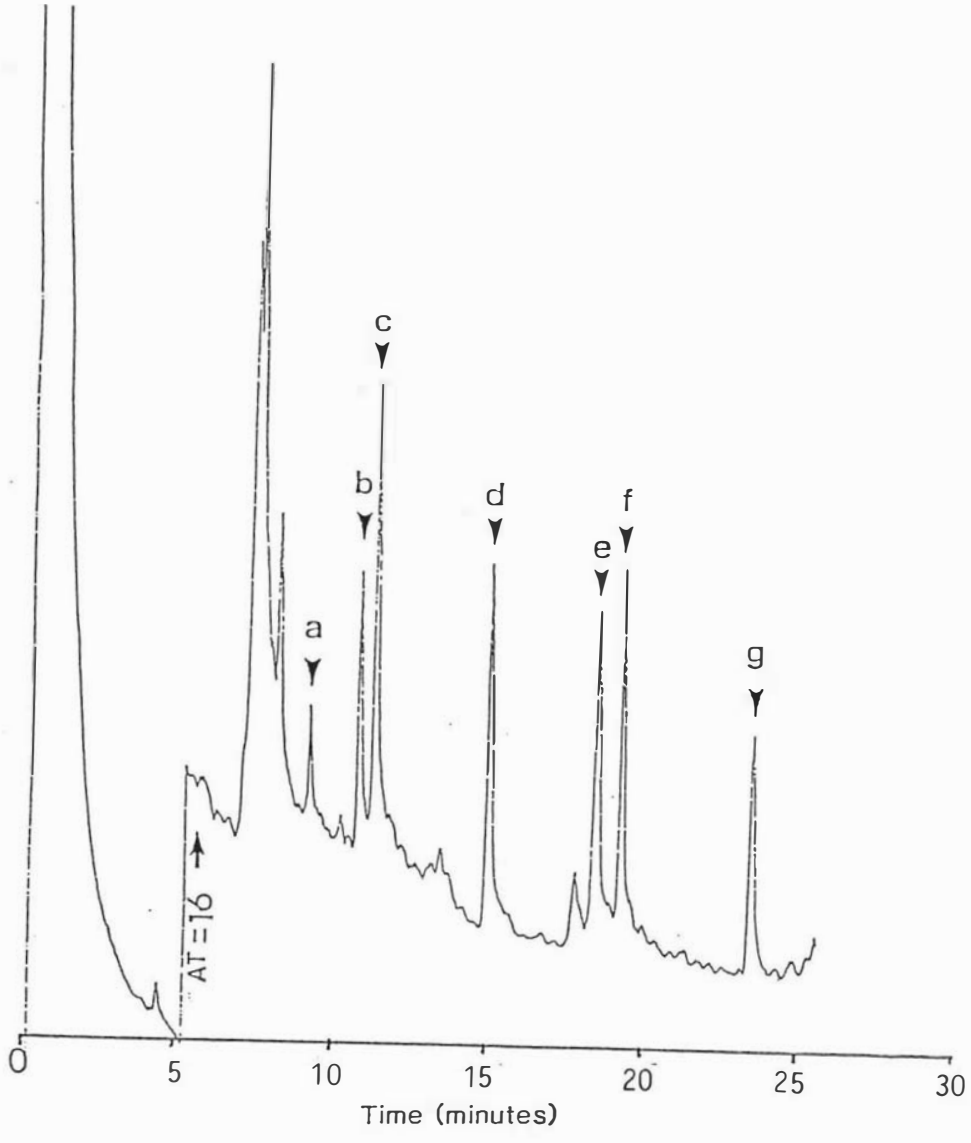


Figure 3-13: Gas chromatogram on 12 m capillary column, silylated extracts from naturally-contaminated maize sample B3.
Peak identification:
a = DON-TMS, b = DAS-TMS, c = C_{24}, d = C_{26},
e = T-2-TMS + unknown, f = C_{28} + ZEA-TMS
(Attenuation was changed from 64 to 16.)

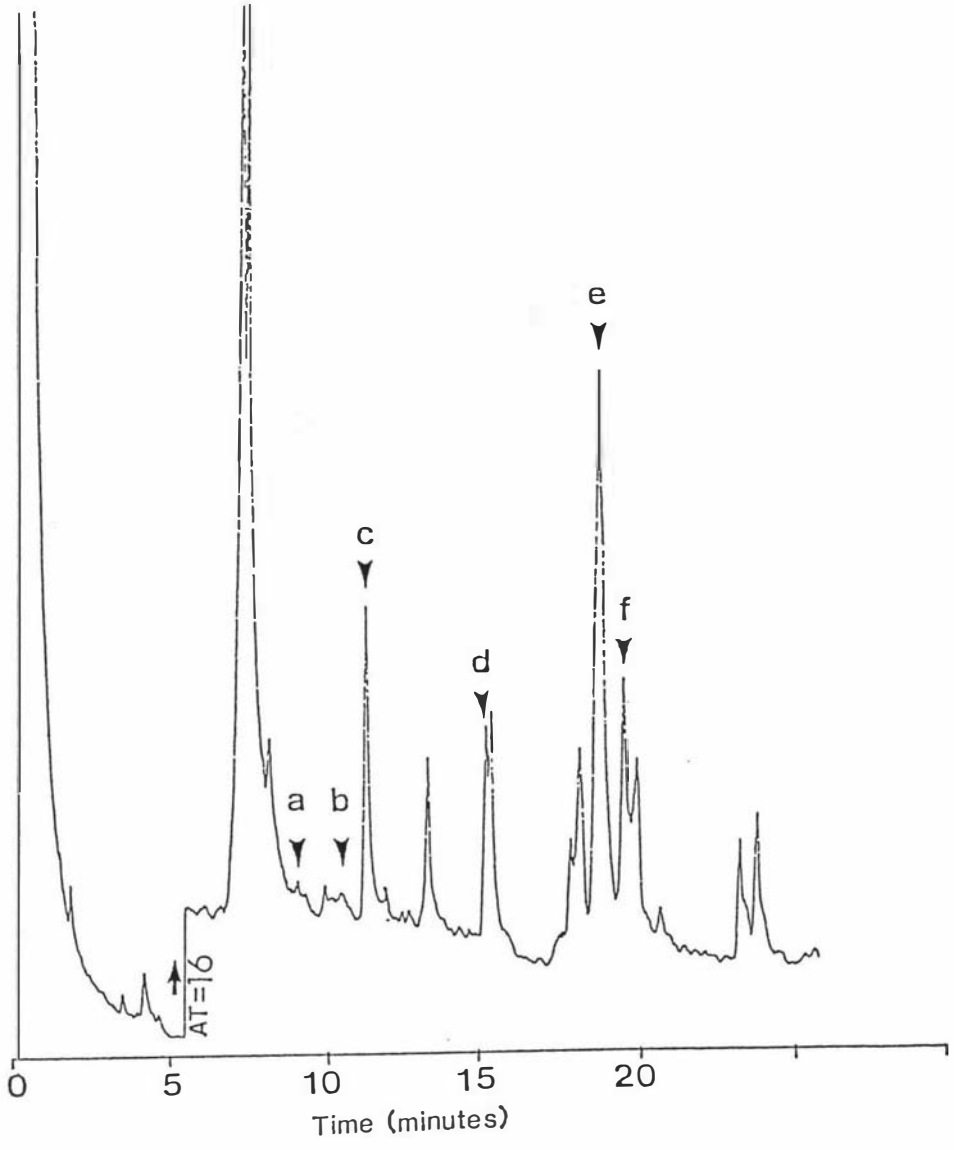


Figure 3-14: Sample J2 contaminated with DON, DAS, T-2 toxin and ZEA. Fewer impurities are apparent. Peak identification: a = DON-TMS, b = DAS-TMS, c = C_{24}, d = C_{26}, e = T-2-TMS and f = C_{28} + ZEA-TMS.
(Attenuation was changed from 64 to 16.)

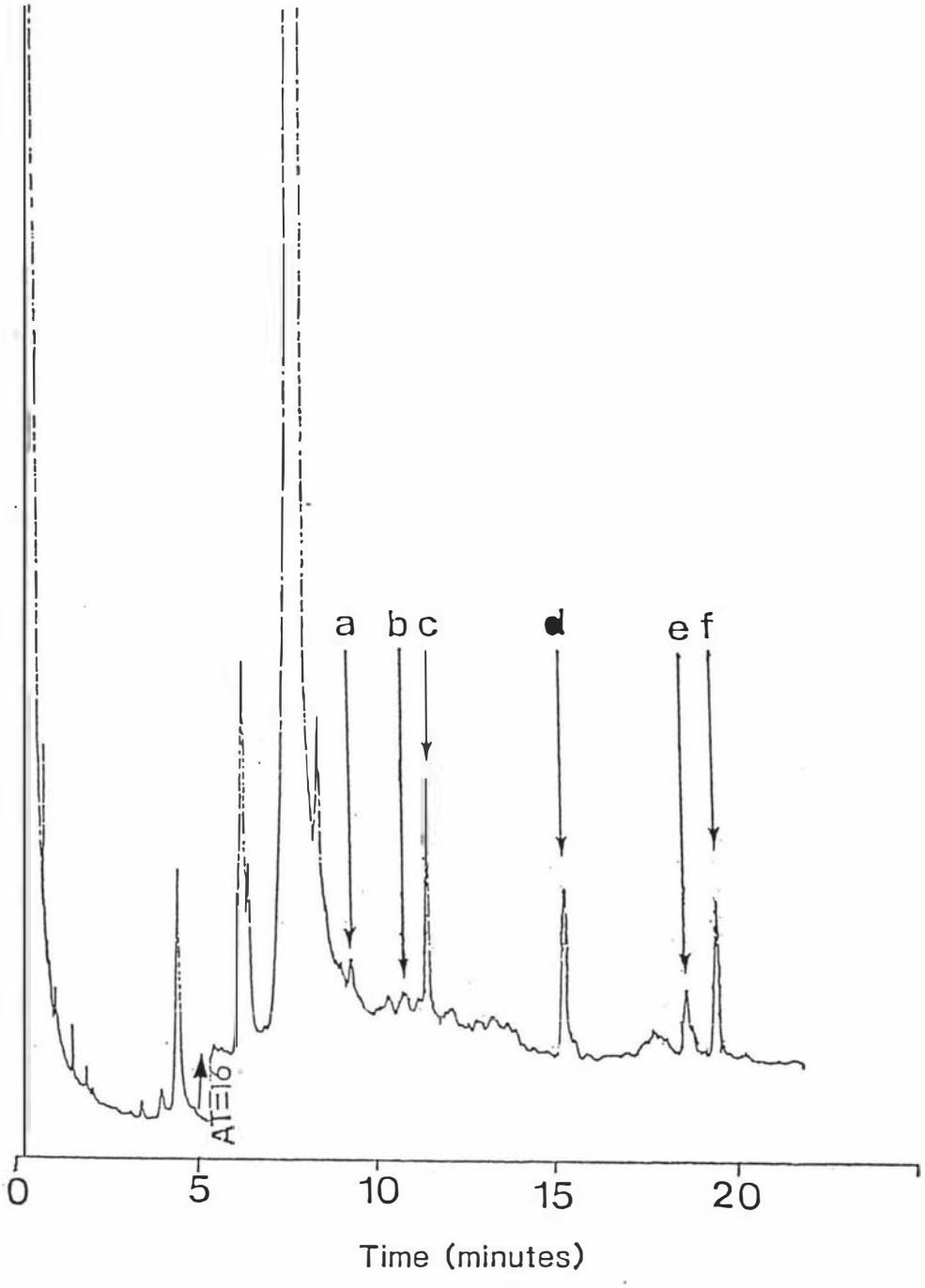


Figure 3-15: Gas chromatogram of TMS-derivatives of mycotoxin standard on 50 m capillary column. Peak identification:
a = DON-TMS, b = DAS-TMS, c = C_{24}, d = C_{26}
e = T-2-TMS, f = C_{28} g = ZEA-TMS (Attenuation 64).

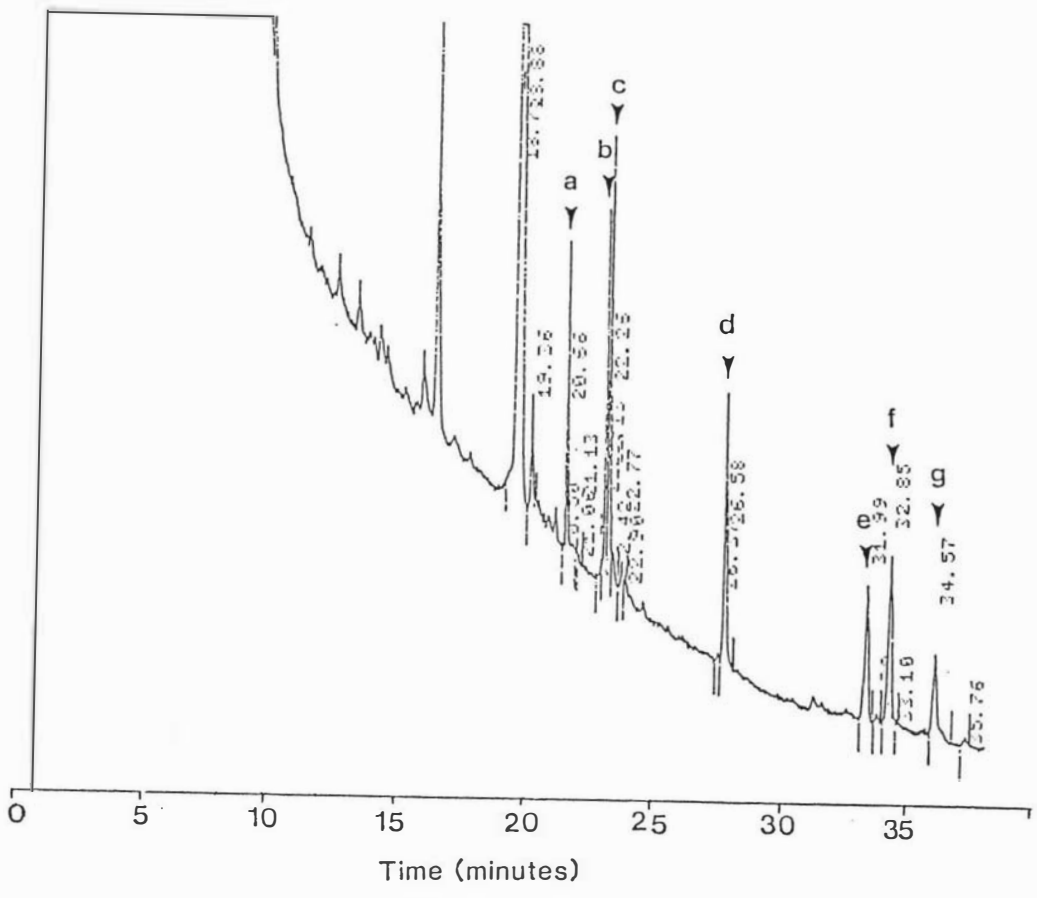
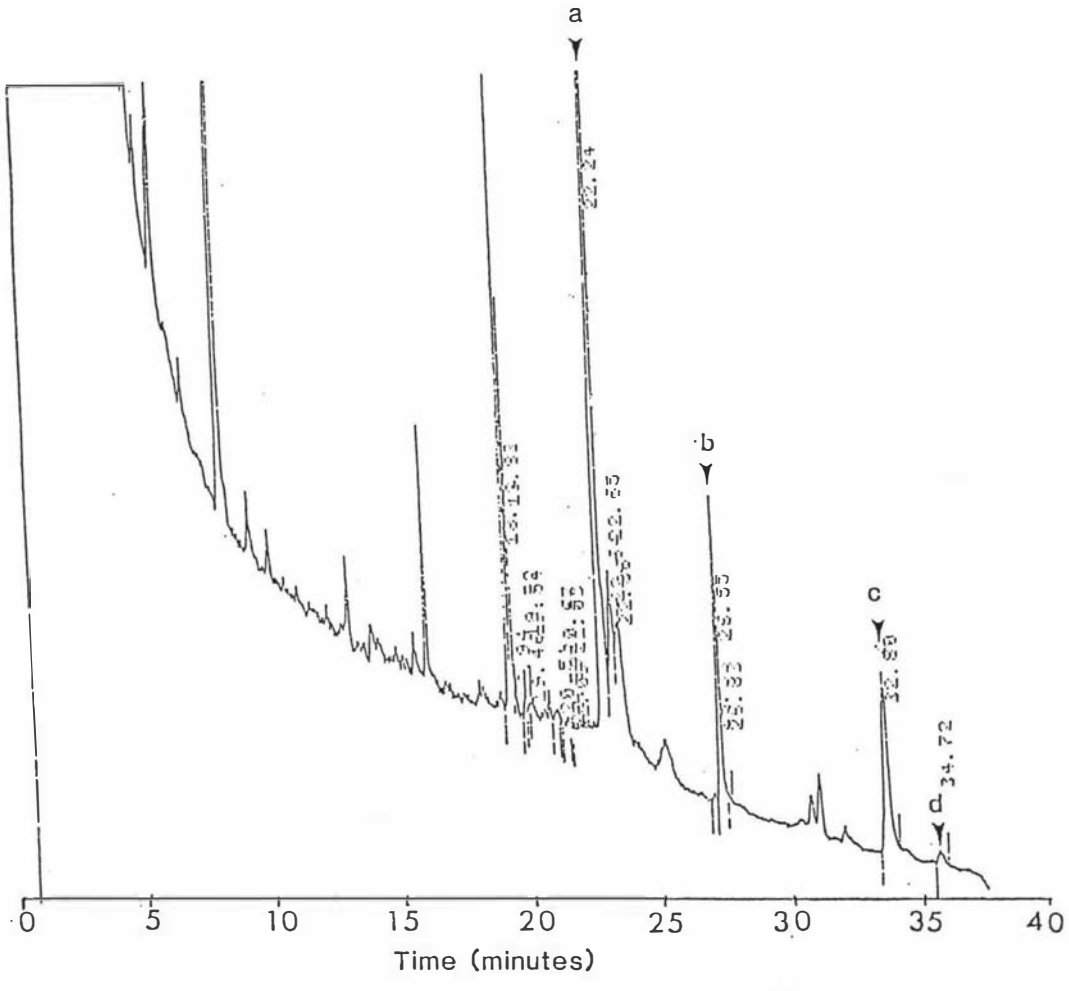


Figure 3-16: Gas chromatogram of naturally-contaminated sample (AN-2) after derivatisation with TMS, on 50 m capillary column.
Peak identification:
a = C_{24}, b = C_{26}, c = C_{28}, d = ZEA-TMS
(Attenuation was 64 except for peak a, which was 32.)



The retention times for peaks, both in mycotoxin standard mixtures and some of the samples (as determined by the computer) are summarised in Table 3-39. It can be seen that there are considerable differences between the two columns with regard to separations obtained. For examaple, ZEA and C_{28} were not separated on the 12 m column but were widely separated on the 50 m column. Such differences may have been due to degradation of the stationary phase on the 12 m column (compared with the Forest Research Institute 12 m column, Figure 3-17). The level of T-2 toxin estimated was frequently higher than that obtained by GC-MS (Section 3.2.3). This was evidently due to the presence of an interfering compound in the samples. No such problem was observed with the 50 m column, on which a large peak in the samples often occurred before the T-2 toxin peak.

Table 3-39: Retention time (min) of the peaks of the mycotoxin standard mixture (MTM) and some of those from the samples, (computer analysis).

A = on short column

Sample or MTM	DON	DAS	C_{24}	C_{26}	T-2	C_{28}	ZEA	Date of run
MTM	8.97	10.43	10.97	14.69	17.99	18.81	18.81	27.2.86
B3	8.95	10.44	10.92	14.67	18.08	18.81	18.81	"
J2	8.86	10.34	10.95	14.65	17.96	18.79	18.79	"
MTM	8.93	10.45	10.92	14.61	18.02	18.76	18.76	25.2.86

B = on long column

MTM	20.63	22.09	22.22	26.53	31.91	32.77	34.67	27.6.86
AN-2	20.67	-	22.24	26.55	-	32.80	34.74	"
MTM	20.66	22.13	22.26	26.58	31.99	32.85	34.57	30.6.86

Figure 3-17: Gas chromatography of mycotoxin standard on 12 m capillary column using Hewlett Packard GC system at Forest Research Institute. Peaks a, b, e and g for DON, DAS, T-2 toxin and ZEA derivatives respectively, with lesser, unknown peaks being by-products, peaks c, d, f and h internal standard. (Attenuation was 16.)

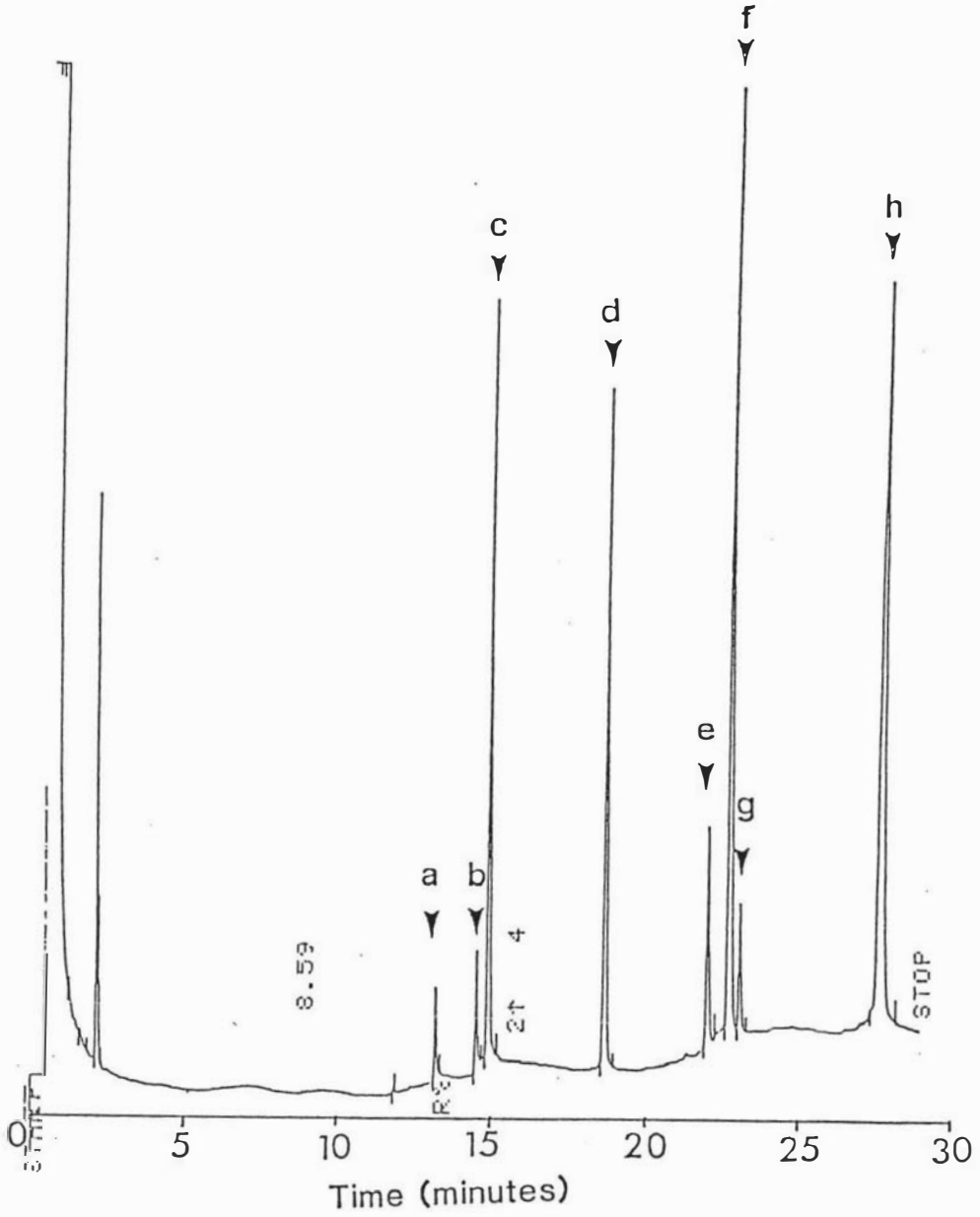


Table 3-40 shows the semiquantitative results for the four Fusarium mycotoxins DON, DAS, T-2 toxin and ZEA. Results obtained from the GC-FID indicated a very high frequency of occurrence of the above Fusarium mycotoxins in comparison to the result of the TLC method (Table 3-38). The most common was ZEA (70.8% of samples) and the least common was DAS (16.7% of samples). DON and T-2 toxin were detected in 58.3% and 62.5% of samples respectively. Four maize samples (J2, T3, T4 and B3) were contaminated with four mycotoxins each, another four samples were contaminated with three mycotoxins each while ten samples contained two mycotoxins each. The negative samples were from poultry rations (GC2, GC3 and GC4), together with one maize sample (T1H).

The lowest apparent level of toxin detected was 0.009 ppm for DON in sample B3. T-2 toxin and ZEA contaminated some samples at high levels. Samples B3 and RH gave 37 and 25 ppm respectively of apparently T-2 toxin, and sample A1 gave 17 ppm of ZEA. These were later found to be false positives.

Table 3-40: Semiquantitative assessment of four Fusarium mycotoxins in 24 samples of maize and poultry rations, using the GC-FID method.

Sample code	Level of mycotoxin (ppm)				No. of toxins detected
	DON	DAS	T-2 toxin	ZEA	
J2	0.3	1.3	0.18	1.8	4
T3	1.5	4.2	1.7	3.0	4
T4	0.3	0.3	0.6	3.1	4
T1	0.3	-	trace	trace	3
J1	4.9	-	-	trace	2
T2	-	-	-	trace	1
T1H	-	-	-	-	0
KH	-	-	13.0	3.9	2
T2H	0.14	-	-	0.5	2
RH	-	-	25.0	3.8	2
T4H	0.09	-	trace	-	2
J2H	1.3	-	8.0	3.0	3
T3H	-	-	1.2	1.4	2
C2	-	-	0.02	-	1
D1	0.08	-	0.08	-	2
B3	0.009	0.4	37.0	6.6	4
A1	0.14	-	4.7	17.0	3
A3	-	-	3.1	4.2	2
SA.1	0.6	-	-	1.2	2
SA.2	0.12	-	-	1.7	2
GC1	0.4	-	6.7	2.9	3
GC2	-	-	-	-	0
GC3	-	-	-	-	0
GC4	-	-	-	-	0
Total +ve	14	4	15	17	
% +ve	58.3	16.7	62.5	70.8	

3.2.3. Gas Chromatography - Mass Spectrometry

Detection, quantitation and confirmation of the natural occurrence of Fusarium mycotoxins in maize samples were more reliably achieved by using the GC-MS system as described in Section 2.2.3.1,B. Figure 3-18 shows the ion chromatograms of five selected ions for each mycotoxin standard under the conditions described in Section 2.2.6.3. The TMS derivatised standard mixtures of the four mycotoxins were injected at the level of 80 ng of DON and 120 ng for each of DAS, T-2 toxin and ZEA. Using an open split interface, only 90% of the amount of injected material is analysed by the mass spectrometer. SIM ion chromatograms A, B, C and D (Figure 3-18) show the approximate retention times of DON-TMS (12.55 min), DAS-TMS (13.9 min), T-2-TMS (21.2 min) and ZEA-TMS (22.3 min). The peaks on five selected ion chromatograms for each derivative have the same retention time.

The data stored in the computer system can be analysed and presented in different forms. Figure 3-19 shows the mass spectrum of the selected ions for each mycotoxin standard derivative as well as the whole SIM ion chromatogram of the mixture. Full scan mass spectra of the TMS derivatives of each mycotoxin standard were obtained by using the total ion monitoring mode (Figure 3-20) to compare with SIM mode for absolute confirmation.

The chromatograms obtained from an extract of a spiked maize sample after derivatisation are shown in Figure 3-21. SIM ion chromatograms of the four mycotoxins were identical to those of authentic mycotoxin standards, in both retention time and the ratios of ion intensities. This chromatogram was unusual in that a second peak appeared with a similar fragmentation pattern to ZEA-TMS but an an earlier retention time (19.55 min). The identity of the peak is unknown and it did not appear in any of the samples.

Typical natural contamination with all four Fusarium mycotoxins is exemplified by sample J2. This maize sample was contaminated with DON, DAS, T-2 toxin and ZEA. The chromatograms of the mycotoxins are shown in Figure 3-22. The SIM ion chromatograms were identical to those of the authentic mycotoxin standards. Two ions (M/Z 173 and 122) from T-2 toxin showed a second peak corresponding to an unknown

component. Quantitation of the level of contamination gave 0.3, 0.9, 0.2 and 2.4 ppm for DON, DAS, T-2 toxin and ZEA respectively (Table 3-41).

Figure 3-23 shows two different amounts of ZEA in contaminated samples AN-1 and T2. Calculations revealed the level of contamination to be 0.33 and 1.3 ppm for AN-1 and T2 samples respectively (Table 3-41).

Results from the GC-MS method for detection and quantitation of DON, DAS, T-2 toxin and ZEA, as summarised in Table 3-41, indicated that T-2 toxin had the highest frequency of occurrence among the 24 samples examined. DAS was the least common. The levels of occurrence of DAS, DON, ZEA and T-2 toxin were 25%, 41.7%, 50% and 54.2% respectively. Two samples, J2 and T4, were contaminated with four mycotoxins and eight samples were contaminated with two mycotoxins. No Fusarium mycotoxins were found in the four poultry ration samples (GC1, GC2, GC3 and GC4). The limit of detectability for the four toxins was estimated to be between 20-40 ppb (20-40 ng/kg).

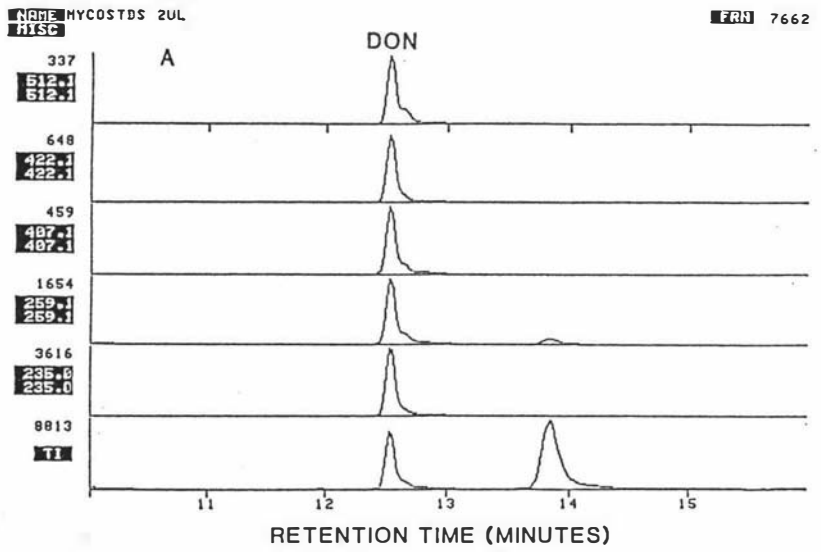
Figure 3-18: SIM ion chromatograms of mycotoxin standards

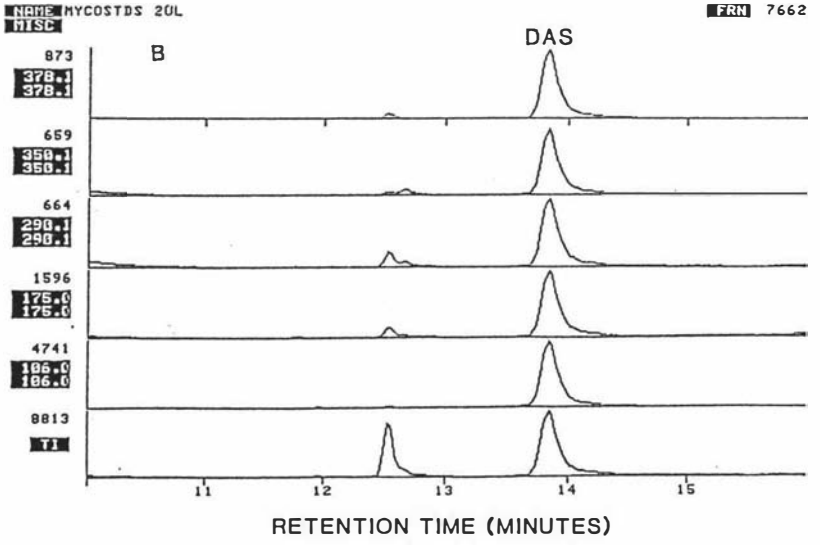
(A) DON-TMS;

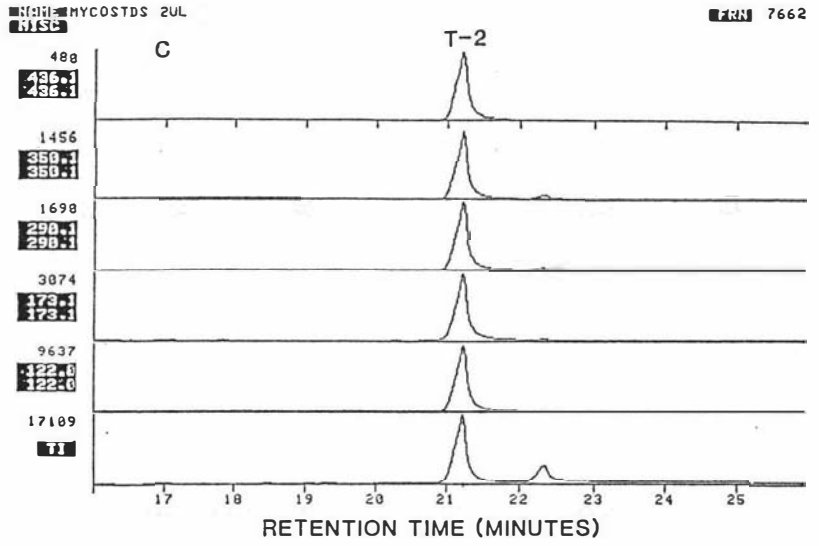
(B) DAS-TMS;

(C) T-2-TMS;

(D) ZEA-TMS
with retention times of 12.55, 13.9, 21.2 and 22.3 min respectively.


NAME MYCOSTDS 20UL
MISC
FRN 7662
A
DON
337
512.1
512.1
648
422.1
422.1
459
407.1
407.1
1654
259.1
259.1
3616
235.0
235.0
8813
TI
11
12
13
14
15
RETENTION TIME (MINUTES)


NAME MYCOSTDS 20UL
MISC
FRN 7662
B
DAS
873
378.1
378.1
659
350.1
350.1
664
290.1
290.1
1596
175.0
175.0
4741
186.0
186.0
8813
TI
11
12
13
14
15
RETENTION TIME (MINUTES)


NAME MYCOSTDS 20UL
MISC
FRN 7662
C
T-2
488
436.1
486.1
1456
350.1
350.1
1698
290.1
290.1
3074
173.1
173.1
9637
122.0
122.0
17109
TI
17
18
19
20
21
22
23
24
25
RETENTION TIME (MINUTES)

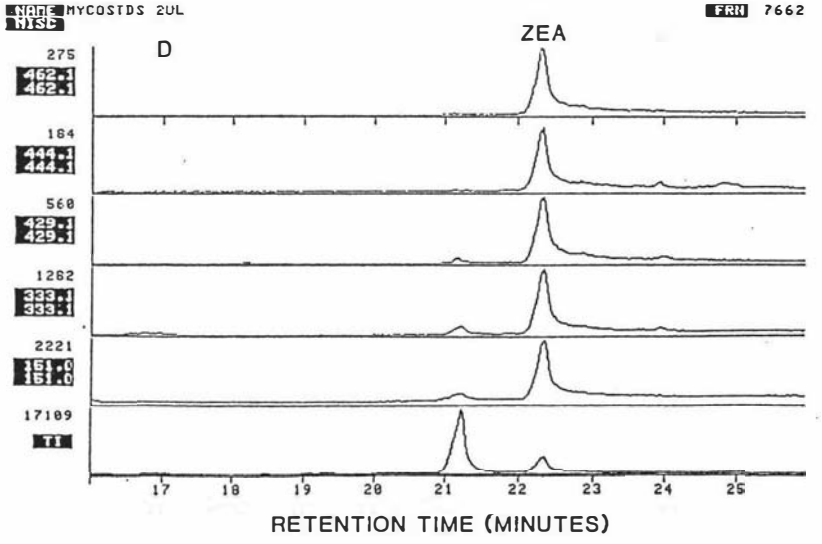

NAME MYCOSTDS 20UL
MISC
FRN 7662
D
ZEA
275
462.1
462.1
184
441.1
441.1
560
429.1
429.1
1282
333.1
333.1
2221
151.0
151.0
17109
TI
17
18
19
20
21
22
23
24
25
RETENTION TIME (MINUTES)

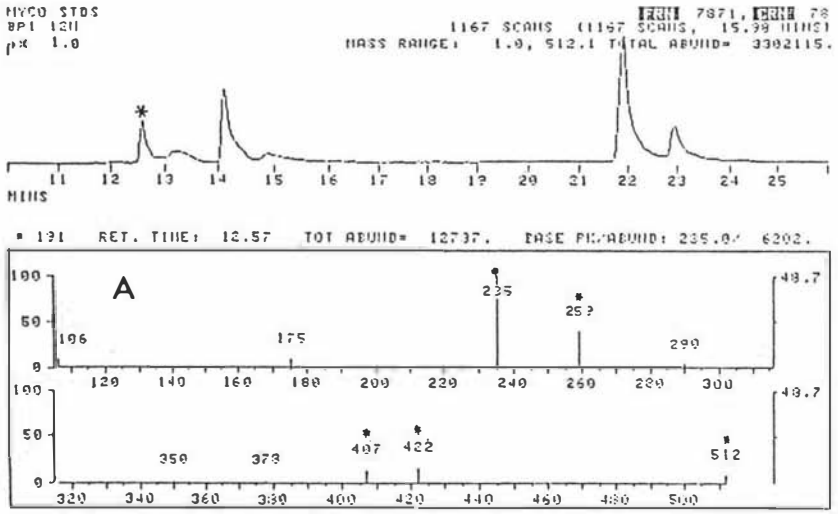
Figure 3-19: SIM mass spectra of mycotoxin standards of

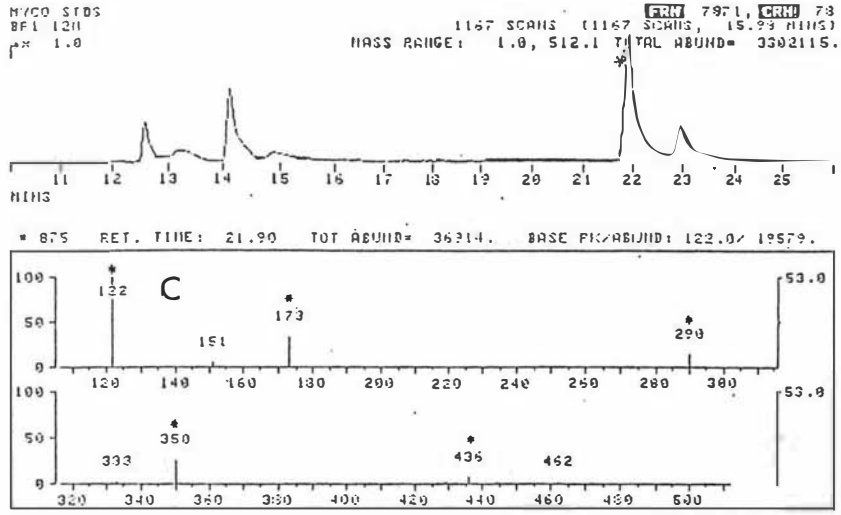
(A) DON-TMS;

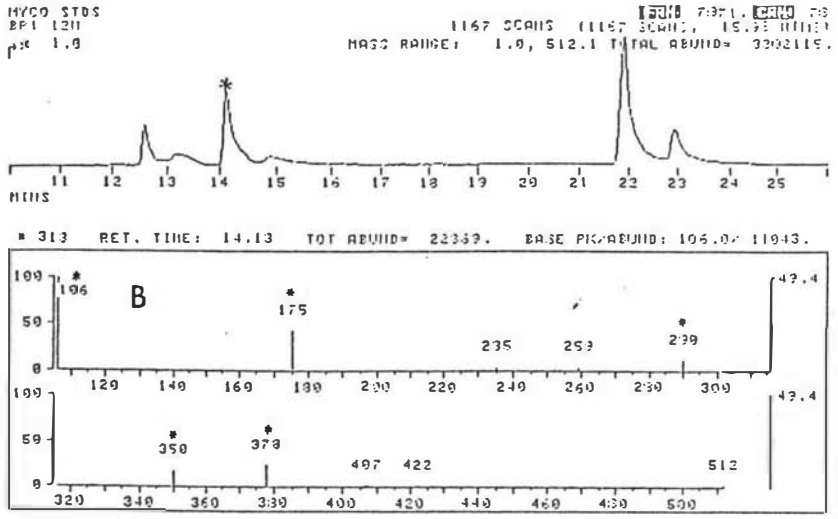
(B) DAS-TMS;

(C) T-2-TMS;

(D) ZEA-TMS. (* = selected ion.)


MYCO STDS
BPI 120
1167 SCANS
MASS RANGE: 1.0, 512.1
TOTAL ABUND= 3302115.
MINS
191 RET. TIME: 12.57 TOT ABUND= 12737. BASE PK/ABUND: 235.0/ 6202.
A
106
175
235
259
290
407
422
512
48.7


MYCO STDS
BPI 120
1167 SCANS
MASS RANGE: 1.0, 512.1
TOTAL ABUND= 3302115.
MINS
875 RET. TIME: 21.90 TOT ABUND= 36314. BASE PK/ABUND: 122.0/ 19579.
C
122
151
173
290
333
350
436
462
53.0


MYCO STDS
BPI 120
1167 SCANS
MASS RANGE: 1.0, 512.1
TOTAL ABUND= 3302115.
MINS
313 RET. TIME: 14.13 TOT ABUND= 22369. BASE PK/ABUND: 106.0/ 11943.
B
106
175
235
259
290
350
378
407
422
512
42.4

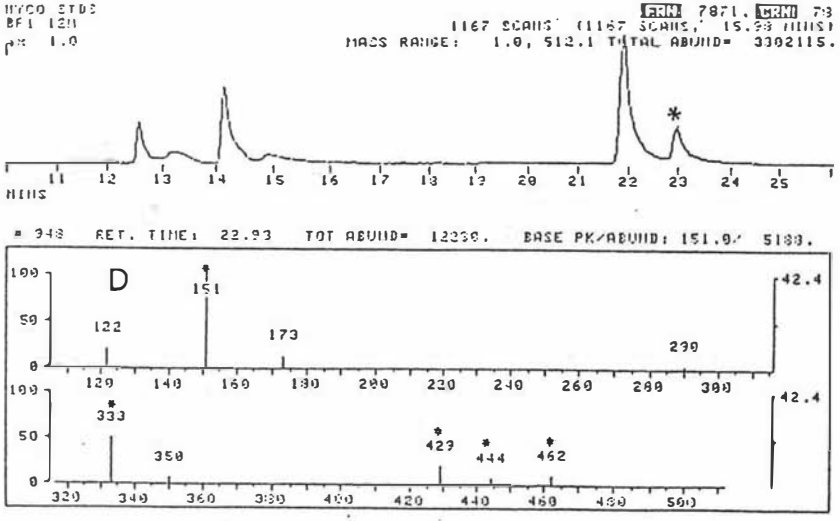

MYCO STDS
BPI 120
1167 SCANS
MASS RANGE: 1.0, 512.1
TOTAL ABUND= 3302115.
MINS
948 RET. TIME: 22.93 TOT ABUND= 12230. BASE PK/ABUND: 151.0/ 5188.
D
122
151
173
290
333
350
429
444
462
42.4

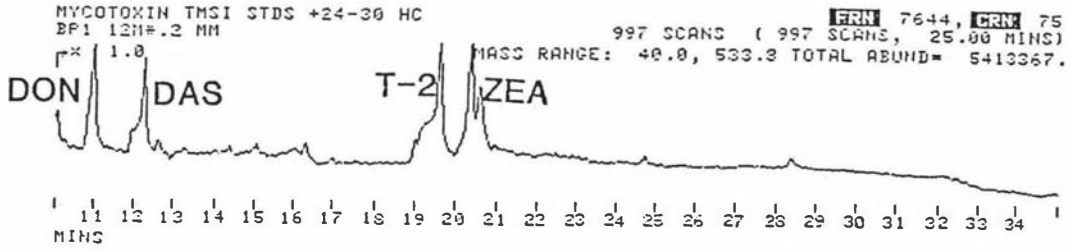
Figure 3-20: Total ion chromatogram of 4 standard mycotoxins; top from left to right: TMS derivatives of DON, DAS, T-2 and ZEA at retention times of 11.1, 12.2, 19.5 and 20.5 min respectively, and full scan mass spectra of TMS-derivatives of

(A) DON;

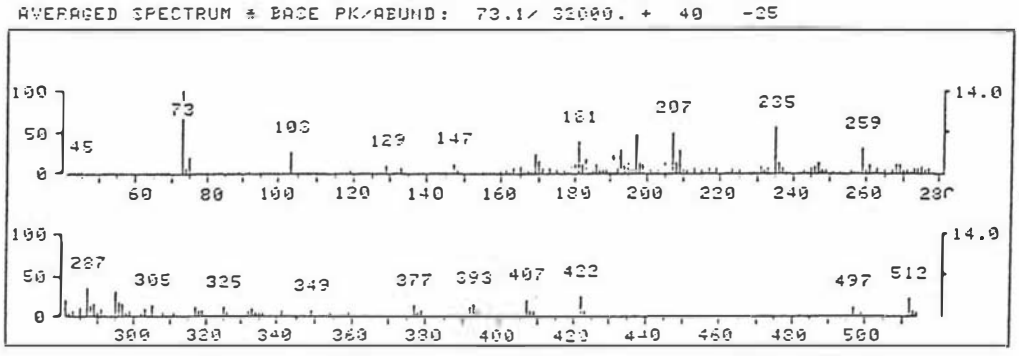
(B) DAS;

(C) T-2 toxin;

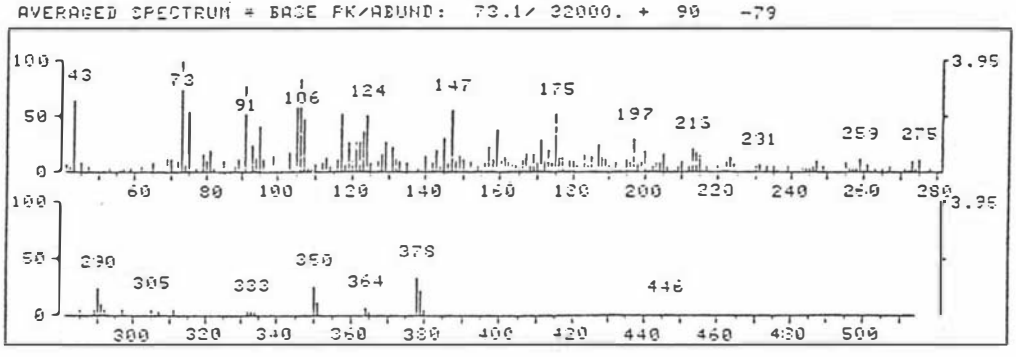
(D) ZEA.


MYCOTOXIN TMSI STDS +24-30 HC
BP1 12N#.2 MM
997 SCANS (997 SCANS, 25.00 MINS)
MASS RANGE: 40.0, 533.8 TOTAL ABUND= 5413367.
DON
DAS
T-2
ZEA
MINS

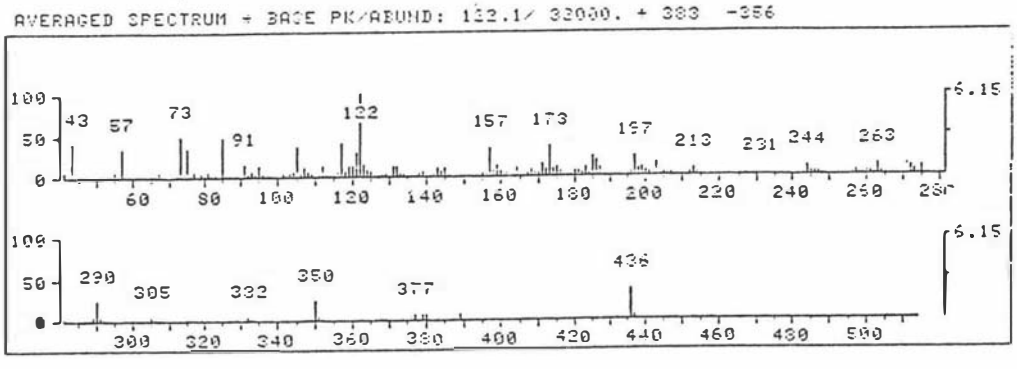
A


AVERAGED SPECTRUM # BASE PK/ABUND: 73.1/ 32000. + 40 -25

B


AVERAGED SPECTRUM # BASE PK/ABUND: 73.1/ 32000. + 90 -79

C


AVERAGED SPECTRUM # BASE PK/ABUND: 132.1/ 32000. + 383 -356

D

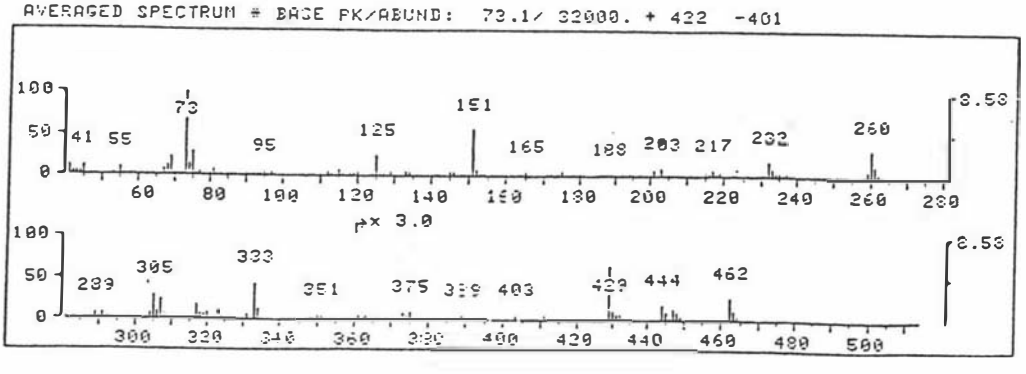

AVERAGED SPECTRUM # BASE PK/ABUND: 73.1/ 32000. + 422 -401

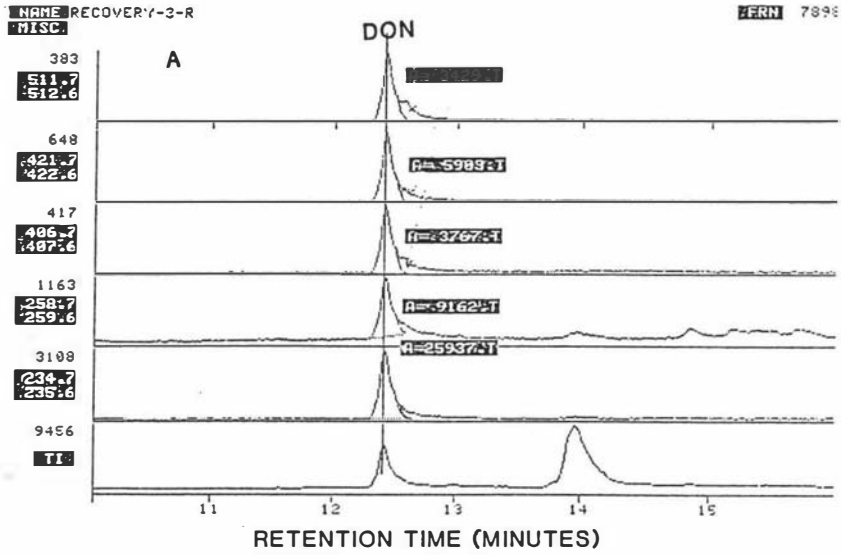
Figure 3-21: SIM ion chromatograms of mycotoxins recovered from spiked sample:

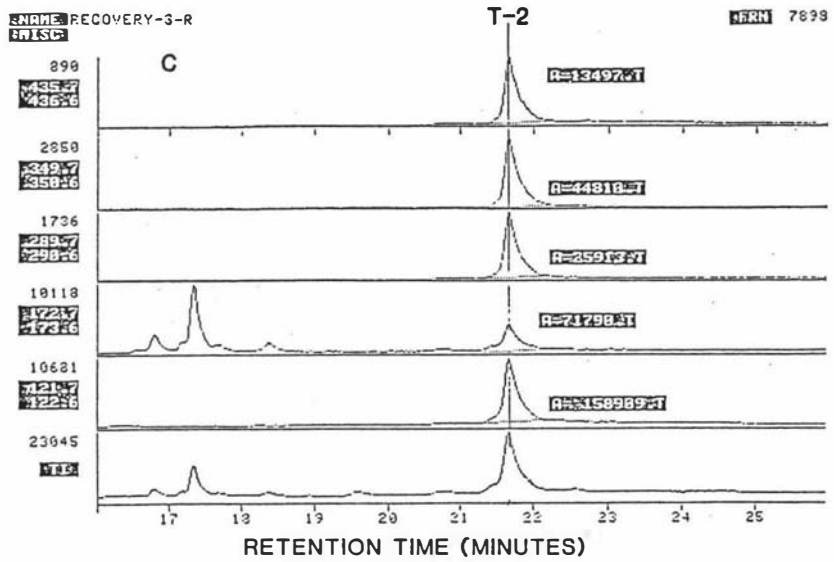
(A) DON-TMS;

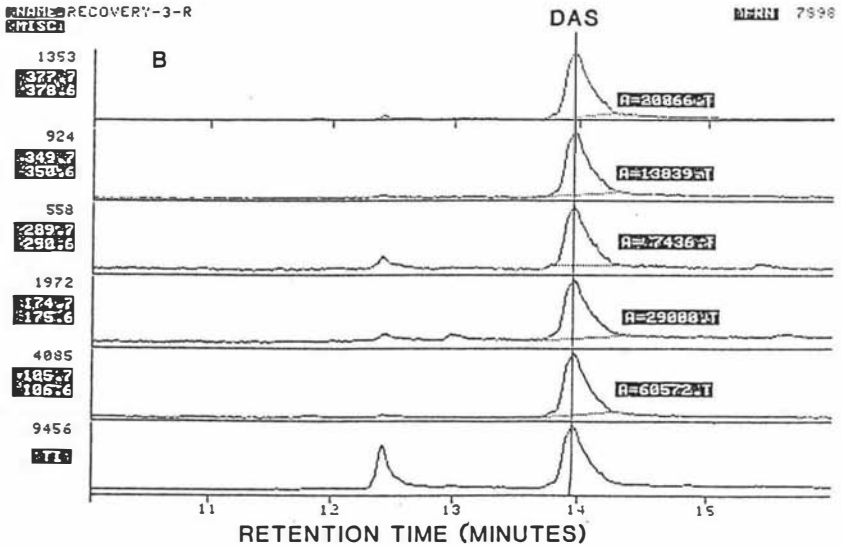
(B) DAS-TMS;

(C) T-2-TMS;

(D) ZEA-TMS,
with retention times of 12.4, 13.9, 21.6 and 22.6 min respectively.


NAME RECOVERY-3-R
MISC
DON
A
RETENTION TIME (MINUTES)


NAME RECOVERY-3-R
MISC
T-2
C
RETENTION TIME (MINUTES)


NAME RECOVERY-3-R
MISC
DAS
B
RETENTION TIME (MINUTES)

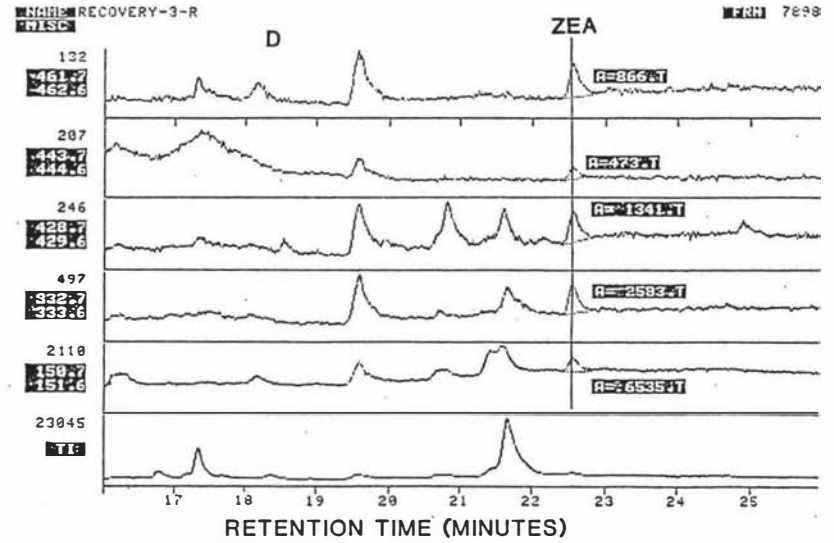

NAME RECOVERY-3-R
MISC
ZEA
D
RETENTION TIME (MINUTES)

Figure 3-22: SIM ion chromatograms of naturally-contaminated maize (sample J2).

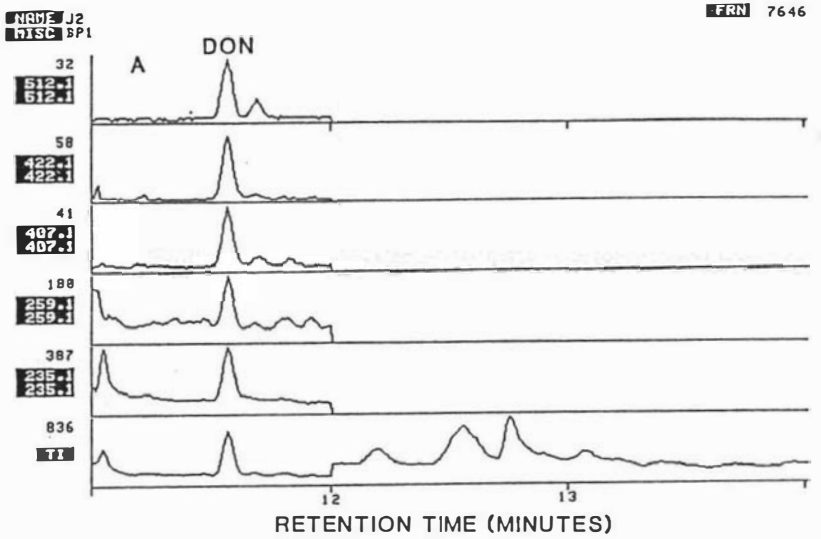
(A) DON-TMS;

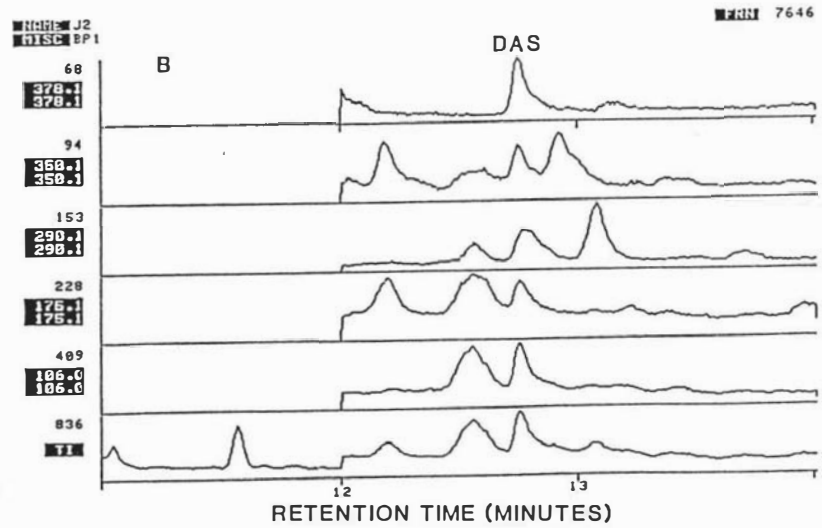
(B) DAS-TMS;

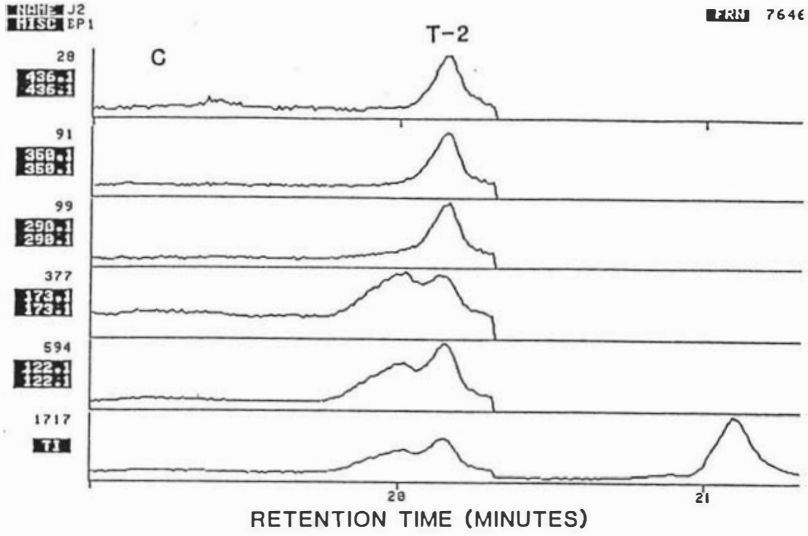
(C) T-2-TMS;

(D) ZEA-TMS

with retention times of 11.58, 12.77, 20.12 and 21.07 min respectively.


NAME J2
MISC BP1
FRN 7646
A
DON
32
512.1
612.1
58
422.1
422.1
41
407.1
407.1
188
259.1
259.1
387
235.1
235.1
836
TI
12
13
RETENTION TIME (MINUTES)


NAME J2
MISC BP1
FRN 7646
B
DAS
68
378.1
378.1
94
350.1
350.1
153
290.1
290.1
228
176.1
176.1
409
186.0
186.0
836
TI
12
13
RETENTION TIME (MINUTES)


NAME J2
MISC BP1
FRN 7646
C
T-2
28
436.1
436.1
91
368.1
368.1
99
298.1
298.1
377
173.1
173.1
594
122.1
122.1
1717
TI
20
21
RETENTION TIME (MINUTES)

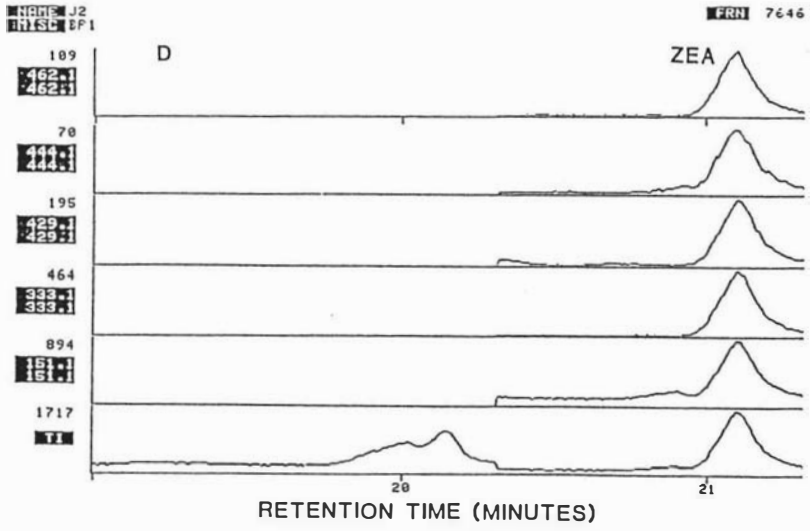
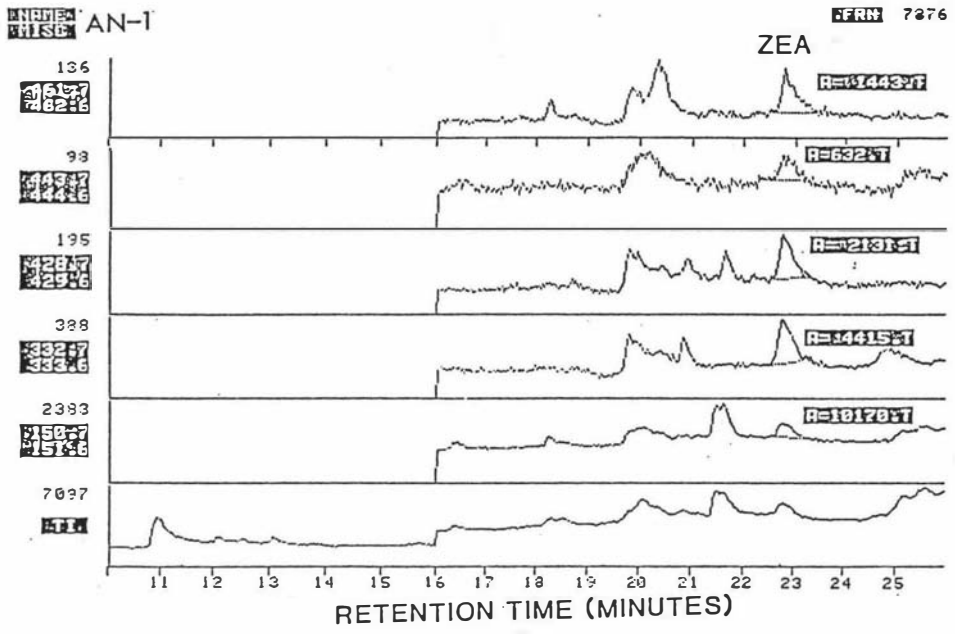
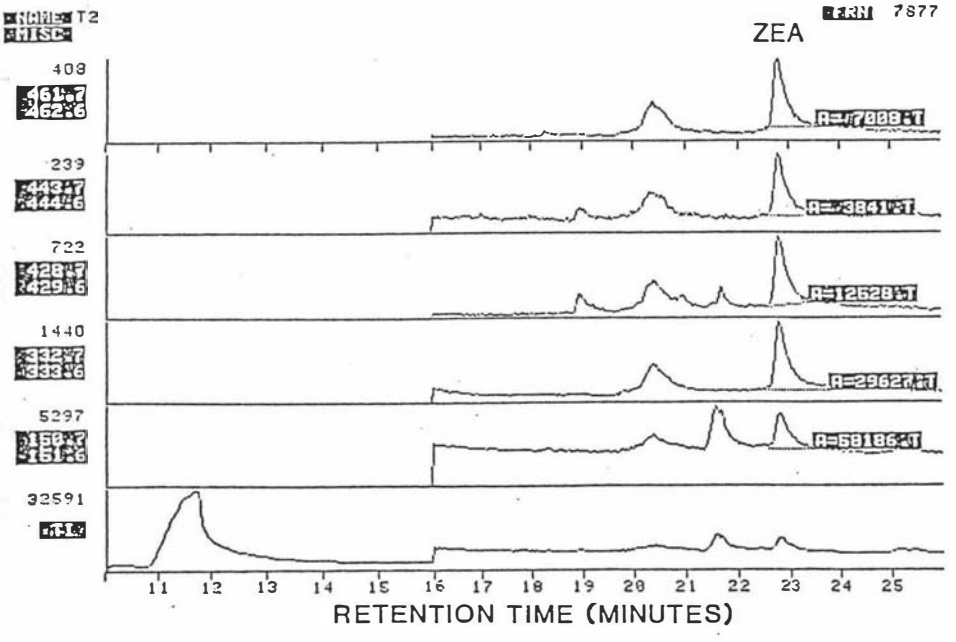

NAME J2
MISC BP1
FRN 7646
D
ZEA
109
462.1
462.1
70
443.1
443.1
195
429.1
429.1
464
333.1
333.1
894
151.1
151.1
1717
TI
20
21
RETENTION TIME (MINUTES)

Table 3-41: Semiquantitative assessment of Fusarium mycotoxins in 24 samples of maize and poultry rations, using the GC-MS method.

Sample code	Level of mycotoxins (ppm)				No. of toxins detected
	DON	DAS	T-2 toxin	ZEA	
J2	0.3	0.9	0.2	2.4	4
T3	-	trace	-	0.5	2
T4	0.2	0.5	0.12	trace	4
T1	0.3	0.5	0.1	-	3
J1	-	-	-	-	0
T2	-	-	0.007	1.3	2
T1H	-	-	0.005	2.0	2
KH	-	-	-	-	0
T2H	-	-	0.05	-	1
RH	0.09	-	0.04	0	2
T4H	-	0.03	0.2	0.08	3
J2H	0.8	-	0.1	0.08	3
T3H	-	-	-	3.8	1
C2	-	-	-	-	0
D1	0.02	-	0.05	0.2	3
B3	0.1	-	0.06	-	2
A1	0.1	-	0.05	0.2	3
A3	0.016	-	0.14	-	2
AN-1	0.024	-	-	0.33	2
AN-2	-	0.13	-	0.6	2
GC1	-	-	-	-	0
GC2	-	-	-	-	0
GC3	-	-	-	-	0
GC4	-	-	-	-	0
Total +ve	10	6	13	12	
% +ve	41.7	25.0	54.2	50.0	

Figure 3-23: SIM ion chromatograms of maize samples (AN-1 and T2) naturally contaminated) with ZEA. The retention time was 22.75.


AN-1
ZEA
136
98
195
388
2383
7097
TIC
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
RETENTION TIME (MINUTES)


T2
ZEA
408
239
722
1440
5297
32591
TIC
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
RETENTION TIME (MINUTES)

3.2.4. TLC and TLC Densitometry Screening for Moniliformin

The MON results were separated from the other four mycotoxins because different analytical methods were used. One method, using TLC plates without fluorescent indicator (Section 2.2.6.1) was used to attempt to detect MON in sample extracts. According to this method, ten samples were contaminated with MON (Table 3-42). MON was also produced by the two F. moniliforme and one F. subglutinans isolates used as positive controls. However, using the more accurate technique of densitometry of the 2,4-DNPH derivatives only the two isolates NRRL 13088 and 6022 produced MON (at levels of 638 and >500 mg/kg [ppm] respectively) (Table 3-42). No MON was detected in any of the maize samples examined. and all the samples were therefore regarded as negative.

Table 3-42: Presence of moniliformin in samples of maize and poultry rations using TLC and TLC densitometry of MON-2,4-DNPH derivatives. Also included were three known MON-producing strains of F. subglutinans.

	Sample code	Direct visual method	Densitometry of 2,4-DNPH derivatives
	J2	+	-
	T3	+	-
	T4	-	-
	T1	+	-
	J1	-	-
	T2	+	-
	T1H	-	-
	KH	-	-
	T2H	+	-
	RH	+	-
	T4H	+	-
	J2H	+	-
	T3H	+	-
	C2	-	-
	D1	-	-
	B3	+	-
	A1	-	-
	A2	-	-
	AN-1	-	-
	AN2-2	-	-
	GC1	-	-
	GC2	-	-
	GC3	-	-
	GC4	-	-
+ve control	NRRL 13088	+	638 mg/kg
	NRRL 6022	+	>500 mg/kg
	NRRL 5860	+	-

3.2.5. Final Assessment of the Analyses

Table 3-43 shows the percentage frequency of occurrence of the four *Fusarium* mycotoxins (excluding MON). The data are also presented as a histogram (Figure 3-24). There was reasonable agreement between the GC and GC-MS methods for all mycotoxins, except that with ZEA there was a closer relationship between TLC and GC-MS than with the GC and GC-MS method.

The numbers of mycotoxins per sample which could be detected by the different methods are listed in Table 3-44. This table shows there was reasonable agreement between the GC and GC-MS methods. The maximum number of mycotoxins detected in any one sample by TLC was three, from one sample only, while four mycotoxins were detected by GC in four samples and in two samples by GC-MS. It was apparent, nevertheless, that a high proportion of samples were contaminated with more than one mycotoxin.

Table 3-43: Frequency of occurrence of four *Fusarium* mycotoxins in 24 samples of maize and poultry rations as recorded by the three analytical methods.

Method of analysis	% occurrence (n=24)			
	DON	DAS	T-2	ZEA
TLC	29.0	0	25.0	54.2
GC	58.3	16.7	62.5	70.8
GC-MS	41.7	25.0	54.2	50.0

Table 3-44: Number of samples contaminated with one or more *Fusarium* mycotoxins as recorded by the three analytical methods.

Method of analysis	Number of mycotoxins in sample (n=24)				
	0	1	2	3	4
TLC	6	11	6	1	0
GC	4	2	10	4	4
GC-MS	7	2	8	5	2

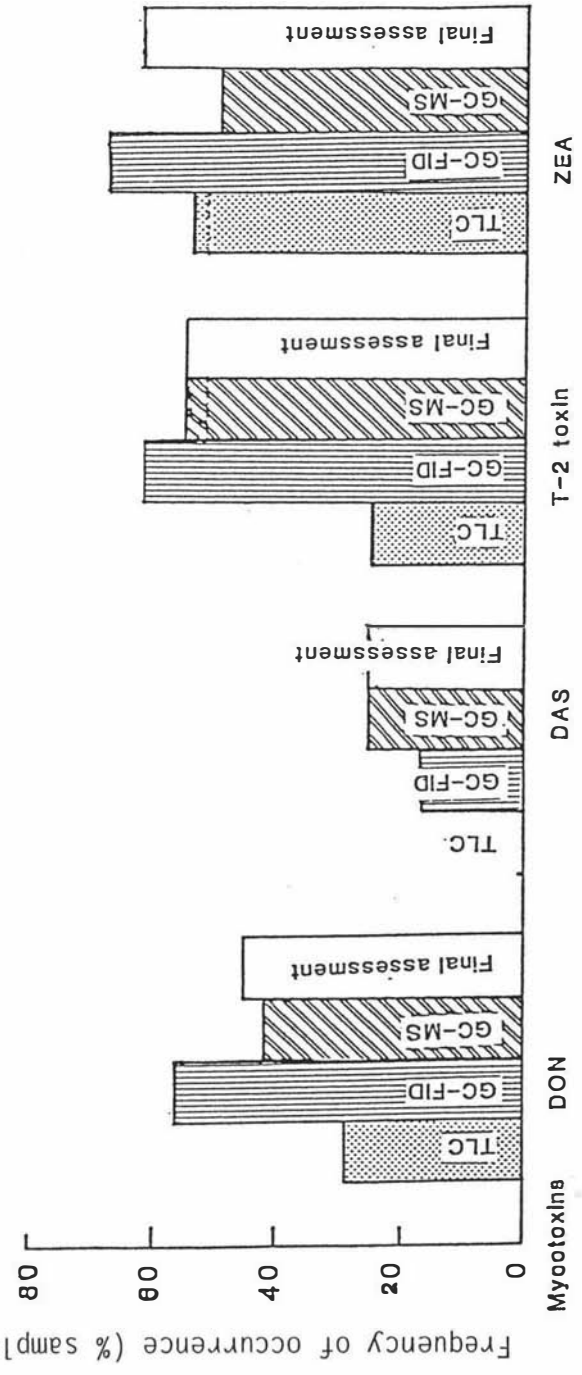


Figure 3-24: Comparison between three analytical methods, TLC, GC and GC-MS for the detection of mycotoxins.

Final judgement of "contaminated sample" status was determined using the following parameters:-

a - Every mycotoxin detected by GC-MS method on a SIM ion chromatogram using an identical five (on a few occasions four) ions was regarded as positive.

b - Detection and quantitation of ZEA was considered best by the TLC method, but with confirmation by GC-MS.

c - If GC-MS revealed only two or three of the five required peaks but the missing ones may have been masked because of high background interference from another compound eluting at a similar retention time, and if the results of TLC or GC analysis also suggested the presence of the mycotoxin, the result was considered positive.

d - Quantitation and confirmation of MON depended solely on the TLC densitometry of derivatives of MON prepared with 2,4-DNPH. Occasionally UV spectrophotometry was also used for further confirmation (discussed later).

On the basis of the above criteria, the final assessment of samples is listed in Table 3-45. ZEA was the most frequent mycotoxin (62.5% of samples). The highest level of contamination was recorded for sample J2H (16 ppm). The lowest level of detection by TLC was 0.1 ppm. Four samples were contaminated at levels of over 2 ppm. The frequency of occurrence of T-2 toxin and DAS did not differ from the results obtained by GC-MS (54.2% and 25% of samples respectively). The frequency of DON was adjusted to 45.8% of samples instead of 41.7% by GC-MS or 58.3% by GC. None of the samples were considered positive for MON.

The final results indicated that three samples were contaminated with four mycotoxins each, seven samples showed three mycotoxins each, five samples had two mycotoxins each and two samples had one mycotoxin each.

Table 3-45: "Contaminated sample status" and semiquantitative assessment (ppm) of DON, DAS, T-2 toxin, ZEA and MON in maize and poultry ration samples.

Sample code	Mycotoxin					No. of toxins detected
	DON	DAS	T-2 toxin	ZEA	MON	
J2	0.3	0.9	0.2	0.32	-	4
T3	-	0.01	-	0.5	-	2
T4	0.2	0.5	0.12	2.2	-	4
T1	0.2	0.5	0.1	0.4	-	4
J1	-	-	-	0.4	-	1
T2	-	-	0.007	1.9	-	2
T1H	-	-	0.005	2.6	-	2
KH	-	-	-	-	-	0
T2H	-	-	0.05	-	-	1
RH	0.09	-	0.04	2.9	-	3
T4H	-	0.03	0.2	0.2	-	3
J2H	0.03	-	0.07	16.0	-	3
T3H	-	-	-	-	-	0
C2	-	-	-	-	-	0
D1	0.02	-	0.05	0.2	-	3
B3	0.1	-	0.06	-	-	2
A1	0.12	-	0.05	0.1	-	3
A2	0.03	-	0.02	0.1	-	3
AN-1	0.02	-	-	0.8	-	2
AN-2	0.02	0.13	-	0.6	-	3
GC1	-	-	-	-	-	0
GC2	-	-	-	-	-	0
GC3	-	-	-	-	-	0
GC4	-	-	-	-	-	0
Total +ve	11	6	13	15	-	17
% +ve	45.8	25.0	54.2	62.5	-	70.8

3.3. PRODUCTION OF TOXINS BY *FUSARIUM* SPP ISOLATED FROM MAIZE FIELDS

3.3.1. Isolates Tested

A total of 40 *Fusarium* isolates belonging to 13 different species obtained from maize, husk, litter and soil, were cultivated on maize (Section 2.2.4) to screen for the production of five mycotoxins: DON, DAS, T-2 toxin, ZEA and MON. Brief information about these species, including their field of origin and their relation to specific substrates, is given in Table 3-46. These isolates were randomly selected from those obtained from different fields and substrates, but the majority were from fields T4, T3 and J2, from which 15, 11 and 9 isolates respectively were obtained. Table 3-46 also indicates that the majority of tested isolates belonged to one of two major species, *F. graminearum* (11 isolates) and *F. subglutinans* (15 isolates).

Table 3-46: Fusarium isolates tested for toxin production.

Species	Isolate code	Origin: Field	Origin: Substrates
F. graminearum	SN1	T1	Maize - harvest
	SN2	T3	Maize - field
	SN5	J2	" "
	SN9	J2	Maize - harvest
	SN13	T3	Husk
	SN15	J2	Maize - field
	SN37	T3	Maize - harvest
	SN39	T4	" "
	SN40	J2	Litter
	SN41	J2	Maize - harvest
	SN42	T1	Maize - field
F. subglutinans	SN3	T4	Maize - field
	SN14	T3	Maize - harvest
	SN17	T4	Husk
	SN18	T4	"
	SN19	T4	Maize - field
	SN21	J1	" "
	SN22	J1	" "
	SN23	T4	" "
	SN24	T3	Maize - harvest
	SN25	T3	" "
	SN27	T4	Maize - field
	SN28	T4	" "
	SN29	J2	Husk
	SN30	J2	Maize - stored
	SN31	J2	" "
F. avenaceum	SN7	J2	Maize - harvest
	SN38	T4	Litter
F. culmorum	SN26	T4	Maize - harvest
	SN35	T3	Maize - field
F. equiseti	SN6	T4	Litter
	SN34	T3	Husk
F. merismoides	SN36	T4	Soil
F. moniliforme	SN4	T4	Maize - field
F. crookwellense	SN8	T4	Litter
F. stilboides	SN12	T3	Soil
F. acuminatum	SN16	T3	Husk
F. oxysporum	SN20	J2	Soil
F. sambucinum	SN32	T4	Litter
F. oxysporum	SN33	T3	Maize - field

3.3.2. Mycotoxin Assay

These isolates were cultured on maize medium (Section 2.2.4) and treated similarly to the maize samples for extraction, clean-up steps and assay methods for any mycotoxins (Sections 2.2.5 and 2.2.6). The three assay techniques (TLC, GC and GC-MS) were applied but not all the isolates were assayed by all three methods.

Table 3.47 reports the results of TLC assay of 28 different Fusarium isolates screened for DON, DAS, T-2 toxin and ZEA. These results are expressed as toxin present (+ve) or absent (-ve), except for ZEA for which an estimation as ppm of ZEA was made.

Over 78% of the isolates were ZEA-producers, at levels ranging from traces (<0.1 ppm) to about 96 ppm. All F. graminearum isolates (11/11) were ZEA-producers. No ZEA was detected in the cultures of the three isolates of F. subglutinans.

Using the TLC method, T-2 toxin was detected from 35.7% of cultures, closely followed by DON 32.1%. No DAS was detected by this method. Two isolates of F. graminearum and one isolate of F. equiseti were found to produce three Fusarium toxins (DON, T-2 toxin and ZEA).

Only 12 Fusarium cultures were assayed by the GC method. The majority (seven isolates) were F. graminearum. Other species were represented by single isolates only (Table 3-48). The GC method was employed for both detection and quantitation of the toxin.

Using GC all the 12 isolates were found to be producers of ZEA and T-2 toxin, 58.3% were DON producers. Only one isolate (F. graminearum, SN39) produced DAS in addition to the DON, T-2 toxin and ZEA. ZEA production ranged from a high of 85 ppm from F. graminearum isolate SN40 to a low of 0.9 ppm for F. acuminatum isolate SN16. The maximum production of T-2 toxin was from F. graminearum isolate SN39, which yielded 38.0 ppm. Lowest T-2 production (0.6 ppm) was from F. acuminatum SN16.

Five out of the seven isolates of F. graminearum were DON-producers. DON was usually produced at low levels, the maximum being

5.6 ppm from F. graminearum isolate SN13 and the lowest 0.007 ppm from F. sambucinum SN32.

Twenty-five different cultures of Fusarium isolates were assayed quantitatively and qualitatively by the GC-MS method and the results are reported in Table 3-49. Of the tested isolates, 11 were of F. graminearum. The total ion chromatograms of Fusarium toxins produced by isolate SN39 are shown in Figure 3-25 as an example (DON, DAS and ZEA).

GC-MS analysis indicated that more than half of the tested isolates were T-2 toxin producers, but at very low levels, ranging from 0.02 ppm from isolate F. sambucinum SN32 to 1.1 ppm from F. crookwellense SN8. ZEA was found to be produced by 44% of the isolates tested. The amount of ZEA produced was higher than that of the other mycotoxins. Amounts ranged from about 31 ppm by isolate F. graminearum SN40 to only 0.09 ppm from isolate F. graminearum SN2. DON and DAS were produced by 24% of the isolates. Production levels were very low for both toxins ($\leq$1.0 ppm).

Three isolates (SN5, SN41 and SN42) of F. graminearum and one isolate of F. sambucinum (SN32) produced the four mycotoxins, while two other F. graminearum isolates (SN2 and SN39) and F. avenaceum SN38 produced three Fusarium toxins each. The rest of the isolates produced only one toxin each.

Table 3-47: Production of DON, DAS, T-2 toxin and ZEA by Fusarium spp grown on maize kernels, as detected by TLC assay of culture extracts.

Species	Isolate code	Mycotoxin			
		DON	DAS	T-2	ZEA (ppm)
F. graminearum:	SN1	+	-	+	0.1
	SN2	-	-	-	trace
	SN5	-	-	+	16.0
	SN9	-	-	-	trace
	SN13	-	-	-	96.0
	SN15	+	-	+	38.0
	SN37	-	-	+	20.5
	SN39	+*	-	-	22.0
	SN40	-	-	-	>64.0
	SN41	+	-	-	38.4
	SN42	+*	-	-	4.8
F. subglutinans:	SN3	-	-	-	-
	SN14	-	-	-	-
	SN25	-	-	+*	-
F. avenaceum:	SN7	-	-	-	-
	SN38	+*	-	-	16.0
F. culmorum:	SN26	-	-	+	4.3
	SN35	+	-	+	2.6
F. equiseti:	SN6	+	-	+	3.2
	SN34	-	-	-	0.2
F. merismoides:	SN36	-	-	-	0.3
F. moniliforme:	SN4	-	-	-	0.1
F. crookwellense:	SN8	-	-	-	-
F. stilboides:	SN12	-	-	-	trace
F. acuminatum:	SN16	-	-	-	1.0
F. oxysporum:	SN20	-	-	+*	0.64
F. sambucinum:	SN32	-	-	+	0.43
F. oxysporum:	SN33	+	-	-	-
Total +ve		9	0	10	22
% +ve		32.1		35.7	78.6

*: doubtful

Table 3-48: Semiquantitative assay by GC analysis of *Fusarium* mycotoxins produced by *Fusarium* spp grown on maize kernels.

Species	Isolate code	Mycotoxin (ppm) DON	DAS	T-2	ZEA
F. graminearum:	SN9	-	-	6.0	1.9
	SN13	5.6	-	6.4	51.2
	SN15	1.0	-	12.8	5.5
	SN39	0.9	0.1	38.0	15.5
	SN40	0.026	-	2.8	85.0
	SN41	-	-	5.2	10.8
	SN42	0.4	-	0.7	2.9
F. acuminatum:	SN16	-	-	0.6	0.9
F. oxysporum:	SN20	0.7	-	8.3	1.2
F. oxysporum:	SN33	-	-	1.5	3.3
F. sambucinum:	SN32	0.007	-	2.7	4.2
F. avenaceum:	SN38	-	-	3.0	12.2
		7	1	12	12
		58.3%	8.3%	100%	100%

Table 3-49: Production of DON, DAS, T-2 toxin and ZEA by Fusarium spp grown on maize kernels, as detected by GC-MS analysis.

Species	Isolate code	DON	DAS	T-2	ZEA
F. graminearum:	SN1	-	-	0.24	-
	SN2	0.12	-	0.2	0.09
	SN5	0.02	0.06	0.14	22.0
	SN9	-	-	0.6	-
	SN13	-	-	*	*
	SN15	-	-	-	-
	SN37	-	-	-	-
	SN39	0.2	0.02	-	9.0
	SN40	-	-	-	31.0
	SN41	0.5	1.0	0.14	3.0
	SN42	0.024	0.2	0.3	7.8
F. subglutinans:	SN14	-	-	-	-
F. avenaceum:	SN7	-	-	0.2	-
	SN38	-	0.05	0.2	1.1
F. culmorum:	SN26	-	-	0.04	-
	SN35	-	-	-	-
F. equiseti:	SN6	-	-	0.1	-
	SN34	-	-	-	-
F. merismoides:	SN36	-	-	-	0.9
F. moniliforme:	SN4	-	-	-	-
F. crookwellense:	SN8	-	-	1.1	-
F. stilboides:	SN12	-	-	0.4	11.8
F. oxysporum:	SN20	-	-	-	2.8
F. sambucinum:	SN32	0.5	0.2	0.02	7.8
F. oxysporum:	SN33	-	-	-	-
Total producers		6	6	13	11
% producers		24	24	52	44

*: not determined

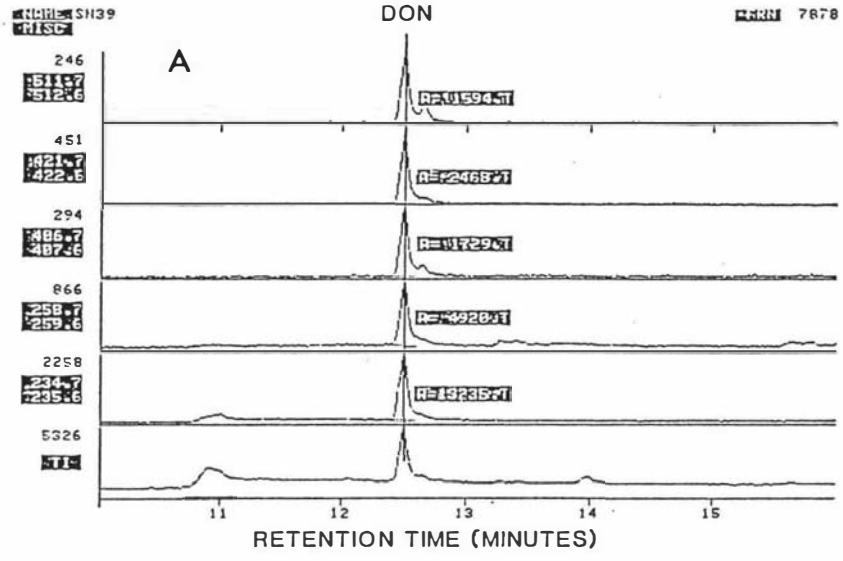
Figure 3-25: SIM ion chromatograms of three toxins produced by F. graminearum isolate SN39 :

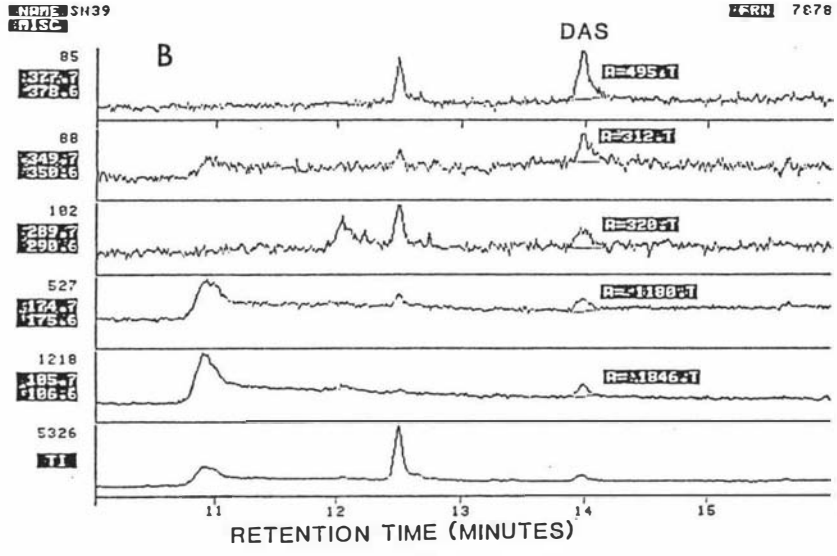
(A) DON-TMS,

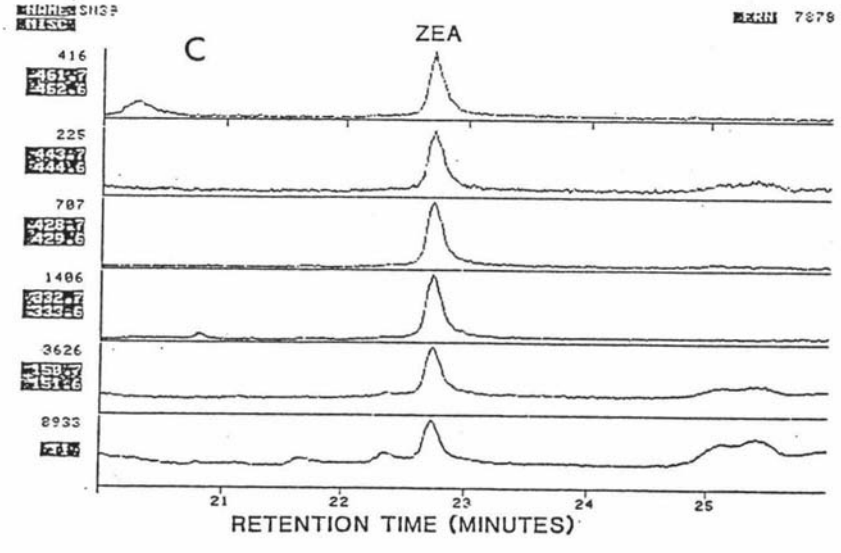
(B) DAS-TMS and

(C) ZEA-TMS

with retention times of 12.5, 13.9 and 22.75 min respectively.


DON
A
RETENTION TIME (MINUTES)


DAS
B
RETENTION TIME (MINUTES)


ZEA
C
RETENTION TIME (MINUTES)

3.3.3. Final Assessment of the Analyses

Tables 3-50 and 3-51 summarise the overall data from the analyses, after following the criteria for assessment as defined for maize samples (see Section 3.2.5). Twenty-two out of the 28 isolates (78.6%) produced ZEA in amounts ranging from 96 ppm from F. graminearum SN13 to 0.09 ppm from F. graminearum SN2. Fourteen of the 22 isolates (63%) were found to produce ZEA at >2.0 ppm. All F. graminearum isolates (11/11) had the ability to produce ZEA. The limit of detection for ZEA ranged between 20 and 50 ppb (ng/g).

T-2 toxin was produced by 13 out of the 28 tested isolates (46.4%). The level of production was in general very low, ranging from 1.7 ppm from F. avenaceum isolate SN38 to only 0.04 ppm from F. culmorum isolate SN26. Over 50% of F. graminearum isolates were T-2 toxin producers.

DON and DAS were produced by fewer isolates, only 21.4% of those tested being found capable of producing these two toxins and most of these producers were F. graminearum isolates.

F. avenaceum isolate SN38 produced DAS, T-2 toxin and ZEA. Four isolates (SN5, SN39, SN41 and SN42) of F. graminearum were found to produce both DON and DAS simultaneously. Levels of production of these two toxins were very low, ranging from 1.0 ppm to 0.02 ppm. Some of the tested isolates (4/28) were found to have the ability to produce four toxins simultaneously; three of the 28 produced three toxins and five produced two toxins.

Table 3-50: Production of DON, DAS, T-2 toxin and ZEA by Fusarium isolates. Results as semiquantitative (ppm) assessment from the three analysis methods.

Species	Isolate code	Mycotoxin (ppm) DON	DAS	T-2	ZEA
F. graminearum:	SN1	-	-	0.24	0.1
	SN2	0.12	-	0.2	0.09
	SN5	0.02	0.06	0.14	19.0
	SN9	-	-	1.6	0.6
	SN13	-	-	-	96.0
	SN15	-	-	-	38.0
	SN37	-	-	-	20.5
	SN39	0.5	0.02	-	15.5
	SN40	-	-	-	60.0
	SN41	0.5	1.0	0.14	24.6
	SN42	0.4	0.2	0.3	5.2
F. subglutinans:	SN3	-	-	-	-
	SN14	-	-	-	-
	SN25	-	-	-	-
F. avenaceum:	SN7	-	-	0.2	-
	SN38	-	0.05	1.7	10.0
F. culmorum:	SN26	-	-	0.04	4.3
	SN35	-	-	-	2.6
F. equiseti:	SN6	-	-	0.1	3.2
	SN34	-	-	-	0.2
F. merismoides:	SN36	-	-	-	0.6
F. moniliforme:	SN4	-	-	-	0.1
F. crookwellense:	SN8	-	-	1.1	-
F. stilboides:	SN12	-	-	0.4	3.9
F. acuminatum:	SN16	-	-	-	1.0
F. oxysporum:	SN20	-	-	-	0.64
F. sambucinum:	SN32	0.5	0.2	1.36	4.0
F. oxysporum:	SN33	-	-	-	-
Total producers		6	6	13	22
% producers		21.4	21.4	46.4	78.6

Table 3-51: Numbers of Fusarium isolates producing toxins.

Species	Total examined (n=28)	No. producing toxins			
		DON	DAS	T-2	ZEA
F. graminearum	11	5	4	6	11
F. subglutinans	3	-	-	-	-
F. avenaceum	2	-	1	2	1
F. culmorum	2	-	-	1	2
F. equiseti	2	-	-	1	2
F. merismoides	1	-	-	-	1
F. moniliforme	1	-	-	-	1
F. crookwellense	1	-	-	1	-
F. stilboides	1	-	-	1	1
F. acuminatum	1	-	-	-	1
F. oxysporum	2	-	-	-	2
F. sambucinum	1	1	1	1	1
Total producers		6	6	13	22
% producers		21.4	21.4	41.4	78.6

3.3.4. MON Production

Tables 3-52 and 3-53 report the results of the screening of 40 Fusarium isolates for MON production using TLC densitometry after derivatising the samples with 2,4-DNPH (Section 2.2.6.5). Twelve isolates (30%) were found to produce amounts of MON ranging from 64 ppm from F. oxysporum SN20 to 0.4 ppm from F. subglutinans SN25. The average amount detected was 14 ppm. Seven out of 15 of the F. subglutinans isolates were MON producers. No MON was detected from any of the 11 isolates of F. graminearum. Table 3-53 also includes two isolates, F. moniliforme and F. subglutinans known to be MON producers (NRRL 6022 and NRRL 13088) which were employed here as positive controls for checking the production and assaying methods. These two isolates were found to produce amounts in excess of 500 ppm.

For confirmation of the results, peak heights were determined at three wavelengths (400, 480 and 540 nm) and the ratios of the peaks compared with those from the authentic MON standard. In some cases, further confirmation was obtained by UV spectrophotometry (Section 2.2.6.6). Figure 3-26 shows the UV spectrum of both the authentic MON standard and the MON yielded by F. acuminatum isolate SN16.

Table 3-52: Numbers of Fusarium isolates producing moniliformin.

Species	No. tested (n=40)	No. positive*	No. negative
F. graminearum	11	-	11
F. subglutinans	15	7	8
F. avenaceum	2	-	2
F. culmorum	2	-	2
F. equiseti	2	1	1
F. merismoides	1	-	1
F. crookwellense	1	-	1
F. stilboides	1	-	1
F. acuminatum	1	1	-
F. sambucinum	1	-	1
F. oxysporum	2	2	-
F. moniliforme	1	1	-
Total producers		12	28
% producers		30	70

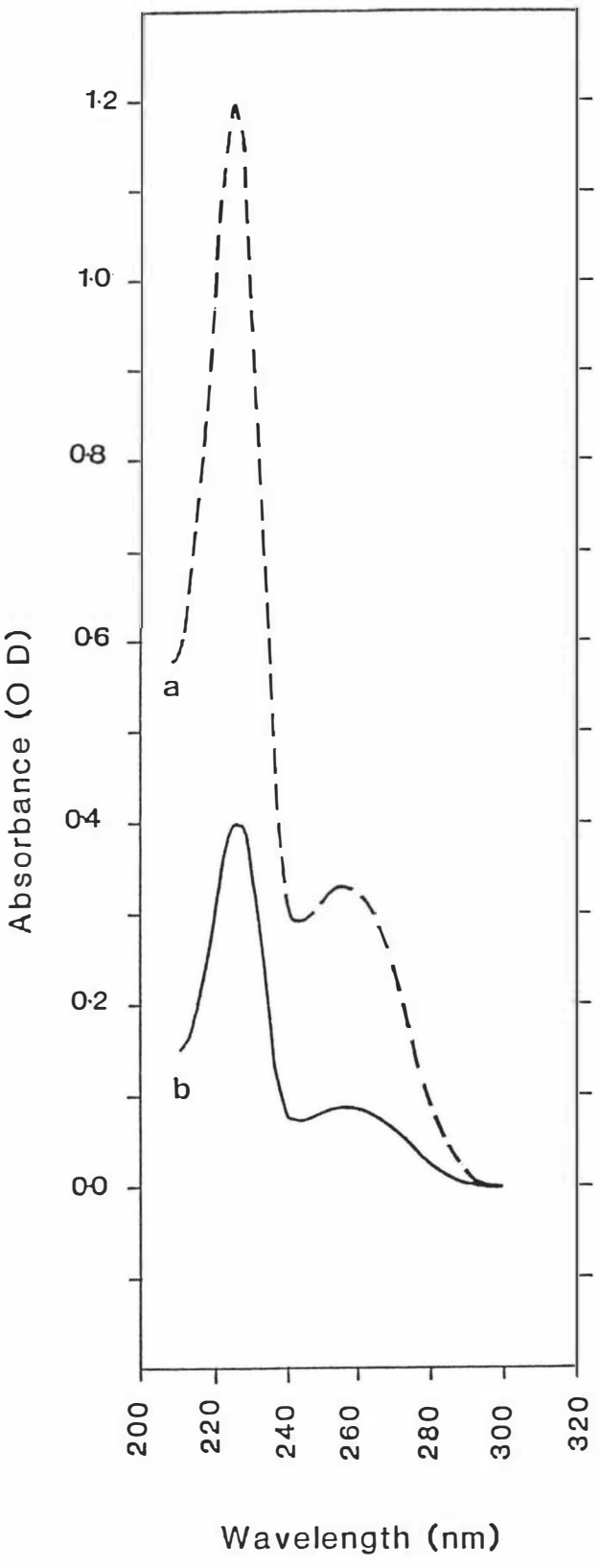
*: levels are included in Table 3-53

Table 3-53: Moniliformin production by various Fusarium spp.

Isolate code	Species	Level of MON (ppm)
SN17	F. subglutinans	7.7
SN18	"	3.0
SN21	"	18.0
SN23	"	3.0
SN25	"	0.4
SN28	"	13.5
SN30	"	5.7
Sn6	F. equiseti	1.4
SN4	F. moniliforme	0.8
SN16	F. acuminatum	45.0
SN20	F. oxysporum	64.0
SN33	F. oxysporum	5.8
NRRL 13088	F. subglutinans *	638.4
NRRL 6022	F. moniliforme	>500.0

*: received as F. moniliforme var subglutinans

Figure 3-26: UV spectrum of (a) moniliformin from F. acuminatum isolate and (b) moniliformin authentic standard



CHAPTER 4.
DISCUSSION

The attributes of the genus Fusarium have drawn considerable attention from taxonomists, plant pathologists and mycotoxicologists throughout the world. The fusaria are the most common destructive fungi of cereal grains (Gordon, 1952; Uoti and Ylimaki, 1974; Neish et al., 1982) and several species can cause serious mycotoxin problems in various countries (Miller et al., 1983). A better understanding of the ecology of the genus could form the basis for developing strategies aimed at preventing mycotoxin contamination of grains and feedstuffs. Apart from our limited knowledge of general fungal ecology, no basis exists for predicting the presence of particular Fusarium mycotoxins in grains. Therefore, in the present study all maize samples were analysed for the presence of potentially toxigenic species and simultaneously for the presence of a broad range of Fusarium mycotoxins, with the purpose of determining the extent to which mycological and toxicological analyses can give complementary results.

In New Zealand most mycological studies of the relationship of Fusarium spp to maize have involved the parasitic species. There have been no comprehensive surveys of the general mycoflora of maize stands in the field or at harvest time. There is no doubt that cereal crops in the field are regularly contaminated with a variety of fungi, mostly field fungi. Even if these are saprophytic fungi they cannot be ignored because the specific composition of this flora may give some indication of the general condition of the grain. Furthermore, a knowledge of the overall fungal flora is also important when samples for fodder and industrial purposes are examined, because the presence of mould on grains and feedstuffs may imply a risk of mycotoxin contamination (Ylimaki, 1981). To allow consideration of the above problems the level of occurrence of both pathogenic and saprophytic fusaria and other fungi associated with maize, and their possible reservoirs, were recorded during this study. Thus the survey of the numbers and kinds of fungi present in maize kernels, husk, litter and soil was undertaken to answer questions about the diversity and origins of Fusarium spp associated with maize kernels, particularly those species which could be involved in toxin production.

The taxonomy of the genus Fusarium (discussed in Section 1.1.1) is a critical problem for all mycologists, particularly those who are interested in its ecology and toxigenicity. The classification of Fusarium in the present study was largely based on the system of Burgess and Liddell which was described by those authors as a composite of the Snyder and Hansen system with that of Wollenweber and Reinking. However, a few modifications were made. F. graminearum, divided into two groups by Burgess and Liddell, was considered as one species. The two species F. merismoides and F. stilboides were classified according to Booth (1971) and as described by Gerlach and Nirenberg (1982). F. crookwellense (Burgess, Nelson and Toussoun, 1982, sp. nov.), recently reported from New Zealand pasture (Lauren, 1986) was, in the present study, found on all substrates in maize fields. This species shows characters between F. culmorum and F. graminearum and was most likely earlier classified within the above taxa. Burgess et al. (1982) consider the species to belong to the section Discolor (see Section 1.1.1).

Media and cultural conditions used for the identification of fusaria are very critical factors (Burgess and Liddell, 1983). The growth and sporulation of Fusarium spp depend a great deal on the cultural conditions. Using a carbohydrate-rich medium such as PDA causes delays in sporulation and also may stimulate non-uniform, misshapen conidia (Fisher et al., 1982). The carnation leaf agar (CLA) used in the present investigation was an excellent medium, giving good growth and sporulation. The original culture type of the Fusarium isolates was maintained. Sporulation occurred within 14 days with very few exceptions, eg. F. avenaceum strains could take over three weeks. Another useful condition employed to stimulate sporulation was exposure to black light at a fluctuating temperature (20-24^{o}C). Such conditions were favourable for all Fusarium spp. Black light has been used commonly by Fusarium taxonomists (Toussoun and Nelson, 1976; Burgess and Liddell, 1983).

Techniques for Ecological Studies

Of the major problems which can be encountered when studying fungal ecology, the total lack of standard methods and the limitations on the amount of time and effort which can be expended are perhaps the

most important (Price, 1984). Many reports, as well as the present study, have indicated that the validity of techniques for sampling for fungi (and their mycotoxins) in grain, either in the field or in storage, largely depends on the sample size and sampling procedure.

It has been recommended that the required sample size of maize to provide reliable results is about 10 lb (±5 kg) (Davis et al., 1980). Nevertheless, in practice this recommendation has rarely been followed because of the cost of transport and storage facilities etc. The present study recognised the above suggestion and the maize sample size ranged between 3 and 5 kg.

The size of other substrates is apparently not critical (Nash and Snyder, 1962; Smith and Snyder, 1975; Sturz and Johnston, 1985). In the case of soil, about 1 kg has most commonly been used (Wearing and Burgess, 1978). In the present study approximately 750 g samples were collected. Sample size for the two other substrates (litter and husk) were measured by number of pieces. Windels and Kommedahl (1984) considered that 50 pieces were more than enough to represent the field, but in these investigations litter and husk sample size was much larger (A 1 g sample of litter or husk could contain hundreds of pieces).

The second major factor governing validity of results, actual sampling procedure, has been considered by many to be the most important (Davis et al., 1980; McMullen and Stack, 1983). Sampling procedure is determined by the stage of maturity at which the sample is collected - in the field, during harvesting etc. Each stage has its own problems. Davis et al. (1980) were of the opinion that it is better to sample shelled maize rather than ear maize and it is better to sample ground maize rather than shelled maize. But often investigators do not mention the method of sampling from the field. Sturz and Johnston (1983) for example, collected 240 ears each of barley and wheat from 16 plots without mentioning the method used for the collection. In the present study, techniques for sampling from the field as well as at harvest were considered to be extremely important as Fusarium spp more easily contaminate substrates in the field than in storage. So great care was taken to obtain representative samples from the field for all substrates. Taking random samples in large

fields is a formidable task, and the method used here of 25 site samplings (Figure 2-2), with sites located among five circular plots covering the entire field, was considered better than other previously-suggested patterns (W-shaped, X-shaped, V-shaped, diagonal etc.) (Basu et al., 1977, Figure 1-2). The method employed also fulfilled the recommendation of other investigators (Francis and Burgess, 1975; Davis et al., 1980), that sampling is better when widely distributed throughout the field.

The sampling site factor has rarely been considered (Dickinson and Kent, 1972). Since it is impossible to rely on one site or even plot sample in a field being representative, bulking plot samples is the method of choice where the processing of all individual plot samples is impossible because of both financial and time costs. Bulking field samples is, of course, a common practice (Wearing and Burgess, 1978; Burgess and Liddell, 1983; Sturz and Johnston, 1985). Although authors do not agree about the size and number of samples, they do agree about pooling the samples.

Investigations concerning sampling and sub-sampling techniques in the present study as reported in Chapters 2 and 3 determined the variation between plot samples. Maize, husk and litter showed heterogeneous distribution of fungi among the five plots sampled from both test fields, with significant differences between samples. Fusaria surprisingly were also unevenly distributed in maize samples from both fields but not in litter and husk samples. Only soil gave homogenous results for both total fungi and the fusaria (Section 3.1.1.1). This study has also shown that total fungal propagules as well as propagules of Fusarium spp in litter and husk are very much higher than those in soil.

The maize kernels had the lowest counts (field J1). The uneven distribution of fungi particularly within the maize samples may be due to uneven exposure of the maize kernels to fungal propagules. Shredding of the husk and injuries to the ear could play very important roles as factors predisposing to increased levels of fungal contamination (Sutton et al., 1980).

A second variable involved in the uneven distribution of fungal propagules in both maize and husks could be the maize variety itself. It was found that at the first sampling maize of variety XL45 from plots B and D of field T3 (Section 3.1.1.1) showed no mycelial fungi when examined by the dilution plating technique. Using direct plating, the kernels from these two plots were contaminated with Fusarium spp at rates of 4 and 1% respectively compared to 57, 97 and 89% (mean of two media) from plots A, C and E respectively, which were of variety PX 442. Plots B and D were resampled again 5 weeks later and this time gave very low fungal viable counts by dilution plating (500 CFU/g). By direct plating a 100% kernel contamination rate was noted, about 25% of these fungi being Fusarium*. Similarly husk samples of variety XL45 showed somewhat lower counts for both total fungi and Fusarium (Table 3.3 and 3.4) compared to the husks of other varieties.

Making a major contribution to these differences could be the time of maturation. Variety XL45 matures about 2 months after PX 442. Thus sampling field T3 at the beginning of May resulted in samples of XL45 being taken about 3 months before harvest while PX 442 was just 5 weeks away from harvest. Such a delayed development of the mycoflora was similar to the findings of Miller et al. (1983) who isolated no fungi from kernels until about 6 weeks before harvesting. Similarly, Tuite et al. (1974) recommended examining maize fields for ear (cob) rot approximately 4-6 weeks before the beginning of the maize harvest.

The information obtained in the present study indicated that overall numbers of Fusarium spp or other fungi in the soil, unlike the other substrates, could be estimated from just one plot sample (one of the four quadrants or the central one) to give a reliable representative result for the whole field. Similar results for soil samples have been reported by Nash and Snyder (1962 and Miller et al., (1983). But sometimes total fungal viable counts can be meaningless when compared to individual viable counts of some toxigenic species. Thus, particularly in regard to individual Fusarium spp, there can be an uneven distribution within a field (Wearing and Burgess, 1977). Relying on one plot sample would therefore create bias and bulking of several samples is essential.

* These data are not presented in the Results section.

Subsampling is another factor which needs to be carefully considered. Methods which have been routinely used for subsampling (particularly of grain) have been reviewed in Section 2.4 and suitable procedures can be achieved in several ways. In the present study a mechanical mixing device was used. The results obtained indicated that 70 rotations (2 min in the mixer) gave the highest viable counts, but it was clear that there was no significant difference ($P>0.05$) between viable counts obtained following any of the four periods of rotation tested (35, 70, 105 and 140 rolls). For convenience, it was decided to use the two minute rotation for all other maize samples throughout the project.

Sampling of soil, litter and husk is described in Section 2.1.3.1. No attempt was made to assess the efficiency of the subsampling method of those three substrates. It was assumed that only partially filling large polythene bags with the sample allowed the husk or litter segments to mix freely within the bag.

Employing two media and two isolation techniques in this investigation improved the recovery of Fusarium spp from all the examined materials (maize, husk, litter and soil). The two factors of medium and technique will now be examined through the data collected from this study, to determine their efficiency.

Although the medium factor has been found by some authors to not significantly affect mould counts or their recovery rates (Jarvis, 1973; McMullen and Stack, 1983), using a selective medium to facilitate screening for fungi such as Fusarium is considered desirable (Andrews and Pitt, 1986). The most widely used selective (semi-selective) medium for fusaria has been the Nash-Snyder PCNB agar (Lim, 1974; Kommedahl et al., 1975; McMullen and Stack, 1983). In the present study this medium was used but was supplemented with streptomycin sulphate and chlorotetracycline HCl.

The other commonly-used medium, PDA, is used routinely by mycologists as a medium for the isolation and identification of numerous fungi and has been commonly used for Fusarium spp. This medium as well as other carbohydrate-based media pose the problem of supporting fast-growing fungi such as Rhizopus and Mucor spp, and two

modifications were introduced in the present study. First, freshly-prepared PDA (non-commercial) was used to enhance sporulation, following the suggestion of other researchers (Toussoun and Nelson, 1976; Ogawa *et al.*, 1978). Second, dichloran was added to the PDA to restrict the diameter of the most rapidly growing colonies, so aiding counting and preventing overgrowth of the slower-growing strains. Thus PDA-D was used as a general medium as well as a semi-selective medium.

Results from the present investigation indicated that the general fungal population as well as that of *Fusarium* spp from maize kernels was slightly higher when determined on PDA-D than on PCNB using the dilution plating technique. Using the direct plating technique, PDA-D still gave the highest recovery of total fungi but not of *Fusarium* spp (Tables 3-28 and 3-29). Although PCNB was apparently superior to PDA-D in terms of the number of different *Fusarium* spp recorded, such a difference was not significant ($P>0.05$). The numbers of other fungi were significantly lower ($P<0.01$) on PCNB (Table 3-30 and Figure 3-6). Similar results were reported by Francis and Burgess (1975) who employed three media (CLA, PDA and PCNB) for isolating fusaria as well as other fungi from maize stalks. These authors isolated a total of 11 *Fusarium* spp on the three media used. The highest frequency of isolation of *Fusarium* spp was on PCNB agar while other fungal species showed a significantly lower recovery on this medium.

Several techniques have been used for the isolation of different fungal species from different host substrates, but the two techniques employed in the present studies (dilution plating and direct plating) are perhaps the most common (Flannigan, 1977). Most mycologists, particularly plant pathologists and mycotoxicologists, dealing with the mycoflora associated with grain seeds and plant parts (stalk, stem, leaves etc.) prefer using direct plating. This is because the internal mycoflora is more important than that on the outer surface in terms of plant invasion and mycotoxin production. Thus direct plating after surface sterilization is considered a most useful technique (Lacey *et al.*, 1980). However, when a wide range of fungal genera as well as individual *Fusarium* spp are to be studied and compared from different habitats, as in the present study, employing a technique which does not involve surface sterilisation is essential. The

dilution plating technique provided results which were satisfactory for assessing the overall mycoflora and the general occurrence of Fusarium spp amongst all the fungi present.

Results reported earlier did indicate, however, that fungi which do not produce abundant spores, such as Alternaria, Epicoccum, Fusarium, Nigrospora, Chaetomium and Trichoderma give a smaller population on dilution plating. The presence of highly sporulating genera such as Penicillium, Rhizopus and Acremonium may also suppress those less sporulating fungi. These results therefore agree with the conclusion of Lacey et al. (1980) that by dilution plating only a limited range of species can be enumerated accurately.

The actual number of different Fusarium spp recovered from maize kernels by direct plating was significantly higher than that recorded by dilution plating ($P<0.001$). Direct plating of maize kernels was superior to dilution plating when examining the frequency of occurrence of both Fusarium spp and other fungal genera within the kernels (Table 3-31 and Figure 3-7). The data from husks and litter was less, but it is nevertheless possible to draw a conclusion regarding the techniques as applied to these substrates also. Husk gave similar results to maize but litter showed the opposite effect. Both the total number of genera as well as the numbers of Fusarium spp recovered from litter samples were higher by dilution plating than by direct plating. This could be because the litter segments, being decayed, absorbed the sodium hypochlorite used for surface sterilization, with the result that some of the internal Fusarium mycelium was killed (Burgess and Liddell, 1983).

In the case of soil, blending of samples aims to obtain the release of the maximum number of fungal propagules from the soil and its organic matter, and it reduces the time and costs involved in alternative sieving and washing techniques. Such blending techniques have been successfully used by several workers, for example Smith and Snyder (1975) recovered approximately seven times more F.oxysporum from barley roots by using blending as opposed to the shaking method. The technique seemed to be particularly useful in the present investigations, blending during preparation of the inoculum resulting in

the recovery of a high number (10) of Fusarium spp from the samples after assay by dilution plating.

Differences between Fusarium populations obtained after assay by the two techniques of dilution plating and direct plating have been reported as being due solely to the isolation technique (Smith and Snyder, 1975; McMullen and Stack, 1983), but such an explanation must be considered as uncertain. The high populations sometimes recorded by direct plating could be due to habitat (host) factors, as it has also been found that debris fragments are a greater reservoir of fungi than the soil itself (Burgess, 1981). Indeed, the results from the present study indicate that differences obtained by the two techniques may be only partially due to the isolation technique as the habitat factor also seemed to have an influence. Such a conclusion can be made as the two techniques were applied to the same single substrates. There is no doubt that the use of two isolation techniques and two media in the present study led to an increase in the frequency and density of fungal species, 15 species of Fusarium and 30 other fungal genera being isolated.

Dilution plating provided an opportunity for an overall assessment of the mycoflora and the place of Fusarium spp within it, although it is recognised that this technique determines the total numbers of viable fungal propagules, whether they are actively growing or not. These studies indicated that both total Fusarium counts and the number of different species were significantly lower than those noted by the direct plating technique (Tables 3-13, 3-14 and 3-31). Such low results could be because species of this genus are less competitive on the isolation plates with those more highly sporulating genera which are also present.

Direct plating after surface sterilisation, on the other hand, provides information on the internal fungi of the substrate, more of which can be assumed to be actively growing within that substrate. The technique allows isolations of fungi which might otherwise be masked by superficial species, not all of which would be active (i.e. isolations originating from transient spores etc.). The true proportion and importance of Fusarium spp can then be assessed. The

results showed that 75.6, 69.0 and 43.3% of kernels, husk segments and litter segments were actually contaminated with this genus.

The General Mycoflora of Field Maize

Rarely has screening been carried out for such a wide range of fungi from such a variety of substrates. Most investigators have concentrated their studies on specific pathogens (Sutton, 1982), although Christensen and Kaufmann (1969) reported on several genera (Alternaria, Cladosporium, Nigrospora, Helminthosporium, Fusarium and Trichoderma) invading cereal grains in the field. Results from the present study partially agree with those of Christensen and Kaufmann but no Helminthosporium was found and Penicillium was common. From the U.S.A., Hesseltine and Bothast (1977) recorded four genera, Fusarium, Acremonium, Nigrospora and Cladosporium (in descending order of frequency). Penicillium and Mucor were also found in the field very early as contaminating the maize silks.

The most frequent fungi from all materials examined were Fusarium, Acremonium, Cladosporium and Penicillium. Five other genera, Mucor, Alternaria, Epicoccum, Verticillium, and Phoma occurred at moderate frequencies. Soil and litter gave the greatest variety of genera (19 each, Table 5.13). Fusarium was the predominant genus in maize kernels in 3 out of the 4 field samples examined, while in the fourth sample, Mucor was the most frequent isolate (Table 5.13). Penicillium was the predominant genus in two each of the samples of husk and soil but was isolated less frequently from maize and litter. Acremonium was the predominant genus in the greatest number of samples of husk, litter and soil (2/4, 3/4 and 2/4 samples respectively), but not of maize. Verticillium was the predominant genus in only one litter sample. Cladosporium was the second most frequent genus overall, but was not predominant in any sample.

An unexpected result was that three "field fungi", Alternaria, Epicoccum and Nigrospora, were mostly recovered from the husk and litter, but were rather uncommon from maize in the field. These genera are said to be late season colonizers of maize (Hesseltine and Bothast, 1977). In the present study, Epicoccum and Nigrospora were not isolated from soil samples in spite of their occurrence on other

substrates, particularly husks and litter. Alternaria was also isolated most frequently from husk and litter (Table 3-11).

No Aspergillus spp were detected from any of the samples examined (except for the storage samples from Silo C). Other workers have failed to detect any Aspergillus spp in field maize. Hesseltine and Bothast (1977) examined over 4,500 individual kernels of maize but no Aspergillus spp were found. Similarly, Rambo et al. (1974) did not find any A. flavus in 156 field samples of maize from Indiana from the 1971 crop while only 0.08% from 369 samples were contaminated with this species in 1972. However, there can occasionally be a serious problem of contamination of maize in the field with A. flavus and consequent aflatoxin production. Such problems mainly occur in high temperature areas (over 34^{o}C) and under drought stress (Payne et al., 1986). So the cooler climate in New Zealand could be the main factor leading to a lack of Aspergillus spp in maize fields.

Fusaria in Maize Fields

The genus Fusarium was of the most interest in the present study. Its high frequency of occurrence and its high level of contamination (75.6%, Table 3-12) of maize in the field has not been reported before in this country.

The studies on the occurrence of Fusarium spp from all the substrates in the fully-studied maize fields T3, T4, J1 and J2 indicated that five fusaria (in decreasing order: F. culmorum, F. graminearum, F. acuminatum, F. oxysporum and F. subglutinans, Table 3-15) were present at high frequencies. It is appropriate to discuss these findings and to compare the results of the present study with those of other reports.

F. culmorum was found at the highest level (14/15 of total examined samples from the four substrates) which is probably the result of its saprophytic rather than its parasitic activities. It was noted in all samples of soil, litter and husk and in five maize kernel samples out of six. The species is typically a soil-based fungus (Burgess, 1981), which possesses a high competitive saprophytic ability, but also causes a variety of diseases on a wide range of

cereals and grasses in most countries of the world (Jones and Clifford, 1978). Later results showed that this species occurred at a frequency of 100% in harvest samples, but was absent in storage samples (Table 3-24). F. culmorum has not been frequently reported in New Zealand maize before, possibly because investigators classified it as F. roseum (Fowler, 1985). Hampton (1980) found this species to be the predominant one in wheat seeds and it has also been recorded in freshly-harvested barley (Chong and Sheridan, 1982; Lee, 1986). It has commonly been found in pasture grass and may produce zearalenone in the grass leaves (Gallagher, 1985; Lauren, 1986).

Although F. culmorum showed the highest frequency of occurrence, (an expression of the number of all samples positive for this species), the actual level of contamination of the substrates (the frequency of isolation from a sample, as estimated from all plates of both techniques) was lower. The level of contamination of maize and litter by F. culmorum was second to F. graminearum, while in husks and soil F. culmorum was third and fourth respectively (Figure 3-2).

F. graminearum was second in frequency of occurrence (13/15 samples). It was present in only one of the three soil samples. In terms of level of contamination, F. graminearum was the most significant species in both maize and husks and was third in the litter samples. These results suggest that the main inoculum of F. graminearum in the field was disposed in the aerial parts of the plants from which spread to the soil and litter could occur. Such a conclusion agrees with that of Kommedahl et al. (1979) and Miller et al. (1983). At harvest time and after harvest, plant debris in the soil could then be the main reservoir of this species.

The low level of occurrence of F. graminearum in the soil was not unexpected as a similar result had been reported by several authors (Wearing and Burgess, 1978; Windels and Kommedahl, 1974). Windels and Kommedahl (1974) detected F. graminearum in only 30% of soil samples. According to these authors such a low level was because this species rarely produces chlamydospores for survival in soil. But the technique used (dilution plating) could be another reason for such a low level. This species was not detected by using dilution plating of

several hundred soil samples from Canadian fields (Gordon, 1956). In the present study, F. graminearum was detected in one out of three soil samples but at the low population count of 390 CFU/g.

Most of the isolates of F. graminearum obtained from this study produced perithecia on CLA medium and this suggests that the isolates belonged to the "Group 2" of Francis and Burgess (1977) (see Section 1.1.2). This group contains air-borne isolates which cause disease of aerial plant parts such as stalk and ear rots of maize and head blight of wheat, barley and oats (Windels and Kommedahl, 1984). F. graminearum group 2 has been found to survive mostly in plant tissue (eg. debris) and to be seldom isolated from soil (Windels and Kommedahl, 1974; Burgess et al., 1981). The aetiological studies of Windels and Kommedahl (1984) suggest that this species could build up its inoculum potential in debris, on or near the soil surface, and in standing maize. The present results supported the observation that soil is probably not an important inoculum source of this species, while the organic matter in soil (old stalk, maize ear, stubble etc.) could be the principal one. The present study also reveals that the husks and kernels can also be an important source of inoculum.

In New Zealand F. graminearum is apparently not important as a plant pathogen in maize and in fact it is not well known in New Zealand maize, although as its teleomorphic stage (G. zeae) it has been reported as being involved in stalk and cob rots (Fullerton, 1978), particularly in wetter areas. F. graminearum is better known in other crops, eg. in barley (Chong and Sheridan, 1982; Lee, 1986) and in grass (Lauren, 1986).

A review by Sutton (1982) noted that F. graminearum is responsible for cob rot world-wide, with reports from most maize-producing countries (Canada, U.S.A., Southern and Eastern Europe, Central and Southern Africa and U.S.S.R.). There is a great deal of concern about such a high level of contamination by F. graminearum in maize, because isolates of it are well known to produce several important toxins such as DON, ZEA, NIV and FUS-X (Ichinoe and Kurata, 1983; Mirocha, 1984).

The results of the present investigation agree that F. graminearum is common in maize, but it apparently occurs as a saprophytic organism producing no symptoms on the plant.

The third most frequent Fusarium species was F. acuminatum (11/15 samples). It was present in all maize and litter samples and its population in maize and litter was the highest among those species present there (3.2×10^3 and 3.7×10^5 CFU/g respectively). It was also common in husk samples, with a level of contamination second after F. graminearum. It was not found in soil. These results support those of Kommedahl et al. (1979) who found that F. acuminatum was the predominant Fusarium in maize stalk debris. F. acuminatum could be significant as a mycotoxin-producer as it is known that several mycotoxins (T-2 toxin, HT-2 toxin, DAS and NEO) can be produced by strains of this species (Ichinoe and Kurata, 1983).

Amongst the other frequently-occurring fusaria was F. oxysporum (9/15 samples). It was the predominant Fusarium spp in all soil samples, while it was second in husk and litter. No F. oxysporum was recorded from maize.

Fusarium oxysporum is one of the most prevalent soil-borne fusaria and is highly variable. Some varieties are very pathogenic, causing wilt, crown and root rots while the saprophytic strains are common colonisers of senescent or damaged plant tissue (Burgess, 1981). The results of the present study support previous studies from Canada (Gordon, 1954), from the U.S.A. (Windels and Kommedahl, 1974) from Australia (Wearing and Burgess, 1977) and from New Zealand (Falloon, 1982) that F. oxysporum is the most common isolate from soil and roots.

Although the level of contamination of maize kernels with F. oxysporum was not significant, there is a possibility of some mycotoxins being produced under suitable conditions in grain. Results from other investigators show that some isolates of this species can produce T-2 toxin, ZEA and MON (Ichinoe and Kurata, 1983; Rabie et al., 1982).

Fusarium subglutinans was found at a moderate frequency (8/15 samples). Its population level was high in soil and its regular isolation from soil samples (Wearing and Burgess, 1978) has led to the soil being considered its most important inoculum source. F. subglutinans and F. moniliforme are often lumped together and in the past have caused confusion, but several investigators now regard them as two distinct species (Marasas et al., 1979c) (this will be discussed later).

A few other Fusarium spp also occurred at reasonably high frequencies or population levels. These species were F. poae, F. solani and F. crookwellense. F. poae was common in the husk and kernels while none was detected from litter or soil. Overseas reports indicate that F. poae can be recovered from soil, but mostly at a low level of occurrence (Domsch et al., 1980). It is common in temperate regions where it is considered as a weak parasite or saprophyte, but may be associated with some plant diseases such as cob rot in maize in France, Poland and North America (Booth, 1971).

Fusarium solani was isolated from soil samples only but at moderate population levels (Results not included in Table 3-16). This species is a common soil-borne fungus (Gordon, 1956; Wearing and Burgess, 1977). It includes parasitic and saprophytic groups. The parasitic members can be important in root and crown rots (Burgess, 1981). The present results agree with the opinions of other investigators that F. solani is rarely found in cereal grain (Domsch et al., 1980).

Fusarium crookwellense occurred at almost equal frequency from all substrates. Although its overall frequency of occurrence was low (5/15 samples), it could be important as a toxigenic species. It has been recovered from grass pasture in New Zealand and some isolates have been found to be strongly toxigenic, producing a large variety of toxins (Lauren, 1986). The ecology of this species was discussed in Section 1.1.2.

The Mycoflora of Maize at Harvest

The general mycoflora of maize kernels in the field and during harvest have a special importance in regard to the invasion of the kernels by fungi and the possible release of mycotoxins (Sutton et al,, 1980). The frequency of occurrence of Fusarium spp from the harvest samples examined was almost the same as that from field samples (Table 3-24), but the actual level of contamination of the maize kernels was lower at harvest time for most species, particularly F. graminearum, F. culmorum and F. acuminatum. Similar results to these have been reported by other investigators (Hesseltine and Bothast, 1977; Miller et al., 1983). Miller and his colleagues experimentally inoculated maize ears with F. graminearum 79 days after planting the maize. They noticed that the propagules of F. graminearum present as the maize developed increased after inoculation for six weeks and then started to decrease, and they suggested that such a decline was due to end-product inhibition by DON, although it could also be attributed to the lack of moisture and nutrition. Hesseltine and Bothast (1977) also reported that the numbers of Fusarium-infected maize kernels were lower in the last few weeks before harvesting, but no explanation for this was given.

In the present study the decrease of the Fusarium population could well be due to the decrease of moisture content of the kernels. The MC of the field maize averaged 32.2%, which would support good growth of Fusarium spp. According to Sutton (1982) F. graminearum grows vigorously at 35% MC but sparsely near 20-22% MC. Therefore the dropping of MC to 22% (average) at harvest time as observed in the present study could be the main factor causing the decrease in the population level of most Fusarium spp. Supporting this concept was the observation that the levels of F. subglutinans remained constant. This species has the ability to continue growth at low MCs (Russel et al., 1982).

Regarding the level of contamination by Fusarium spp, the results, as well as the results of other workers (e.g. Neish et al., 1983) indicate that it is difficult to rely on analysis of grain at harvest to assess the significance of Fusarium spp on that grain. Assay of the grain in the field must be performed. This also supports

the conclusion of Sturz and Johnston (1983), that the early occurrence of toxin-producing fusaria in the grain should not be overlooked when considering the amount and type of mycotoxin present in cereal grain at the end of the growing season. Fusaria isolated from the ears late in the season are not necessarily the only producers of mycotoxins which may be present in contaminated seed.

The maize kernel samples at harvest showed a greater variety of fungi overall than did field samples (a total of 17 genera from seven harvest samples, compared to only 13 genera from six field samples). Four genera (Acremonium, Epicoccum, Fusarium and Penicillium) occurred at the highest frequency, 7/7 samples each. Nigrospora, Cladosporium and Mucor were also common (Table 3-20). However, in terms of population level, Fusarium was the most abundant (58.3%) followed by Penicillium (27.7%) (Table 3-19). These results agree well with those of Marasas and Smalley (1972) who isolated 17 genera from freshly-harvested (but mouldy) maize. Cladosporium, Fusarium, Acremonium and Penicillium were the most common.

Most other investigators have also found that Fusarium is one of the most common genera from freshly-harvested maize. Bothast et al. (1973) found that 55 to 77% of whole maize kernels (freshly harvested) were infected with various fungi, but two genera, Fusarium and Penicillium, were predominant. Aspergillus, Helminthosporium, Nigrospora and Trichoderma were also found at significant levels.

The Mycoflora of Stored Maize

All maize under investigation was artificially dried to below 14% MC and then stored under controlled conditions (by aeration), except for the samples from Silo C, which had been stored for about 12 months in an unaerated silo before the first sample was collected. The controlled storage conditions prevented most fungi from multiplying, with a few exceptions (e.g. Penicillium), while many genera decreased greatly (e.g. Fusarium). Species of Penicillium were present in greatest amounts in Silos A, B and C (23, 12.3 and 43% respectively) and the genus was also the most frequent among all the genera present. Another two genera found at high levels were Nigrospora (31%) from Silo B and Aspergillus (35.6%) from Silo C. Both Penicillium and

Nigrospora were common at harvest time and apparently the drying process did not affect them. The remainder of the 17 genera from all 12 samples occurred very rarely. The frequency of occurrence of Fusarium spp was 9/12 samples, second to Penicillium (11/12), but the fusaria were found at very low levels of contamination (<1% in all samples).

Many Fusarium spp may be killed during the drying and storage process (Miller et al., 1983). Only three (F. subglutinans, F. graminearum and F. poae) were found in the samples examined. These species can be classified as storage fungi (Marasas et al., 1979c; Jackson et al., 1974).

Silo C showed the highest level of contamination (81%) and Aspergillus spp were particularly numerous. This could be due to the long storage of the grain in this silo (12 months). Grain from Silo B showed a high level of contamination (51%), perhaps because the maize in this silo came from field J2 which had shown grain with high mould levels; the MC was also higher at 14.5%.

The observations of other investigators studying stored maize vary greatly and many factors such as MC, storage period and storage conditions can influence results. For example, Marasas and Smalley (1972) reported a very high fungal population (5×10^7) from very moist (30%) mouldy maize which was stored at 10^{o}C. The authors reported that four genera (Cladosporium, Acremonium, Fusarium and Penicillium) were present in large numbers particularly at the beginning of storage. As the storage progressed the level of Fusarium fell. However, Ciegler (1978) reported that if grain was stored at a MC above 25%, Fusarium spp could become a major concern, while at about 15% MC Aspergillus and Penicillium were common. Marasas et al. (1979c) reported a controversial result in that they detected three Fusarium spp (F. subglutinans, F. moniliforme and F. graminearum) from South African maize at harvest time, where the MC was below 13%. They found that the level of contamination by all Fusarium spp was 19% (mean of all samples). But this level of contamination increased to 34% after eight months' storage, although the MC was only 14%. Such results could indicate a weak relationship between MC and contamination by Fusarium spp.

General Comments Regarding the Isolation of Certain Fusaria

An important finding in the present study was the detection of F. subglutinans, while F. moniliforme was found only once. F. subglutinans was a common isolate in the majority of samples and it was third in frequency of occurrence, after F. graminearum and F. culmorum (Table 3-35). It is worth noting that the frequency of this species increased in maize kernels from the field to storage and it was the most common Fusarium spp to be isolated from stored samples.

Because of misidentification, and lumping of this species with F. moniliforme until recently, little is known about its true prevalence and geographical distribution (Marasas et al., 1979c; Neish et al., 1983). According to Neish et al. (1983), results from most studies carried out in North America show that F. subglutinans has not been distinguished from other varieties of F. moniliforme, so it is not known whether F. subglutinans was predominant in that area or not. Similarly, little has been reported on F. subglutinans in New Zealand. Most of the literature mentions F. moniliforme (Dingley, 1965; Fowler, 1985; Lee, 1986).

The two species are widely isolated from plants, soil and seeds but F. subglutinans is common in cooler areas with a high rainfall and is often found to cause cob and kernel rots in maize, while F. moniliforme is found in areas which have a subtropical climate (warm and dry) and is often reported as causing stalk rot of maize plants (Booth, 1971; Francis and Burgess, 1975).

The New Zealand climate (temperate) should be expected to support F. subglutinans better than F. moniliforme and the present results support such an idea. Fullerton (pers. comm.) considers F. subglutinans as the most common species isolated from New Zealand maize with stalk and cob rots.

No species belonging to the section Sporotrichiella were found except for low levels of F. poae. Such results agree with the view of Vesonder and Hesseltine (1981), who found the above species were less frequent on maize in temperate zones.

Unlike results of other researchers in New Zealand (e.g. Hampton, 1980; Lee, 1986) F. nivale ces ex. Sacc. [for which the name Gerlachia nivalis has been suggested (Gams and Muller, 1980) or more recently Microdochium nivale, included in the Hyponectriaceae, (Samuels, 1984)], was not found during this study. This could be because the incubation temperature (i.e. 24^{o}C) was not suitable. Toussoun and Nelson (1976) recommend a 5-10^{o}C incubation temperature for this species.

The present study has shown that fusaria are the predominant isolates from maize both in terms of frequency and level of contamination. Such a result has not been previously reported for New Zealand maize grain. The majority of isolates were of saprophytic species and so may have been ignored by plant pathologists. It is not uncommon to find a high level of contamination by one or more Fusarium spp with little or no disease apparent (Kommedahl et al., 1979). Similar results were reported by McKenzie and Taylor (1983) who found a very large Fusarium population in pasture soil, particularly of F. culmorum, with little evidence of parasitic invasion of the roots.

Analysis of Maize for Mycotoxins

The economic significance of individual mycotoxigenic fungi and their mycotoxins may be gauged according to their prevalence in the food or feed supply in conjunction with the nature and degree of toxicity of the mycotoxin to a certain animal species. In New Zealand the poultry and pig industries are the most likely to face problems with Fusarium toxicoses following animal consumption of contaminated products. Maize constitutes about 63% of the feed grain consumed by both poultry and pigs in the North Island. Although acute mycotoxicoses are probably rarely found, and are usually suspected and diagnosed by veterinarians, the chronic and non-lethal effects of mycotoxins are the most common and these are not recognised easily. Thus the approach where the presence of toxigenic fungi in a particular commodity such as maize is determined and the toxins they produce identified is to be recommended. It can provide useful information in predicting mycotoxin problems, which may occur at the chronic level.

In the present investigation Kamimura's method (Sections 2.2.5 and 2.2.6) was followed when screening for five Fusarium toxins (DON, DAS, T-2 toxin, ZEA and MON) in maize samples as well as in Fusarium cultures. These mycotoxins (except MON) are possibly the most commonly found in nature (Ueno, 1983). MON was assayed because of the level of maize contamination with F. subglutinans (Section 3.1.5.2) which is a well-known MON-producer.

Although this method has been acknowledged by several authors (Scott, 1982; Naoi, 1983) for its low detection limits (<100 ug/kg), good recovery and its ability to analyse several trichothecenes simultaneously, it does not include a mycotoxin confirmation stage. Thus, in the present study the GC-MS technique was included for both confirmation and quantititation. All the final results for trichothecenes were based on GC-MS, so that results from other techniques (TLC and GC-FID) could be assessed for false negatives or false positives by comparing all three methods.

Quantitative analysis of foods and feeds for multimycotoxins remains a challenging problem, particularly with the poor sensitivity and reliability of many current methods (Scott, 1982). In fact the sensitivity of any analytical method will depend on the quality of the sample, which is limited by the ratio of interfering substances to the toxin present (Ilus et al., 1981). The maize samples examined in the present study were extensively contaminated with a large number of interfering substances (lipids, pigments etc. and possibly unknown mycotoxins). Such problems are common in natural products and the two-step clean-up employed did not seem to help too much. There is currently no adequate clean-up procedure.

The TLC method was found to be simple and less time-consuming than other described methods for the detection of Fusarium toxins. It was also useful in that it was particularly inexpensive. But it is not sensitive enough to detect low levels of toxins, especially trichothecenes (Eppley, 1982). So the results obtained by TLC confirmed that this technique is not a suitable one for low levels of trichothecenes (below 100 ppb). Most of the results for T-2 toxin, DAS and DON by TLC were suspiciously positive, the main problem being the presence of large amounts of unknown fluorescent compounds with

similar Rf values to the toxin. It was also difficult to differentiate between T-2 toxin and DAS. Pathre and Mirocha (1977) also found that the Rf values of these two toxins were similar in most solvent systems. Thus the TLC method was of limited value.

Generally, the Rf values noted during the present study were higher than those reported by Kamimura et al. (1981), using the same developing solvents. This could have been due to the washing of the TLC plates with solvent before spotting was done (Section 2.2.6.1). Nevertheless the Rf values found by most workers vary considerably. Such variability could be due to the silica gel used, its thickness, degree of activity, atmospheric moisture, room temperature etc.

In situations where there is no alternative to the TLC technique, several steps could be taken to improve both the specificity and sensitivity. Using different development solvents, developing the TLC in two dimensions and spraying the plate with more than one visualisation reagent could all help. However, the main object of using the TLC method in the present study was as a primary screening tool, to give an initial indication of the kind of contamination of New Zealand maize by Fusarium toxins. Such initial information is obviously important because of the lack of such information about the status of Fusarium toxins in this country. As has been mentioned previously, the TLC method was used for detection of trichothecenes without any attempt at quantitation, but it was also used successfully for the detection and quantitation of ZEA and MON, as will be discussed later.

It is difficult to decide what detection limit can be achieved by the TLC method from naturally contaminated commodities as most of the assessments of this technique (as well as of other techniques) depend on the use of authentic standards or artificially contaminated samples. Obviously, the assessment criteria for naturally contaminated samples differ from those for authentic standards. Thus many authors are doubtful about the sensitivity of TLC for natural extracts as it is difficult to interpret the results obtained by the TLC method (Chaytor and Saxby, 1982).

The low sensitivity for trichothecenes in particular, as obtained in the present study, has been generally agreed amongst investigators

in this field and the detection limits reported are highly variable. Chaytor and Saxby (1982) stated that the TLC detection limit was several hundred parts per billion (ppb) in naturally contaminated foods. Eppley et al. (1984) achieved a limit of detection of 200 ppb for DON from naturally contaminated grain after improving the clean-up procedures and using $AlCl_3$ as spraying reagent. Later the same method was employed by ten collaborative laboratories and this study indicated that the limit of detection was about 300 ppb (Eppley et al., 1986).

Scott (1982) considered that the method reported by Kamimura et al. (1981) was the method which achieved the best sensitivity (for spiked samples) using 20% $AlCl_3$ spray reagent for type B trichothecenes. Kamimura and his colleagues obtained 20-50 ppb sensitivity, while trichothecenes of type A gave 100-500 ppb sensitivity after spraying with 20% H_2SO_4.

Notwithstanding the difficulties encountered with the trichothecenes, the TLC method proved very useful for the detection and quantitation of ZEA and MON in the present study and this is in agreement with the findings of other investigators. Kamimura et al. (1981) reported a high sensitivity using the TLC method for ZEA, detecting 10-50 ppb of ZEA in spiked samples. A level of 40 ppb was achieved in the present study although the actual smallest concentration of ZEA reported from samples was 100 ppb. A sensitivity of 10 ppb (Kamimura et al., 1981) has not been recorded by any other investigator, but a sensitivity of 100 ppb is supported by the results of the collaborative study of Shotwell et al. (1976), four collaborators out of 16 detecting ZEA in samples containing 100 ppb. However, five out of the 16 collaborators missed even the 300 ppb level of contamination.

In the present study the ZEA concentration was assessed visually before spraying with $AlCl_3$ solution. This is a very common method for quantitation of mycotoxins by the TLC method (Shotwell et al., 1976; Eppley et al., 1986). An alternative method for visual assessment is to use a densitometer, by which more consistent results may be achieved (Shotwell et al., 1976). However Eppley et al. (1986) in a study of the detection of DON on TLC concluded that even using

densitometers results can be unsatisfactory. They comment that densitometers from different manufacturers have various combinations of mono-chromatic and/or filter components, so the instrument parameters would have to be carefully specified in any comparative studies.

MON was also detected and quantitated by the TLC method using densitometry, after being derivatised with 2,4-DNPH. A good alternative to TLC is HPLC (Shepherd and Gilbert, 1986) and UV spectrometry has very often been used for confirmation and quantitation (Rabie et al., 1978). In the present study UV spectrometry was used for confirmation of MON (Figure 3-26).

When TLC is used for analysis of MON, the toxin can be visualised by quenching of fluorescence indicator (a dark spot on a fluorescent plate). But false positives due to quenching of fluorescence by interfering substances having the same Rf value as the MON were noted. These substances were found in both maize samples and from cultures of Fusarium. Use of the 2,4-DNPH derivative gave excellent differentiation between MON and these interfering substances.

Another assay method which was used in the present study was GC-FID, which gave more positive results for all Fusarium toxins than did the TLC method (Table 3-40). But when the GC-FID results were compared with those of GC-MS, closer comparative results were obtained in spite of there being false positives for T-2 toxin and DON (ranging between 12-27%) with GC-FID. The main disadvantage of GC-FID was the co-chromatography of interfering substances. Also GC-FID has been found to be less sensitive than GC-ECD, at levels ranging between 2.5 fold for T-2 toxin and DAS and 50 fold for DON (Kamimura et al., 1981). Because of the interference problem some samples and isolates showed an apparently high amount of toxin, but when the GC-MS technique was used, actual amounts were found to be a few parts per billion. False positives with either GC-FID or GC-ECD are commonly found and have been reported by several authors (Scott et al., 1981; Scott, 1984; Gilbert et al., 1984 etc.). Even the GC-ECD technique, which is superior to GC-FID, was found to give 15% false positives by Gilbert et al. (1984). Scott et al. (1981) found that two out of six samples positive for DAS by GC were false positives when tested by

GC-MS. Again Scott (1984) reported false positives for DAS, NEO and NIV by GC and he recommended the use of GC-MS (SIM) for confirmatory purposes.

GC is used for the detection of Fusarium mycotoxins after derivatizing these toxins with suitable derivative reagents. Using an internal standard as well as a mycotoxin reference standard improves the specificity, while using different derivative reagents and employing capillary columns improve both the sensitivity and specificity of this technique (Gilbert, 1984). Even the length of column or the age of it affects the sensitivity significantly. Szathmary et al. (1980) found that by using a 20 m capillary column the resolution capacity was considerably improved compared to a 10 m column. Similarly it was found in the present study that a 50 m column was better than a 12 m column. The main problem with the above comments (i.e. different capillary columns and derivatives etc.) is the expense of the chemical materials.

Investigators have reported variable results regarding detection limits and recovery percentages when using GC. Osborne and Willis (1984), using GC-ECD reported that the limit of detection of DON, DAS and T-2 toxin were 0.2, 0.1 and 0.25 mg/kg respectively and the recoveries were 80, 40 and 48% respectively. Kamimura et al. (1981) reported 0.002 mg/kg for DON and 0.08 mg/kg for DAS and T-2 toxin with GC-ECD. By using GC-FID, these authors obtained detection limits of 0.1 mg/kg for DON and 0.2 mg/kg for T-2 toxin and DAS. The recovery rates for both GC techniques were 93, 103 and 83% for DON, DAS and T-2 toxin respectively. The detection limit of these three toxins in the present study, using capillary column GC-FID showed an improvement compared to Kamimura's results (GC with FID). The detection limits were estimated at about 0.08 mg/kg for DON and 0.1 mg/kg for DAS and T-2 toxin although some samples showed a lower limit (0.009 mg/kg DON). Apparently using a capillary column causes such an improvement (Szathmary et al., 1980).

Obviously, most investigators depend for their calculation of the detection limit on an analysis either of pure mycotoxin standards or spiked samples. But any analysis of a naturally contaminated sample could give a different result because of the interfering substances

present. Even though Kamimura et al. (1981) reported very low detection limits for DON and NIV (2 ppb) in spiked samples, when their method was applied to naturally contaminated grain, they expressed low levels as "a trace - unknown amount". Gilbert (1984) was of the opinion that background interference, particularly in cereals, makes for uncertainties in detection below a 50 ppb level even using the GC-ECD.

The GC method is capable of giving quantitative results, but analysis by GC without mass spectrometry could lead to the misidentification of toxins when an interfering substance has the same retention time as the toxins. The identification, quantitation and confirmation by the usual peak enhancement techniques would not be valid in this latter situation. Mirocha et al. (1976a) detected two lipid components (1-glyceryl-mono-oleate and 1-glyceryl monolinoleate) produced by both fusaria and the plants, which when derivatized with TMS have the same retention time as T-2 TMS on a 3% OV-1 column. Mirocha and his colleagues avoided this interference either by using different derivatives such as the trifuoracetyl derivatives or by subjecting the sample to TLC prior to analysis by GC.

In the present study variation of the GC response for mycotoxin standards and the internal standard was noticed from time to time. Such variation could be due to degradation of the capillary column by the derivatising reagents (Gilbert et al., 1985) or fluctuation in detector performance (Romer et al., 1978). Romer and his co-workers noticed the detector response varied from day to day so they recommended the application of internal standards daily. These authors obtained 0.1 mg/kg for T-2 toxin and 0.025 mg/kg for DAS as the lowest detection limits by using GC-ECD with HFBI derivative.

There are innumerable variations which can be faced during screening procedures for naturally contaminated cereal grains. Some of these variables are easy to control while others are not. In the present study, the difference between the GC systems has been observed to affect the chromatographic separation of the mycotoxin derivatives.

Gas chromatograms of mycotoxin standards obtained from a Pye gas chromatographic system showed a large number of unidentified by-

product peaks (Figure 3-15) while Figure 3-17 obtained from an Hewlett-Packard GC system did not show such by-products. The high level of by-products may be due to the presence of degradation products such as excess of the TMS derivative in the first column (old) while the second column was newly applied. This finding was similar to that of Kientz and Verwej (1986) who noticed three unidentified by-product peaks in addition to TFA-DAS derivative peaks when using GC on a Pye GCV while no by-product peaks were noticed when using a Packard Becker GC system. The authors attributed that as being due to differences in the system as well as being caused by excess of derivatising reagent.

If a positive result for mycotoxins is obtained by either TLC or GC a good confirmatory test should be performed before the sample is judged to contain these mycotoxins (Romer et al., 1978). Most authors agree that the best confirmatory test is the mass spectrometric method (Scott, 1982; Gilbert t al., 1984). Using the GC-MS in the present study allowed precise identifications of DON, DAS, T-2 toxin and ZEA by fragmentation of their TMS-derivatives. These mycotoxins are unlikely to be masked by interfering substances with identical retention times as happened with GC-FID. The mass spectrum was focused on selected characteristic fragmentations of the mycotoxins. This technique (SIM) is very useful for the detection of small amounts of mycotoxins. It allows confirmation of suspected positives without resorting to full-scale mass spectrometry, a technique that requires more material. Sensitivity of the instrument is also improved in the SIM mode. Chaytor and Saxby (1982) point out that GC-MS is extremely sensitive as the instrument monitors only a few ions at a time.

In the present study five selected ions were chosen to give maximum specificity. Other investigators (e.g. Scott et al., 1981; Cohen and Lapointe, 1982) have used just a single ion for confirmation but Rosen and Rosen (1984) and others regard single ion monitoring as not being a truly confirmatory method. A minimum of three selected ions is highly recommended for quantitation and confirmation of Fusarium mycotoxins (Mirocha et al., 1974; Rosen and Rosen, 1984), although two have not uncommonly been used (Vesonder et al., 1981). Mirocha et al. (1974) detected as low as 10 ng/g ZEA in samples, using three selected ions and Rosen and Rosen (1984) obtained 20-40 ng/g

sensitivity for TMS derivatives of T-2 toxin, HT-2 toxin, DAS and ZEA by using GC-MS with three selected ions.

In this study, applying five selected ions assisted confirmation of the toxins' identity, and also gave high sensitivity. The limit of detection achieved was normally around 10 ng/g for each trichothecene, although 2 maize samples (T1 and T2) were observed to be contaminated with T-2 toxin at levels of 5 and 7 ng/g respectively. But with ZEA the sensitivity was lower by a factor of ten because of derivative deterioration.

Further advantages of the technique used were the use of a capillary column and a mass spectrometer with quadropole, which assisted in increasing sensitivity. However, as Romer et al. (1978) comment, some disadvantages also exist. The type of mass spectrometer can affect the sensitivity level and whenever analyses are carried out at low levels (i.e. ppb) in complex matrices such as naturally contaminated grains or food, various interferences may occur. This problem was noted in the present study several times. For example, an unknown component having a similar M/Z as the five SI of ZEA but at a retention time of about 20.2, was found in samples AN-1 and T2 (Figure 3-23). There may also be variations in quantitation caused by instability of instrumental electronics (Rosen and Rosen, 1984). But perhaps the main problem of this technique is that it is not freely available because of cost (Gilbert et al., 1984).

The results of the present work are presented in Table 3-41 as percentage frequencies of occurrence as well as the levels of contamination for the three trichothecenes and ZEA. The prevalence of DON, T-2 toxin and ZEA were lower than that reported by the GC method but DAS was detected by GC-MS at levels higher than by GC. But in general the GC-MS results were closer to the GC results than to the TLC results, with the exception of ZEA (Table 3-43 and Figure 3-24).

Although quantitation of these mycotoxins by the three different methods indicated overall agreement, particularly for DON and DAS, discrepancies did exist. Even though precautions are taken during the analytical procedure, errors are still unavoidable. The collaborative study reported by Shotwell et al. (1976) showed that errors within

each laboratory were 64% while errors between collaborating laboratories were 36%.

Eppley et al. (1984) claimed comparibility between the GC-ECD and TLC methods for detecting DON in wheat samples contaminated at levels of over 0.5 mg/kg. However, when their results are analysed, there are obvious differences in the quantitation levels of the two methods (Table 4-1).

Table 4-1: Comparison of TLC and GC-ECD methods for detecting DON, as reported by Eppley et al. (1984).

Wheat sample	DON (µg/g) by TLC	DON (µg/g) by GC-ECD	Difference
1	0.5	1.7	-1.2
2	4.0	3.5	0.5
3	8.5	4.6	3.9
4	0	0.2	-0.2
5	1.4	1.4	0
6	3.0	1.0	2.0

In this study ZEA-TMS was detected and confirmed by GC-MS but could not be satisfactorily quantitated by either the GC or GC-MS methods, due to the instability of ZEA-TMS derivatives (Rosen and Rosen, 1984). This problem is apparently not restricted to TMS ethers. Kientz and Verwej (1986) noticed highly irreproducible results for ZEA on a GC system when using different derivatization reagents.

Prevalence of Mycotoxins in Maize

This is the first report about the natural occurrence of Fusarium toxins in samples of maize in New Zealand. Factors affecting the production of Fusarium toxins include the existence of toxigenic strains, a suitable substrate (i.e. maize) and a favourable climate. Results from this limited survey are summarised in Table 3-45 as frequency of occurrence (percentage) as well as by a semiquantitation

of the level of Fusarium toxin contaminating the samples. The overall frequency of contamination with one or more Fusarium toxins was about 85% of the samples examined (excluding the poultry feed samples). Although this is a highly significant frequency, it is not surprising, in comparison with the results of other investigators (Blaney et al., 1984; Yoshizawa, 1984). However, the present contamination was with low levels of individual toxins and is probably not significantly important (except ZEA in some samples) if the combined effects (additive or synergistic) of more than one Fusarium toxin are ignored. Low levels of mycotoxin in animal and human food chains are commonly found and it is still a matter of debate whether such an occurrence constitutes a real long-term hazard to human and animal health (Smith et al., 1984).

The predominant occurrence of ZEA in the present study is in agreement with most of the results from other parts of the world. ZEA was found to be the most frequent Fusarium mycotoxin in maize and feed samples in the U.S.A. (Mirocha et al., 1976b; Pier, 1981; Bennett et al., 1985), in Canada, particularly from maize in Southern Ontario (Sutton et al., 1980) and in Australia (Blaney et al., 1984). In New Zealand, a survey of wheat grain (no work has been done on maize samples) showed 100% of the samples examined were contaminated with ZEA (Agnew et al., 1986).

The frequently reported occurrence of ZEA, as found in the present study, is probably due to two main factors, namely the common incidence of Fusarium spp that are capable of releasing ZEA (e.g. F. graminearum and F. culmorum) and the simplicity of the detection of ZEA, particularly by TLC. In the New Zealand maize examined, the frequency of occurrence was 75% at levels ranging between 0.1 and 16 ppm. The frequency of occurrence in Australian maize reported by Blaney et al. (1984) was 85%, at levels ranging between 0.25 and 2.0 ppm.

The only other report about the natural occurrence of ZEA in New Zealand in addition to that of Agnew and his colleagues is that of di Menna et al. (1985), who detected ZEA in pasture leaves. They examined 61 samples, 16% of which were contaminated with ZEA at levels ranging between 0.2 to 2.6 ppm.

Although the level of ZEA contamination in the present study was generally low, ranging between 0.1 to 2.9 ppm, one sample (J2H) showed a high level of contamination of 16.0 ppm by the TLC method. Analysis by GC-MS gave only 0.08 ppm in this sample and 3.0 ppm by GC-FID. The 16.0 ppm detected by TLC could be due to the ZEA being mixed with an interfering substance having similar characteristics to ZEA on TLC, but when fast Violet B was employed for confirmation, the result did agree with the previous one.

In the present study T-2 toxin attracts special attention because of its unusually high frequency of occurrence (65%) in the maize samples. This toxin is capable of causing severe systemic disease in both humans and animals, either alone or in combination with other trichothecenes. Fortunately the level of contamination by T-2 toxin was very low (<0.2 ppm) and probably no risk to animal and human health is to be expected.

There are only sporadic reports about the natural occurrence of T-2 toxin world-wide, originating from the U.S.A., Canada, France and Italy (Ueno, 1983). Although the toxin was reported at low frequencies (except from Italy), most occurrences were associated with outbreaks of mycotoxicoses either in humans or animals (Hsu et al., 1972; Greenway and Puls, 1976; Joff, 1978). The results of the Italian survey showed that T-2 toxin can be the most common of the trichothecenes (compared to DAS and NIV), particularly in imported grains (Cirilli, 1983). The Italian investigators reported that T-2 toxin occurred at high frequency in imported barley from Australia (32%) while imported maize from Argentina and Canada were also significantly contaminated at levels of 22% and 15% of samples respectively. Domestic maize was contaminated at the 3% level.

The poor sensitivity of T-2 toxin for most chemical assay methods (Scott, 1982) could be the main reason that samples naturally contaminated with small amounts of the toxin remain undetected. For example, Hintikka (1983) failed to find any trichothecenes in Finnish grain by the TLC technique but by using GC, small amounts (10-50 ppb) of T-2 toxin were detected.

The frequency of occurrence of DON in the New Zealand maize samples was also significantly high (50%), with contamination levels ranging between 0.02 to 0.3 ppm. DON has been isolated from maize and mixed feed more frequently than other trichothecenes in overseas studies and reports have shown that DON commonly occurs in cereal grains in the U.S.A. (Vesonder et al., 1978), Canada (Scott, 1984), U.K. (Gilbert, 1984) and Japan (Yoshizawa, 1984). In Canada, DON was second in importance to ZEA (Andrews et al., 1981) and Scott (1984) found DON contaminating wheat grain at levels of 0.13 to 0.74 ppm. In the U.K. 16% of home-grown wheat samples were contaminated at levels of 0.2 to 0.4 ppm (Gilbert, 1984). Recently, Tanaka et al. (1986) reported 65% of U.K. wheat samples were DON-positive, but at low levels (0.031 ppm). The highest level of DON has been reported from pre-harvest maize in the U.S.A., 24 out of 52 samples being contaminated with DON at 0.5 to 10 ppm (Vesonder et al., 1978).

The frequency of occurrence of DAS (25% of samples) was the lowest amongst the Fusarium mycotoxins detected in the present study, but such a result was not unexpected. There is only scarce information available in the literature about the frequency of occurrence of this toxin. Mirocha (1983) stated that DAS was found infrequently in nature. In Italian grains and feedstuff DAS was found less frequently than other Fusarium toxins (Cirilli, 1983) and none was found as a natural contaminant of either Canadian grains (Neish et al., 1982) or U.K. grains (Osborne and Willis, 1984). However, DAS is considered to be the most toxic among the trichothecenes (Mirocha, 1983).

All the samples were screened for MON by the TLC method (Section 2.2.6.1), and a number appeared positive for MON as assessed by the appearance of dark absorption spots or bands under 254 nm UV light which had an identical Rf value to the MON standard. But spraying the TLC with 2,4-DNPH did not give a brick-red colour as described by Kamimura et al. (1981), and TLC of the DNPH derivatives (Section 2.2.6.5) showed no detectable MON (detection limit ca 0.1 ppm) in any sample tested (Table 3-42). Only two papers seem to have been published on the subject of natural contamination by this toxin. Thiel et al. (1982) was the first to detect MON in two maize samples (levels of 16 and 25 ppm) from South Africa. Thiel et al.

(1986) again reported the detection of MON as well as fusarin C from one maize sample from the U.S.A.

The majority of samples were contaminated with a single mycotoxin, but some were contaminated with three or four different mycotoxins. Three samples (J2, T4 and T1) were contaminated simultaneously with four Fusarium toxins. Few investigators have detected so many mycotoxins simultaneously. One of the few reports is that of Jemmali et al. (1978) from France. These authors found NIV, DON, T-2 toxin and ZEA in maize samples.

In the present study seven of the samples were contaminated with three Fusarium toxins, particularly DON, ZEA and T-2 toxin. Mirocha et al. (1979) also reported the above three mycotoxins from corn stalk samples, while DON, DAS and ZEA were detected from mixed feed by Mirocha et al. (1976b). Cirilli (1983) reported three Fusarium toxins (DAS, T-2 toxin and ZEA) from Italian grain and foodstuff samples.

Under laboratory conditions, up to ten toxins have been found to be produced by a single Fusarium strain (Mirocha, 1984) and the natural occurrence of more than one toxin in cereal grains, such as maize, is probably not uncommon. The failure of most investigators to detect several mycotoxins could be because most have screened their samples for single mycotoxins or have used methods which either were not sensitive enough to detect small amounts of mycotoxins (e.g. trichothecenes) or were not suitable for detecting all of the mycotoxins which could be present. For example, in U.K cereals, only one Fusarium toxin (DON) was detected, at low frequencies (<16% of samples), during several surveys (Osborne and Willis, 1984). But more recently when Tanaka et al. (1986) employed more than one technique with an improved method, three toxins (DON, NIV and ZEA) were simultaneously detected, at frequencies of 55, 65 and 13% respectively.

Mycotoxin Production by Fusarium Isolates in Culture

As mentioned earlier there are three factors, the fungal strain, the substrate (medium) and the cultural conditions, which can influence toxin production by strains of Fusarium in culture. It has proved difficult to find a single substrate and single temperature

suitable for toxin production by all species of Fusarium (Greenhalgh et al., 1984; Bottalica et al., 1984). However, the substrates and conditions used for testing the Fusarium isolates obtained in the present investigation were chosen on the general recommendations of other investigators. The Fusarium isolates to be tested were chosen randomly without any previous knowledge as to whether any were positive.

The mycological data described in Section 3.1.5.2 note that 15 different Fusarium spp were found in samples of maize, husk, litter and soil, but not all these species are toxicologically important. The studies of other investigators have shown that most of the toxigenic fusaria belong to five main sections, namely Liseola, Sporotrichiella, Discolor, Gibbosum and Roseum (Palti, 1978; Samuels, 1984). The mycological surveys which have investigated the incidence of various fusaria in samples of grains and pasture grass in New Zealand (Hampton, 1980; Fowler, 1985; di Menna et al., 1985), as well as the results of the present study, show that species belonging to the Sporotrichiella section are rare in this country. It is the opinion of Vesonder and Hesseltine (1981) that this is true of all temperate regions.

Species belonging to the sections Liseola and Discolor are considered the most serious contaminants of New Zealand maize. Two species, F. graminearum and F. culmorum (section Discolor), were found to be the predominant species both in frequency of occurrence and level of contamination of the samples examined (Section 3.1.5.2). The toxigenicity of these species has been studied extensively in various parts of the world, especially with regard to the oestrogenic and emetic effects observed in swine and some other animals (Palti, 1978), and their toxin-producing ability in culture has also been widely studied (Miller et al.,1983; Naik et al., 1978; Sutton, 1982). Isolates belonging to the species F. graminearum have been found to be good ZEA and DON producers (Palti, 1978; Neish and Cohen, 1981; Neish et al., 1982). Neish and Cohen (1981) found that all nine F. graminearum isolates examined produced both ZEA and DON, at levels ranging from 0.1 to 6.8 ppm for DON and from 0.4 to 90.6 ppm for ZEA. However, in the present study, all isolates (11) of F. graminearum examined were ZEA producers, while only 45% of these isolates were

both ZEA and DON producers. Some of these isolates were also found able to produce T-2 toxin and DAS. Three isolates were found producing T-2 toxin, DAS, DON and ZEA simultaneously.

The ability of F. graminearum to produce T-2 toxin and DAS is not well known and no explanation can be suggested for the findings reported here other than that it might be due to confusion caused by the classification system used by different invesigators. For example, F. roseum (of the Snyder and Hansen system) is a collective name of many species including F. graminearum and F. culmorum and has been reported to produce both DAS and T-2 toxin (Pathre et al., 1976; Pier, 1981; Allen et al., 1982). Only recently has direct evidence been presented that isolates of F. graminearum produce DAS and T-2 toxin (Rabie et al., 1986; Chatterjee et al., 1986).

The ability of about 45% of F. graminearum isolates to produce T-2 toxin, even though in small amounts, is a most interesting finding of the present study and is the first report of this property in Australasia. F. graminearum strains producing T-2 toxin have previously been isolated in Europe (Bottalica et al., 1984), in North America (Scott et al., 1980), in Asia (Tseng, 1983) and recently in Africa (Rabie et al., 1986). Rabie et al. considered that the geographical distribution of toxigenic strains of the fungus could be an important factor, in addition to the kind of analytical technique(s) used.

The influence of the geographical factor in the distribution of toxigenic strains was studied by Joffe and Yagen (1977). These authors concluded that a certain toxigenic Fusarium strain may be more prevalent in a specific geographical area. They also found that strains of the Sporotrichiella section from the Orenburg district of the U.S.S.R. produced a much higher level of T-2 toxin under identical conditions in culture than did similar isolates from other areas. Hesseltine (1977) stated that because some fusaria can grow over a wide range of temperatures, a certain Fusarium strain may be found to produce several different toxins, each at a different time depending on the substrate and temperature. This could explain why some strains of F. graminearum have been found to be T-2 toxin producers in New Zealand but are rarely reported in other countries.

Even so, the T-2 toxin in the present study, either from F. graminearum isolates or from other isolates, was produced at a low level. Several factors could contribute to this finding, such as the toxigenicity of the strains, the incubation temperature used, the sensitivity of the analytical method etc. Regarding the incubation temperature, Burmeister (1971), Bottalico et al. (1984) and Scott et al. (1980) found that the optimum temperature for the production of T-2 toxin was comparatively low (8 to 14^{o}C) with the yield being much lower or negligible at temperatures of 25^{o}C and above. Botallico et al. (1984) obtained only 20 ppm of T-2 toxin at 27^{o}C while much more (467 ppm) was obtained after incubation at 12^{o}C. Conversely, Rabie et al. (1986) found that all 11 F. acuminatum strains produced T-2 toxin at 25^{o}C with two isolates producing a very large amount (about 2600 ppm). Thus the low production of T-2 toxin in the present study could be partially due to the incubation temperature, although other factors might also be involved.

F. subglutinans (Liseola section) was another Fusarium spp studied extensively in the present study because this species was commonly found in maize and has also been found to be a common MON producer (Marasas et al., 1986). About 47% of the examined isolates were found to produce MON in amounts ranging between 0.4 to 64 ppm. Although the level of MON produced in culture was high, this toxin was not found as a natural contaminant in the samples examined. But in view of the high toxicity and the massive amount of MON which can be produced by these fusaria it appears very important to avoid exposure to this hazard (Kriek et al., 1977).

Single isolates of five other Fusarium spp in addition to the seven of 15 F. subglutinans strains tested were found to be MON-producers under laboratory conditions (Table 3-52). All these species have been reported previously by other workers as MON-producers (Thiel et al., 1982). In the case of F. oxysporum which has also been reported as a MON-producer, one isolate (SN20) was found to produce the highest amount of MON (64 ppm) in the present study.

The confusion in the classification of F. subglutinans and F. moniliforme (discussed earlier) has resulted in misjudgement of the toxigenic ability of these two taxa. However, apparently all

investigators agree that F. subglutinans is the species most often shown to be capable of producing MON (Marasas et al., 1979c; Kriek et al., 1979; Rabie et al., 1978). Marasas noted that only 22% of toxic strains of F. moniliforme produced MON, compared to 81% of strains of F. subglutinans.

A large amount of MON can be produced on maize substrate under laboratory conditions. Steyn et al. (1978) obtained yields of between 2 and 16,000 ppm on autoclaved maize from F. subglutinans isolates. Rabie et al. (1982) reported 33,700 ppm of MON on maize from an isolate of F. moniliforme. Rabie and his colleagues also found large amounts of MON (up to 645 ppm) in a maize field after inoculation of the maize ears with a number of Fusarium isolates known to produce MON.

In this study the amounts of MON detected as being produced by New Zealand isolates were low when compared with the results from other investigators. This could be because either the New Zealand isolates are weak MON-producers or because a significant amount of MON was lost during the clean-up steps. Shepherd and Gilbert (1986) claimed that extensive losses of the toxin could occur during the several evaporation steps of the clean-up processing. However, according to Kamimura et al. (1981), the recovery of MON from spiked maize at 2 ppm was 76%. Amounts of MON from the two positive controls (NRRL 6022 and NRRL 13088) used in this study were reasonably high; they produced amounts greater than 500 ppm. Thus Shepherd and Gilbert's speculation could be inapplicable.

Only two isolates of F. culmorum were screened for their toxin-producing ability, and both were found to produce ZEA. One also produced T-2 toxin. This species is well known to New Zealand investigators as a ZEA producer (Gallagher, 1985; Lauren, 1986).

Because of the high cost of the analysis and the time involved in the work, only one or two isolates from the remainder of the fusaria isolated were tested for toxin production. These species (e.g. F. avenaceum, F. equiseti etc.) occurred either at a low frequency or at a low level of contamination (Section 3.1.5.2). However, some of these species gave interesting results; for example, the highest

amount of MON was produced by an isolate of F. oxysporum and one isolate of F. sambucinum was found to produce four mycotoxins. In addition to the F. sambucinum strain, four toxins were also produced by three F. graminearum isolates. It seems that two or three toxins are not uncommonly reported by other investigators (Bottalico et al., 1984; Greenhalgh et al., 1983), but it is quite rare to record four mycotoxins produced simultaneously.

Significance of Natural Mycotoxin Contamination

The general significance of mycotoxins to animal health has been reviewed in Section 1.4.3, but some further comments are now appropriate in view of the results reported in this thesis.

The level of Fusarium toxins which were found to be naturally contaminating maize samples examined in the present study apparently are not significantly important as individual toxins, since, except for ZEA, they were found at low levels. But before giving any judgement about the safety of commodities, three presently unresolved points should be considered. The first is that the actual amount of any one mycotoxin required to cause symptoms of mycotoxicosis in farm animals is uncertain (Pathre and Mirocha, 1979). The second point is the possibility of the existence of unknown Fusarium toxins in addition to the presently known toxins. And thirdly, the combined effect of more than one toxin either in additive or synergistic form, particularly those structurally-close trichothecenes (e.g. T-2 toxin and DAS), is still not clearly known (Pathre and Mirocha, 1979).

General factors such as species, sex, age, general health etc. can affect the susceptibility of animals to any particular toxin. For instance, gilts and sows are highly susceptible to ZEA compared to other animals. Oestrogenism can be produced in gilts fed rations containing only 1 to 5 ppm ZEA (Pier, 1981). Some authors only accept a nil value for ZEA in grain fed to swine (Sherwood and Peberdy, 1974).

Results from the present study as well as other studies carried out by New Zealand investigators (Gallagher, 1985; di Menna et al., 1985; Agnew et al., 1986) demonstrate that ZEA could be the most

important Fusarium toxin in this country, both in its frequency of occurrence and level of contamination. The level of ZEA detected in the present study is of more concern to the pig industry than to the poultry industry. Chickens in general are more resistant to Fusarium toxins, particularly ZEA. However, caution should still be exercised as most often feed containing ZEA will include other toxins produced by Fusarium spp such as T-2 toxin, DON, DAS (Bryden, 1986).

While there have been few reports of natural outbreaks of trichothecene toxicoses in poultry, those reports made mention especially of T-2 toxin as the major cause (Bryden, 1986). There is still no clear understanding regarding a tolerance level of T-2 toxin in chicken rations. Some studies have found that feeding pure T-2 toxin to poultry at a level of 4 ppm could cause oral necrosis, neural effects, hepatic haematoma and reduced weight gain, while feeding 8 ppm caused coagulopathy. Feeding laying hens 20 ppm of T-2 toxin decreased egg production and shell quality (Bryden, 1986). In contrast, Cirilli (1983) found that a small amount of T-2 toxin (0.04-0.03 ppm) in maize caused serious symptoms such as hepatic necrosis, nephrosis and death in broiler chickens. Such apparent differences in toxicity could possibly be explained by the presence of other mycotoxins in addition to T-2 toxin.

Mycotoxicologists are particularly conscious of the toxic interaction of two or more substances (Kangsadalampai et al., 1981) but little is known about the synergestic or the additive effect of different Fusarium toxins. Other mycotoxins (particularly aflatoxin, ochratoxin and citrinin) have been studied more extensively (Vesela et al., 1983; Huff and Doerr, 1981). It is worth noting that Fusarium mycotoxins, particularly the trichothecene group, are still not fully explored, because of a lack of methodology and unavailability of standards. During the last decade the number of known trichothecenes has steadily increased from 34 (Mirocha et al., 1976b) to 75 (Gilbert, 1984).

CONCLUSION

The findings reported in this thesis emphasise the high frequency of occurrence of several significant Fusarium species and of several Fusarium mycotoxins in maize in New Zealand. That these mycotoxins had been formed in apparently healthy maize grain in the reasonably mild conditions of 1984 is of particular concern. In less clement conditions, particularly, perhaps, when harvesting has to be delayed, it is likely that more Fusarium growth would occur and higher concentrations of mycotoxins be produced.

Similar research to the present work was commenced by the Ministry of Agriculture and Fisheries at the Ruakura Agricultural Research Centre at the beginning of 1984 (Lauren, pers. comm.). That project involves a series of trials to examine the incidence and build-up of Fusarium spp on pasture leaves in different districts, the kinds of Fusarium spp present and the ability of these species to produce known mycotoxins. It has also investigated the relationship between ZEA dosing and the reproductive performance of ewes. Some published results of this work have been commented on in this thesis. The project continues and it is intended that it will be expanded to include cereal grains and other feedstuffs. The results of the present investigations lend considerable support to these proposals.

It is important that feed producers and farmers, pig farmers in particular, are made aware of the potential economic losses which may occur if their feed becomes contaminated with mycotoxins. The routine detection and monitoring of fungal problems in feed and feedstuffs is the first step in preventing outbreaks of mycotoxicoses. Furthermore, there is a need for research on the toxic interactions between mycotoxins and a need for increased awareness that mixed mycotoxicoses may occur in the pig and poultry industries (and even probably in humans) in this country.

However, as has been noted in this thesis, it is difficult to assess the significance of mycotoxins to human and animal health because data on their incidence in nature is lacking. Thus, further large-scale surveys of maize and also of other cereal grains such as wheat and barley are strongly recommended. It would be preferable to

carry out regular tests in the field and at harvest time of the three common cereal grains, wheat, maize and barley, when these grains are grown under wet weather conditions, particularly when this results in a delay in harvesting. In such future investigations other Fusarium toxins, particularly NIV, could be added to those already studied. NIV could be specially important and has recently been found in New Zealand wheat (Agnew et al., 1986).

It must be pointed out that there seems to be little risk of fresh contamination of maize with Fusarium mycotoxins during storage periods, at least when modern storage systems, particularly those of large-scale operations, are employed. An exception could be contamination with moniliformin, as discussed in Chapter 4. These comments apply to the Fusarium mycotoxins only; they may not be true for toxins produced by other species which have not been reported on in this project. It can be noted that one species which can be of concern on maize overseas, Aspergillus flavus, was present only in samples from Silo C. The maize in this silo had been stored for 12 months without aeration. Several samples from this silo were assayed for aflatoxin by a TLC method (Hussein, 1984) but no toxin was detected. However, 14% of the A. flavus isolates tested for aflatoxin production in culture were found to be capable of producing aflatoxin B1. It is likely that aflatoxin is rarely found under a climate which supports the growth of fungi such as Fusarium spp (Andrews et al., 1981).

Zearalenone is apparently the most worrying toxin, particularly for pig farmers, and was found to be very common in maize. Thus where there are any seasonal cases of disease characterised by precocious sexual development, particularly in gilts, with ramefaction of the vulva and enlargement of the mammary glands as the most obvious signs, and if other causative agents are not incriminated, ZEA toxicosis could be involved. This would be true even if the grain used was not obviously mouldy. Many authors have emphasised that mouldiness of grain is not always necessary for mycotoxin to be present. Indeed, Osborne (1982) was also of the opinion that when feed supports obvious, vigorously-growing mould, then it is unlikely to be contaminated with mycotoxin. The dangerous foods are those where active mould growth has ceased and secondary biosynthesis may have taken over.

Besides being formed in the kernels, *Fusarium* mycotoxins could conceivably contaminate other parts of the maize plant (husk, stem, stalk etc.). There are only a few reports about the natural occurrence of *Fusarium* toxins in these parts as most research has concentrated on the kernels. Mirocha *et al*. (1979), after finding that three important *Fusarium* toxins (ZEA, DON and T-2 toxin) contaminated maize stalks naturally, concluded that such contamination may contribute to mycotoxicoses of farm animals when the stalks are eaten by grazing animals or fed to them as silage. Similar warnings have come from Miller *et al*. (1983), after they detected three *Fusarium* toxins (DON, 15-ADON and ZEA) in the husk and stalk of maize plants and from Bottalico *et al*. (1984) who found ZEA, DON and zearalenol to be produced in crops infected with foot and stalk rot. The above reports increase the concern about the possibility of contamination of various parts of the maize plant under New Zealand conditions and the findings of the present investigation that several potentially toxigenic *Fusarium* species can be isolated from husks etc. suggest that further study of this possibility should be undertaken.

When cereal crops have become infected with *Fusarium* spp and *Fusarium* toxin contamination can be expected, forecasting systems based on epidemiological factors and meteorological information may become feasible and allow warnings to be issued to growers to harvest promptly, aerate and dry damp grain and have samples of suspect grain and, if appropriate, other plant parts, examined for mould and toxin contamination. The work reported in this thesis has established suitable techniques and laid a foundation for such studies.

APPENDIX A

Fusarium spp and other genera deposited at Plant Diseases Division, D.S.I.R., Auckland.

i) Fusarium spp

	PDD No. (Culture collection)	PDD No. (herbarium)	Source of isolate
F. equiseti (Corda) Sacc.	8850	47550	Maize
F. poae (Peck) Wr.	8851	47549	"
F. subglutinans (Wr. Reink) Nelson, Toussoun, Marasas	8852	47551	"
F. solani (Mart.) Appel and Wollenw.	8853	47552	"
F. moniliforme Sheldon	8854	47553	"
F. lateritium Nees	8855	47554	"
F. crookwellense Burgess, Nelson, Toussoun	8856	47555	Litter
F. equiseti (Corda) Sacc.	8857	47556	Maize
F. sambucinum Fuckel	8858	47557	Litter
F. merismoides Corda	8859	47558	Soil
F. avenaceum (Fr.) Sacc.	8860	47559	Maize
F. stilboides Wollenw.	8993	47560	"
F. oxysporum Schlecht.	8994	47561	"
F. sambucinum Fuckel	8995	47562	Litter
F. acuminatum Ell. and Ev.	8996	47563	"
F. culmorum (W.G. Smith) Sacc.	8997	47564	Soil
F. graminearum Schwabe	8998	47565	Maize

ii) Other genera

Genus	PDD No.	Source of isolate
Fusicoccum	47589	Maize
Ascochyta	47582	Litter
Sependonium	47590	Soil
Phoma	47584	Litter
Scopulariopsis	47581	"
Giocladium	47593	Soil
Verticillium	47583	"
Preussia	47592	"
Arthrinium	47587	Litter
Coniothyrium	47591	Soil
Pythium	47588	"
Acremonium	47579	Litter
Chrysosporium	47586	"
Scopulariopsis	47580	Maize
Alternaria	47567	Litter
Mucor	?	Soil
Trichoderma	47569	Litter
Acremoniella	47571	Maize
Chaetomium	47575	Litter
Schizophyllum	47577	Maize
Nigrospora	47568	Litter
Rhizopus	?	Maize
Epicoccum	47574	Litter
Cladosporium	47566	"
Beauvaria	47572	Maize
Myrothecium	47576	Litter
Paecilomyces	47573	Soil
Penicillium	47570	Maize

APPENDIX B

Fusarium spp deposited in American Type Culture Collection (ATCC).

Fusarium spp	Local code	Code no. at ATCC	Source of isolate
F. subglutinans (Wr. Reink/ (Nelson,	MU1018	60887	Husk
F. subglutinans (Toussoun, (Marasas	MU1020	60888	Maize
F. poae (Peck) Wr.	MU1024	60882	"
F. poae (Peck) Wr.	MU1026	60883	"
F. graminearum Schwabe	MU1027	60880	"
F. graminearum Schwabe	MU1028	60881	"
F. stilboides Wollenw.	MU1029(SN12)*	60885	Soil
F. crookwellense (Burgess,	MU1031	60878	Husk
F. crookwellense (Nelson (Toussoun	MU1032	60879	"
F. sambucinum Fuckel	MU1002(SN32)	60280	Litter
F. moniliforme Sheldon	MU1003(SN4)	60278	Maize
F. merismoides Corda	MU1004(SN36)	60318	Soil
F. avenaceum (Fr.) Sacc.	MU1005(SN7)	60273	Maize
F. equiseti (Corda) Sacc.	MU1006(SN34)	60276	Husk
F. lateritium Nees	MU1008	60277	Soil
F. solani (Mart.) Appel	MU1009	60299	"
F. acuminatum Ell. and Ev.	MU1010	60272	Litter
F. culmorum (W.G. Smith) Sacc.	MU1014(SN35)	60275	Maize
F. oxysporum Schlecht	MU1015(SN33)	60279	"
F. stilboides Wollenw.	MU1030	60886	Soil

* Isolates tested for toxin production

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