INCIDENCE AND CHARACTERIZATION OF *Fusarium* SPECIES FROM MYCOTIC KERATITIS INFECTIONS

By

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ABSTRACT

Mycotic keratitis has been found to account for 6% to 50% of all cases of ulcerated keratitis. *Fusarium* species, and in particular *Fusarium solani*, is the most frequent cause of mycotic infections of the cornea. These infections lead to a marked loss of vision and eventually a complete perforation of the cornea if not correctly diagnosed and treated. *Fusarium* species produce toxic mycotoxins that are known to exert adverse health effects in humans and animals. However, very few attempts have been made to establish the mycotoxin-producing capabilities of clinical isolates of *Fusarium* species from keratitis infections or any other human infections for that matter.

Twenty-nine *Fusarium* isolates from mycotic keratitis patients at the King Edward VIII Hospital were identified to species level. *F. solani* was the predominant species isolated, accounting for 72.4% (21/29) of all cases. Other species identified included two isolates each of *F. moniliforme* and *F dimerum* and one isolate each of *F. oxysporum*, *F. semitectum*, *F. chlamydosporum*, and *F. lateritium.* Previous literature supports the isolation of *F. solani, F. moniliforme, F. dimerum* and *F. oxysporum* from mycotic keratitis infections. However, this is the first report of *F. semitectum, F. chlamydosporum* and *F. lateritium* isolation from mycotic keratitis infections.

The ability of the *Fusarium* isolates to produce fusaric acid, moniliformin, and fumonisin B_1 was accomplished using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). HPLC results showed that 69% (20/29)

of the *Fusarium* isolates were capable of fusaric acid production *in-vitro* on a maize-meal substrate at concentrations ranging from 0.32 μg/g to 7.78 μg/g. Of these, 85% (17/20) were *F. solani*- producers, and one isolate each of *F. oxysporum*, *F. moniliforme* and *F. lateritium* produced the toxin. Moniliformin was produced by four *Fusarium* isolates including *F. moniliforme* (2 isolates), *F. oxysporum* and *F. chlamydosporum* at concentrations ranging from 1.04 μg/g to 4.70 μg/g. Fumonisin B_1 was produced by the two *F. moniliforme* isolates at concentrations of 3.07 μg/g and 4.90 μg/g. Toxin recovery studies of the extraction and purification methods used revealed mean percentage recoveries of 84.5%, 84.8% and 72.4% for fusaric acid, moniliformin and fumonisin B_1 respectively, which compared favourably with previous studies. The detection limits of the thin layer chromatography method were 50, 100 and 500 ng for fusaric acid, moniliformin and fumonisin B_1 respectively as compared to the much more sensitive method of high- performance liquid chromatography, with detection limits of 25, 1.0 and 100 ng for fusaric acid, moniliformin and fumonisin B_1 respectively.

The cytotoxic effects of fusaric acid, moniliformin and fumonisin B_1 on A549 (lung carcinoma) cells and VK (monkey kidney) cells were established using the MTT assay and crystal violet test. A comparison of both assays using the student's t-test returned a probability of 0.6465 (< 0.05) for A549 cells and a probability of 0.7480 for VK cells indicating no significant difference in the mean percentage cell viabilities. All three toxins were capable of inducing a cytotoxic effect on the A549 and VK cells. At 0.5 mg/ml, fusaric acid and moniliformin exhibited a greater degree of toxicity to the

A549 cells (2.7% and 2.9% respectively) than fumonisin B_1 (24.3% cell viability) in the MTT assay. However, VK cells were much more susceptible to fumonisin B_1 (11.5% cell viability) than to fusaric acid and moniliformin (30% and 14.7% cell viability, respectively). Purified extracts of fusaric acid, moniliformin and fumonisin B_1 extracts from the clinical *Fusarium* isolates exhibited a marginal degree of toxicity to both cell lines due to the low toxin concentrations produced by the isolates in culture. However, the greatest inhibition of cell viability was exhibited by the purified fusaric acid extract on A549 cells (41.3% cell viability) due to the highest fusaric acid concentration produced in culture (7.78 μg/g).

A retrospective study of eight patients with fusarial keratitis indicated a relationship between the severity of the corneal ulcers and the toxins produced by the *Fusarium* isolates. Corneal ulcers with no hypopyon (pus) formation in the anterior chamber showed *F. dimerum* as the causative organism with no *in-vitro* toxin production. Corneal ulcers that formed a hypopyon in the anterior chamber and increased ulcer sizes showed *F. solani* as the causative organism, which was capable of producing fusaric acid *in-vitro*. The most severe corneal ulcer symptoms in one case included hypopyon formation in the anterior chamber with the patient only able to perceive light. Eventually, this led to a complete perforation of the cornea. The causative organism was *F. moniliforme* that was capable of producing all three toxins in culture.

Although the production of fumonisin B_1 from *Fusarium* strains isolated from mycotic keratitis has been reported previously, this is the first report on the production of fusaric acid and moniliformin from *Fusarium* isolates from mycotic keratitis patients

and the cytotoxic effects of the three toxins on lung carcinoma and monkey kidney cells. Also, this is the first report indicating a possible involvement of fusaric acid, moniliformin and fumonisin B_1 production in the severity of mycotic keratitis infections.

PREFACE

This study represents original work by the author and has not been submitted in any form to another university or institute. Where use was made of the work of others it has been duly acknowledgement in the text.

The research described in this dissertation was carried out at the Department of Biological Sciences, M. L. Sultan Technikon, under the supervision of Dr. B. Odhav.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDICES

CHAPTER ONE: INTRODUCTION

1.1 PREAMBLE

Mycotic keratitis of the eye, because of its increasing occurrence, has become important in ophthalmology (Cuero, 1980). The importance of mycotic keratitis in Columbia was reported for the first time by Greer *et al.* (1972), who related its severity to the general use of steroids or antibiotics in eye infections and emphasized trauma as a predisposing factor. *Fusarium* species, which have a worldwide distribution in soil, have been found in both plants and animals. Members of the genus have been among the fungi most frequently isolated from patients with mycotic keratitis and recently, its occurrence in deep tissues and disseminated infections, has greatly increased (Guarro and Gene, 1995).

Despite comprehensive reviews of the taxonomy, especially those of Gerlach and Nirenberg (1982) and Nelson *et al.* (1983), the identification of *Fusarium* species is difficult and often confusing even for specialists. Probably for this reason in more than half of the published articles reporting *Fusarium* infections, the species are not identified. *Fusarium* species produce mycotoxins that have been associated with outbreaks of human and animal mycotoxicoses (Fink-Gremmels, 1999). However, there have very few attempts to clarify the ability of clinical isolates of *Fusarium* species to produce mycotoxins (Sugiura *et al.*, 1999). It was thus hypothesized that mycotoxin production by *Fusarium* species is related to the severity of a mycotic keratitis infection. The aim of this study was to identify *Fusarium* species isolated from mycotic

keratitis patients and to determine the role of mycotoxins produced by these isolates in the severity of keratitis infections.

This project was divided into four phases. The first phase involved a clinical study of eight patients diagnosed with fusarial keratitis. This included the patients' medical history (age, sex, symptoms of the disease, trauma to eye, previous ocular surgery and systemic diseases) and an ocular examination of the fungal ulcer (visual acuity, ulcer size, site and depth, inflammatory response of anterior chamber and antifungal treatment).

The second phase involved the identification of twenty- nine clinical isolates of Fusaria to species level from patients diagnosed with mycotic keratitis. This was accomplished using a taxonomic key for *Fusarium* species (Nelson *et al.*, 1983). Light microscopy was used to identify cultural characters such as phialides and conidia while scanning electron microscopy was used to identify ultrastructural characters such as apical and basal cells of macroconidia.

The third phase involved the investigation of the mycotoxin- producing capabilities of the clinical *Fusarium* isolates. Maize-meal patty cultures were used to produce fusaric acid, moniliformin and fumonisin B_1 *in-vitro*. Toxins were purified using the appropriate extraction and purification procedures. Detection was achieved using thin layer chromatography (TLC) and detection and quantification achieved by high-performance liquid chromatography (HPLC). Extraction efficiency and detection limits of each of the toxins were also investigated.

The fourth phase involved the investigation of the cytotoxic effects of the mycotoxins on lung adenocarcinoma cells and monkey kidney cells. To achieve this, two cytotoxicity assays for determining cell viabilities after toxin exposure, namely the MTT- cleavage assay and the crystal violet test, were used. Pure standards of fusaric acid, moniliformin and fumonisin B_1 and purified toxin extracts were tested for their ability to induce a cytotoxic effect on A549 (lung adenocarcinoma) cells and VK (monkey kidney) cells.

1.2 LITERATURE REVIEW

1.2.1 Taxonomy of *Fusarium* species

Fungal infections, especially those caused by opportunistic species, have become substantially more common in recent decades. Numerous species cause human infections, and several new human pathogens are discovered yearly. This situation has created an increasing interest in fungal taxonomy (Guarro *et al.*, 1999).

Recently, *Fusarium* species have received increasing attention as emerging human pathogens in immunocompromised patients. The differentiation of *Fusarium* species from other filamentous fungal opportunists is important in establishing the correct clinical diagnosis, in view of their different susceptibilities to antifungal drugs. Because *Aspergillus, Fusarium*, zygomycetes, and *Pseudallescheria boydii* are morphologically similar in tissue, they cannot reliably be identified by histopathology alone. *Fusarium* species are most resistant to available chemotherapy, thus early diagnosis is of prime importance (Sugiura *et al*, 1999).

1.2.1.1 *Fusarium* cultural characteristics

Knowledge of the taxonomy of *Fusarium*, and the appropriate procedures for identification are basic to most studies of the genus. Although *Fusarium* includes some populations, which are quite variable, the identification of most species can be accomplished if consistent and appropriate media, culturing procedures and incubation conditions are adopted (Burgess *et al.*, 1988).

The genus *Fusarium*, classified in the class Hyphomycetes (subdivision Deuteromycetes) (Hawksworth *et al.*, 1995), is now widely considered an anamorphic genus affiliated with the Hypocreales (Ascomycetes). *Fusarium* is characterized by the production of slimy, hyaline, septate, sickle-shaped conidia (known as macroconidia) (Fig. 1.1), that in most species are produced in fruiting-structures called sporodochia. In addition to this, some species also produce distinctly different conidia in the aerial mycelium often referred to as microconidia (Fig. 1.2).

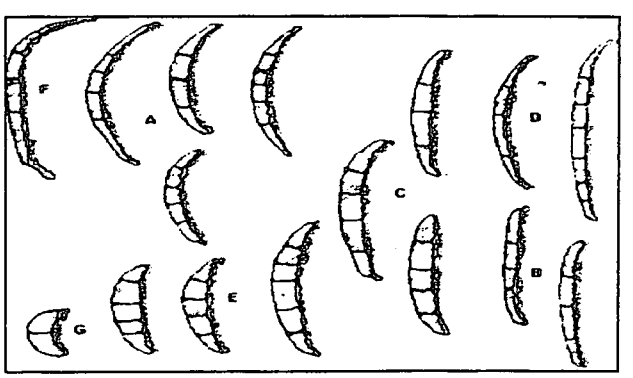


Fig. 1.1 Typical sickle-shaped, septate *Fusarium* macroconidia (Burgess *et al.*, 1988)

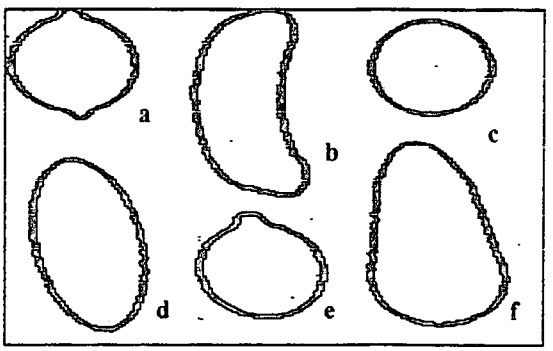


Fig. 1.2 Typical *Fusarium* microconidia. Microconidia may be (a) spindle- shaped, (b) reniform (kidney-shaped), (c) circular, (d) oval, (e) napiform or (f) obovoid (Burgess *et al.*, 1988).

According to the species and/or the ecological situation, either macroconidia or microconidia may dominate on the natural substrate. Chlamydospores (Fig. 1.3) are also produced by some species (Burgess *et al.*, 1988).

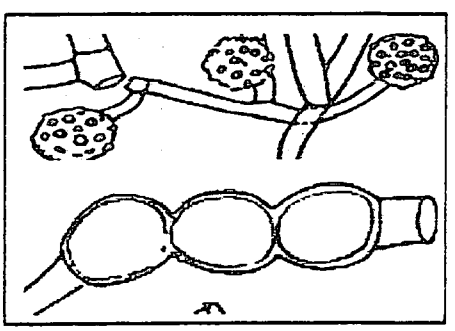


Fig 1.3 Rough- and smooth- walled *Fusarium* chlamydospores (Burgess *et al.*, 1988).

Because one must see all of these anamorphic forms in order to identify a *Fusarium* species with certainty, most modern *Fusarium* taxonomy is based on cultural characters. The important cultural characters of *Fusarium* species (Burgess *et al.*, 1988), which are used in conjunction with a *Fusarium* taxonomic key for identification is outlined in Table 1.1.

Table 1.1 Cultural characteristics of *Fusarium* species and a description of each of the characteristics used in its identification (Burgess *et al.*, 1988)

Cultural characteristics used to identify *Fusarium* species	**Description of *Fusarium* cultural characters**
Shape of the macroconidium	A macroconidium may be stout or slender. It can be falcate or relatively straight. The walls can be parallel or curved and appear thick or thin. The number of septa usually varies. The apical cell can be short and blunt (rounded) or hooked (beaked), papillate (dolphin-nosed), tapered to a point or very long (filamentous) or even curved or coiled.
Presence or absence of microconidia	Produced in the aerial mycelium. Presence or absence of microconidia are important diagnostic features.
Shape and mode of formation of microconidia	Are mostly 1-or 2-celled, are 0-3 septate and are generally smaller than macroconidia. Are formed in chains or false-heads.
Nature of the conidiogenous cell bearing microconidia	Most *Fusarium* species produce monophialides. Other species produce polyphialides in addition to monophialides.
Presence or absence of chlamydospores	The presence of chlamydospores is a useful criterion while their absence is not.
Colony diameters on PDA	They can be extremely useful in distinguishing some populations.
Colony morphology on PDA	Colony morphology provides a useful secondary criterion in *Fusarium* taxonomy.

1.2.1.2 *Fusarium* identification keys

Identifying cultures of *Fusarium* species requires careful observation and attention to detail. The presently available *Fusarium* identification manuals provide a series of synoptic keys (Nelson *et al.*, 1983), dichotomous keys (Booth, 1971), tabular keys (Burgess *et al.*, 1988) or no key at all, (Gerlach and Nirenberg, 1982).

There are basically two types of keys: branched and synoptic (numerical) keys. Branched keys contain steps that are numbered in sequence. The user follows the numbers according to the features that match the organism being identified. Dichotomous keys are the simplest branched keys, with only two choices at each step (Booth, 1971). Synoptic keys (also known as summarizing or outline keys) are set out in a table form. The illustrated identification manual of Nelson *et al.* (1983) provides synoptic keys to both the sections and species of 30 *Fusarium* species.

1.2.2 Toxicity of mycotoxins

Mycotoxins comprise a group of chemically diverse compounds originating from secondary metabolism of moulds (filamentous fungi). As of yet more than 300 mycotoxins have been identified to induce signs of toxicity in mammalian species. Their global occurrence is considered to be a major risk factor affecting human and animal health as it is estimated that 25% of the world's crop production is contaminated to some extent with mycotoxins (Fink-Gremmels and Georgiou, 1996, Kuiper-Goodman, 1998).

In animals, mycotoxin exposure via feed may result in acute intoxication (Cheeke, 1995). Moreover, chronic exposure to low doses may even remain undetected and result in reduced weight gain, diminished productivity and increased susceptibility to infections (Charmley *et al.*, 1995). Like animals, humans are susceptible to mycotoxins. Exposure originates from the consumption of contaminated plant commodities, but might also occur via a secondary following the consumption of meat, milk and eggs, containing residual amounts of mycotoxins ingested by food- producing animals (Fink-Gremmels, 1996). This carry over- linking animal production to public health- has been observed for virtually all mycotoxins and is based on their high lipophilicity.

1.2.3 *Fusarium* mycotoxins

The genus *Fusarium* is one of the most economically important genera of fungi and include many pathogenic species which cause a wide range of plant diseases (Nelson *et al.*, 1981b). Some species are highly mycotoxigenic (Marasas *et al.*, 1984, Burgess, 1985), and a number of species are known to cause opportunistic infections in humans and other animals (Rebell, 1981).

The major fusarial mycotoxins and their effects are listed in Table 1.2. Trichothecenes, zearalenone and fumonisins comprise the majority of *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains, animal feeds and forages. Other important *Fusarium* mycotoxins include moniliformin and fusaric acid. Depending on their chemical structure, mycotoxins exert a broad variety of biological effects. These toxic effects include nausea and vomiting caused by deoxynivalenol, inhibition of protein synthesis

and immunosuppression caused by T2-toxin, equine leucoencephalomalacia (ELEM) and implications of carcinogenesis in humans caused by fumonisin B_1, affected brain and pineal neurotransmitters caused by fusaric acid, and decreased immune function caused by moniliformin.

Table 1.2 Toxic effects of some *Fusarium* mycotoxins in humans and animals

***Fusarium* mycotoxins**	**Toxic effects in humans and animals**	**References**
Deoxynivalenol	Nausea, vomiting, visual disturbances, chills, vertigo and feed refusal in animals. Acute gastrointestinal illness in humans.	Childress *et al.* (1990) Luo (1988), Bhat *et al.* (1989)
T2-toxin	Immunosuppressor, strong inhibitor of protein synthesis in mammalian cells.	Rotter *et al.* (1994)
Fumonisin B_1	Equine leucoencephalomalacia, porcine pulmonary edema. Associated with human esophageal cancer.	Marasas (1995) Rheeder *et al.* (1992) Munkvold and Desjardins (1997)
Fusaric acid	Affects brain and pineal neurotransmitters Hypotensive reaction in dogs and cats	Porter *et al.* (1990, 1995)
Moniliformin	Decreases performance and immune function of broiler chicks	Li *et al.* (2000)

It was recently found that at least one *Fusarium* metabolite has been detected in the urine samples of 93% of patients with chronic idiopathic paraparesis (associated with HTLV-virus infection) suggestive of toxicity of *Fusarium* mycotoxins in this disease (Leon *et al.*, 1998). In the Netherlands, marram grass-planters showed lesions of the skin and mucous membranes suggesting a toxic reaction. *F. culmorum* dominated the mycoflora of these marram grass culms used for planting. The *Fusarium* toxin deoxynivalenol was detected in the suspect marram culms and isolated *F. culmorum* strains were able to produce this toxin *in vitro* (Snidjers *et al.*, 1996).

The ability of clinical isolates of *Fusarium* species to produce mycotoxins is limited to a few studies. *F. solani* and *F. oxysporum* isolated from corneal scrapings from mycotic keratitis patients were found to produce trichothecenes, nivalenol, deoxynivalenol, diacetoxyscirpenol and T-2 toxin (Raza *et al.*, 1993; Raza *et al.*, 1994). Nelson *et al.* (1991) demonstrated fumonisin B_1 production by *F. moniliforme* from patients with mycotic keratitis, or with various types of cancer. However, Sugiura *et al.* (1999) found that none of the 33 strains of *Fusarium* species isolated from blood, the brain, lungs, skin, eye and wound sites throughout the body produced detectable amounts of trichothecenes (deoxynivalenol, 3-acetoxynivalenol, nivalenol, fusarenon-X, T-2 toxin, diacetoxyscirpenol), whereas two strains of *F. moniliforme* and two strains of *F. proliferatum* were capable of producing fumonisins B_1, B_2 and B_3.

No literature is available on the fusaric acid and moniliformin-producing capabilities by human clinical isolates. Fusaric acid is produced by *F. solani*, *F. oxysporum* and *F.*

moniliforme (Bacon *et al.*, 1996), all of which are etiological agents of mycotic keratitis (Rebell, 1981, Guarro and Gene, 1995 and Garg *et al.*, 2000). Moniliformin and fumonisin B_1 are also produced by *F. moniliforme*. Therefore, these three toxins have been discussed in greater detail, and their occurrence and toxicological effects are also described.

1.2.3.1 Fusaric acid

Fusaric acid (5-butylpicolinic acid) (Fig. 1.4) is one of the most common mycotoxins produced by *Fusarium* species (Bacon *et al.*, 1996). This mycotoxin has been isolated from *Fusarium*-infected corn, wheat, and other suspect toxic feeds, and is suggested to enhance the activity of some of the *Fusarium* mycotoxins (Bacon *et al.*, 1995, 1996; Porter *et al.*, 1995, 1996). It is produced predominantly by *F. solani, F. moniliforme* and *F. oxysporum*. This metabolite is different from other mycotoxins synthesized by various *Fusarium* species, such as moniliformin and fumonisin B_1, which are limited to only a few taxonomic entities among a species population (Bacon *et al.*, 1996). Although not acutely toxic to the developing fetal, neonate, juvenile or adult rat, fusaric acid is passed from the feed of nursing dams (via the milk) to the suckling neonate at levels found in *Fusarium*-contaminated corn, wheat and barley (Porter *et al.*, 1996). These results are consistent with observed reduced weight gains in the suckling neonate within the first week after parturition (at 200 parts per million).

Fusaric acid also alters brain and pineal gland concentrations of tyrosine, dopamine, norepinephrine and serotonin, N-acetylserotonin and melatonin in the pineal gland of

juvenile weanlings from dams maintained on diets containing this mycotoxin (Porter *et al.*, 1995; Rimando and Porter, 1997). Alterations of these neurochemical parameters would strongly support fusaric acid predisposing mammalian systems to adverse effects in growth, maturation and behaviour.

Furthermore, brain weight and brain weight-testicular weight ratios in juvenile and adult males are altered, and brain and body weight are reduced in adult female rats after ten months on fusaric acid diets (Porter and Rimando, 1997). It is not known if these effects are due to exposure *in utero*, or the perinatal or postpartum exposure of the neonates to the mycotoxins during lactation, or the chronic effects of toxin consumption.

Recent studies have demonstrated that fusaric acid enhances the toxicity of fumonisin B_1 in the fertile leghorn egg test. Other studies have shown fusaric acid enhances the toxicity of diacetoxyscirpenol, deoxynivalenol (other *Fusarium* toxins) and selected pesticides to certain insects; the neuroendocrine effects of deoxynivalenol in swine (i.e. emetic/feed refusal responses which are serotonergic reactions) are also enhanced by fusaric acid (Dowd, 1988; Smith *et al.*, 1997). Current investigations reveal that a combination of zearalenone and fusaric acid in the diet of lactating rats (at ten and two parts per million respectively) increased the passage of both mycotoxins into the milk of the suckling neonate at two to five times that of their individual concentrations (Porter *et al.*, 1998).

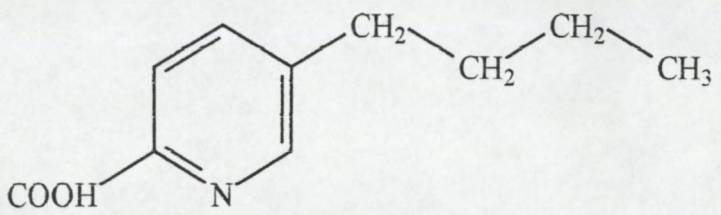
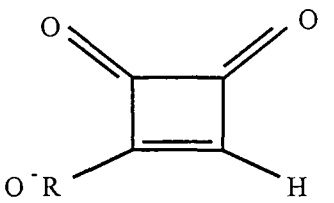


Fig. 1.4 Structure of fusaric acid (Burmeister *et al.*, 1985)

1.2.3.2 Moniliformin

Moniliformin is the sodium or potassium salt of 1-hydroxycyclobut-1-ene-3, 4-dione (Fig. 1.5) produced by *F. moniliforme* (Cole *et al.*, 1973). It occurs in corn grown in Transkei (Thiel *et al.*, 1982) and the Federal Republic of Germany (Thalmann *et al.*, 1985). Moniliformin has plant-growth regulating and phytotoxic effects (Cole and Cox, 1981). Toxicological analysis disclosed that this mycotoxin also causes rapid death in animals without severe cellular damage. Moniliformin has recently been found to decrease performance and immune response in broiler chicks (Li *et al.*, 2000). An interesting finding was the occurrence of acute degenerative lesions in the myocardium and other tissues, which indicated an involvement of suppressed ATP-derived transmembrane transport mechanisms (Ueno, 1984).

At the molecular level, moniliformin does not inhibit eukaryotic protein synthesis and was not mutagenic in the Ames test. This toxin, however, is a potent inhibitor of mitochondrial pyruvate and α-ketoglutarate oxidation (Ueno and Shimada, 1974, Thiel, *et al.*, 1982).



$R = Na^{+}$ or K^{+}

Fig. 1.5 Structure of moniliformin (Cole *et al.*, 1973)

1.2.3.3 Fumonisin B_1

The most recently discovered *Fusarium* toxin is fumonisin B_1, a representative of the fumonisin group produced predominantly by *F. moniliforme*. Among the various categories of mycotoxins identified until now, fumonisin B_1 is assuming a growing significance among highly toxic substances for animals and man. Fumonisin B_1 (Fig. 1.6) is a diester of tricarballylic acid and polyhydric alcohols, and is structurally similar to cellular sphingolipids. This similarity led to the hypothesis that fumonisins may interfere with sphingosine metabolism. Sphingosine is the chemical backbone of sphingolipids, which are believed to play critical roles in a number of cellular functions, including cell-cell communication and cell growth and transformation (Fink-Gremmels, 1999).

In horses (and other equines), fumonisin B_1 causes leucoencephalomalacia (ELEM), a disease characterized by the degeneration of neurons ('hole in the head' disease), while in pigs, the main symptom of fumonisin B_1 exposure is pleural edema (PPE-porcine pulmonary edema) impairing respiratory and heart function (Diaz and Boermans, 1994).

In laboratory animals, species including rats, mice and rabbits, fumonisin B_1 was found to induce hepatotoxicity and nephrotoxicity and exerts tumour-promoting activity.

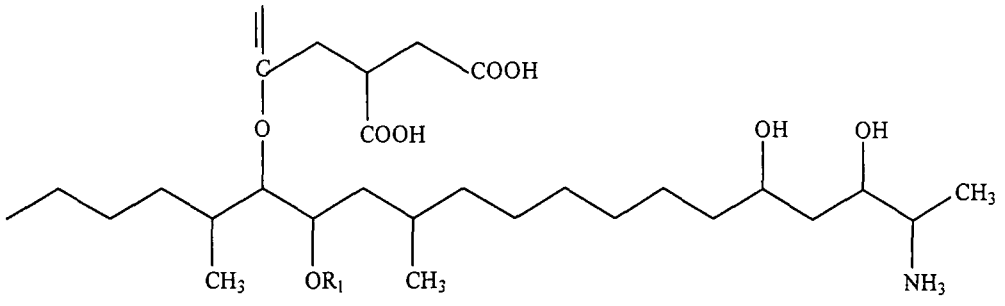


Fig. 1.6 Structure of fumonisin B_1 (Fink-Gremmels, 1999)

1.2.4 Mycotic infections in humans

A large number of different genera and species of fungi cause human infections. These mycotic infections are classified as superficial, cutaneous, subcutaneous and opportunistic infections (Male, 1990). Superficial infections caused by fungi such as *Piedra* and *Trichosporum* are limited to the outer layers of the skin and hair. Cutaneous infections caused by *Trichophyton* and *Microsporum* generally involve the deep epidermis and keratinized body areas (skin, hair and nails). *Sporothrix* are the main etiological agents of subcutaneous infections of the dermis, subcutaneous tissues and muscle. Systemic infections caused by fungi such as *Histoplasma* generally originate in the lungs and spread to many organs. Finally, opportunistic infections caused by fungi have a low potential for virulence and involve a variety of body sites. These include *Aspergillus* and *Fusarium* species (Table 1.3).

Table 1.3 Types and sites of mycotic infections in the human body including the diseases caused and the etiological agents (Male, 1990)

Type and Sites of Infection	Representative Diseases	Representative Genera
Superficial: Limited to outer layers of skin and hair.	Black or white piedra	*Piedra, Trichosporum*
Cutaneous: Involves deep epidermis and keratinized body areas (skin, hair and nails).	Dermatophycoses	*Trichophyton, Microsporum Epidermophyton*
Subcutaneous: Involves dermis, subcutaneous tissues and muscle.	Sporotrichosis	*Sporothrix*
Systemic: Originate in lungs, spreads to many organs.	Histoplasmosis Blastomycosis	*Histoplasma capsulatus* *Blastomyces dematitidis*
Opportunistic: These organisms generally have a low potential for virulence but can produce severe disease involving a variety of body tissues.	Candidiasis Aspergillosis Fusariosis	*Candida albicans* *Aspergillus* *Fusarium*

The capacity of *Fusarium* species to cause opportunistic infections in man and animals has developed rather recently, and the number of reports of such infections is increasing. The highlights of *Fusarium* infections in man are:

i) the inclusion of *Fusarium* species among those fungi which, in patients with compromised host resistance, exhibit a tendency to invade blood vessels, leading to fatal haematogenous dissemination of the infection;

ii) the occurrence of *F. oxysporum* as a cause of white onychomycosis (nail infection);

iii) the occurrence of white grain mycetoma due to *Fusarium* species

iv) mycotic keratitis (corneal ulcers) of which *Fusarium* species, especially *F. solani*, are the most common cause (Rebell, 1980).

1.2.4.1 Mycotic Keratitis

Mycotic infections of the eye have been observed and reported with increasing frequency during the past few decades due to better diagnostic methods, and recognition of the fact that several species of fungi, which are usually considered as non- pathogenic or saprophytic have the ability to infect the eye. Such fungi have been termed opportunistic pathogenic fungi (Gugnani *et al.*, 1978 and Guarro and Gene, 1995). Eye infections caused by fungi are generally divided into three sections including endogenous oculomycosis, extension oculomycosis and mycotic keratitis/ keratomycosis (McGinnis, 1980). Endogenous oculomycosis is an infection of the inside of the eye and is associated with hematogenous dissemination of fungi such as *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*. The mycosis involves the orbit, retina, optic nerve, sclera, conjunctiva, and adjacent tissue. Extension oculomycosis is a special form of rhinocerebral zygomycosis and occurs as a result of trauma, immunosuppression, and extension from paranasal sinuses or the nasal septum (McGinnis, 1980, Binder, 1986). The infection may continue to spread to the central nervous system. The zygomycete most often isolated is *Rhizopus arrhizus*. Mycotic keratitis is an opportunistic fungal infection of the cornea. The cornea, and the sclera makes up the outer wall of the eye and is composed of five layers including the epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelium (Fig. 1.7) (Mcginnis, 1980). Infections can arise either due to a reduction of local defence mechanisms or due to injuries of the ocular epithelium caused by twigs, plants or other materials from the soil, together with a sufficient amount of mycotic spores. The resulting infection is called "true mycotic keratitis" (Mino de Kaspar *et al.*, 1991).

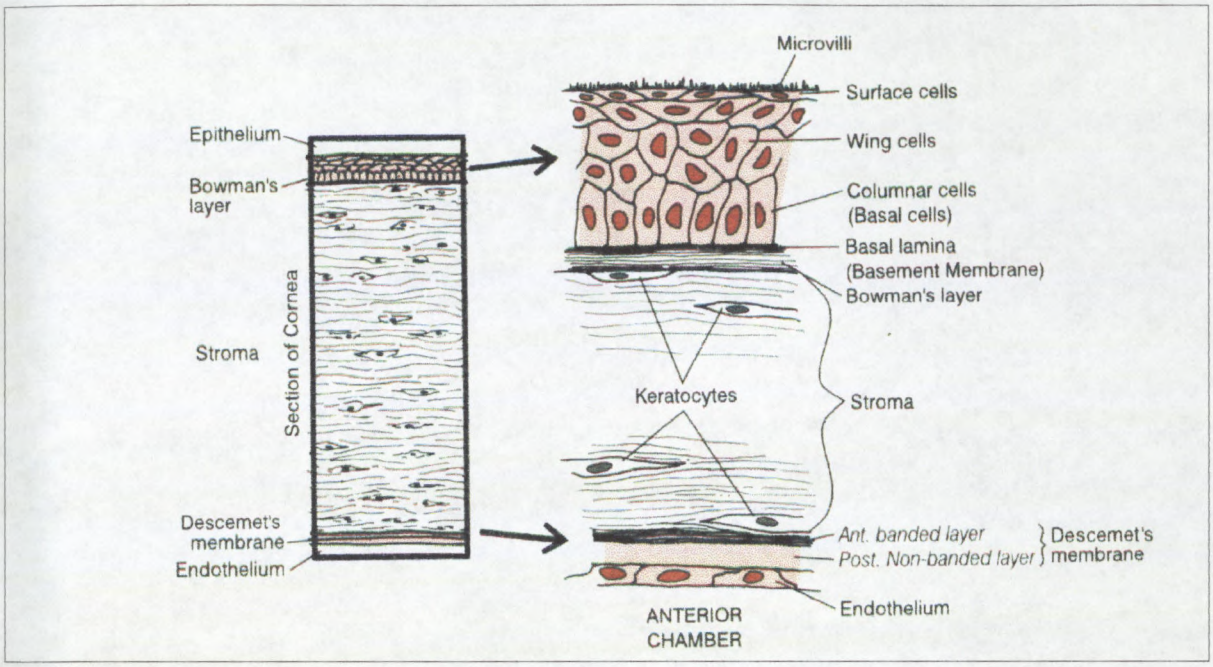


Fig. 1.7 Diagrammatic representation of the cornea illustrating the five layers i.e. epithelium, Bowman' layer, stroma, Descemet's membrane and endothelium (McGinnis, 1980).

1.2.4.1.1 Etiology

Approximately 60 fungal genera have been identified as etiologic agents of infectious keratitis (Jones, 1998). The predominant etiological agents are listed in Table 1.4 and are usually divided into five types i.e. non-pigmented filamentous fungi (Moniliaceae), pigmented filamentous fungi (Dematiaceae), non-septate filamentous fungi, the yeasts and dimorphic fungi.

Table 1.4 Common etiological agents of mycotic keratitis (Jones, 1998)

Moniliaceae (Non-pigmented filamentous fungi)
Fusarium
Aspergillus
Acremonium
Penicillium
Paecilomyces
Dematiaceae (Pigmented filamentous fungi)
Curvularia
Sphaeropsidales
Melanconiales
Alternaria
Drechslera
Non-septate Filamentous Fungi (Rare)
Absidia
Mucor
Dimorphic Fungi
Blastomyces
Cryptococcus
Yeasts
Candida
Geotrichum

The capacity of *Fusarium* species to cause opportunistic ocular infections in man has developed rather recently, and the number of reports of such infections is increasing. In Madurai, South India, 434 patients with central corneal ulceration were evaluated. A history of previous corneal injury was present in 284 patients (65.4%). The most common fungal pathogen isolated was *Fusarium* species, representing 47.1% of all positive fungal cultures, followed by *Aspergillus* species (16.1%) (Srinivasan *et al.*, 1997). *Fusarium* species and in particular *F. solani*, is the most frequent cause of mycotic infections of the

cornea (Rebell, 1981, Mselle, 1999). Over half of the cases of fungal keratitis in the United States are caused by *F. solani* (McGinnis, 1980). In Tanzania, (October 1994 to October 1995), *Fusarium solani* accounted for 75% of cases (Mselle, 1999).

1.2.4.1.2 Epidemiology

The literature on fungal infections of the cornea is quite extensive (Liesegang and Forster, 1980, Poria *et al.*, 1985, Upadhyay *et al.*, 1991, Dunlop *et al*, 1994, Rosa *et al.*, 1994, and Panda *et al.*, 1997). These studies indicate that although corneal infections have worldwide distribution, the incidence of fungal infections is higher in tropical and semitropical areas (Garg *et al.*, 2000).

Epidemiological characteristics of ocular infections due to filamentous and yeast-like fungi are different. Overall, *Fusarium* and *Aspergillus* species appear at a higher frequency in corneal infections in tropical and subtropical climates, whereas infections due to yeasts predominate at temperate and colder climates (Alfonso and Rosa, 1997 and Viera, 1997, Jones, 1998). *Fusarium* has been the most frequent agent of mycotic keratitis in Latin American countries (Cuero, 1980) and in the Unites States (Rosa *et al.*, 1994, Wilhelmus *et al.*, 1988).

1.2.4.1.3 Predisposing factors

Any situation that promotes loss of corneal epithelium integrity is considered a risk factor for keratitis (Alfonso and Rosa, 1997, Viera, 1997). Common risk factors for the development of fungal keratitis include trauma (contact lenses, foreign body); topical

corticosteroid use, corneal surgery (such as penetrating keratoplasty) and chronic keratitis (due to herpes simplex, herpes zoster, or vernal keratoconjunctivitis). Trauma caused by accidental injury appears to be the most common predisposing factor (Tanure, 2000). Mycotic keratitis may also be influenced by factors such as the sex, age, occupation, immune status of the host, as well as geographic conditions. Sandhu and Rattan (1981) found that men between 20 and 40 years of age are more prone to mycotic keratitis, especially if they are farmers or labourers.

1.2.4.1.4 Pathogenesis

Although the majority of corneal epithelial abrasions heal without incident, breakdown of the epithelial barriers allows any opportunistic microorganism present to infect the cornea. Stained sections of the cornea showed the presence of fusarial hyphae and cells that had penetrated the corneal layers (Fig. 1.8) (Bridgens, 1998). Fungi gain access into the corneal stroma through a defect in the epithelium, then multiply and cause tissue necrosis and an inflammatory reaction. The epithelial defect is usually due to trauma, for example, from contact lens wear, foreign material or prior corneal surgery. The organisms can penetrate an intact Descemet's membrane and gain access into the anterior chamber or posterior segment. Mycotoxins and proteolytic enzymes may augment the tissue damage (Rebell, 1981). Filamentous fungi usually invade and interdigitate between collagen lamellae and lead to disruption of the normal collagen fiber arrangement. They cause one of three distinct tissue responses: chronic inflammation (scarring and accumulation of lymphocytes), granulomatous inflammation (collections of modified epithelial cells and lymphocytes) and acute suppurative inflammation (vascular

congestion, exudation of plasma and accumulation of polymorphonuclear leukocytes (PMLs) (Naumann *et al.*, 1967). This mechanism for pathogenicity is shown in Fig. 1.9.

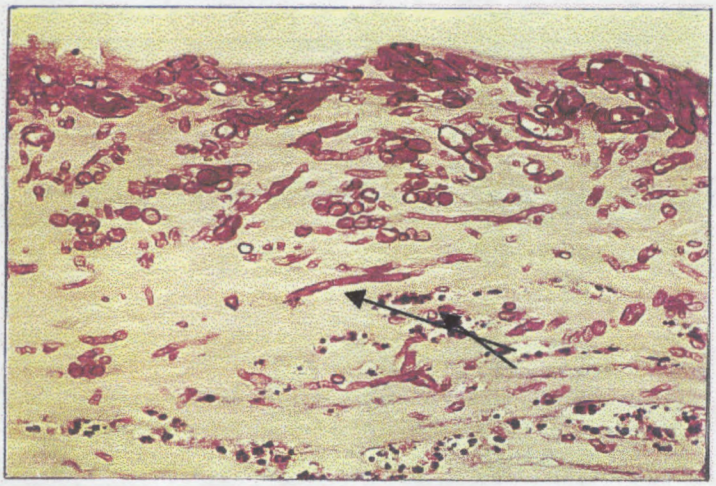


Fig. 1.8 Stained section through the cornea. The arrowheads point to fusarial hyphae and cells that penetrated the corneal layers (Bridgens, 1998).

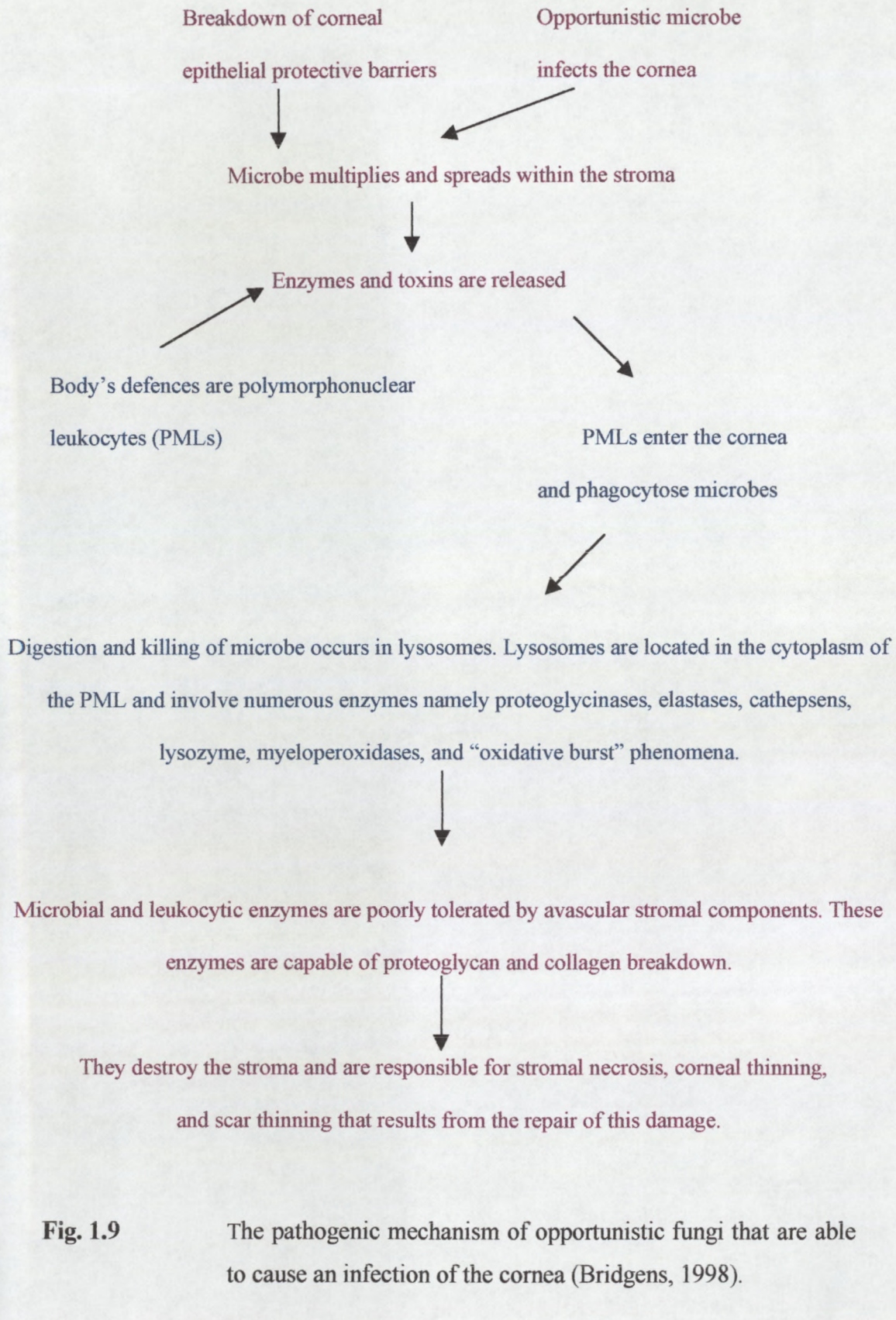


Fig. 1.9 The pathogenic mechanism of opportunistic fungi that are able to cause an infection of the cornea (Bridgens, 1998).

It is suggested that the ocular virulence of *Fusarium* is a result of its propensity to invade and occlude intraocular vascular channels by secondary infarction, hemorrhage and necrosis (Patel, 1994). *Fusarium solani* has also been thought to cause extensive ocular destruction because it liberates proteolytic enzymes or mycotoxins (Jones, 1978). However, *in vitro* tests to date have not revealed fungal collagenase activity or other factors that account for the pathogenicity of *F. solani* (or other fungi) in the apparently healthy cornea following abrasion or trauma (Rebell, 1981). There are also no reports of mycotoxin involvement.

1.2.4.1.5 Clinical features

Infectious ulcerative keratitis is a serious ocular problem that can result in corneal scarring and opacification with concomitant severe visual disability. Even relatively minor infections in the visual axis can cause a serious loss of vision, and severe infections can penetrate the cornea with loss of the eye within 24 hours (Bridgens, 1998).

The most common physical signs upon slit-lamp examination are nonspecific and include epithelial defect, suppuration, stromal infiltration, anterior chamber reaction and hypopyon. Presenting clinical features that are specific to fungal keratitis include an infiltrate with feathery margins, rough texture, raised borders, associated endothelial plaque, and satellite lesions. A deep stromal infiltrate with an intact epithelium may also be present (Florakis *et al.*, 1997). The corneal ulcer is characterized by a raised epithelium with a white shaggy border. There is frequently a radiating margin from the

ulcer. Other characteristics include satellite lesions, sterile hypopyon (pus in the anterior chamber), and late vascularization if steroid treatment was used earlier (McGinnis, 1980).

The clinical features of corneal ulcers vary with the type of etiological agent responsible for the infection (Bridgens, 1998). An ulcer caused by *Fusarium* and *Aspergillus* has typically feathered margins and possibly satellite lesions whereas a ring precipitate usually surrounds an ulcer caused by *Acanthamoeba*. The stroma surrounding an ulcer that is caused by *Staphylococcus* is relatively clear whereby in a *Pseudomonas* infection, the surrounding stroma is hazy with a ground-glass-like appearance (Fig. 1.10a-d).

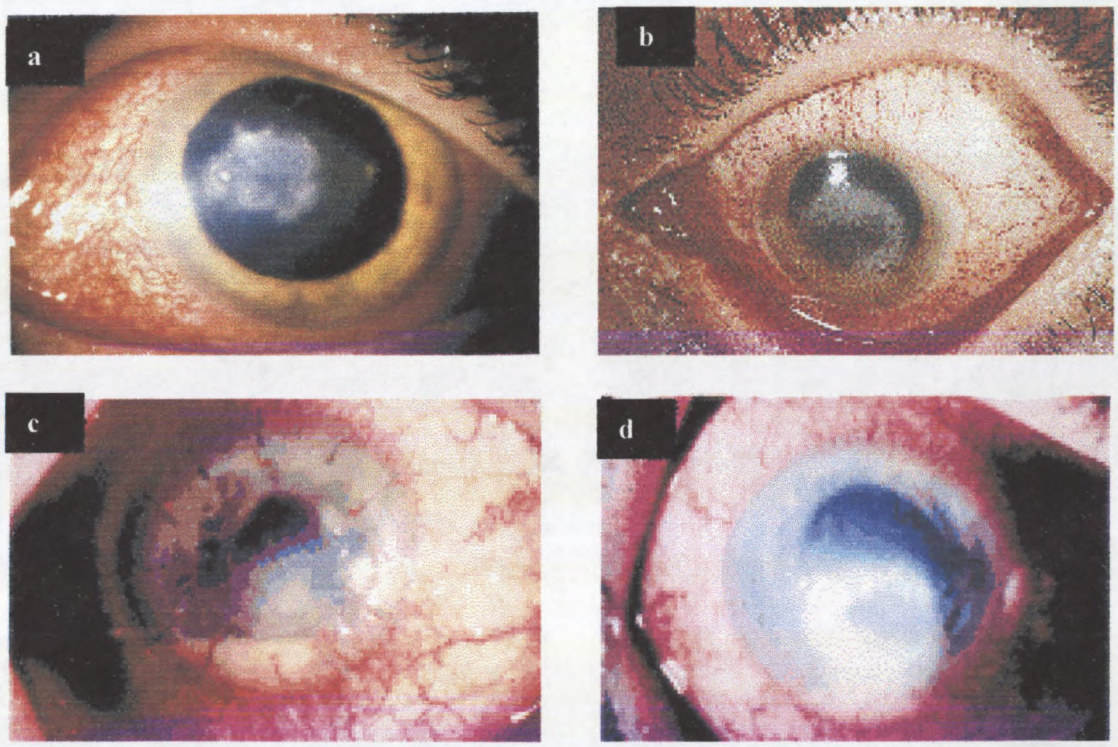


Fig. 1.10 Characteristic clinical features of corneal ulcers caused by different microbial agents. (a) Typical gray, feathery margins of ulcer caused by *Aspergillus*, (b) ring precipitate of ulcer caused by *Acanthamoeba*, (c) clear stroma of ulcer caused by *Staphylococcus* and (d) hazy stroma and ground-glass-like appearance of ulcer caused by *Pseudomonas* (McGinnis, 1980, and Bennet *et al.*, 1998).

Clinically, keratitis caused by *Fusarium* is similar in appearance to that caused by other genera, but it has a worse prognosis, often resulting in rapid corneal sloughing and marked loss of vision, and it is difficult to treat (Richardson and Warnock, 1993). The course of infection is progressive beginning with an increase in the size of the corneal infiltrate, and then leading to hypopyon formation in the anterior chamber (Fig. 1.11a-d). Perforation of the cornea occurs in some cases resulting in blindness of the infected eye within a few weeks (Rebell, 1981).

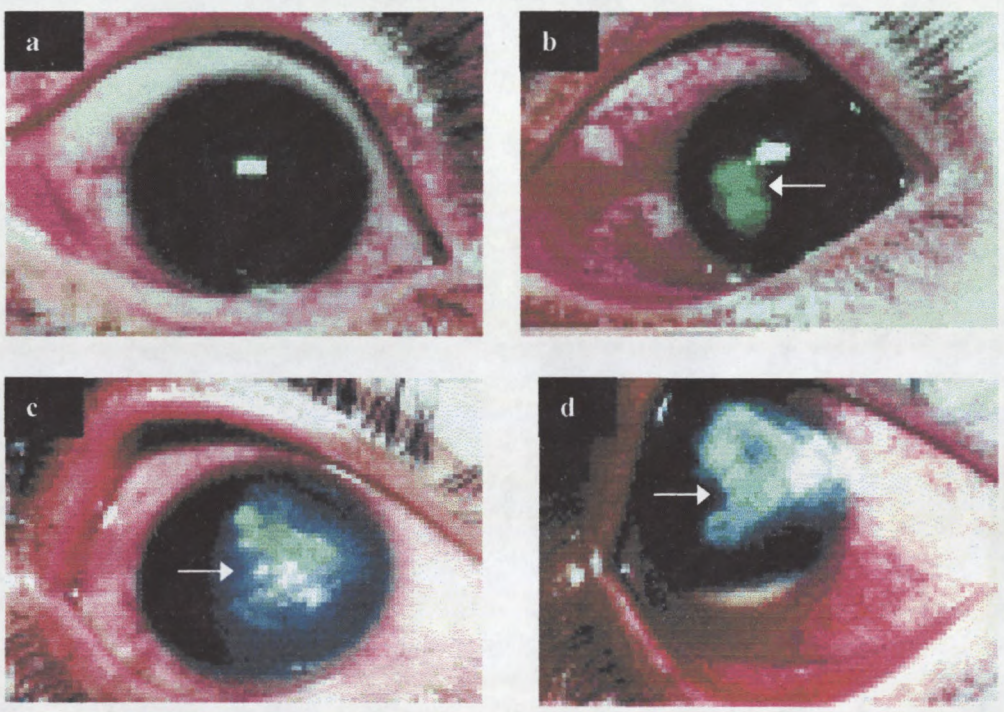


Fig. 1.11 The progression of corneal ulcer formation in a fusarial keratitis infection. (a) No corneal infiltrate (ulcer) formation, (b) formation of corneal infiltrate, (c) increase in size of corneal infiltrate and (d) larger corneal ulcer evident and the formation of hypopyon in the anterior chamber (Rahman *et al.*, 1998).

1.2.4.1.6 Antifungal treatments

Antifungal agents are classified into polyenes, azole derivatives and fluorinated pyrimidines. Polyenes include the antifungal agents natamycin, nystatin, and amphotericin B. They disrupt the cell by binding to fungal cell wall ergosterol and are effective against both filamentous and yeast forms. Amphotericin B is the first choice agent against fungal keratitis due to yeasts. Natamycin has a broad-spectrum of activity against filamentous organisms. The penetration of topically applied amphotericin B is found to be less than that of topically applied natamycin through the intact corneal epithelium (Guzek *et al.*, 1998).

Azoles (imidazoles and triazoles) include the antifungal agents ketoconazole, miconazole, fluconazole, itraconazole, econazole, and clotrimazole. Azoles inhibit ergosterol synthesis at low concentrations, and at higher concentrations appear to cause direct damage to cell walls. Oral fluconazole and ketoconazole are systemically absorbed with good levels in the anterior chamber and cornea and therefore should be considered in the management of deep fungal keratitis.

Fluorinated pyrimidines such as flucytocine are other antifungal agents. Flucytocine is converted into a thymidine analog that blocks fungal thymidine synthesis. It is usually administered in combination with an azole or amphotericin B.

Treatment is instituted promptly with topical fortified antifungal drops, initially every hour during the day and every two hours over night. Subconjunctival injections may also

be used in cases of severe keratitis, keratoscleritis or when poor compliance exists. An oral antifungal (such as ketoconazole or fluconazole) is normally considered in cases of deep stromal infection. Antifungal therapy is maintained for 12 weeks and closely followed up. Fluconazole has been shown to penetrate better into the cornea after systemic administration compared to other azoles and may be associated with fewer side effects.

While keratitis due to *Aspergillus*, *Candida* and dematiaceous fungi can be successfully treated with polyene and azoles, the treatment of *Fusarium* keratitis still requires the use of natamycin or econazole. Natamycin, as a 5% suspension, is the only FDA-approved antifungal agent for ocular use in the United States. It has a good spectrum against filamentous fungi, and is most effective against superficial *Fusarium* infections (Thomas, 1994).

CHAPTER TWO: MATERIALS AND METHODS

2.1 OVERVIEW

The first part of this study involved the collection of 29 *Fusarium* isolates from mycotic keratitis patients. This included the diagnosis of a mycotic keratitis infection and the isolation of Fusaria from these infections. A clinical study of eight patients with fusarial keratitis including the patients' medical history and ocular examination of the infected eye was performed by an ophthalmologist.

The second part of this study involved the identification of these *Fusarium* isolates to species level. Isolates were initially cultured on the appropriate identification media. Taxonomic characteristics of *Fusarium* species such as the presence or absence and shape of macroconidia, microconidia, chlamydospores and phialides were observed by light microscopy and were identified using a synoptic key for *Fusarium* species (Nelson *et al.*, 1983). Ultrastructural characteristics such as the shape of the apical and basal cells of macroconidia were examined using scanning electron microscopy (SEM).

The third part of this study involved determining the mycotoxin- producing potential of these *Fusarium* species. This was accomplished by culturing the isolates on sterilized maize patties and extraction and purification of fusaric acid, moniliformin and fumonisin B_1 using the appropriate procedures. Extraction and detection procedures were optimized for mycotoxins by determining the mean percentage recoveries and standard deviations of each the mycotoxins using commercial standards. Detection limits of the mycotoxins by thin layer chromatography (TLC) and high-performance liquid chromatography

(HPLC) were also determined using commercial standards. Fusaric acid, moniliformin and fumonisin B_1 production by the *Fusarium* isolates were then detected using TLC. Confirmation and quantification were accomplished using HPLC.

The final part of the study involved the evaluation of the cytotoxic effects of fusaric acid, moniliformin and fumonisin B_1 commercial standards and purified toxin extracts from the *Fusarium* isolates, on A549 (human lung adenocarcinoma) cells and VK (monkey kidney) cells. This was accomplished by determining the percentage cell viabilities of the A549 and VK cells after exposure to the toxins using the MTT-cleavage assay and the crystal violet test. The correlation coefficient was determined to compare the values obtained from both these assays.

2.2 *Fusarium* SPECIES IDENTIFICATION

2.2.1 *Fusarium* isolates

Diagnosis of all mycotic keratitis infections was made by the ophthalmologist at the King Edward VIII Hospital- Eye Clinic. Ulcerative keratitis was diagnosed by the presence of a corneal stromal infiltrate with an overlying epithelial abnormality (ulceration). At the baseline visit (day 1), a complete medical history (i.e. age, sex, race, trauma, previous ocular surgery, underlying systemic disease and nutritional status) and ocular examination (visual acuity, size, site and depth of ulcer, inflammatory reaction in the corneal anterior chamber and the types of antifungal treatments used) was obtained from patients. The clinical questionnaire (Table 2.1) was administered by the ophthalmologist.

Table 2.1 Clinical questionnaire including the patients' medical history and ocular examination completed by the ophthalmologist

Medical History	
Age	**Sex**
Symptoms	
Previous ocular surgery	**Systemic diseases:** Diabetes mellitus (DM), Tuberculosis (TB), Hypertension, Other
Trauma to eyes **Location:** household, agricultural, industrial, other **Type:** wooden, metal, vegetable matter, other	
A. Ocular Examination	
Visual Acuity: CF (Count fingers), HM (Hand movements), PL (Perception of light)	**Side of ulcer:** **Right eye, left eye**
Size of ulcer (mm)	**Site of ulcer:** Central, marginal, satellite lesions
Depth of ulcer: Epithelial, stromal, Descemetocoele, perforation	**Anterior chamber reaction:** **Cells, flare, hypopyon**
Antifungal treatment (Topical treatment): gentamycin kefzole (GK), subconjunctival miconazole (SM), natamycin miconazole (NM) drops	

Corneal scrapings were taken from the infected eye using a sterile platinum spatula for microscopic examination. The Gram stain and potassium hydroxide preparation (Rebell, 1981) of the specimens was used to determine the presence of fungal elements by light microscopy. The scraping was also inoculated on Sabouraud Dextrose Agar (SDA) (Sigma) (Appendix 1) and incubated for 24 hours at 25°C for fungal growth.

Identification of Fusaria as the etiological agent of keratitis was made at the Mycology Unit - University of Natal Medical School by observing macroconidia, microconidia and

chlamydospores, characteristic of the genus *Fusarium*, by light microscopy. Presumptive *Fusarium* isolates were subcultured onto SDA, and incubated for 14 days at 28°C.

2.2.2 Culture Media

To achieve distinct colony colours, which was a secondary criterion in the identification of *Fusarium* species, the isolates were cultured on the carbohydrate-rich medium, potato dextrose agar (PDA) (Nelson *et al.*, 1983). PDA was prepared using baking-grade, white-skinned potatoes. The unpeeled potatoes were washed, sliced and 250 g added to 500 ml of distilled water. A flask containing 20 g of agar (Merck) in 500 ml of distilled water was also prepared and sterilized along with the flask of potatoes in an autoclave for 30 min at 121°C. The potato broth was strained through several layers of cheesecloth into the flask of molten agar. The remaining potato pulp was squeezed through several layers of cheesecloth until a half-cup of potato pulp was obtained. This pulp and 20 g glucose was added to the flask containing the melted agar and potato broth. The total volume was adjusted to one liter with distilled water. The medium was autoclaved at 121°C for 15 min and dispensed into petri dishes after cooling.

Carnation leaf agar (CLA), a minimal media which enhances sporulation (Nelson *et al.*, 1983), was prepared with young carnation leaves, cut into pieces approximately 5 mm^2, and dried in an oven at 45°C for 2 h. The dried leaf-pieces were sealed in plastic packets and sterilized with 2.5 megarads of gamma irradiation at Gamwave, Prospecton. CLA was prepared by aseptically transferring several sterile leaf-pieces to a petri dish containing 2% water agar (WA) (Appendix 1) cooled to 45°C. The medium was left at

room temperature for 24 h before use, to check for possible contaminants from the leaf-pieces.

2.2.3 Single-spore culture

Suspensions of *Fusarium* conidia were prepared by adding 10 ml sterile distilled water to the *Fusarium* cultures (cultured on SDA) and scraping the surfaces with a sterile surgical blade until a turbid suspension was formed. The conidial suspensions were poured over the entire surface of solidified 2% WA. The plates were incubated at room temperature for 24 h and examined with a dissecting microscope (Nikon). Small squares of the agar containing single germinating conidia were cut out with a sterile surgical blade and inoculated aseptically to PDA and CLA. The PDA plates were incubated for 14 days at 25°C whilst the CLA plates were incubated for 3 days at 25°C. Cultures were used for microscopic observation.

2.2.4 Microscopy

2.2.4.1 Light microscopy

The wet-mount method (Pelzar *et al.*, 1993) was used for the microscopic examination of the *Fusarium* cultures, using a dissecting microscope (Nikon) at a 100X magnification. Culture material was removed from the CLA plates using a sterile dissecting needle and transferred to a clean glass slide containing a small drop of lactophenol-cotton-blue stain (Appendix 1). The material was teased evenly into the stain with two dissecting needles and covered with a cover- slip. Cultural characteristics i.e. macroconidia, microconidia, chlamydospores and phialides were observed using a light microscope (Nikon) at 400X

magnification. These characteristics were used in the synoptic key (Appendix 2) for the characterization of *Fusarium* species (Nelson *et al.*, 1983).

2.2.4.2 Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was carried out at the Electron Microscope Unit-University of Natal, Umbilo. Single carnation leaf-pieces containing *Fusarium* culture were removed from the CLA plates and transferred to glass vials. A 2% glutaraldehyde solution (Appendix 3) was added to cover the leaf-pieces and fixed twice for 2 h in this solution. The leaf-pieces were then washed three times for 5 min each in phosphate buffer (pH 7.0) (Appendix 3). The buffer was removed from the vials and the leaf-pieces were fixed in 0.5% osmium tetroxide (Appendix 3) for 1 h, and washed three times with distilled water for 5 min each. They were dehydrated in a series of alcohol treatments-30%, 50%, 75% (for 5 min each) and 100% ethanol (for 10 min). Each alcohol treatment was repeated twice. The leaf-pieces (still submerged in the final alcohol treatment) were then transferred into mesh baskets and placed in a Hitachi HCP –1 Critical Point Dryer overnight. Drying was achieved by flushing liquid nitrogen through the mesh baskets. After drying, double- sided tape was used to fix the dried leaf- pieces onto metal stubs. The metal stubs were then placed in the SEM Coating Unit E5100-Polaron Sputter until the leaf-pieces were coated in colloidal gold. They were viewed using the scanning electron microscope unit at various magnifications for the presence and shape of microconidia, macroconidia (including apical and basal cell structures) and chlamydospores.

2.3 MYCOTOXIN ANALYSIS

The ability of the clinical *Fusarium* isolates to produce fusaric acid, moniliformin and fumonisin B_1 was analysed. This was achieved by producing the toxins on maize patties, extraction and clean- up of these mycotoxins, toxin detection using TLC and detection and quantification using HPLC. Extraction efficiency and detection limits of each of the toxins were also investigated.

2.3.1 *Fusarium* culture conditions

Cultures were tested for fusaric acid, moniliformin and fumonisin B_1 production on maize- patties (Thiel *et al.*, 1993). A mix of maize flour with distilled water (1mg/ml) was prepared and 30 g of maize dispensed into glass petri-dishes. The petri-dishes were then wrapped in aluminum-foil and autoclaved for 30 min at 121°C. The 29 *Fusarium* isolates were inoculated aseptically onto the maize patties. Square blocks of the *Fusarium* cultures, approximately 5 mm^2 in size, were cut out using a sterile surgical blade, and transferred aseptically to the autoclaved maize-patties. The cultures were incubated in the dark for 28 days at 25°C to allow for mycotoxin production. After incubation, the maize patty cultures were tested for presence of fusaric acid, moniliformin and fumonisin B_1 using the appropriate extraction and purification procedures.

2.3.2 Extraction and clean-up of mycotoxins

2.3.2.1 Fusaric acid

Fusaric acid was extracted using a modification of the procedure of Smith and Sousadias (1993). Five grams of the contaminated maize patties was weighed out in 45 ml

centrifuge tubes (Corning) and shaken thoroughly to a uniform consistency with 25 ml of methanol-1% KH_2PO_4 (1:1, v/v) (pH 3.0) (Appendix 4). The samples were centrifuged at 10 000 × *g* for 20 min at ambient temperature, and the pH of the supernatant adjusted to 3.0 with 1 M HCl (Appendix 4).

The acidified supernatant was extracted (sequentially) three times using 20 ml dichloromethane in a separatory funnel. The extracts were pooled, and the volume reduced *in vacuo* to less than 10 ml on a Buchi Rotavapor RE 120 rotary evaporator. The dichloromethane was extracted twice with 5 ml of aqueous 5% $NaHCO_3$ (Appendix 4). The dichloromethane extract was discarded, and the aqueous $NaHCO_3$ solutions were pooled. The pH of the $NaHCO_3$ was adjusted to 3.0 with 1 M HCl, and the solution extracted twice with dichloromethane. The dichloromethane extracts were combined, and evaporated *in vacuo* at 40°C on the rotary evaporator. The resulting residue was stored at 4°C for TLC and HPLC analysis.

2.3.2.2 Moniliformin and Fumonisin B_1

Bond- Elut strong anion exchange (SAX) resin cartridges were used for the extractions of both moniliformin and fumonisin B_1 (Whatman, 1991, Thiel *et al.*, 1993). 5 g of the contaminated maize sample was extracted with 10 ml of methanol/water (3:1) for 2 h. The slurry was filtered using Whatman No. 4 filter paper and the pH of the supernatant was adjusted to pH 6 with 1 M HCl. Clean- up of the toxins were performed as follows:

a) SAX cartridges (Bond- Elut) were preconditioned with 1 ml of methanol.

b) Methanol/water (3:1) (1 ml) was applied to the SAX cartridges.

c) A 2 ml aliquot of the filtered extract was applied to the SAX cartridge at a flow rate of 1ml per minute.

d) The cartridge was washed with 1 ml methanol/water (3:1), followed by 1 ml of methanol.

e) For moniliformin, the eluate was collected with 2 ml 1% HCl in methanol solution (Appendix 4), and for fumonisin B_1, the eluate was collected with 2 ml 1% acetic acid in methanol (Appendix 4).

f) The eluates were evaporated to dryness under a stream of nitrogen, and kept in a vial at 4 °C until further use.

2.3.3 Detection by thin- layer chromatography

Fusaric acid detection using TLC was carried out according to the method of Burmeister *et al.* (1985). Stock solutions (100 µg) of fusaric acid standard (Sigma) were prepared (Appendix 4) and stored at 4 °C. For TLC analysis, 100 µg of fusaric acid standard was reconstituted in 1 ml of methanol (100 µg/ml). Samples were also reconstituted in 1 ml methanol.

Samples and standard (10 µl) were spotted on pre-coated 0.25-mm-thick Silica Gel 60 F-254 TLC plates (Merck). The solvent system used was isopropyl alcohol: ethyl acetate: water: acetic acid (40: 38: 20: 2, v/v/v). Detection was achieved under shortwave UV light (254 nm).

Moniliformin detection was performed according to the method of Desjardins *et al.* (1997). Stock solutions of the moniliformin standard (Sigma) were prepared (Appendix 4) and 10 µl of the sample and standard spotted onto pre-coated silica gel F-254 TLC plates. The solvent system used was acetonitrile: water (85: 15, v/v) and detection was achieved under shortwave UV light (254 nm).

The TLC method of Thiel *et al.* (1993) was used for fumonisin B_1 detection. Stock solutions of fumonisin B_1 standard (Sigma) were prepared as for moniliformin. An aliquot of 10 µl of the samples and the standard was spotted on pre-coated Silica-Gel 60 TLC plates. The solvent system used was ethyl acetate: acetic acid: H_20 (60: 30: 10, v/v/v). The dried TLC plate was sprayed with ρ-anisaldehyde visualizing reagent (Appendix 4), and heated at 110°C for 5 min.

2.3.3.1 Detection limits

The detection limits for each of the mycotoxins were determined by spotting 10 µl each of the toxin standards on a 0.25mm Silica Gel 60 F-254 TLC plate for fusaric acid and moniliformin and on a pre- coated Silica- Gel 60 TLC plate for fumonisin B_1. The lowest standard that produced a spot was the detection limit for the technique. Each toxin concentration was analysed in duplicate.

Fusaric acid : 10, 5, 4, 3, 2, 1, 0.5, 0.25 and 0.1 µg/ml

Moniliformin : 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1 and 0.05 µg/ml

Fumonisin B_1 : 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25 and 0.1 µg/ml

For the detection of toxins, migration rates of the samples were compared to the authentic standards using the following equation:

$$R_f = \frac{\text{Distance of sample from baseline}}{\text{Distance of solvent from baseline}}$$

2.3.4 Detection and quantification by high- performance liquid chromatography

HPLC was performed using a Merck La Chrom liquid- chromatograph system. The sample volume was 50 µl. The mobile phase flow rate was constant at 1 $ml.min^{-1}$.

Fusaric acid was analysed according to the method of Venter and Steyn (1998) under reversed phased conditions using a Merck RP-C18 column (3.4 × 250 mm, 5 µm-particles). The mobile phase consisted of 40% methanol and 60% of an aqueous solution of 0.62 mM Na_2EDTA and 2% H_3PO_4 (Appendix 4), and the elution time was 10 min. Peaks were detected at a fixed wavelength of 254 nm using a Merck L7400 UV lamp.

Moniliformin was analysed according to the method of Faber (1988), using a Merck RP-C18 column. The mobile phase consisted 10 mM phosphate buffer with 5 mM tetra-butyl-ammonium bromide (pH 7):methanol (92:8:v/v). Detection was achieved at 215 nm using a Merck L7400 UV lamp (elution time was 10 min).

Fumonisin B_1 was analysed according to the method of Thiel *et al.* (1993) under reversed phase conditions. The mobile phase consisted of methanol: 0.1 M sodium dihydrogen phosphate (Appendix 4) (80: 20, v/v), and was adjusted to pH 3.3 with phosphoric acid. Samples were derivatized prior to analysis. Samples were prepared immediately prior to injection, by the addition of 450 µl of the *ortho*-phthaldialdehyde (OPA) derivatizing reagent (Appendix 4) to 50 µl of purified toxin extract. Detection was accomplished using a Merck-L7480 Fluorescence detector at excitation and emission wavelengths of 335 and 440 nm respectively.

2.3.4.1 Detection limits

Fusaric acid, moniliformin and fumonisin B_1 standards were purchased from Sigma Chemicals and diluted in methanol for detection by HPLC in the following concentrations:

fusaric acid : 5, 2.5 1.0, 0.5, 0.2, 0.10 and 0.05 µg/ml

moniliformin : 5, 2.5, 1.0, 0.5, 0.1, 0.05, 0.02 and 0.01 µg/ml

fumonisin B_1 : 20, 10, 5, 2, 1, 0.5, 0.2 and 0.10 µg/ml

The lowest concentration that produced a peak area that was three times the signal of the noise level was considered to be the detection limit for the toxin (Ravindranath, 1989). Samples were analysed in duplicate.

For the quantification of toxins, fusaric acid, moniliformin and fumonisin B_1 concentrations was calculated from the peak areas using the following equation:

$$A\ (ng) = G/H \times S$$

Where A = ng toxin present in test extract injected into HPLC

G = toxin peak area in test sample

H = toxin peak area of standard

S = Amount of toxin injected into HPLC

The concentration (C) of toxin present in the test extract (ng/g) was calculated using the following equation:

$$C\ (ng/g) = \frac{A \times T \times D}{I \times W}$$

Where A is calculated above

T = total volume of sample

D = dilution volume used

I = injection volume used

W = test portion equivalent weight (Thiel *et al.*, 1993)

2.3.5 Recovery studies

Extraction efficiency was investigated by determining the recovery of fusaric acid, moniliformin and fumonisin B_1 standards by spiking the maize samples with 5 μg/g of each of the toxins. Each experiment was performed in triplicate. The percentage recovery was calculated from the following equation (Ravindranath, 1989):

$$\text{Percentage (\%) Recovery} = [C_{calculated} - C_{untreated})/C_{initial\ spiked}] \times f \times 100$$

Where,

$C_{calculated}$ = Concentration of toxin (μg/ ml) detected by the experimental technique in the spiked sample

$C_{untreated}$ = Concentration of toxin (μg/ml) detected by the experimental technique in untreated sample

$C_{initial\ spiked}$ = Initial concentration of toxin (μg/ml) spiked in the sample prior to extraction.

f = dilution factor, where f = 1 for the liquid-liquid extraction, and f = 5 for the SAX- cartridge extraction method.

2.4 CYTOTOXICITY TESTS

All cytotoxicity tests were performed at the Virology Department - University of Natal Medical School.

2.4.1 Materials

In this study, A549 (lung carcinoma) cells and VK (monkey kidney) cells were used to evaluate the cytotoxic effects of fusaric acid, moniliformin and fumonisin B_1. A description of each of the cell lines used is given in Table 2.2.

Table 2.2 Description of cell lines used

CELLS	ORIGIN	SPECIES	LINEAGE	CELL TYPE
A549	*ATCC CCL 185	Human	Lung carcinoma	Epithelial
VK	Primary culture	Rhesus monkey	Kidney	Epithelial, Fibroblasts

*ATCC = American Type Culture Collection

Reagents and culture media were purchased from Polychem S.A. Eagles Minimum Essential Medium (EMEM) supplemented with Earles' salts, Hepes, non- essential amino acids and L-glutamine was routinely used as the cell culture medium. To enhance cellular growth, 5% or 10% foetal calf serum (FCS) was added to the culture medium. The antibiotics penicillin, amicasin and fungizone (100 units/ml, 100 μg/ml and 2.5 μg/ml, respectively) were added to the culture medium to prevent microbial contamination.

2.4.2 Manipulation of cell lines

2.4.2.1 A549 (lung carcinoma) cells

EMEM supplemented with 10% FCS was used for growing and maintaining the A549 cell line. A cryovial of frozen cells was removed from the -70°C biofreezer and thawed rapidly by placing in a basin of tap water warmed to 37°C. After the contents had thawed, the vial was swabbed with 70% alcohol to prevent contamination. The vial was opened and the contents transferred aseptically to a sterile centrifuge tube. EMEM (15 ml) supplemented with 5% FCS and 1% penicillin/amicasin/fungizone mixture was added slowly to the centrifuge tube to dilute the freezing agent. The cells were harvested by centrifugation at 1 500 rpm for 10 min. The supernatant was decanted and the cell pellet resuspended in 5 ml EMEM supplemented with 10% FCS and 1% penicillin/amicasin/fungizone mixture. The flasks were incubated in a horizontal position at 37°C. The medium was replaced with fresh growth medium after 24 hours.

2.4.2.2 VK (monkey kidney) cells

Kidneys of a one year-old Rhesus monkey were obtained from the University of Durban-Westville (UDW). To establish a primary culture of kidney cells (epithelial and fibroblasts), kidneys were rinsed with PBS (pH 7.2) (Appendix 5) and sliced using a sterile blade. The kidney pieces were rinsed twice in PBS (pH 7.2) to remove red blood cells and chopped very finely until minced. PBS (pH 7.2) (100 ml) was then added and the kidney particles allowed to settle at the bottom of the flask. The remaining cloudy liquid was discarded. This procedure was repeated until the liquid became clear.

For collection of cells, 100 ml of trypsin (Appendix 5) was added to the flask and stirred for 15 min at 37°C. A 1% trypsin solution (Appendix 5) was used instead of 0.25% trypsin (Appendix 5) since cells were trypsinized from a whole organ instead of the normal monolayer). After 15 min, the liquid containing cells was separated from any solid particles present by filtering through a sterile muslin cloth placed on a funnel. The cells were collected in a sterile 500 ml Schott bottle. The trypsinization procedure was repeated at 15, 30, 45 and 60 min until sufficient cells were collected. The cell suspension was transferred into 50 ml centrifuge tubes (Corning) and centrifuged at 2000 rpm for 5 min. The cell pellets were resuspended in 5 ml growth medium (EMEM supplemented with 10% FCS and 1% penicillin/amicasin/fungizone mixture) and transferred to culture flasks. The flasks were incubated at 37°C and the medium changed after 24 h.

Cells not immediately required for cytotoxicity testing were stored by freezing. Cells were removed from a confluent monolayer by trypsinization and suspended in a preservative (EMEM supplemented with 20% DMSO and 20% FCS). Aliquots of 1 ml each were prepared in cryovials and placed in a thermo-flask before storing at -70°C.

2.4.3 Trypsinization of cells

Flasks were inspected under an inverted microscope daily until they reached confluency and then passaged at a 1:20 split ratio. The medium was decanted from the culture vessels and the cells washed twice with pre-warmed PBS (pH 7.2). The rinse medium was poured off and 2 ml of 0.25% trypsin added for 5 min to allow trypsin digestion to take place. The trypsinization efficacy was monitored with the inverted microscope by

observing for cell rounding. Cells were dislodged by tapping the flask firmly against the palm of the hand. EMEM (20 ml) supplemented with 5% FCS was added to the flask and aliquoted to the recommended split ratio.

2.4.4 Cytotoxicity assays

Flasks of confluent A549 and VK cells that were trypsinized and resuspended in culture media (EMEM supplemented with 5% FCS) were adjusted to give a cell number of 2 $\times 10^5$ cells/ml using a Neubar counting chamber (Appendix 5). An aliquot of 100 µl of the cell suspension was dispensed into each well of a 96-well microtitre plate.

Mycotoxin standard solutions (0.5 mg/ml) (Appendix 5) were diluted serially (two-fold dilutions) so that a range of concentrations from 0.004 to 0.5 mg/ml were obtained. A volume of 100 µl of each dilution was transferred to each well except eight wells, which served as a cell control. For each mycotoxin concentration 5 replicates were performed.

The purified toxin extracts containing the highest concentrations of fusaric acid, moniliformin and fumonisin B_1 were reconstituted in 1 ml PBS (pH 7.2) and 100 µl was transferred to each well of a 96-well microtitre plate Four replicates were performed for each toxin tested. Plates were incubated in a moist chamber at 37°C for 24 h.

Cytotoxicity was assessed using the MTT assay (Hanelt *et al.*, 1994) and the crystal violet test (Landegrand, 1984). In the MTT assay, a volume of 20 µl of the MTT stock solution (Appendix 5) was added after 24 h to each well. The plates were incubated for

another 4 h. The supernatants were removed using a multi-channel micropipette and 100 µl DMSO was added to each well to dissolve the dark formazan crystals. The optical density of each well was measured spectrophotometrically with an ELISA-reader at an absorbance value of A_{570} nm and a reference filter of A_{650} nm.

In the crystal violet test, the cell culture medium was decanted from the microtitre plate after 24 h and the cells fixed by adding a drop of ammonium oxalate crystal violet dye so that the bottom of the well was completely covered. After staining, cells were washed extensively using tap water and dried. The remaining crystal violet was extracted with 2-methoxy-ethanol and left for 1 h at room temperature. Absorbance was monitored at 595 nm and compared to the results of the MTT assay.

For both assays, the percentage cell viability was calculated as follows:

$$\text{Cell Viability (\%)} = \frac{\text{absorbance of test sample}}{\text{absorbance of media}} \times 100$$

The mean percentage cell viabilities of A549 and VK cells in the MTT-cleavage assay were compared to the values obtained in the crystal violet test for any significant difference using the Student's t-test (Microsoft Excel package).

CHAPTER THREE: RESULTS

3.1 Clinical details of fungal ulcer study

Clinical specimens from patients with mycotic keratitis that attended the Eye Clinic-King Edward VIII Hospital and had presumptive Fusaria isolated from these infections, were identified to species level. Twenty- one samples were taken from 1986 to 1998 and eight samples were taken in 1999. The latter specimens were part of a fungal ulcer study and complete clinical investigation that included the patients' medical history, symptoms, risk factors for the infection (age, sex, trauma), visual acuity, characteristics of the ulcer (size, site and depth), inflammatory response in the anterior chamber (cells, flare, hypopyon) as well as the type of antifungal treatment used, was recorded by the clinician. Table 3.1 outlines these results.

The age of the patients ranged from 36 to 67 years. The male to female sex ratio was 1:1. Trauma was a major risk factor for the infection (80% of the patients' infections were injury-induced), caused either by industrial, agricultural or household items. Visual acuity was compromised in all the patients. Only one patient exhibited a marginal ulcer. The remaining seven patients presented with central ulcers with satellite lesions included in three patients. The depth of the ulcer extended into the corneal stroma in six cases whereas a complete perforation of the cornea occurred in two cases. The anterior chamber inflammatory response included hypopyon formation in four cases. The antifungal treatment included gentamycin kefzole for all the patients, subconjunctival drops for four patients and natamycin miconazole drops was included in the treatments of seven patients.

Table 3.1 Medical history and fungal ulcer study of eight patients with fusarial keratitis.

Isolate no.	Age	Sex	Symptoms	Trauma to eyes (Location/ Type)	Other ocular pathology/ Previous surgery	Systemic diseases
1	53	Male	Painful	None	None	None
3	36	Female	Painful, red	Agricultural	None	None
4	53	Female	Painful	Industrial	None	None
5	60	Female	Painful	Agricultural	None	None
6	39	Male	Painful, red	Other	None	None
10	60	Male	Painful	Household	None	Diabetes mellitus
11	42	Male	Painful	Industrial	None	None
14	67	Female	Painful	None	None	None

Isolate no.	Visual Acuity		Side of ulcer	Size of ulcer (mm)	Site of ulcer	Depth of ulcer	Anterior chamber inflammatory response	*Antifungal treatment
	R	L						
1	6/12	*CF	L	2×2	Central, satellite lesions	Stromal	None	GK, SM, NM
3	6/5	6/36	L	4×4	Central, satellite lesions	Stromal	Cells, flare	GK, SM, NM
4	6/6	CF	L	Large central ulcer	Central	Perforation	Cells, flare, hypopyon	GK, NM
5	6/18	CF	L	3×3	Central, satellite lesions	Epithelial, stromal	Cells, flare, hypopyon	GK, SM, NM
6	6/12	6/5	R	1×1	Central	Stromal	Flare	GK, NM
10	CF	6/12	R	Large central ulcer	Central	Epithelial, stromal	Cells, flare	GK, NM
11	6/9	*HM	L	3×3	Marginal	Stromal	Hypopyon	GK, SM, NM
14	6/36	*PL	L	Large central ulcer	Central	Epithelial, stromal, perforation	Cells, flare, hypopyon	GK

*Antifungal treatments: GK = Gentamycin Kefzole; SM = Subconjunctival Miconazole; NM = Natamycin miconazole drops

*CF = counting fingers, *HM = hand movements, *PL = perception of light

3.2 Identification of *Fusarium* species

3.2.1 Cultural characteristics

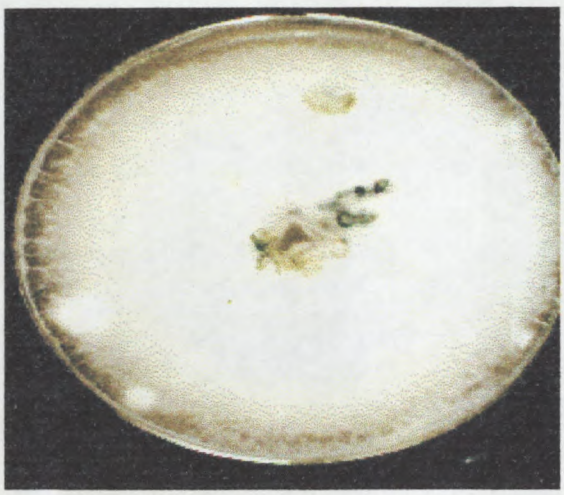
The characterization of Fusaria to species level was initiated in CLA and PDA. CLA is a minimal media with gamma- irradiated leaves acting as a nutrient source. This medium induced sporulation of all the *Fusarium* cultures. Fig. 3.1 shows a mass of fusarial hyphae grown on sterile carnation leaf- pieces.

Fig. 3.1 *Fusarium solani* cultured on CLA

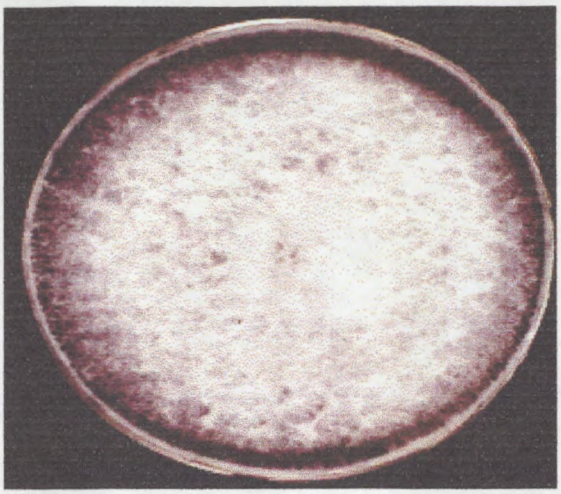
PDA is a carbohydrate rich media used to distinguish colony morphology and colour of *Fusarium* species. Characteristic colonial features of the *Fusarium* isolates are described in Table 3.2. Fig 3.2a-g shows the characteristic colours for the seven *Fusarium* species described in Table 3.2.

Table 3.2 Characteristic colony colours on PDA of the twenty- nine clinical isolates of Fusaria identified to species level.

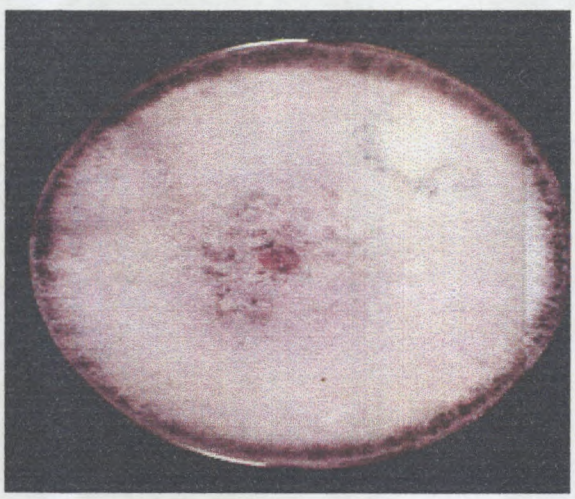
***Fusarium* species**	**Colony colours on PDA**	**No. of clinical isolates with similar colony colours and characteristics of seven *Fusarium* species identified**
F. solani	Abundant white to cream mycelial growth. Green exudates produced. Cream undersurface (Fig. 3.2a).	21
F. oxysporum	Abundant, white, cottony mycelium with lilac to purple tinge. Dark purple undersurface (Fig. 3.2b).	1
F. moniliforme	Cottony, white mycelium with purple tinge. Violet undersurface (Fig. 3.2c)	2
F. dimerum	Mycelium scarce. Growth yeast- like. Orange sporodochia colour the surface. Undersurface orange to tan (Fig. 3.2d).	2
F. semitectum	White to cream cottony mycelia with yellow to tan exudates produced. Undersurface tan to dark- brown (Fig. 3.2e)	1
F. chlamydosporum	White mycelial growth with surface gradually becoming tan to dark- brown. Undersurface tan (Fig. 3.2f).	1
F. lateritium	White to cream mycelia, sparse. Slow growth. Cream undersurface (Fig. 3.2g).	1



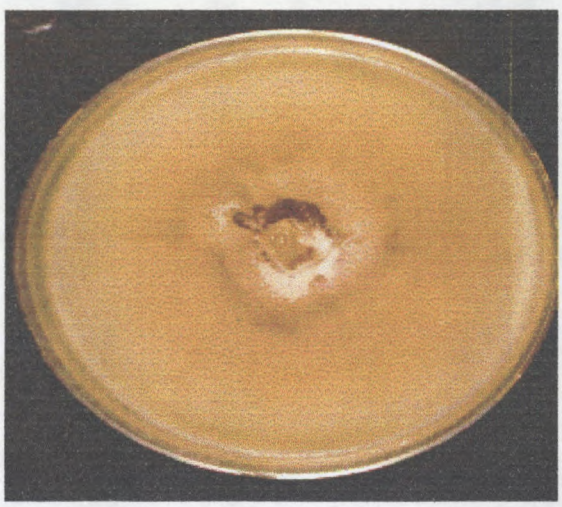
(a) *F. solani*



(b) *F. oxysporum*



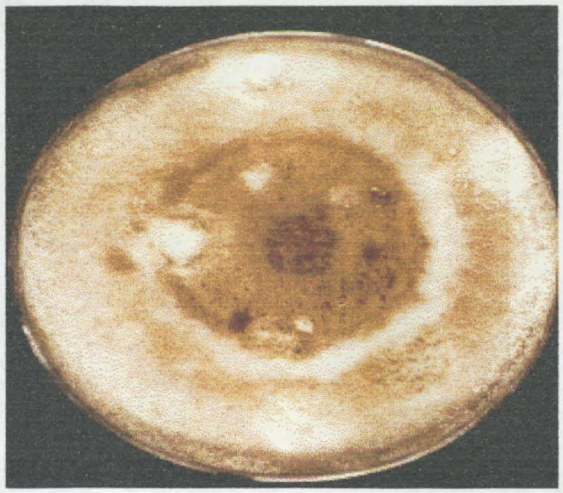
(c) *F. moniliforme*



(d) *F. dimerum*

Fig. 3.2(a-d) Mycelium formation and colony pigmentation of the *Fusarium* isolates on PDA. Colony characteristics of the following *Fusarium* species were observed including (a) *F. solani,* (b), *F. oxysporum*, (c) *F. moniliforme*, (d) *F. dimerum.*

(e) *F. semitectum*



(f) *F. chlamydosporum*

(e) *F. lateritium*

Fig. 3.2(e-f) Mycelium formation and colony pigmentation of the *Fusarium* isolates on PDA. Colony characteristics of the following *Fusarium* species were observed including (e) *F. semitectum*, (f) *F. chlamydosporum* and (g) *F. lateritium*

3.2.2 Morphological characteristics

The morphological characteristics of the 29 cultures grown on CLA were examined by light microscopy using the wet-mount method. The spore-bearing structures (phialides), distinguishes certain species of Fusaria from each other. In this study, the short monophialide characteristic of *F. oxysporum* was observed in one isolate. (Fig. 3.3a), whilst the long, slender monophialide characteristic of *F. solani* was observed in 21 isolates (Fig. 3.3b). Fig. 3.4a shows a branched monophialide characteristic of *F. moniliforme* whilst Fig. 3.4b shows a branched polyphialide characteristic of *F. semitectum.*

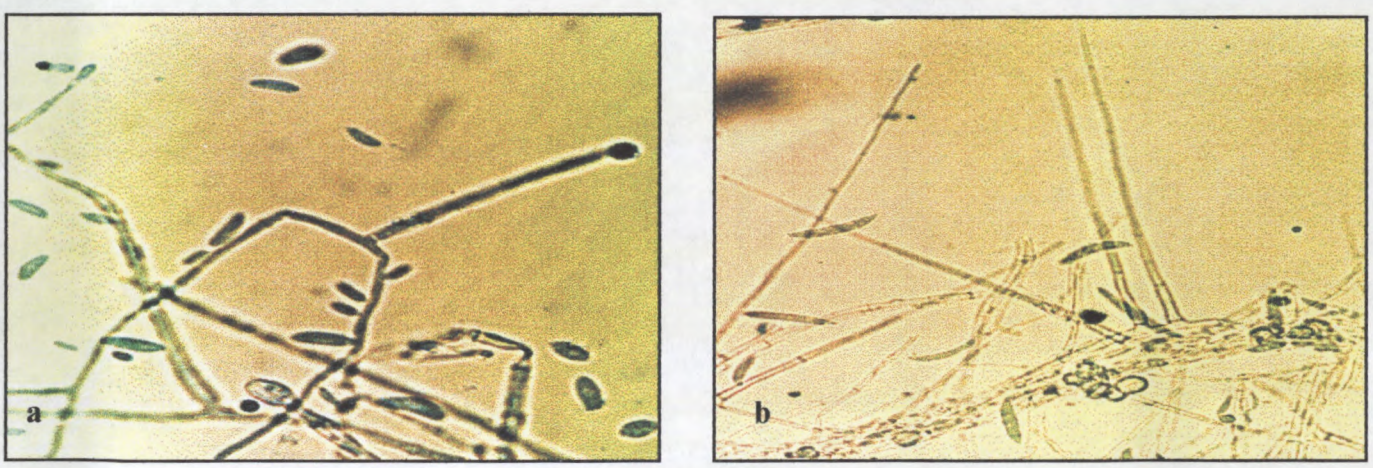


Fig. 3.3 Photomicrographs (400X magnification) showing (a) a short *Fusarium oxysporum* monophialide and (b) long, slender monophialides of *F. solani.*

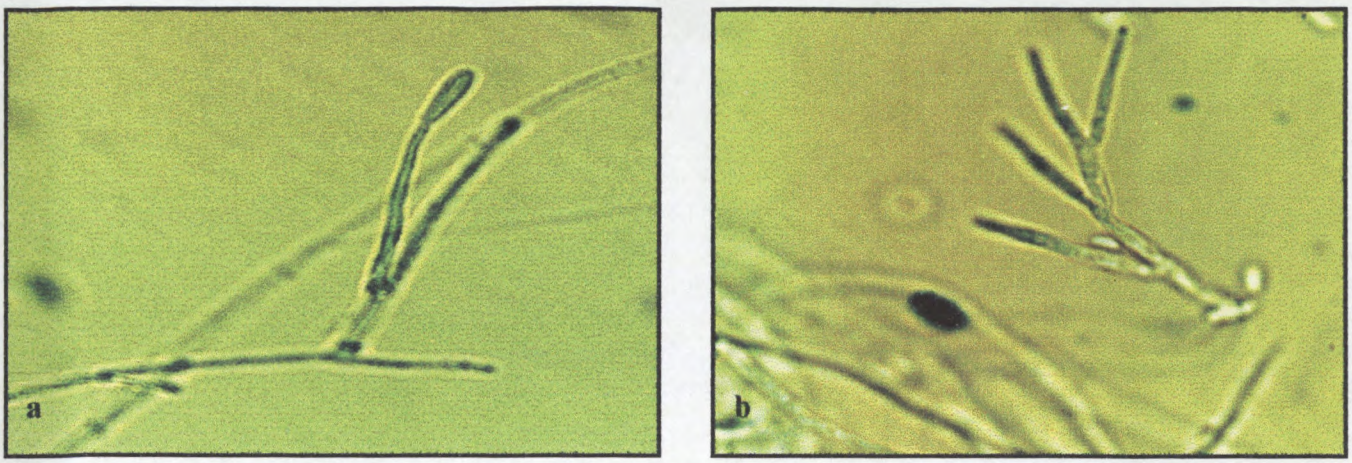


Fig. 3.4 Photomicrographs (400X magnification) of (a) a branched *Fusarium moniliforme* monophialide and (b) a branched polyphialide of *F. semitectum*

3.2.3 Ultrastructural characteristics

Scanning electron microscopy was used to observe the distinguishing features of the three types of *Fusarium* spores, i.e. macroconidia, microconidia and chlamydospores from the isolates cultured on CLA.

The important features of *Fusarium* macroconidia, which are formed in sporodochia (fruiting bodies) (Fig. 3.5), that distinguish the different types of species are shape of the basal and apical cells, length and curvature. The typical foot-shaped basal cell characteristic of *F. solani, F. oxysporum, F. moniliforme* and *F. dimerum* macroconidia was observed in eleven isolates (Fig. 3.6). Macroconidia with blunt or rounded ends of both the apical and basal cells characteristic of *F. solani* were seen in 21 isolates (Fig. 3.7). The hooked apical cell characteristic of *F. dimerum* was observed in two isolates (Fig. 3.8).

The differentiation of *Fusarium* species based on microconidia revealed single-celled, reniform microconidia in all the isolates (Fig. 3.9) whereas the single-celled, spindle-shaped microconidia typical of *F. chlamydosporum* was seen in one isolate (Fig. 3.10).

Smooth-walled chlamydospores typical of *F. solani* were observed in twenty isolates. Fig. 3.11a shows typical smooth-walled chlamydospores appearing on false heads whereas in Fig. 3.11b, chlamydospores appear in an intercalary chain. Rough-walled chlamydospores typical of *F. chlamydosporum* species were observed in one isolate (Fig. 3.12).

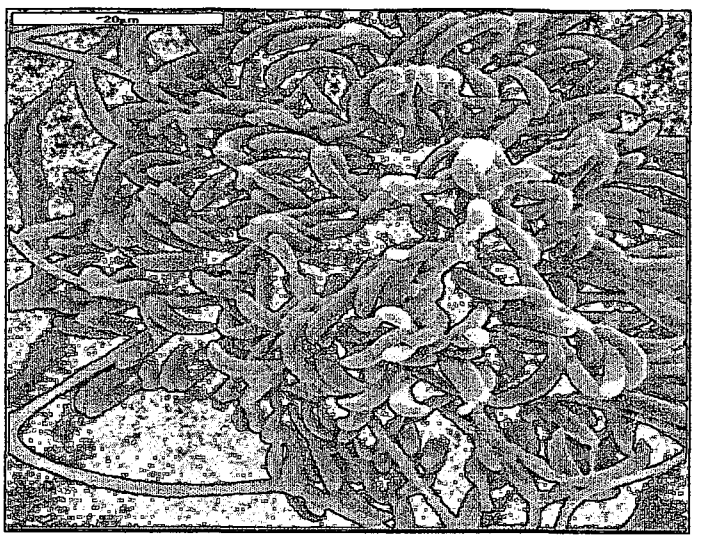


Fig. 3.5 Scanning electron micrograph of a typical sporodochium (X2.0K magnification). Fruiting bodies or spore-bearing structures (sporodochia) were observed in all the *Fusarium* isolates.

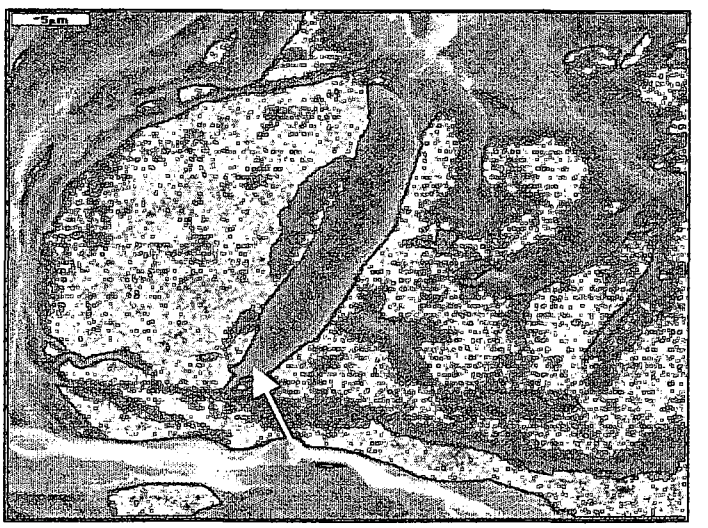


Fig. 3.6 Scanning electron micrograph of a single *F. oxysporum* macroconidium (X4.0K magnification). The arrow points to a foot-shaped basal cell typical of this species.

Fig. 3.7 Scanning electron micrograph of a *F. solani* macroconidium (X5.0K magnification). The arrows point to the apical and basal cells with blunt or rounded ends.

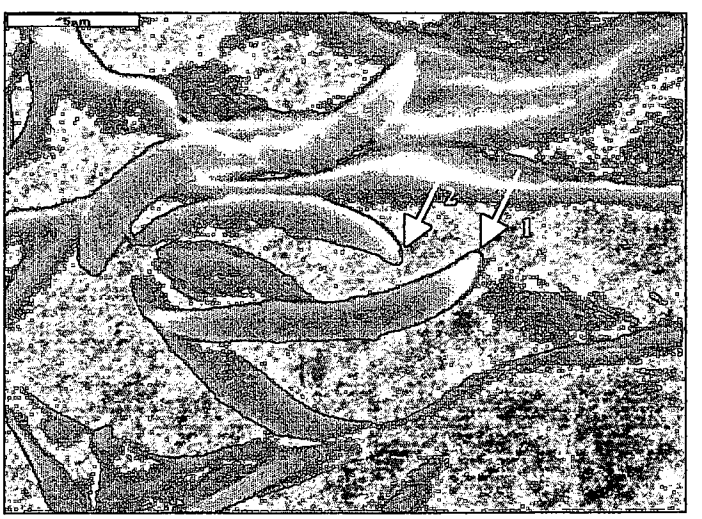


Fig. 3.8 Scanning electron micrograph of *F. dimerum* macroconidia (X4.0K magnification). Macroconidia are small and 2-septate. Arrow 1 points to a hooked apical cell while arrow 2 points to the slightly notched basal cell.

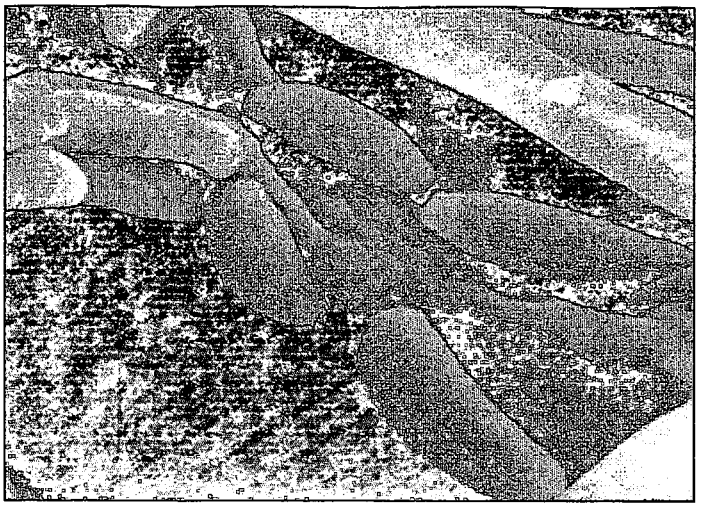


Fig. 3.9 Scanning electron micrograph of single-celled, reniform (X7.0K magnification) *F. solani* microconidia.

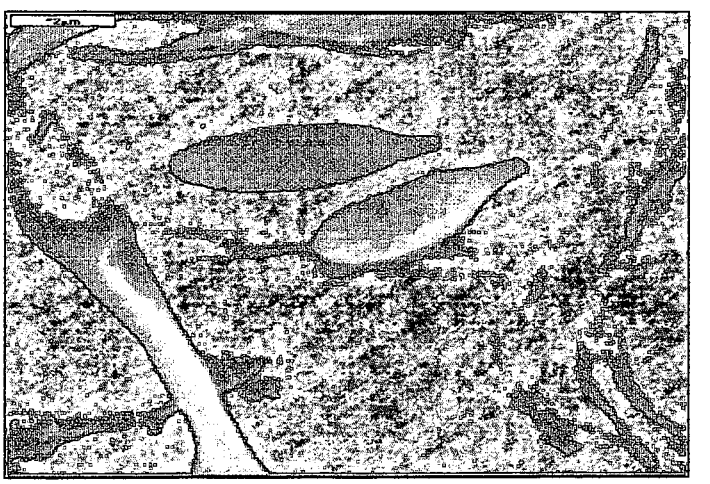


Fig. 3.10 Scanning electron micrograph of single-celled, spindle-shaped *F. chlamydosporum* microconidia (X6.0K magnification).

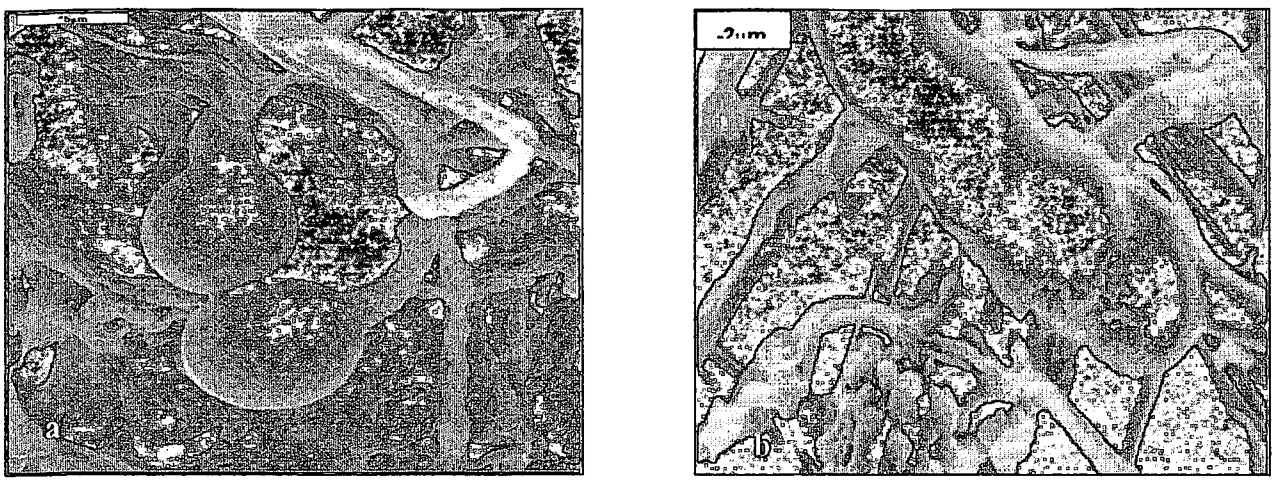


Fig. 3.11 Scanning electron micrographs of *F. solani* chlamydospores. (a) Smooth-walled chlamydospores were observed on false heads (X4.0K magnification) and (b) chlamydospores appearing in an intercalary chain (X4.0K magnification).

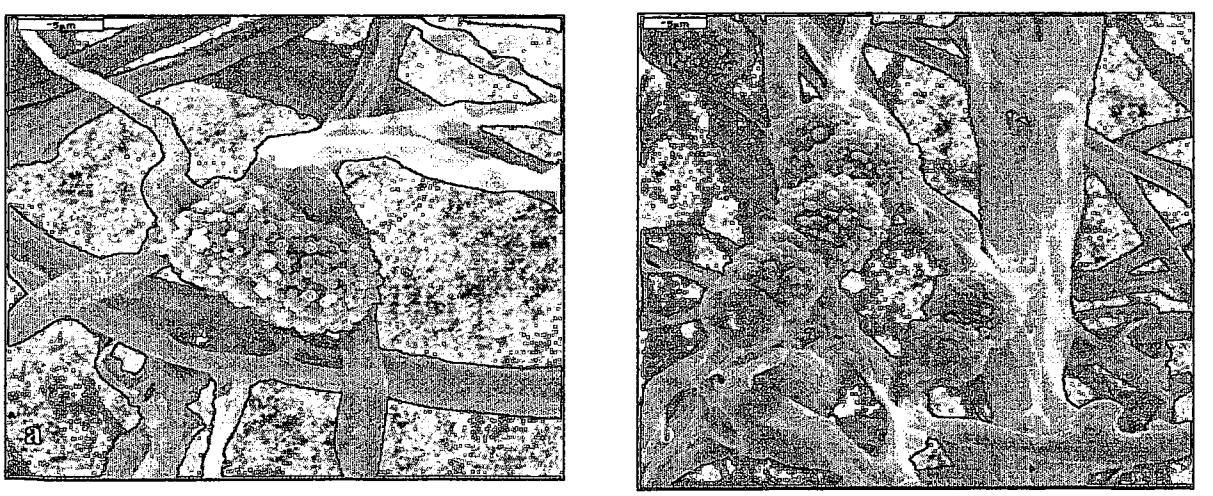


Fig. 3.12 Scanning electron micrographs of *F. chlamydosporum* chlamydospores. (a) Rough-walled chlamydospores appearing in a pair (X3.0K magnification) and (b) a chain of chlamydospores (X3.0K magnification).

3.2.4 Species identification using the synoptic key

The synoptic key (Appendix 2) to the sections and species of Fusaria revealed that the clinical isolates showed similar cultural characteristics of seven *Fusarium* species i.e. *F. dimerum, F. oxysporum, F. solani, F. moniliforme, F. semitectum, F. lateritium and F. chlamydosporum.* Isolates 1 and 24 were identified as *F. dimerum* (Table 3.3); isolate 2 as *F. oxysporum* (Table 3.4); isolates 3 to 23 as *F. solani* (Table 3.5); isolate 28 as *F. semitectum* (Table 3.6), isolate 25 and 26 as *F. moniliforme* (Table3.7); isolate 29 as *F. lateritium* (Table 3.8) and isolate 27 as *F. chlamydosporum* (Table 3.9).

Of the twenty-nine *Fusarium* isolates, twenty-one (72.4%) were identified as *F. solani*, two (6.9%) were identified as *F. dimerum*, two as (6.9%) as *F. moniliforme*, and one (3.4%) each of *F. oxysporum, F. lateritium, F. chlamydosporum* and *F. semitectum* (Fig. 3.13). The *Fusarium* sections and species, to which each of the twenty-nine isolates belongs, are shown in Table 3.10.

Table 3.3 Synoptic key to section and species of isolate no. 1 and 24 *(F. dimerum)*

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
A. Cultural characteristics		**B. Cultural characteristics**	
1. Rate of growth	1	1. Rate of growth	1, 2, 3
2. Aerial mycelium present or absent	1	2. Aerial mycelium present or absent	1, 2, 3
3. Colour of aerial mycelium		3. Colour of aerial mycelium	
4. Colour of colony from below	1, 3, 4, 5, 6, 7, 8, 10, 11, 12	4. Colour of colony from below	1, 2, 3
5. Colour of spore masses	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	5. Colour of spore masses	1, 2, 3
B. Macroconidia from sporodochia		**C. Macroconidia from sporodochia**	
1. Size	1, 3	1. Size	1, 2, 3
2. Shape	1, 3, 4, 5, 6, 7, 8, 11	2. Shape	1, 3, 5
3. Shape of basal and apical cells	1, 3, 4	3. Shape of basal and apical cells	1, 2, 3, 5
C. Microconidia from aerial mycelium		**D. Microconidia from aerial mycelium**	
		1. Present or absent	1, 2, 3, 5
1. Present or absent	1, 3, 5, 6, 7, 8, 9	2. In chains or false heads	
2. In chains or false heads		3. Shape	
3. Shape			
		E. Conidiophores	
D. Conidiophores		1. Type	
1. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		1, 2, 3, 4, 5
		F. Chlamydospores	
E. Chlamydospores		1. Present or absent	
1. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		2, 3
2. Arrangement	1, 4, 9, 11, 12		

Table 3.4 Synoptic key of section and species to isolate no.2 *(F. oxysporum)*

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
A. Cultural characteristics		**A. Cultural characteristics**	
1. Rate of growth	2, 4, 5, 6, 7, 8, 10, 11, 12		
2. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	1. Rate of growth	25, 26, 27, 28, 29, 30
3. Colour of aerial mycelium	10, 11	2. Colour of aerial mycelium	24, 25, 26, 27, 28, 29, 30
4. Colour of colony from below	1, 3, 4, 5, 6, 7, 8, 10, 11, 12	3. Colour of colony from below	25, 26, 27, 28, 29, 30
5. Colour of spore masses	11, 12	4. Colour of spore masses	24, 25, 26, 27, 28, 29
B. Macroconidia from sporodochia		**B. Macroconidia from sporodochia**	
1. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12	1. Size	25, 26, 27, 28, 29, 30
2. Shape	2, 6, 8, 9, 10, 11, 12	2. Shape	25, 26, 27, 28, 29
3. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12	3. Shape of basal and apical cells	
C. Microconidia from aerial mycelium		**C. Microconidia from aerial mycelium**	
		1. Present or absent	24, 25, 26, 27, 28, 29, 30
1. Present or absent	2, 4, 7, 8, 9, 10, 11, 12	2. In chains or false heads	24, 27, 28, 29, 30
2. In chains or false heads	4, 11, 12	3. Shape	24, 25, 26, 27, 29, 30
3. Shape	2, 4, 7, 8, 10, 11, 12		
		D. Conidiophores	
D. Conidiophores		1. Type	29
1. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
		E. Chlamydospores	
E. Chlamydospores		1. Present or absent	29, 30
1. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		
2. Arrangement	1, 4, 9, 11, 12		

Table 3.5 Synoptic key of section and species to isolates 3 –23 *(F. solani)*.

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
A. Cultural characteristics		**A. Cultural characteristics**	
1. Rate of growth	2, 4, 5, 6, 7, 8, 10, 11, 12	1. Rate of growth	25, 26, 27, 28, 29, 30
2. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	2. Colour of aerial mycelium	24, 25, 26, 27, 28, 29, 30
3. Colour of aerial mycelium	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	3. Colour of colony from below	24, 25, 26, 26, 28, 29, 30
4. Colour of colony from below	1, 3, 4, 5, 6, 7, 8, 10, 11, 12	4. Colour of spore masses	29, 30
5. Colour of spore masses	11, 12		
		B. Macroconidia from sporodochia	
B. Macroconidia from sporodochia			
		1. Size	25, 26, 27, 28, 29, 30
1. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12	2. Shape	24, 30
2. Shape	2, 6, 8, 9, 10, 11, 12	3. Shape of basal and apical cells	30
3. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12		
		C. Microconidia from aerial mycelium	
C. Microconidia from aerial mycelium		1. Present or absent	24, 25, 26, 27, 28, 29, 30
		2. In chains or false heads	24, 27, 28, 29, 30
1. Present or absent	2, 4, 7, 8, 9, 10, 11, 12	3. Shape	24, 25, 26, 27, 29, 30
2. In chains or false heads	4, 11, 12		
3. Shape	2, 4, 7, 8, 10, 11, 12	**D. Conidiophores**	
		1. Type	25, 30
D. Conidiophores			
1. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	**E. Chlamydospores**	
		1. Present or absent	29, 30
E. Chlamydospores			
1. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		
2. Arrangement	1, 4, 9, 11, 12		

Table 3 .6 Synoptic key of section and species to isolate no. 28 *(F. semitectum)*

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
F. Cultural characteristics		**G. Cultural characteristics**	
6. Rate of growth	2, 4, 5, 6, 7, 8, 10, 11, 12	6. Rate of growth	6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23
7. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
8. Colour of aerial mycelium	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	7. Colour of aerial mycelium	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20
9. Colour of colony from below	2, 4, 5, 6, 7, 8, 9	8. Colour of colony from below	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20
10. Colour of spore masses	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	9. Colour of spore masses	7, 8, 12, 14, 15, 20
G. Macroconidia from sporodochia		**H. Macroconidia from sporodochia**	
4. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12	4. Shape	11, 12, 13
5. Shape	2, 6, 8, 9, 10, 11, 12	5. Shape of basal and apical cells	12, 13
6. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12		
		I. Microconidia from aerial mycelium	
H. Microconidia from aerial mycelium		4. Present or absent	10, 11, 12, 13, 14, 16, 17, 18, 19, 20, 21, 22, 23
		5. Shape	
4. Present or absent	1, 3, 5, 6, 7, 8, 9		24, 25, 26, 27, 29, 30
5. In chains or false heads		**J. Conidiophores**	
6. Shape		2. Type	
			24
I. Conidiophores		**K. Chlamydospores**	
2. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	6. Present or absent	
			24
J. Chlamydospores			
3. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		
4. Arrangement	4, 6, 7, 8		

Table 3.7 Synoptic key of section and species to isolates 25 and 26 (*F. moniliforme*)

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
A. Cultural characteristics		**A. Cultural characteristics**	
1. Rate of growth	2, 4, 5, 6, 7, 8, 10, 11, 12	1. Rate of growth	25, 26, 27, 28, 29, 30
2. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	2. Colour of aerial mycelium	24, 25, 26, 27, 28, 29, 30
3. Colour of aerial mycelium	10, 11	3. Colour of colony from below	25, 26, 27, 28, 29, 30
4. Colour of colony from below	10, 11, 12	4. Colour of spore masses	
5. Colour of spore masses	11, 12		
		B. Macroconidia from sporodochia	
B. Macroconidia from sporodochia			
		1. Shape	25, 26, 27, 28, 30
1. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12	2. Shape of basal and apical cells	
2. Shape	2, 6, 8, 9, 10, 11, 12		
3. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12	**C. Microconidia from aerial mycelium**	
		1. Present or absent	24, 25, 26, 27, 28, 29, 309
C. Microconidia from aerial mycelium		2. In chains and false heads	25, 26
		3. Shape	24, 25, 26, 27, 29, 30
1. Present or absent			
2. In chains or false heads	2, 4, 7, 8, 9, 10, 11, 12	**D. Conidiophores**	
3. Shape	2, 10	1. Type	25, 30
	2, 4, 7, 8, 10, 11, 12		
D. Conidiophores		**E. Chlamydospores**	
1. Type		1. Present or absent	25, 26, 27, 28
	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
E. Chlamydospores			
1. Present or absent			
2. Arrangement	1, 2, 3, 5, 10		

Table 3.8 Synoptic key of section and species to isolate no. 29 *(F. lateritium)*

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* section	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
A. Cultural characteristics		**A. Cultural characteristics**	
1. Rate of growth	2, 3, 8, 9	1. Rate of growth	24
2. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	2. Colour of aerial mycelium	24, 25, 26, 27, 28, 29, 30
3. Colour of aerial mycelium	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	3. Colour of colony from below	24, 25, 26, 27, 28, 29, 30
4. Colour of colony from below	2, 4, 5, 6, 7, 8, 9	4. Colour of spore masses	24, 25, 26, 27, 28, 29
5. Colour of spore masses	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11		
		B. Macroconidia from sporodochia	
B. Macroconidia from sporodochia			
1. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12	1. Shape	24, 30
2. Shape	2, 6, 8, 9, 10, 11, 12	2. Shape of basal and apical cells	24
3. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12		
		C. Microconidia from aerial mycelium	
C. Microconidia from aerial mycelium		1. Present or absent	24, 25, 26, 27, 28, 29, 309
1. Present or absent	2, 4, 7, 8, 9, 10, 11, 12	2. In chains and false heads	24, 27, 28, 29, 30
2. In chains or false heads		3. Shape	24, 25, 26, 27, 29, 30
3. Shape			
		D. Conidiophores	
D. Conidiophores		1. Type	24
1. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
		E. Chlamydospores	
E. Chlamydospores		1. Present or absent	24
1. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		
2. Arrangement	1, 4, 9, 11, 12		

Table 3.9 Synoptic key of section and species to isolate no. 27 *(F. chlamydosporum)*

SYNOPTIC KEY TO SECTION		SYNOPTIC KEY TO SPECIES	
Characteristics used to identify *Fusarium* sections	**No. indicating *Fusarium* section**	**Characteristics used to identify *Fusarium* species**	**No. indicating *Fusarium* species in the sections Eupionnotes, Spicarioides, and Arachnites**
K. Cultural characteristics		**F. Cultural characteristics**	
11. Rate of growth	2, 4, 5, 6, 7, 8, 10, 11, 12	10. Rate of growth	6, 7, 8, 9 ,10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 23
12. Aerial mycelium present or absent	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12		
13. Colour of aerial mycelium	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	11. Colour of aerial mycelium	8, 9, 11, 12, 13, 14, 15, 20, 22, 23
14. Colour of colony from below	2, 4, 5, 6, 7, 8, 9	12. Colour of colony from below	8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
15. Colour of spore masses	1, 2, 3, 4, 5, 6, 7, 8, 8, 10, 11	13. Colour of spore masses	
L. Macroconidia from sporodochia		**G. Macroconidia from sporodochia**	
7. Size	1, 4, 5, 6, 7, 8, 9, 10, 11, 12		
8. Shape	5, 6, 9,	6. Shape	6, 7, 8, 9, 18, 20, 21, 22
9. Shape of basal and apical cells	2, 4, 5, 6, 7, 8, 9, 10, 11, 12	7. Shape of basal and apical cells	6, 7, 9, 10, 11, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
M. Microconidia from aerial mycelium		**H. Microconidia from aerial mycelium**	
		6. Present or absent	6, 7, 8, 9
7. Present or absent	1, 3, 5, 6, 7, 8, 9	7. Shape	9
8. In chains or false heads			
9. Shape		**I. Conidiophores**	
		3. Type	8, 9, 12, 13, 15
N. Conidiophores			
3. Type	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12	**J. Chlamydospores**	
		7. Present or absent	6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23
O. Chlamydospores			
5. Present or absent	1, 4, 6, 7, 8, 9, 11, 12		
6. Arrangement	4, 6, 7, 8		

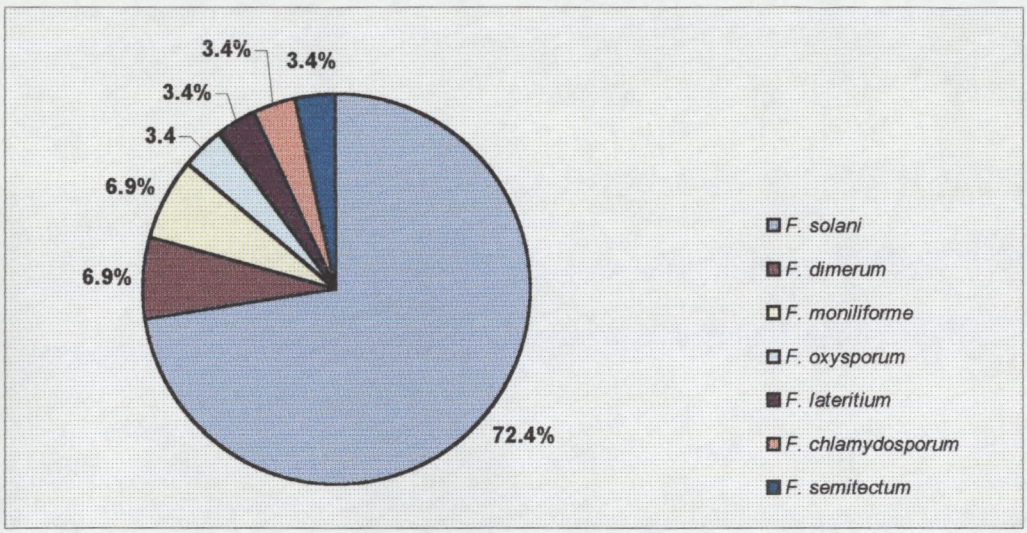


Fig. 3.13 Pie chart showing distribution of different *Fusarium* species isolated from 29 mycotic keratitis patients.

Table 3.10 The 29 clinical isolates identified to *Fusarium* section and species level using the synoptic key for *Fusarium* taxonomy by Nelson *et al.* (1983).

ISOLATE NUMBER	*Fusarium* SECTION	*Fusarium* SPECIES
1	Eupionnotes	*F. dimerum*
2	Elegans	*F. oxysporum*
3	Martiella and Ventricosum	*F. solani*
4	Martiella and Ventricosum	*F. solani*
5	Martiella and Ventricosum	*F. solani*
6	Martiella and Ventricosum	*F. solani*
7	Martiella and Ventricosum	*F. solani*
8	Martiella and Ventricosum	*F. solani*
9	Martiella and Ventricosum	*F. solani*
10	Martiella and Ventricosum	*F. solani*
11	Martiella and Ventricosum	*F. solani*
12	Arthrosporiella	*F. semitectum*
13	Liseola	*F. moniliforme*
14	Martiella and Ventricosum	*F. solani*
15	Martiella and Ventricosum	*F. solani*
16	Martiella and Ventricosum	*F. solani*
17	Martiella and Ventricosum	*F. solani*
18	Martiella and Ventricosum	*F. solani*
19	Martiella and Ventricosum	*F. solani*
20	Martiella and Ventricosum	*F. solani*
21	Martiella and Ventricosum	*F. solani*
22	Martiella and Ventricosum	*F. solani*
23	Liseola	*F. moniliforme*
24	Martiella and Ventricosum	*F. solani*
25	Eupionnotes	*F. dimerum*
26	Lateritium	*F. lateritium*
27	Martiella and Ventricosum	*F. solani*
28	Martiella and Ventricosum	*F. solani*
29	Sporotrichiella	*F. chlamydosporum*

3.3 MYCOTOXIN ANALYSIS

3.3.1 Method optimization

Prior to establishing whether the 29 clinical *Fusarium* isolates in this study were capable of producing mycotoxins, the toxin extraction methods were evaluated. This was accomplished by assessing the efficiency of the extraction methods used (the liquid-liquid extraction method for fusaric acid and strong anion exchange chromatography (SAX) for moniliformin and fumonisin B_1). The sensitivity of the TLC and HPLC systems for mycotoxin detection was determined by establishing toxin detection limits (using commercial standards).

At n = 3, extraction efficiency was 84.5 % for fusaric acid (SD= 4.75), 84.8% (SD= 4.45) for moniliformin and 72.4% (SD= 2.35) for fumonisin B_1 (Table 3.11). Detection limits for fusaric acid, moniliformin and fumonisin B_1 were established at 50 ng, 100 ng and 10 ng respectively using TLC and 10 ng, 5 ng and 0.5 ng respectively using HPLC (Table 3.12).

Table .3.11 Percentage recovery of fusaric acid, moniliformin and fumonisin B_1 from spiked maize patty cultures.

Toxins	**Recovery ± SD (%) (n = 3)**
Fusaric acid (20 µg/ml)	84.5 ± 20
Moniliformin (20 µg/ml)	84.8 ± 17
Fumonisin B_1 (10 µg/ml)	72.4 ± 13

Table 3.12 Detection limits for fusaric acid, moniliformin and fumonisin B_1 using the TLC and HPLC systems

Mycotoxins	**Thin layer chromatography**	**High performance liquid chromatography**
Fusaric acid	50 ng	25 ng at 254 nm
Moniliformin	100 ng	1.0 ng at 229 nm
Fumonisin B_1	500 ng	100 ng

3.3.2 Detection of mycotoxins produced by *Fusarium* isolates

3.3.2.1 Thin- layer chromatography

Fusaric acid standards viewed under shortwave UV light appeared as bright purple spots with a R_f value of 0.78 in lanes 1 and 2 (Fig. 3.14 to 3.16). Bright purple spots with a similar R_f value were observed for the purified extracts of isolates 15, 21, 11 and 26 in

lanes 4, 7, 8 and 11, respectively (Fig. 3.14), isolates 16, 6, 19 and 12 in lanes 4, 6, 7 and 8, respectively (Fig. 3.15) and isolates 28, 4, 14 and 3 in lanes 6, 8, 9 and 10, respectively (Fig. 3.16). The similar R_f values of the fusaric acid standards and the purified extracts indicate fusaric acid production by 12 *Fusarium* isolates.

Moniliformin standards produced bright purple spots under shortwave UV light in lanes 1, 8 and 9 (Fig. 3.17) with a R_f value of 0.86. Bright purple spots with a similar Rf value were observed for the purified extracts of *Fusarium* isolates 14 and 23 in lanes 3 and 6, respectively. The similar R_f values of the moniliformin standards and the purified extracts indicate that moniliformin production was detected for isolates 14 and 23 only.

Fumonisin B_1 standard produced dark violet spots in lanes 1 and 6 with a R_f value of 0.65 (Fig. 3.18). Dark violet spots with a similar R_f value were observed for the purified extracts of isolates 14 and 23 in lanes 4 and 5 respectively. The similar R_f value for the fumonisin B_1 standards and the purified extracts indicate that fumonisin B_1 production was detected for isolates 14 and 23 only.

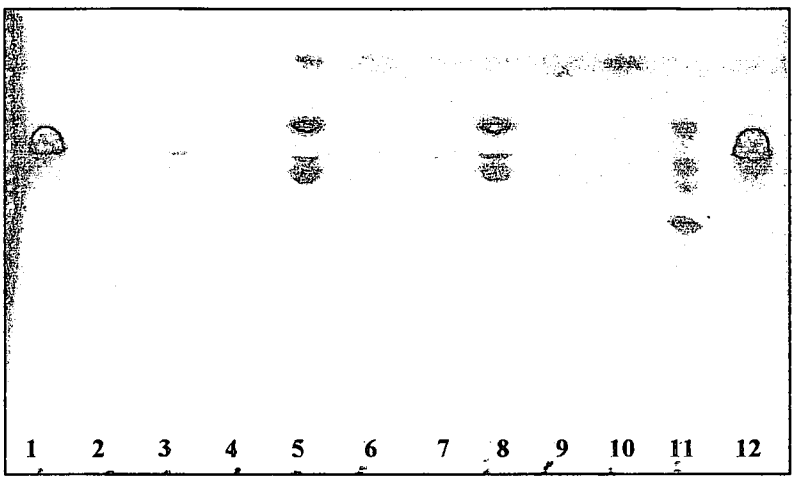


Fig. 3.14 TLC plate showing spots detected under shortwave UV light (254 nm). Lanes 1 and 12 were spotted with fusaric acid standard, lanes 2 to 11 were spotted with purified extracts of isolates 1, 11, 15, 21, 24, 2, 7, 29, 5 and 26, respectively.

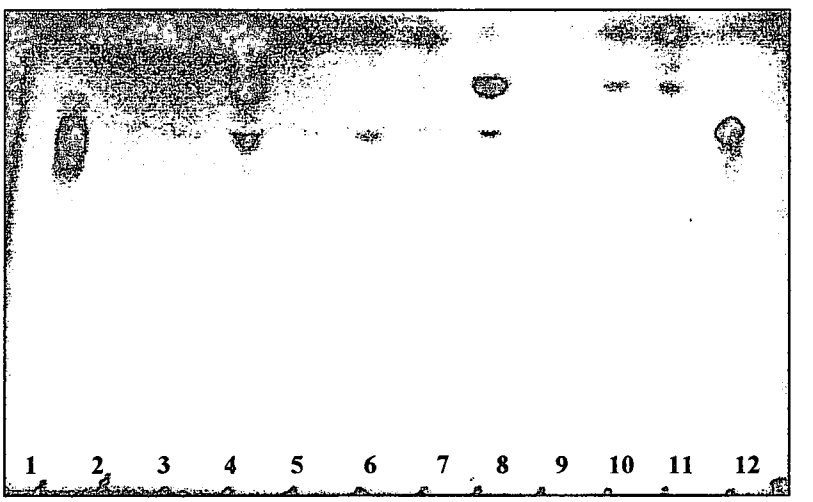


Fig. 3.15 TLC plate showing spots detected under shortwave UV light. Lanes 1 and 12 were spotted with fusaric acid and lanes 2 to 11 were spotted with purified extracts of isolates 22, 23, 16, 25, 6, 19, 12, 17 and 18, respectively.

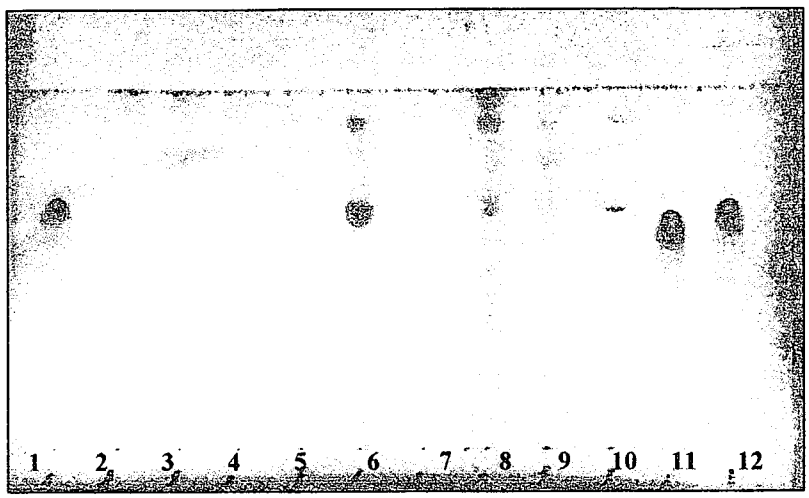


Fig. 3.16 TLC plate showing spots detected under shortwave UV light. Lanes 1 and 12 were spotted with fusaric acid standard, and lanes 2 to 11 spotted with purified extracts of isolates 10, 13, 9, 27, 28, 8, 4, 14, and 3, respectively.

Moniliformin

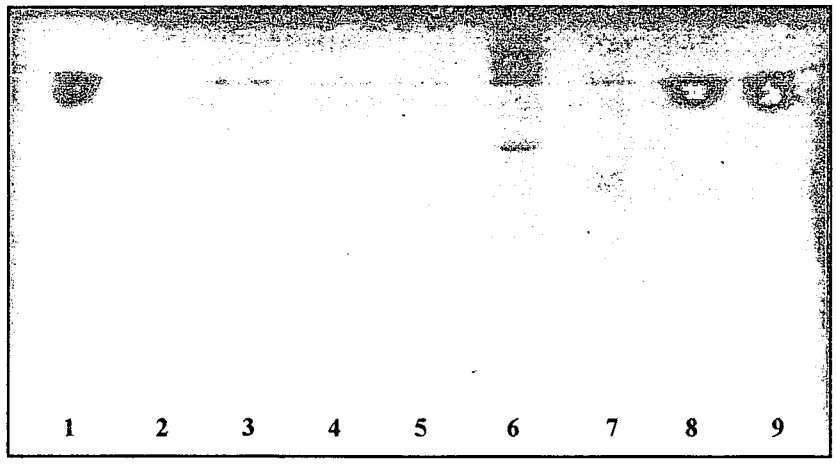


Fig. 3.17 TLC plate showing spots detected under shortwave UV light. Lanes 1, 8 and 9 were spotted with moniliformin standard while lanes 2 to 6 were spotted with purified extracts of isolates 23, 29, 12, 13 and 2, respectively.

Fumonisin B_1

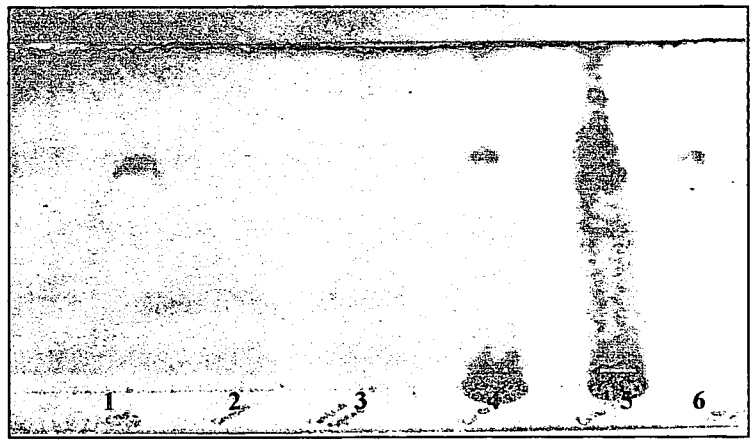


Fig. 3.18 TLC plate with lanes 1 and 6 spotted with fumonisin B_1 standard. Lane 2 was spotted with a control (uninoculated maize patty), lane 3 was spotted with a spiked control while lanes 4 and 5 were spotted with purified extracts of isolates 23 and 14, respectively.

3.3.2.2 High- performance liquid chromatography

Detection and quantification of fusaric acid, moniliformin and fumonisin B_1 was accomplished by HPLC. Verification of the purified toxins was achieved by comparing the retention times of the purified compounds to commercial standards. The peak obtained for 15 µg/ml fusaric acid standard eluted at 3.15 min (Fig. 3. 19a). Fig. 3.19b shows a peak obtained from the purified extracts of isolates 28, which when compared to the standard, eluted at similar retention times. Fig. 3.19d shows a chromatogram obtained from an uninoculated maize patty that represented the control. Peaks with similar retention times as the fusaric acid standard were detected for 20 *Fusarium* isolates.

A peak at a retention time of 2.99 min was eluted after injection of 200 µg/ml moniliformin standard (Fig. 3. 20a). Similarly, peaks obtained for the purified extracts of

isolates 14 and 29 (Fig. 3.20b and 3.20c) eluted at retention times of 3.01 and 3.05 min respectively. Of the 29 *Fusarium* isolates analysed, only the purified extracts of 4 isolates produced peaks at similar retention times. Fig. 3.20d is the control (uninoculated maize patty).

Injection of 600 μg/ml fumonisin B_1 standard into the HPLC system revealed a peak at a retention time 3.55 min. Similarly peaks obtained for the purified extracts of isolate nos. 14 and 23 eluted at retention times of 3.52 and 3.65 min respectively (Fig. 3.21a and 3.21b. These were the only 2 isolates that produced peaks at this retention time.

Toxin levels were quantified using sample and fusaric acid peak areas in a formula method (Thiel *et al.*, 1993). The toxin concentrations (μg/g) for isolates that produced fusaric acid, moniliformin and fumonisin B_1 are shown in Table 3.13. Seven of the twenty-nine *Fusarium* isolates did not produce any of the toxins. These species included the two *F. dimerum* (isolates 1 and 25) and five *F. solani* (isolates 7, 10, 22, 24 and 27). The remaining sixteen *F. solani* isolates were capable of fusaric acid production only. Fusaric acid concentrations ranged from 0.32 μg/g to 7.78 μg/g. *F. oxysporum* (isolate 2) was capable of both fusaric acid and moniliformin production. *F. semitectum* was capable of fusaric acid production only. *F. moniliforme* (isolate 14) was capable of producing all three toxins while another *F. moniliforme* species (isolate 23) was capable of producing moniliformin and fumonisin B_1 only. *F. lateritium* was capable of fusaric acid production only while *F. chlamydosporum* was capable of moniliformin production only.

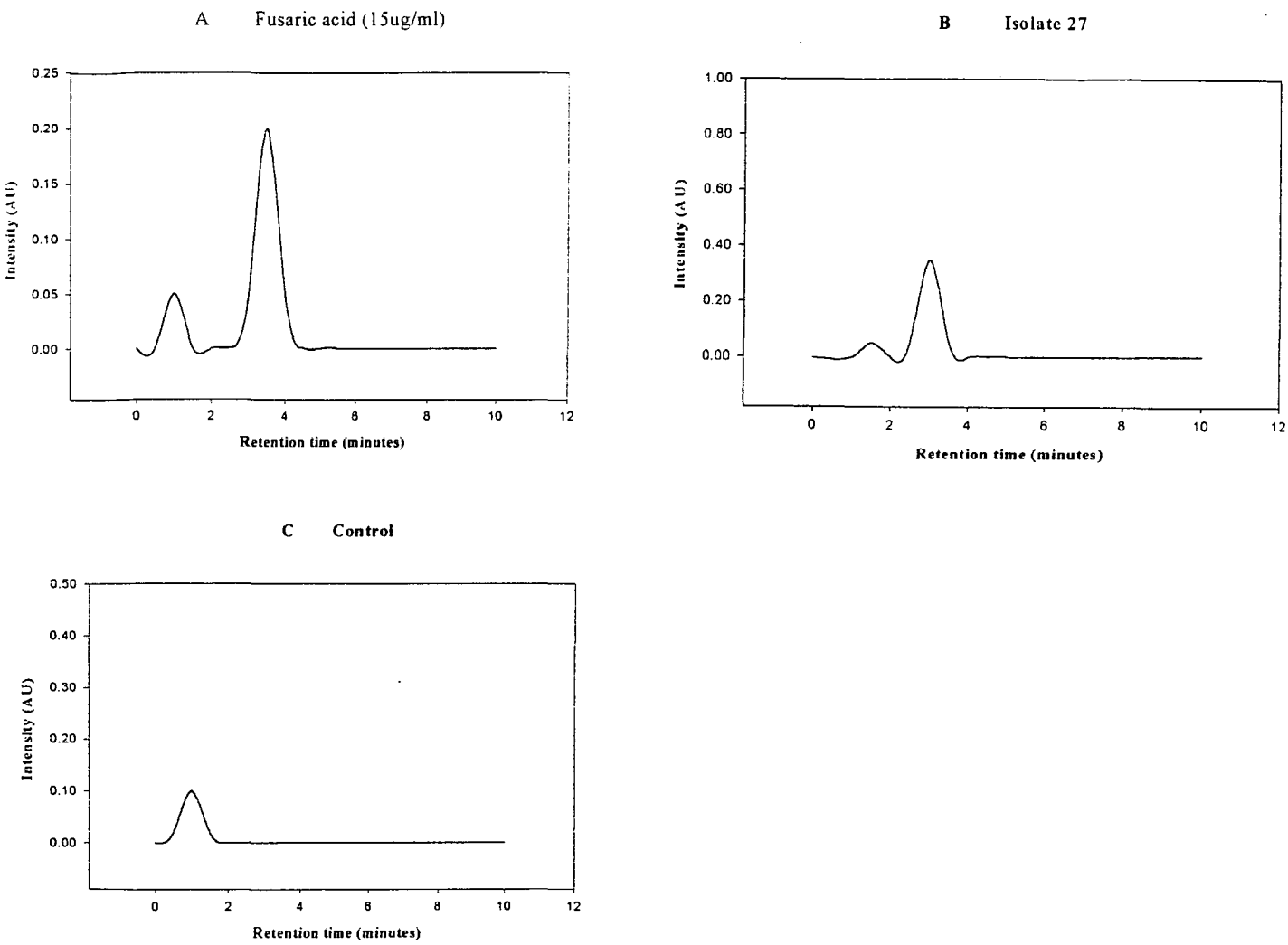


Fig. 3.19 HPLC chromatograms obtained for the analysis of fusaric acid extracted from maize patty cultures. In Fig. 3.19a, a chromatogram for 15 µg/ml fusaric acid standard is shown. In Fig. 3.19b, a chromatogram for isolate 27 is shown and in Fig. 3.19c a chromatogram of the control (uninoculated maize patty) is given.

[Analysis conditions: Mobile phase: 40 % methanol, 60% aqueous 0.62 mM Na_2EDTA, 2% H_3PO_4, Merck RP C- 18 column, 20 µl sample injection, UV- 254nm detection, flow rate = 1ml/min, R_t = 3.1 – 3.5 minutes]

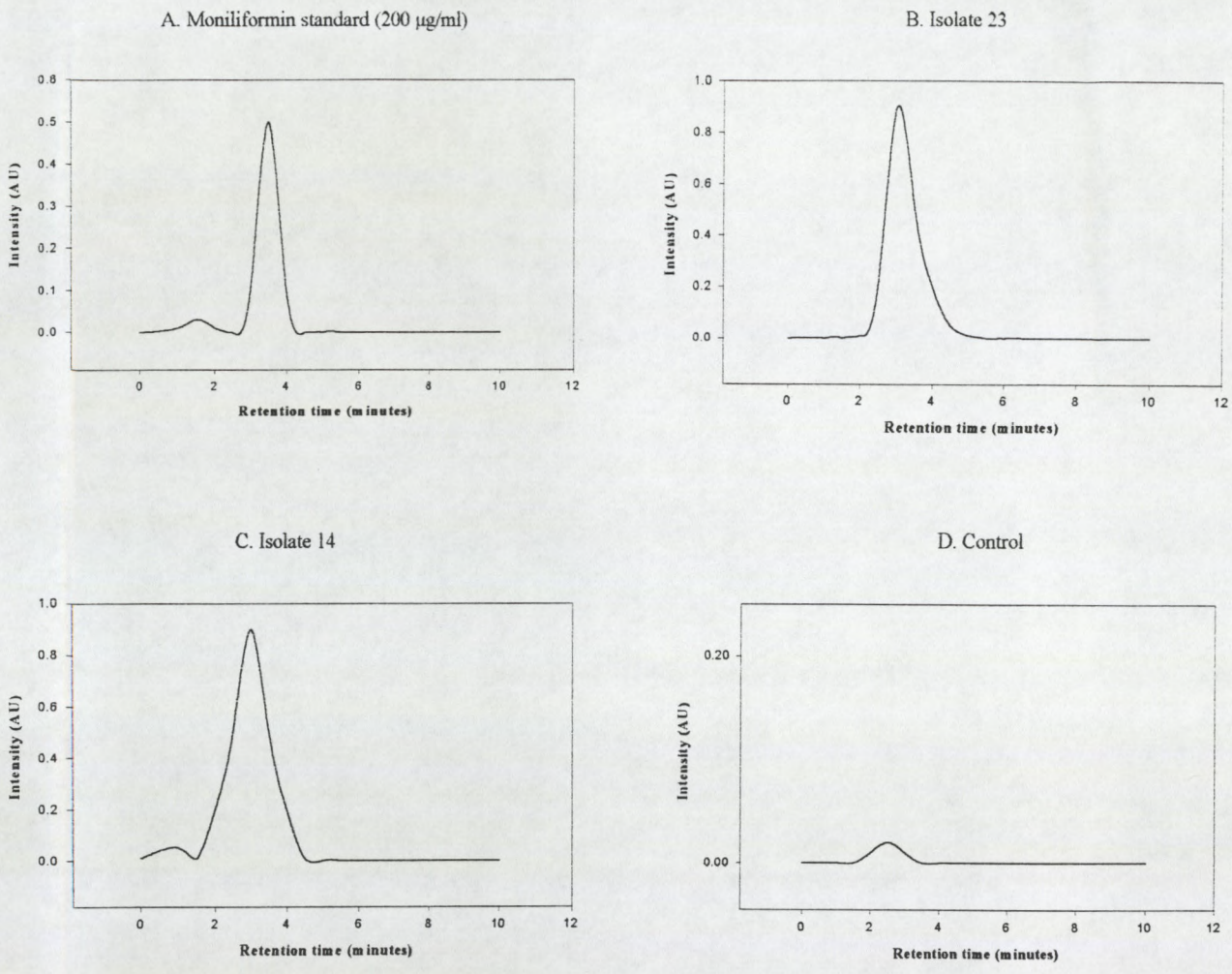


Fig. 3.20 HPLC chromatograms obtained for the analysis of moniliformin. Fig. 3.20a shows a chromatogram for moniliformin standard injected at a concentration of 200 µg/ml. Fig. 3.20b and Fig. 3.20c show chromatograms obtained for the purified extracts of isolates 23 and 14, respectively. Fig. 3.20d shows the chromatogram obtained for the control.

[Analysis conditions: Mobile phase- 10mM phosphate buffer/ 5mM tetra-butyl-ammonium bromide (pH7.0): methanol (92:8 v/v), Merck RP C-18 column, 20 µl sample injection, UV-215 nm detection, flow rate: 1 ml/min., R_t = 2.75- 2.95 minutes]

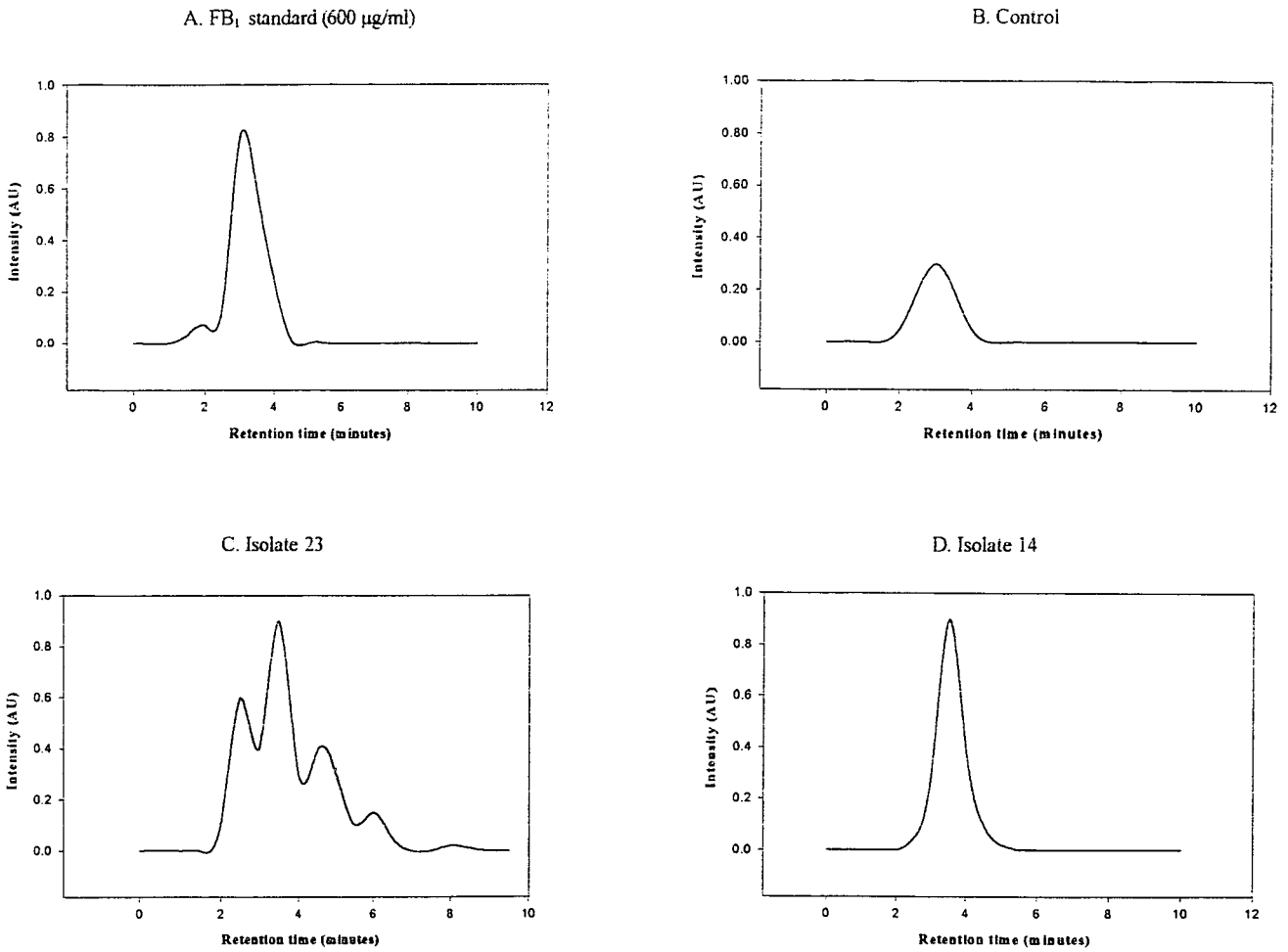


Fig.3.21 HPLC chromatograms obtained for the analysis of fumonisin B_1. Fig. 3.21a shows the chromatogram obtained for toxin standard injected at 600 µg/ml. Fig. 3.21b shows a chromatogram obtained for the control (no toxin), Fig. 3.21c- d are chromatograms obtained for the purified extracts of isolates 23 and 14, respectively.

[Analysis conditions: Mobile phase- methanol: 0.1 M NaH_2P0_4 (80: 20), Merck RP C18 column, OPA- derivatizing reagent (450 µl + 50 µl sample), 50 µl sample injection, fluorescence detection, flow rate 1 ml/min, R_t = 3.5- 3.8 minutes].

Table 3.13 Concentrations (μg/g) of fusaric acid, moniliformin and fumonisin B_1 detected by HPLC. Values given are the mean of two replicates. Standard deviation is given in brackets (SD ≤ 2.62)

	Mycotoxin concentrations (μg/g) detected in maize samples by HPLC (n= 2)			
Isolate No.	**Identified *Fusarium* species**	**Fusaric acid**	**Moniliformin**	**Fumonisin B_1**
1	*F. dimerum*	-	-	-
2	*F. oxysporum*	0.39(±0.35)	2.50 (±2.55)	-
3	*F. solani*	0.86 (±0.66)	-	-
4	*F. solani*	1.82 (±1.41)	-	-
5	*F. solani*	0.52 (±0.23)	-	-
6	*F. solani*	2.98 (±0.60)	-	-
7	*F. solani*	-	-	-
8	*F. solani*	0.57 (±0.60)	-	-
9	*F. solani*	0.46 (±0.23)	-	-
10	*F. solani*	-	-	-
11	*F. solani*	3.81 (±0.42)	-	-
12	*F. semitectum*	4.26 (±0.49)	-	-
13	*F. solani*	0.41 (±0.37)	-	-
14	*F. moniliforme*	0.72 (±0.28)	4.7 (±2.61)	4.90 (±1.56)
15	*F. solani*	0.69 (±0.71)	-	-
16	*F. solani*	3.31 (±0.99)	-	-
17	*F. solani*	0.32 (±0.23)	-	-
18	*F. solani*	0.48 (±0.47)	-	-
19	*F. solani*	0.59 (±1.62)	-	-
20	*F. solani*	0.49 (±0.58)	-	-
21	*F. solani*	6.9 (±0.06)	-	-
22	*F. solani*	-	-	-
23	*F. moniliforme*	-	2.57 (±0.78)	3.07 (±2.62)
24	*F. solani*	-	-	-
25	*F. dimerum*	-	-	-
26	*F. lateritium*	2.43 (±2.26)	-	-
27	*F. solani*	-	-	-
28	*F. solani*	7.78 (±2.61)	-	-
29	*F. chlamydosporum*	-	1.04 (±1.13)	-

3.3.3 Relationship between mycotoxin production and clinical symptoms of fusarial keratitis

The relationship between the clinical symptoms and the type of mycotoxins present is shown in Table 3.14. *F. dimerum* (one isolate) did not produce any of the toxins. The ulcer size for which this species was responsible was 2 × 2mm with the depth of infection being stromal. There was also no inflammatory response detected in the anterior chamber. Only 5 of the 6 *F. solani* isolates identified were capable of fusaric acid production, at concentrations ranging from 0.52 to 3.81 μg/g. The ulcer sizes for which these species were responsible ranged from 1×1 mm to a large central ulcer that covered the cornea surface. A perforation of the cornea occurred in one patient, with the *F. solani* isolate capable of fusaric acid production. *F. solani* was isolated from only those eyes in which previous trauma or injury to the cornea was inflicted. From all the cases from which *F. solani* was isolated, loss of visual acuity was the most severe in one case. The *F. solani* isolate from this case produced the highest fusaric acid concentration (3.81 μg/g). The anterior chamber responded by the formation of a hypopyon in two cases. *F. solani* isolated from these cases were capable of fusaric acid production. In the most severe ulcer in this study, *F. moniliforme* was isolated and was capable of producing all three toxins. Overall, visual acuity was the most compromised in this case, with the patient only able to perceive light. A large central ulcer was observed in this case, with a complete perforation of the cornea. The anterior chamber response included the formation of a hypopyon.

Table 3.14 Relationship between the identified *Fusarium* species, the toxins produced and the clinical study of eight patients with fusarial keratitis

Isolate	*Fusarium* species	Fusaric acid (μg/g)	Moniliformin (μg/g)	Fumonisin B_1 (μg/g)	Visual Acuity	Size of ulcer	Depth of ulcer	Anterior chamber reaction
1	*F. dimerum*	-	-	-	CF	2×2	Stromal	None
3	*F. solani*	0.86	-	-	6/36	4×4	Stromal	Cells, flare
4	*F. solani*	1.82	-	-	CF	large central ulcer	Perforation	Cells, flare, hypopyon
5	*F. solani*	0.52	-	-	CF	3×3	Stromal	Cells, flare, hypopyon
6	*F. solani*	2.98	-	-	6/12	1×1	Stromal	Flare
10	*F. solani*	-	-	-	CF	large central ulcer	Stromal	Cells, flare
11	*F. solani*	3.81	-	-	HM	3×3	Stromal	Hypopyon
14	*F. moniliforme*	0.72	4.7	4.90	PL	large central ulcer	Perforation	Cells, flare, hypopyon

3.4 CYTOTOXICITY OF MYCOTOXINS

The MTT- cleavage assay and the crystal violet test were performed to evaluate the cytotoxic effects of fusaric acid, moniliformin and fumonisin B_1 on the viability of A549 (human lung adenocarcinoma) cells and VK (monkey kidney) cells.

At a toxin concentration of 0.05 mg/ml, fusaric acid and moniliformin was shown to exhibit a greater degree of toxicity to A549 (lung adenocarcinoma) cells (2.7% and 2.9% cell viability) than fumonisin B_1 (24.3% cell viability) in the MTT assay (Fig. 3.22). In the crystal violet test, a 5.1% and 1.2% cell viability was observed for A549 cells after exposure to fusaric acid and moniliformin, respectively, while 30.5% cell viability was observed after exposure to fumonisin B_1 (Fig. 3.23). At a toxin concentration of 0.004 mg/ml, a percentage cell viability of greater than 91% was obtained after exposure of the cells to all three toxins in both the MTT and the crystal violet tests.

At a toxin concentration of 0.5 mg/ml in the MTT assay, fumonisin B_1 was shown to exhibit a greater degree of toxicity to VK (monkey kidney) cells (11.9% cell viability) than fusaric acid and moniliformin (30% and 14.7% cell viability) (Fig. 3.24). In the crystal violet test, VK cells revealed a percentage cell viability of 11.5% after fumonisin B_1 exposure while 33.6% and 21.1% cell viability was observed for fusaric acid and moniliformin, respectively (Fig. 3.25). At 0.004 mg/ml, a percentage cell viability of greater than 94% was obtained after exposure to all three toxins in the MTT and the crystal violet test (Refer to Appendix 6A and 6B for percentage cell viability values).

The percentage cell viabilities of A549 and VK cells after 24 hours exposure to purified fusaric acid extracts from isolates 28, 22, 12, 17 and 4 (at concentrations of 7.78, 6.9, 4.26, 3.31 and 1.82 μg/g, respectively) are shown in Table 3.15. The greatest inhibition of cell viability was obtained for isolate 28 (41.3% cell viability) in the MTT assay. The percentage cell viability of the cells exposed to purified moniliformin extracts of isolates 14, 23, 2 and 29 (at concentrations ranging from 1.04 and 4.7 μg/g) and fumonisin B_1 extracts of isolate 14 and 23 (3.07 and 4.9 μg/g) is shown in Table 3.16. The greatest inhibition of cell viability was obtained for isolate 14 (70% cell viability) after moniliformin exposure in the MTT assay. For fumonisin B_1 percentage cell viabilities were greater than 90%. The % cell viability values for the toxin extracts are the mean of four replicates (SD ≤ 0.05).

A549 cells that were not exposed to the toxins showed a confluent monolayer of epithelial cells with 100% cell viability (Fig. 3.26a). Large gaps could be observed in the monolayer when the cells were exposed to toxins. This was particularly evident after exposure to fusaric acid and moniliformin. Cellular proliferation was almost completely inhibited while fumonisin B_1 had the least effect (Fig. 3.26b-d).

VK cells not challenged with toxins also produced a confluent monolayer of epithelial cells and fibroblasts that resembled" valleys" and "streams" (Fig. 3.27a). Large gaps were also observed in the cell monolayers after toxin exposure. The greatest inhibition of cellular proliferation was observed after fumonisin B_1 exposure while inhibition of cell growth by fusaric acid and moniliformin was not as profound (Fig. 3.27b-d).

The student's t-test returned a probability of 0.65 and 0.75 for A549 and VK cells respectively, (greater than 0.05) indicating that there was no significant difference in the mean % cell viabilities between the MTT- cleavage assay and the crystal violet test.

3.4.1 Effect of mycotoxins on cell viability

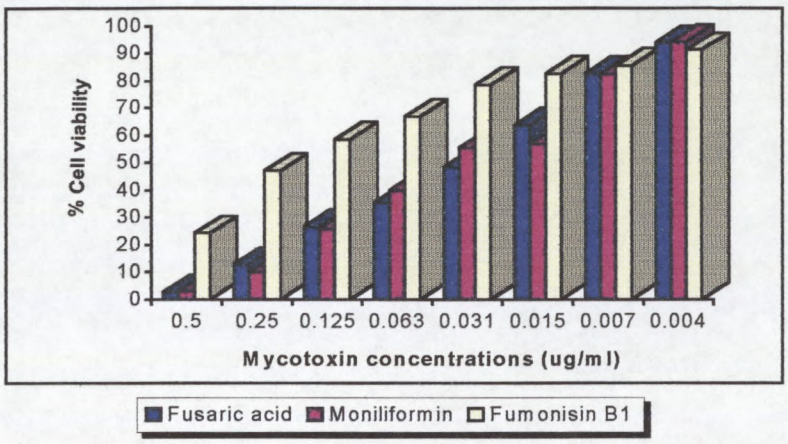


Fig. 3. 22 Bar graph depicting the effect of fusaric acid, moniliformin and fumonisin B_1 (at concentrations ranging from 0.004 to 0.5 mg/ml) on the viability of A549 (lung adenocarcinoma) cells after 24 hours exposure in the MTT assay. Values represent the mean of 5 replicates (SD ≤ 0.05)

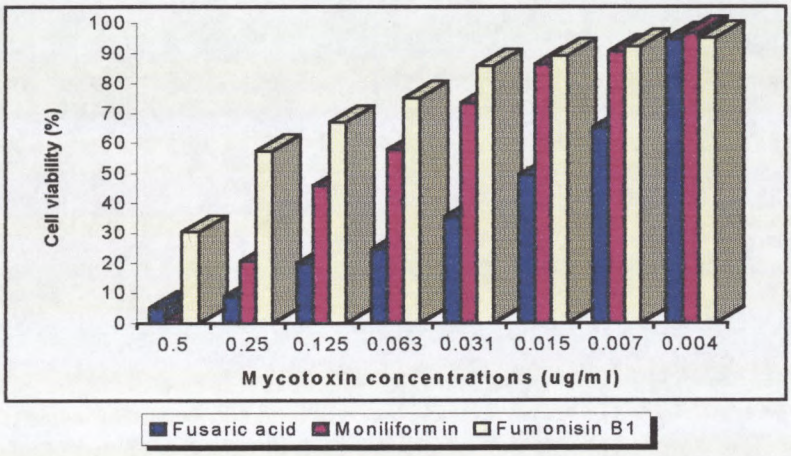


Fig. 3.23 Bar graph showing viability of A549 (lung carcinoma) cells after 24 hours exposure to fusaric acid, moniliformin and fumonisin B_1 (at concentrations ranged from 0.004 to 0.5 mg/ml) in the crystal violet test. Values represent the mean of 5 replicates (SD ≤ 0.05)

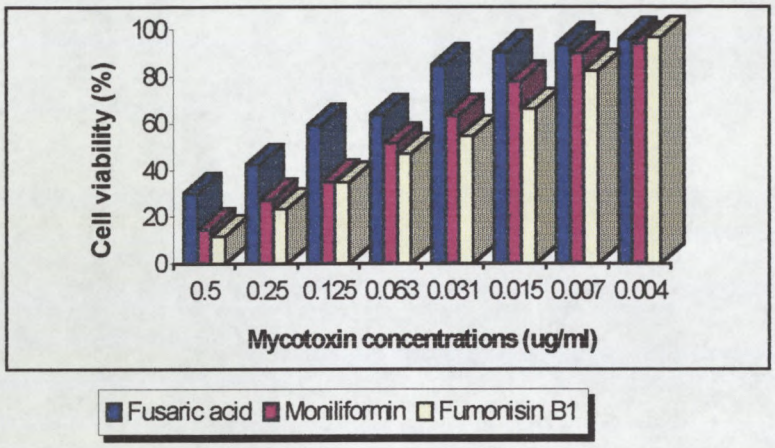


Fig.3. 24 Bar graph showing viability of VK (monkey kidney cells) after 24 hours exposure to fusaric acid, moniliformin and fumonisin B_1 (at concentrations ranging from 0.004 to 0.5 mg/ml) in the MTT assay. Values represent the mean of 5 replicates (SD ≤ 0.05)

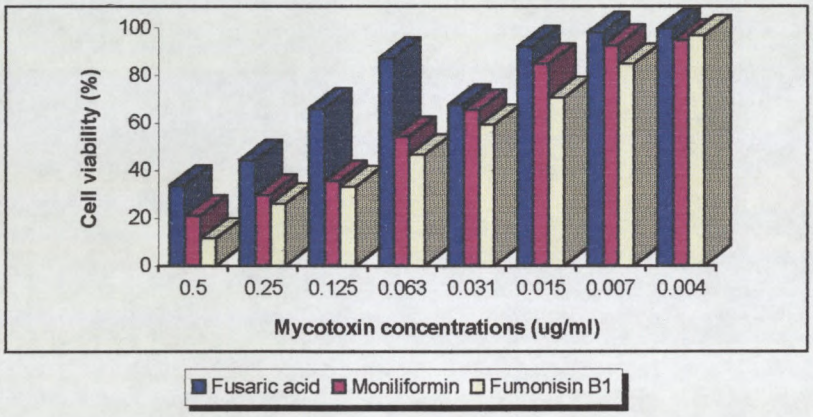


Fig. 3.25 Bar graph showing the effect of fusaric acid, moniliformin and fumonisin B_1 (at concentrations ranging from 0.004 to 0.5 mg/ml) on the viability of VK (monkey kidney) cells after 24 hours exposure in the crystal violet assay. Values represent the mean of 5 replicates (SD ≤ 0.05).

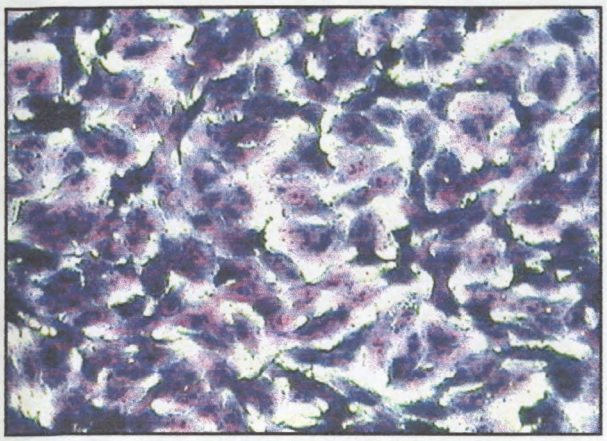
Table 3.15 Cell viabilities (%) of A549 cells and VK cells exposed to purified fusaric acid extracts. Each value is the mean of 4 replicates (SD ≤ 0.05).

Fusarium isolates capable of fusaric acid production	Concentrations of purified toxin extracts (µg/g)	Cell viability (%) of A549 (lung adenocarcinoma) cells		Cell viability (%) of VK (monkey kidney) cells	
		MTT cleavage assay	Crystal violet test	MTT cleavage assay	Crystal violet test
28	7.78	41.3 (±0.035)	57.1 (±0.049)	87.8 (±0.048)	89.0 (±0.029)
22	6.90	58.7 (±0.038)	60.9 (±0.05)	92.9 (±0.026)	96.4 (±0.029)
12	4.26	86 (±0.021)	93 (±0.013)	94.9 (±0.045)	95.9 (±0.032)
17	3.31	96.7 (±0.006)	93.1 (±0.013)	96.5 (±0.028)	98.1 (±0.024)
4	1.82	98.7 (±0.006)	95.7 (±0.035)	98.4 (±0.017)	99.0 (±0.015)

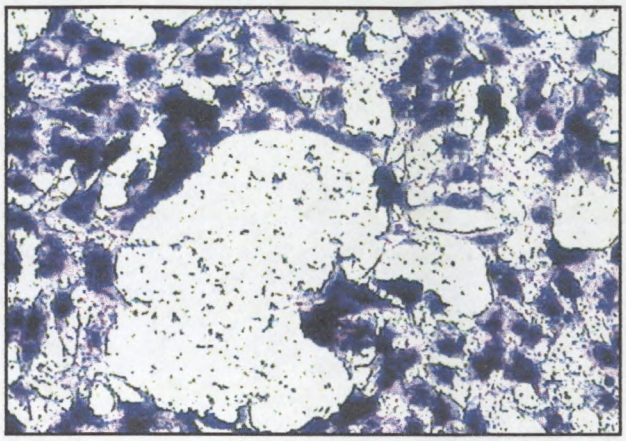
Table 3.16 Cell viability (%) of A549 and VK cells exposed to purified extracts of moniliformin and fumonisin B_1. Each value is the mean of 4 replicates (SD ≤ 0.05).

Mycotoxins	*Fusarium* isolates capable of moniliformin and fumonisin B_1 production	Concentrations of purified toxins (μg/g)	Cell viability (%) of A549 (lung adenocarcinoma) cells		Cell viability (%) of VK (monkey kidney) cells	
			MTT Assay	Crystal Violet Test	MTT Assay	Crystal Violet Test
Moniliformin	14	4.7	70.0 (±0.01)	94.08 (±0.016)	92.3 (±0.018)	93.9 (±0.01)
	23	2.57	82.9 (±0.003)	98.5 (±0.012)	96.7 (±0.031)	95.9 (±0.018)
	2	2.5	87.1 (±0.004)	97.1 (±0.022)	97.0 (±0.03)	96.8 (±0.013)
	29	1.04	94.3 (±0.005)	97.9 (±0.013)	99.1 (±0.013)	97.9 (±0.024)
Fumonisin B_1	14	4.9	90.0 (±0.004)	94.9 (±0.023)	95.4 (±0.025)	98.7 (±0.01)
	23	3.07	91.4 (±0.004)	98.6 (±0.01)	97.0 (±0.016)	98.9 (±0.015)

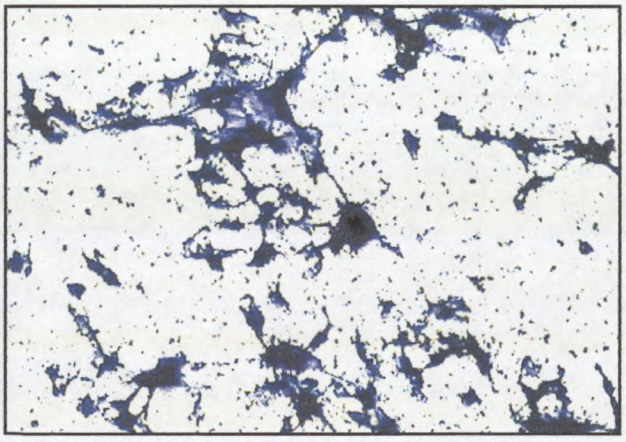
3.4.2 Effect of mycotoxins on viability of A549 (lung adenocarcinoma) cells



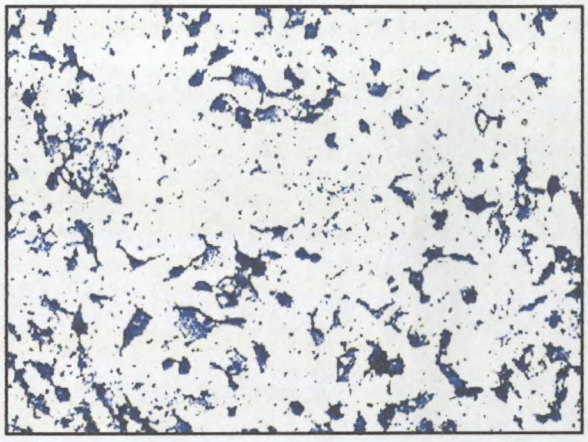
(a) A549 cells (100% cell viability) represent the control



(b) A549 cells exposed to 0.5 mg/ml fumonisin B_1 (30.5% cell viability)



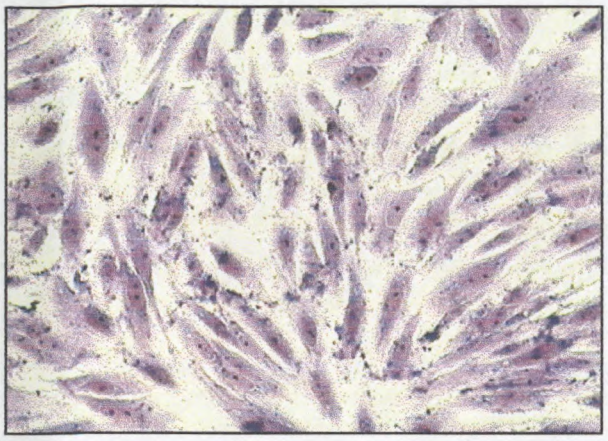
(c) A549 cells exposed to 0.5 mg/ml fusaric acid (5.1% cell viability)



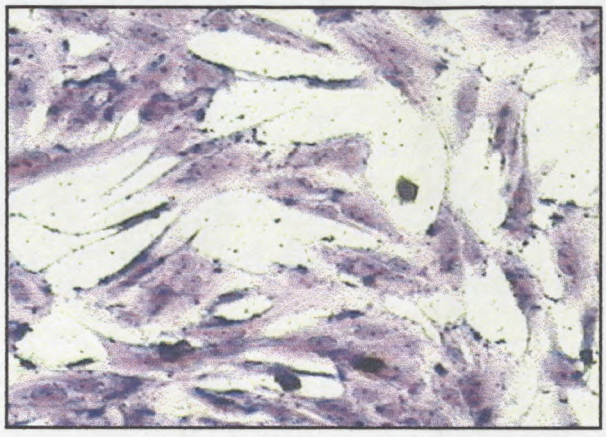
(d) A549 cells exposed to 0.5 mg/ml moniliformin (1.26%)

Fig. 3.26 Photomicrograph of A549 (lung adenocarcinoma) cells stained in the crystal violet test showing (a) cells with 100% cell viability (no toxin exposure) and (b-d) cells exposed to fusaric acid, moniliformin and fumonisin B_1, respectively, at concentrations of 0.5 mg/ml.

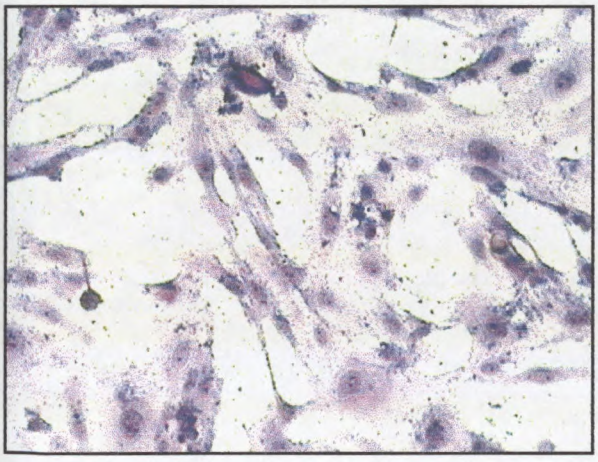
3.4.3 Effect of mycotoxins on the viability of VK (monkey kidney) cells



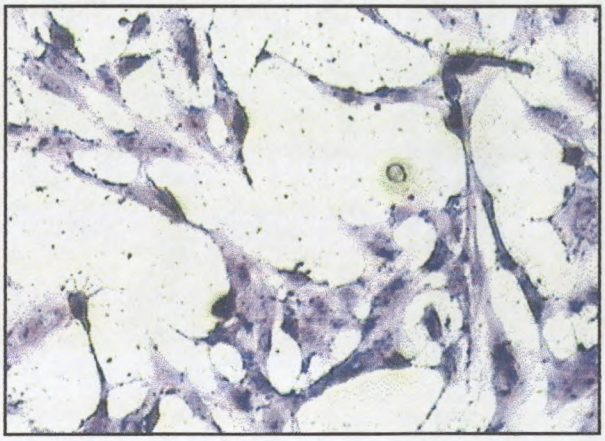
(a) VK cells (100% cell viability) represent the control



(b) VK cells exposed to 0.05 mg/ml fusaric acid (33.6% cell viability)



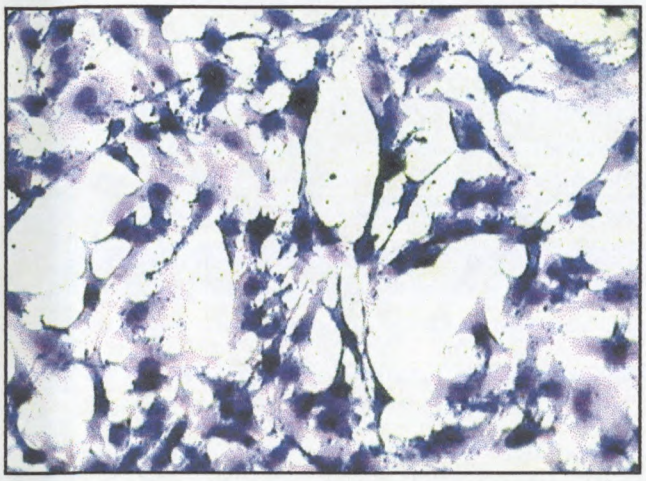
(c) VK cells exposed to 0.05 mg/ml moniliformin (21.1% cell viability)



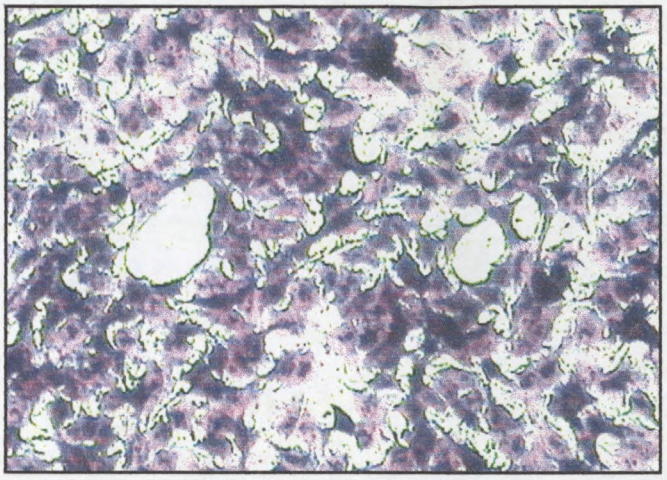
(d) VK cells exposed to 0.05 mg/ml .fumonisin B_1 (11.5% cell viability)

Fig.3.27 Photomicrograph of VK cells stained in the crystal violet test showing (a) cells with 100% cell viability and (b-d) cells exposed to 0.5 mg/ml fusaric acid, moniliformin and fumonisin B_1, respectively.

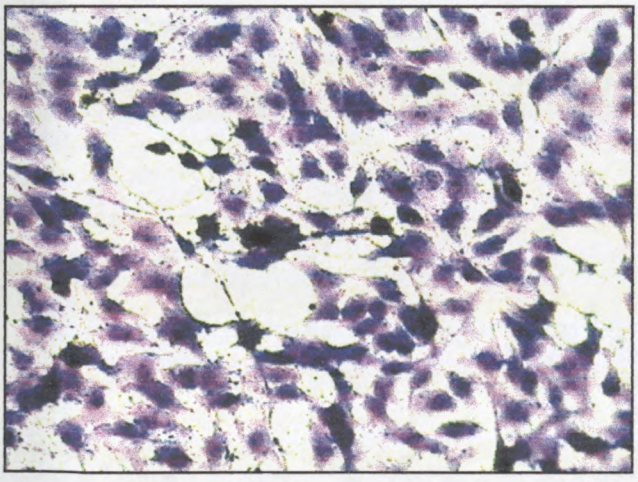
3.4.4 Effect of purified toxin extracts on A549 (lung carcinoma) cells



(a) A549 cells exposed to 7.78 µg/ml fusaric acid (isolate 27) (57.1% cell viability)



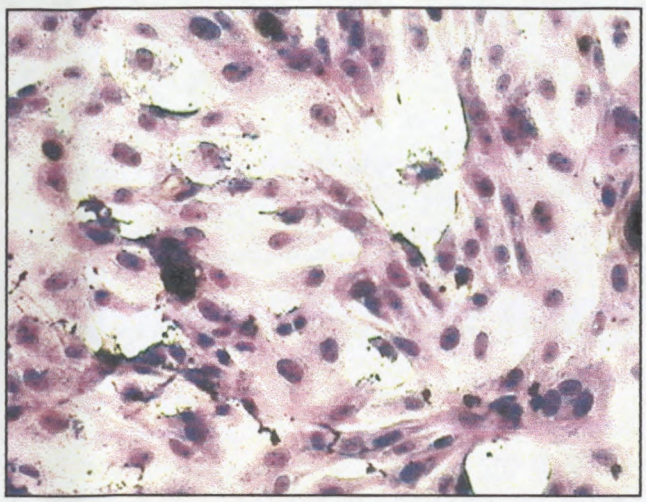
(b) A549 cells exposed to 4.7 µg/ml moniliformin (isolate 14) (94.08% cell viability)



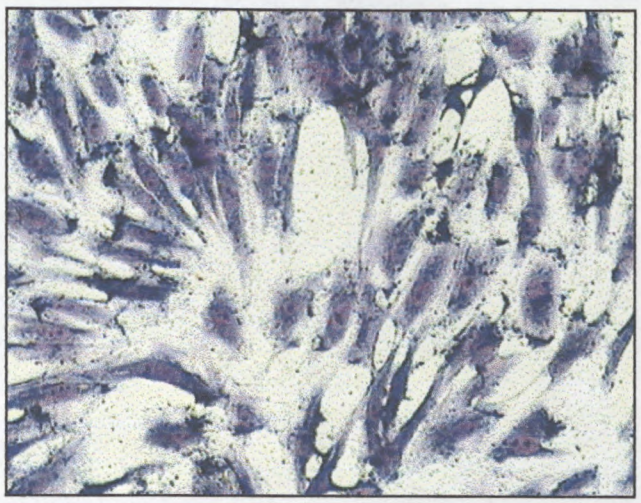
(c) A549 cells exposed to 4.9 µg/ml fumonisin B_1 (isolate 14) (94.9% cell viability)

Fig. 3.28 Photomicrographs showing A549 cells after 24 hours exposure to purified toxins (a-c), fusaric acid, moniliformin and fumonisin B_1, respectively, in the crystal violet test.

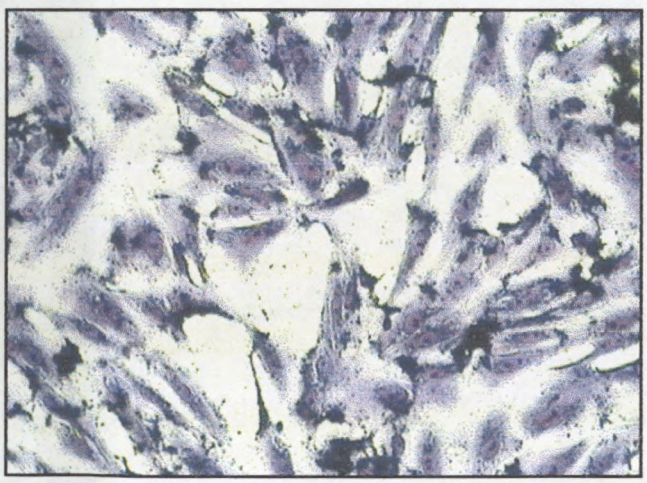
3.4.5 Effect of purified toxin extracts on VK (monkey kidney) cells



(a) VK cells exposed to 7.78 µg/ml fusaric acid (isolate 27) (89% cell viability)



(b) A549 cells exposed to 4.7 µg/ml moniliformin (isolate 14) (93.9% cell viability)



(C) VK cells exposed to 4.9 µg/ml fumonisin B_1 (isolate 14) (98.9% cell viability)

Fig. 3.29 Photomicrographs showing VK cells after 24 hours exposure to (a-c) purified fusaric acid, moniliformin and fumonisin B_1 in the crystal violet test.

CHAPTER FOUR: DISCUSSION

This project was divided into four phases. The first phase involved a clinical study that included the medical history and ocular examination of eight patients diagnosed with mycotic keratitis. The mean age of patients with fusarial keratitis was 51 years (ranging from 36 to 67 years). Tanure *et al.* (2000) reported a mean age of keratitis-infected patients of 59 years (ranging from 19 to 86 years). This study revealed a central infiltrate (ulcer) for 7/8 cases (87.5%) whereas a peripheral (marginal) infiltrate was observed in one case (12.5%). Similar results were reported by Garg *et al.* (2000) i.e., a central infiltrate in 82.8% of cases with a peripheral infiltrate observed in one case (12.5%). The male to female ratio was 1:1 as compared to a ratio of 1.7:1 reported by Mselle (1999). Sandhu and Rattan (1981) concluded that men aged between 20-40 years are more prone to mycotic keratitis, especially if they are farmers or labourers.

A prior history of superficial corneal injury was observed in 75% of cases, thus revealing ocular trauma as the major risk factor for mycotic keratitis. Trauma caused by accidental injury was previously reported as the most common predisposing factor for mycotic keratitis (Sandhu and Rattan, 1981 and Garg *et al.*, 2000).

Visual acuity was compromised in all the patients. Visual acuity in the keratitis-infected eye was 6/12 (one case), 6/36 (one case), CF (or ability to count fingers only) (four cases), HM (or ability to observe hand movements only) (one case), PL (or ability to perceive light only) (one case). The therapeutic regimen included a treatment of the topical antifungal antibiotics, gentamycin kefzole (GK), subconjunctival miconazole

(SM) and natamycin miconazole (NM) drops. Outcome data revealed that none of the patients' eyesight improved after treatment with three patients requiring scleral patches to prevent further deterioration of the entire eye.

The second phase of the study included identification of the 29 *Fusarium* isolates to species level. *F. solani* was the predominant species and was identified in 72.4% (21/29) of the cases. *F. moniliforme* and *F. dimerum* were identified in two cases (6.9%) each and *F. oxysporum, F. semitectum, F. chlamydosporum* and *F. lateritium* reported in one case each (3.4%). It is thus evident that *F. solani* is prevalent in mycotic keratitis infections. This is in agreement with previous studies by Liesegang and Forster (1980), Mino de Kaspar *et al.* (1991), Srinivasan *et al.* (1997) and Garg *et al.* (2000). *F. solani*, clearly, is a frequent cause of mycotic keratitis.

The frequency of *F. solani* keratitis may correlate with the increased prevalence of this fungus in soils in tropical regions (Rebell, 1980). Also, isolates from the cornea appear to share the special property of being able to grow reasonably well at 37°C (Rebell, 1980 and Sugiura *et al.*, 1999).

While *F. solani* is well known as a cause of mycotic keratitis, there are previous reports that support the isolation of *F. moniliforme, F. oxysporum* and *F. dimerum* from mycotic keratitis infections. Torres-Rodriguez (1987) identified *F. moniliforme* and *F. oxysporum* among other principle causative agents of mycotic keratitis. Zapater *et al.* (1972) isolated *F. dimerum* from mycotic keratitis infections. Although *F. chlamydosporum* has been

isolated from a disseminated human fusarial infection (Guarro and Gene, 1995), no literature is available on the isolation of *F. chlamydosporum*, *F. semitectum and F. lateritium* from mycotic keratitis infections. Thus, this is the first known report of these *Fusarium* species as etiological agents of mycotic keratitis.

Fusarium species were identified by the observation of colony morphology and colour and ultrastructural features using a *Fusarium* species synoptic key by Nelson *et al.* (1983). *F. solani* was characterized by the production of white to cream floccose mycelium and confluent bluish-green sporodochia on PDA. *F. solani* is the only known *Fusarium* species that produces this characteristic blue-green pigment (Nelson *et al.*, 1983). Other key characteristics of this species included the abundance of 3-4 septate macroconidia with blunt or rounded apical and basal cells, the presence of single-celled, oval and reniform microconidia and the formation of long, slender monophialides. In comparison, the only taxonomic characteristic that enabled the differentiation of *F. oxysporum* from *F. solani* was the formation of short monophialides in the first species. *F. moniliforme* was identified by the production of white floccose mycelium with a deep-violet tinge. The key taxonomic feature of this species was the presence of long microconidial chains produced from the mycelium and the absence of chlamydospores. The production of confluent orange sporodochia that gave the agar a characteristic deep-orange colour assisted the identification of *F. dimerum* while *F. semitectum* was identified by the characteristic spindle-shaped macroconidia formed on polyphialides. The latter species produced abundant white mycelium that became beige after maturation. *F. chlamydosporum* was identified by the characteristic abundant comma-shaped

microconidia produced from polyphialides. Macroconidia has a characteristic pointed apical cell and a foot-shaped basal cell. Another taxonomic criterion that aided identification was the abundant production of rough-walled chlamydospores occurring singly, in chains and in clusters, that was pale brown in colour. After maturation of the culture, a distinct deep brown colour of the mycelium was produced. *F. lateritium* was identified by its characteristic slow growth. Macroconidia were long with parallel walls, the apical cells had a distinct beak shape while basal cells were foot-shaped. Microconidial shapes varied from being oval, spindle and reniform.

The advantage of using CLA, a minimal media, was that it enhanced sporulation of the isolates which is vital for the identification of primary taxonomic structures such as macroconidia. PDA, a carbohydrate-rich medium, was used because of the distinct colony colours that were produced by the isolates. Colony colour was an important secondary criterion for *Fusarium* species identification. Both CLA and PDA were used by Sugiura *et al.* (1999) in the identification of human clinical isolates of *Fusarium* species.

The differentiation of *Fusarium* species from other filamentous opportunists occurring in mycotic keratitis infections is important in establishing the correct clinical diagnosis, in view of their different susceptibilities to antifungal drugs. *Fusarium* species are most resistant to available chemotherapy; thus early diagnosis is of prime importance (Sugiura *et al.*, 1999).

Because of the limited knowledge about the toxigenicity of *Fusarium* species associated with human infection, the *in vitro* production of fusaric acid, moniliformin and fumonisin B_1 from these clinical *Fusarium* isolates was determined. This formed the third phase of the project. The efficiency of the extraction methods and the sensitivity of the instruments used, i.e. thin layer chromatography and high- performance liquid chromatography, were assessed using authentic fusaric acid, moniliformin and fumonisin B_1 standards prior to mycotoxin analysis from the clinical isolates.

A percentage recovery of 84.5% was achieved after spiking 5 µg/g fusaric acid into maize patties using a liquid-liquid extraction method. Venter and Steyn (1998) obtained a recovery of 96.02% the identical extraction method. However, a difference in the percentage recoveries may be because maize patty cultures were used in this study as opposed to broth cultures used by Venter and Steyn (1998). A percentage recovery of 84.8% was obtained after spiking maize patties with 5 µg/g moniliformin using SAX cartridges as compared to an average 70% recovery reported by Filek and Lindner (1996) from spiked wheat ranging from 0.02- 0.25mg/g using a similar extraction method. Munimbazi and Bullerman (1998) reported 97.8% recovery of moniliformin from maize spiked at 1 µg/g using SAX cartridges. For fumonisin B_1, a recovery of 72.4% was obtained using SAX cartridges. Stack and Eppley (1992) recovered 67% fumonisin B_1 from maize using SAX cartridges while Scott and Lawrence (1992) reported an 84% recovery of fumonisin B_1 from maize spiked at 5 µg/g.

The detection limits for fusaric acid, moniliformin and fumonisin B_1 using TLC was 50, 100 and 500 ng respectively whereas the detection limits using HPLC was 25, 1 and 100 ng respectively. The HPLC methods for the three toxins were two-fold, a hundred-fold and five-fold more sensitive than the TLC methods, respectively. This trend was expected, since HPLC is a more advanced technique, using high- resolution instrumentation.

Fusaric acid produced purple spots under UV-illumination at 254 nm. The R_f value of authentic fusaric acid standard was 0.78. Purified extracts of the *Fusarium* isolates revealed a similar R_f value for isolates. Moniliformin also produced purple spots at 254 nm. A R_f value of 0.86 was established for an authentic moniliformin standard. A similar R_f value was revealed for the purified extract of one *Fusarium* isolate. Detection of fumonisin B_1 revealed a dark-purple spot. A R_f value of 0.65 was obtained for an authentic fumonisin B_1 standard. A similar R_f value was obtained for the purified extract of isolates 23 and 14.

HPLC analysis of fusaric acid at 254 nm revealed a reproducible peak for an authentic fusaric acid standard at a retention time of 3.15 min. Peaks that eluted at retention times ranging from 3.11 min to 3.17 min were obtained for the purified extracts of 20 *Fusarium* isolates. The purified extract of an uninoculated maize patty revealed no peak elution at this retention time.

The UV-detection (229 nm) of an authentic moniliformin standard by HPLC revealed a reproducible peak that eluted at a retention time of 2.99 min. Peaks that eluted at retention times ranging from 2.99 to 3.01 min were obtained for the purified extracts of four *Fusarium* isolates. Fluorescence detection of the *o*-phthaldialdehyde-derivatized fumonisin B_1 at an excitation and emission wavelength of 335 and 440 nm respectively revealed a reproducible peak at a retention time of 4.01 min. Peaks that eluted at similar retention times were obtained for the purified extracts of 2 *Fusarium* isolates.

Fusaric acid was produced by 69% (21/29) of the *Fusarium* isolates. Mycotoxin concentrations ranged from 0.32 μg/g to 7.78 μg/g maize patty. Of the 20 *Fusarium* isolates capable of fusaric acid production, 85% (17/20) were identified as *F. solani*; and one each (5%) as *F. oxysporum, F. moniliforme* and *F. lateritium.* The highest fusaric acid- producer was *F. solani* (7.78 μg/g).

Studies by Betina (1980) and Bacon *et al.* (1996) have already established *F. solani, F. oxysporum, F. moniliforme* and *F. lateritium* to be fusaric acid- producers. There is extensive literature citing the production of fusaric acid by *Fusarium* species in contaminated agricultural commodities (cereal grains, maize, wheat, barley and rice) and its implications in plant pathogenesis is well-documented. However, no literature exists on the ability of clinical isolates of Fusaria to produce fusaric acid.

Moniliformin was produced by four isolates of Fusaria. Two isolates were identified as *F. moniliforme*, one as *F. oxysporum* and one as *F. chlamydosporum.* Moniliformin

concentrations ranged from 1.04 μg/g to 4.7 μg/g. The highest toxin producer was *F. moniliforme* (4.7 μg/g). All three species have been shown to produce the toxin in maize, peanuts, sorghum, millet and soil (Betina, 1980, Cole and Cox, 1981, Rabie *et al.*, 1982, Marasas *et al.*, 1984, Joffe, 1986). However moniliformin production from clinical isolates of Fusaria have not been researched previously.

Fumonisin B_1 was produced by two isolates of Fusaria. Both of the isolates were identified as *F. moniliforme*. Toxin concentrations were 3.07 μg/g and 4.90 μg/g maize patty. *F. moniliforme* and *F. proliferatum* are the only two *Fusarium* species known to produce fumonisin B_1. Nelson *et al.* (1991) showed that *F. moniliforme* isolates from mycotic keratitis infections were able to produce fumonisin B_1. More recently, Sugiura *et al.* (1999), demonstrated the production of fumonisin B_1 from *F. moniliforme* strains isolated from the blood, skin and eye of human fuariosis patients.

The final phase of this project involved the evaluation of the cytotoxic effects of the three toxins on human lung adenocarcinoma cells (A549) and monkey kidney (VK) cells.

In the MTT assay, dimethylsulphoxide (DMSO) was used instead of acidified isopropanol to dissolve the formazan crystals. Reubel *et al.* (1987) showed that formazan was not totally dissolved in acidified isopropanol and that non-reduced MTT remained in the wells. But by subsequent addition of DMSO to the residue, a homogenous solution of the formazan product was achieved, which was essential for an exact photometric

measurement. In this way, low standard deviations (SD ≤0.05) for the samples and cell controls were obtained.

In A549 cells and VK cells, a nearly linear relationship could be observed between the mycotoxin concentrations and the percentage cell viability for fusaric acid, moniliformin and fumonisin B_1, in the range of 0.5 to 0.004 mg/ml. As the mycotoxin concentration increased, a decrease in the percentage cell viability was observed for each of the toxins. In comparison to fusaric acid and moniliformin, fumonisin B_1 was less toxic to the A549 cells whereas in the VK cell line, fumonisin B_1 exhibited a greater degree of toxicity than fusaric acid and moniliformin. In the MTT assay, at 0.05 mg/ml toxin concentration, A549 cells showed a 2.7% and 2.9% cell viability after 24 hours exposure to fusaric acid and moniliformin, respectively, whereas 24.3% cell viability was obtained after exposure to fumonisin B_1. VK cells showed 30% and 14.7% cell viability after 24 hours exposure to fusaric acid and moniliformin, respectively, whereas 11.5% cell viability was obtained for fumonisin B_1.

A comparison between A549 and VK cells in the MTT assay showed that the two cell lines possessed a different susceptibility to the three mycotoxins tested. Main factors may be the ability of the particular mycotoxin to bind to cellular receptors, and/or penetrate cell membranes, which is dependant on the size, structural conformation of the toxin molecules and the polarity of the compound (Thompson and Wannemacher, 1984).

Purified fusaric acid, moniliformin and fumonisin B_1 extracts from the *Fusarium* isolates, detected and quantified by HPLC, were evaluated for their cytotoxic effects on the A549 cells and VK cells. Five purified extracts of fusaric acid of the highest toxin-producers i.e. *F. solani* (four isolates) and *F lateritium* (one isolate) (at toxin concentrations ranging from 1.82 μg/g to 7.78 μg/g) were tested. The greatest inhibition of mitochondrial activity (41.3% cell viability) was achieved in A549 cells after exposure to an extract containing 7.78 μg/g fusaric acid.

The percentage cell viabilities for the three toxins obtained in the MTT- cleavage assay and the crystal violet test revealed values that were almost similar. A comparison of the two assays using the student's t-test returned a probability of 0.6465 (> 0.05) for A549 cells and a probability of 0.7480 (> 0.05) indicating no significant difference between the two assays. Both assays were simple, permitting the testing of multiple replicates and conditions and a rapid recovery of OD values (within 24 hours).

A correlation between toxin production by the *Fusarium* isolates and the severity of the corneal ulcers was formed from eight patients with fusarial keratitis. One *Fusarium* isolate from a corneal ulcer that was 2×2 mm in diameter was identified as *F. dimerum*. Visual acuity was CF (ability to count fingers only) for the patient. The ulcer was limited to the corneal stroma with no hypopyon (pus) formation in the anterior chamber. Neither fusaric acid, moniliformin nor fumonisin B_1, was produced by the *F. dimerum* isolate in culture. The ulcers, from which six *F. solani* species were isolated, showed much more severe symptoms in terms of ulcer size and depth, visual acuity and inflammatory

response in the anterior chamber. Ulcer sizes ranged from 1×1mm to large, central ulcers (covering entire corneal surface). Five ulcers were stromal in depth with a complete perforation of the cornea in one case. The anterior chamber response included cells and flare in 6 cases with hypopyon formation in three of these cases. *F. solani* isolates were shown to produce fusaric acid in all three cases in which a hypopyon formed and in one case, in which the ulcer size was 4×4mm. The most severe symptoms of the ulcer were observed in one patient, from which *F. moniliforme* was isolated. The symptoms included a large central ulcer that covered the entire cornea, the depth of which involved both the epithelium and the stroma. This later led to perforation of the entire cornea. The inflammatory response of the anterior chamber included cells, flare and hypopyon formation. The visual acuity of this patient was PL (ability to perceive light only) which was the most severe case of all the patients in this study. All three mycotoxins were produced from this *F. moniliforme* isolate.

CHAPTER FIVE: CONCLUSIONS

The predominant *Fusarium* species isolated from patients with mycotic keratitis patients was identified as *F. solani.* Other *Fusarium* species identified include *F. moniliforme*, *F. oxysporum* and *F. dimerum*, all of which are common etiological agents of mycotic keratitis infections. However, this is a first report of *F. semitectum*, *F. chlamydosporum* and *F. lateritium* as etiological agents of mycotic keratitis. This highlights the ability of a variety of *Fusarium* species, normally regarded as common soil saprophytes and plant pathogens, to cause opportunistic infections in humans.

Seventy- six percent of the *Fusarium* isolates was capable of mycotoxin production. This is the first report of fusaric acid and moniliformin production by *Fusarium* species isolated from mycotic keratitis infections. However, fumonisin B_1 production has been reported previously (Nelson *et al.*, 1991). Future work should include determining the ability of clinical isolates of Fusaria from mycotic keratitis infections to produce other mycotoxins.

The ability of fusaric acid, moniliformin and fumonisin B_1 to induce a cytotoxic effect on lung adenocarcinoma (A549) cells and monkey kidney (VK) cells was determined. It was observed that both the cell lines are susceptible after exposure to all three toxins in the MTT and crystal violet assays. However, the A549 cells are more susceptible to fusaric acid and moniliformin than to fumonisin B_1. In comparison, VK cells are more susceptible to fumonisin B_1 than to fusaric acid and moniliformin. Purified extracts of the fusaric acid, moniliformin and fumonisin B_1 from the clinical *Fusarium* isolates exposed

to the A549 and VK cells did not exhibit a marked degree of toxicity on cell viability due to the low toxin concentrations produced in culture. However, future work should include determining the cytotoxic effects of these toxins on corneal epithelial and endothelial cells.

A correlation between mycotoxin production and the clinical symptoms of the corneal ulcers were observed in a retrospective study involving eight patients. The corneal ulcer symptoms of one patient from which *F. dimerum* was isolated were not as severe as those symptoms of another patient from which *F. moniliforme* was isolated. The latter species was shown to produce all three toxins, fusaric acid, moniliformin and fumonisin B_1 in culture while the *F. dimerum* isolate did not produce any of the above toxins in culture. It is thus clear from the above observations, that possible mycotoxin production by these *Fusarium* isolates *in vivo* may have resulted in a worsening of ulcer symptoms. Since the *in-vitro* production of mycotoxins by Fusaria isolates from mycotic keratitis infections has been established, future work should include the detection of toxins in eye exudates.

None of the patients showed improvement of visual acuity after a therapeutic regimen of antifungal drugs. All patients received medical examination of the infected cornea in the late stages of infection. Thus, an early diagnosis of a mycotic keratitis infection is required, particularly a fusarial infection because of its known resistance to available drug treatments. The identification of the *Fusarium* species responsible for the keratitis infection is of utmost importance, since the pathogenic potential of each species varies with regard to toxin production.

The results of this study support the hypothesis that the mycotoxins fusaric acid, moniliformin and fumonisin B_1 play a vital role in the pathogenesis of mycotic keratitis. An earlier proposal on the pathogenesis postulated that toxins may be implicated (Fig. 1.9). Although the study included a small number of patients, the evidence is clear that the extent of the infection (Fig. 1.11) is related to the fungal species and the toxins they produce. This study is indicative of the role of toxins in the severity of a fusarial keratitis infection (Table 3.14). The fact that these toxins exhibited varying degrees of toxicity when tested on both human and animal cell lines further lends evidence to the above hypothesis.

REFERENCES

Alfonso, E. C., Rosa, R. H. 1997. Fungal keratitis. *In*: Krachmer, J. H., Mannis, M.J., Holland, E. J. Cornea and external disease: clinical diagnosis and management. St. Louis: Mosby; pp.1253-66.

Bacon, C. W., Porter, J. K., and Norred, W. P. 1995. Toxic interaction of fumonisin B_1 and fusaric acid measured by injection into fertile chicken eggs. *Mycopathologia.* **129**: 29-35.

Bacon, C. W., Porter, J. K., Norred, W. P. and Leslie, J. F. 1996. Production of fusaric acid by *Fusarium* species. *Applied and Environmental Microbiology.* **62** (11): 4039-4043.

Bennett, H. G. B., Hay, J., Kirkness, C. M., Seal, D. V. and Devonshire, P. 1998. Antimicrobial management of presumed microbial keratitis: guidelines for treatment of central and peripheral ulcers. *British Journal of Ophthalmology.* **82**: 137-145.

Betina, V. 1989. Mycotoxins: chemical, biological and environmental aspects. Elsevier Science: New York.

Bhat, R. V. Sashidhar, R. B., Ramakrishna, Y. and Munshi, K. L. 1989. Outbreak of trichothecenes mycotoxicoses associated with consumption of mould- damaged wheat-products in Kashmir Valley, India. *The Lancet.* **1**: 35-37.

Binder, P. S. 1986. Ocular fungal and parasitic infections. *In*: Braude, A. I., Davis, C. E. and Fierer, J. (ed.). *Infectious diseases and medical microbiology.* Philadelphia: WB Saunders.

Booth, C. 1971. The genus *Fusarium.* Commonwealth Mycological Institute, Kew, Surrey, England.

Bridgens, K. I. 1998. Keratitis in the Durban area. Master of Ophthalmology thesis, University of Natal, Durban.

Burgess, L. W. and Liddell, C. M. 1985. Survival of *Fusarium moniliforme* at controlled temperature and relative humidity. *Pathology.* **14** (2): 29-31.

Burgess, L. W., Liddell, C. M. and Summerell, B. A. 1988. Laboratory manual for *Fusarium* research. (2nd ed.). John Wiley and Sons Ltd.

Burmeister, H. R., Grove, M. D., Peterson, R. E., Weisleder, D. and Plattner, R. D. 1985. Isolation and characterization of two new fusaric acid analogs from *Fusarium moniliforme* NRRL 13, 163. *Applied and Environmental Microbiology.* **50**(2): 311-314.

Charmley, L. L., Trenholm, H. L., Prelusky, D. B., and Rosenberg, A. 1995. Economic losses and decontamination. *Natural Toxins.* **3**: 199-203.

Cheeke, P. R. Endogenous toxins and mycotoxins in forage grasses and their effects on livestock. 1995. *Journal of Animal Science.* **73**: 909-918.

Childress, W. L., Krull, I. S., and Selavka, C. M. 1990. *Journal of Chromatographic Science.* **28**: 76-82.

Cole, R. J., Kirksey, J. W., Cutter, H. G. Doupnik, B. L. and Peckham, J. C. 1973. Toxin from *Fusarium moniliforme*: effects on plants and animals. *Science.* **179**: 1324-1326.

Cole, R. J. and Cox, R. H. 1981. Handbook of toxic fungal metabolites. Academic Press, New York.

Cuero, R. G. 1980. Ecological distribution of *Fusarium solani* and its opportunistic action related to mycotic keratitis in Cali, Columbia. *Journal of Clinical Microbiology.* **12** (3): 455-461.

Desjardins, A. E., Plattner, R. D., Nelson, P. E. 1997. Production of fumonisin B_1 and moniliformin by *Gibberella fujikuroi* from rice from various geographic areas. *Applied and Environmental Microbiology.* **63** (5): 1838-1842.

Diaz, G. J. and Boermans, H. J. 1994. Fumonisin toxicosis in domestic animals: a review. *Veterinary and Human Toxicology.* **36** (6): 548-555.

Dowd, P. F. 1988. Toxicological and biochemical interactions of the fungal metabolites fusaric acid and kojic acid with xenobiotics in *Heliothis zea* (F.) and *Spodoptera frugiperda* (J. E. Smith). *Pesticide Biochemistry and Physiology.* **32**: 123-134.

Dunlop, A. A., Wright, E. D. and Howlader, S. A. 1994. Suppurative corneal ulceration in Bangladesh. A study of 142 cases examining the microbiological diagnosis, clinical and epidemiological features of bacterial and fungal keratitis. *Australian and New Zealand Journal of Ophthalmology.* **22**: 105-110.

Faber, J. 1988. Production of moniliformin by Canadian isolates of *Fusarium. Mycopathologia.* **101**: 187- 190.

Filek, G. and Lindner, W. 1996. Determination of the mycotoxin moniliformin in cereals by high- performance liquid chromatography and fluorescence detection. *Journal of Chromatography.* **732**: 291-298.

Fink- Gremmels, J. R. and Georgiou, N. A. 1996 Risk assessment of mycotoxins for the consumer. *In*: Ennen, G., Kuiper, H. A., and Valentin, A. (eds.) Residues of veterinary drugs and mycotoxins in animal products. NL-Wageningen Press. pp. 159-174.

Fink- Gremmels, J. 1999. Mycotoxins: their implications for human and animal. *The Veterinary Quarterly.* **21**: 115-120.

Florakis, G. J., Moazami, G. Schubert, H., Koester, C. J. and Auran, J. D. 1997. Scanning slit confocal microscopy of fungal keratitis. *Archives of Ophthalmology.* **115** (11) 1461-1463.

Garg, M. S., Gopinathan, U., Choudhary, K. and Rao, G. N. 2000. Keratomycosis-Clinical and microbiological experience with dematiaceous fungi. *Ophthalmology.* **107:** 574: 580.

Gerlach, W., Nirenberg, H. I. 1982. The genus *Fusarium-* a pictorial atlas. *Mitteilungen aus der Biologischen Bundesanstalt fur Land und Fortwirtschaft Berlin- Dahlem.* 209: 1-406.

Greer, D. L., Brahim, C., and Gonzalez, L., A. 1972. Queritits micotica en Colombia. Tribuna Medica, Colombia pp. 15-20.

Guarro, J., Gene, J. 1995. Opportunistic fusarial infections in humans. *European Journal of Clinical and Microbiological Infectious Diseases.* **14**:741-754.

Guarro, J., Gene, J., Stchigel, A. M. 1999. Developments in fungal taxonomy. *Clinical Microbiology Reviews.* **12**(3): 454- 500.

Gugnani, H. C., Gupta, S. and Talwar, R. S. 1978. Role of opportunistic fungi in ocular infections in Nigeria. *Mycopathologia.* **65** (1-3): 155-166.

Guzek, J. P., Roosenberg, J. M., Gano, D. L. and Wessels, I. F. 1998. The effect of vehicle on corneal penetration of triturated ketoconazole and itraconazole. *Ophthalmic Surgical Lasers.* **29** (4) 926-929.

Hanelt, M., Gareis, M. and Kollarczik, B. 1994. Cytotoxicity of mycotoxins evaluated by the MTT- cell culture assay. *Mycopathologia.* **128**: 167-174.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C., and Pegler, D. N. 1995. Ainsworth and Bisby's Dictionary of the Fungi. (8th edition)CAB International University Press, Cambridge.

Joffe, A. Z. 1986. *Fusarium* species: their biology and toxicology. Wiley Interscience, New York. pp. 9-79.

Jones, D. B. 1978. Pathogenesis of bacterial and fungal keratitis. *Transactions of Ophthalmological Society of the United Kingdom.* **98**: 367- 371.

Jones, D. B. Diagnosis and management of fungal keratitis. 1998 *In*: Tasman, W., Jaeger E. A., (eds.) Duanes' Clinical Ophthalmology. Philadelphia: J. B. Lippincott, CD-ROM edition.

Kuiper- Goodman, T. 1998. Food safety: mycotoxins and phytotoxins in perspective. *In*: Miraglia, M., van Egmond, H., Brera, C., and Gilbert, J. (eds.). Mycotoxins and phytotoxins- developments in chemistry, toxicology and food safety. pp. 25- 48.

Landegran, U. 1984. Measurement of cell numbers by means of the endogenous hexosaminadase. Applications to the detection of lymphokines and cell surface antigens. *Journal of Immunological Methods.* **67**: 379: 388.

Leon, F. E., Carpintero, M., Bayona, J., Gaffga, N., Ocampo, L. ans del Castillo, N. 1998. Lack of air conditioning and mycotoxin poisoning are basic co-factors in tropical spastic paraparesis. *Review of Neurology.* **152**: 564-567.

Li, Y. C., Ledoux, D. R., Bermudez, A. J., Fritsche, K. L. and Rottinghaust, G. E. 2000. Effects of moniliformin on performance and immune function of broiler chicks. *Poultry Science.* 79 (1): 26-32.

Liesegang, T. J. and Forster, R. K. 1980. Spectrum of microbial keratitis in South Florida. *American Journal of Ophthalmology.* 30 (1): 38- 47.

Luo, X. Y. 1988. *Fusarium* toxin contamination of cereals in China. *Proceedings of the. Japanese Association of Mycotoxicology Supplement.* 1: 97-98.

Male, O. 1990. The significance of mycology in medicine. *In*: Hawksworth, D. L. (ed.). *Frontiers in Mycology.* Wallingford: C..A.B. International. pp. 131-156.

Marasas, W. F. O, Nelson, P. E. and Tousson, T. A. 1984. Toxigenic *Fusarium* species: identity and mycotoxicology. Pennsylvania State University Press: University Park.

Marasas, W. F. O. 1995. Fumonisins: their implications for human and animal health. *Natural Toxins.* 3: 193-198.

McGinnis, M. R., Tilton, R. C. 1980. Mycotic infections of the eye and the ear. *In*: Howard, B. (2nd ed.). *Clinical and pathogenic microbiology.* St. Louis: Mosby- Year Book, Inc. pp. 625-626.

Mino de Kaspar, H., Zoulek, G., Paredes, M.E., Alborno, R., Medina, D., Centurion de Morinigo, M., Ortiz de Fresco, M. and Aguero, F. 1991. Mycotic keratitis in Paraguay. *Mycoses.* 34: 251-254.

Mselle, J. 1999. Fungal keratitis as an indicator of HIV infection in Africa. *Tropical Doctor.* 29 (3): 133-135.

Munimbazi, C. and Bullerman, L. B. 1998. High performance liquid chromatographic method for the determination of moniliformin in corn. *Journal of AOAC International.* **81**(5): 999- 1004.

Munkvold, G. P. and Desjardins, A. E. 1997. Fumonisins in maize. *Plant Disease.* **81**(6) 556-565.

Naumman, G., Green, W. R. and Zimmerman, L. E. 1967. Mycotic keratitis, a histopathological study of 73 cases. *American Journal of Ophthalmology.* **69:** 458-466.

Nelson, P. E., Toussoun, T. A and Cook, R. J. 1981b. *Fusarium*: diseases, biology and taxonomy. The Pennsylvania University Press, University Park, London.

Nelson, P. E., Tousson, T. A. and Marasas, W. F. O. 1983. *Fusarium* species: an illustrated manual for identification. Pennsylvania State University Press: University Park.

Nelson, P. E., Plattner, R. D., Shackelford, D. D. and Desjardins, A. E. 1991. Production of fumonisins by *Fusarium moniliforme* strains from various substrates and geographic areas. *Applied and Environmental Microbiology.* **57**: 2410-2412.

Panda, A., Sharma, N., Das, G., Kumar, N. and Satpathy, G. 1997. Mycotic keratitis in children: epidemiological and microbiological evaluation. *Cornea.* **16** (3): 295-299.

Patel, A. S., Hemady, R. K. Rodriguez, M., Rajagopalan, S. and Elman, M. J. 1994. Endogenous endophthalmitis in a patient with acute lymphocytic leukemia. *American Journal of Ophthalmology.* **117**: 363-368.

Pelzar, M. J., Chan, E. C. S. and Krieg, N. R. 1993. Microbiology, concepts and applications (Int. ed.). McGraw Hill: New York.

Poria, V. C., Pharad, V. R., Dongre, D. S. and Kulkarni, M. V. 1985. Study of mycotic keratitis. *Indian Journal of Ophthalmology.* **33**: 229- 231.

Porter, J. K., Bacon, C. W. and Norred, W. P. 1990. Effects of *Fusarium moniliforme* and corn associated with equine leukoencephalomalacia on rat transmitters and metabolites. *Proceedings of the Society for Experimental Biology and Medicine.* **194**: 265-269.

Porter, J. K. Wray, E. M., Bacon, E. W., Hagler, W. M., Jr. 1995. Fusaric acid in *Fusarium moniliforme* cultures, corn and feeds toxic to livestock and the neurochemical effects in the brain and pineal glands of rats. *Natural Toxins.* **3**: 91: 100.

Porter, J. K., Wray, E. M., Rimando, A. M., Stancel, P. C., Bacon, C. W., and Voss, K. A. 1996. Lactational passage of fusaric acid from the feed of nursing dams to the neonate rat and effects on pineal neurochemistry in the F1 and F2 generations at weaning. *Journal of Toxicology and Environmental Health.* **49**: 101-115.

Porter, J. K. and Rimando, A. M. 1997. Early effects of fusaric acid in rats and long range implications. *Chronobiology International.* **14**: 137.

Porter, J. K., Wray, E. M., Eppley, R. M., and Hagler, W. M., Jr. 1998a. Zearalenone and fusaric acid in the diet of nursing dams enhances both mycotoxins' lactational transfer to the suckling neonate. Unpublished results, 112th AOAC International Annual Meeting and Exposition, Montreal, Quebec, Canada, September 13-17.

Rabie, C. J., Marasas, W. F. O., Thiel, P. G., Lubben, A., Vleggaar, R. 1982. Moniliformin production and toxicity of different *Fusarium* species from southern Africa. *Applied and Environmental Microbiology.* **43**: 517-521.

Rahman, R. M., Johnson, G. J., Husain, R., Shahe, H. M. and Minassian, D. C. 1998. Randomized trial of 0.2% chlorhexidine gluconate and 2.5% natamycin for fungal keratitis in Bangladesh. *British Journal of Ophthalmology.* **82**: 919-925.

Ravindranath, B. 1989. Principles and practice of chromatography. John Wiley and Sons, New York, 228-456.

Raza, S. K., Howell, S. A. and Mallet, A. I. 1993. Identification of mycotoxins in keratomycosis- derived *Fusarium* isolates by gas chromatography- mass spectrometry. *Journal of Chromatography.* **620**: 243-249.

Raza, S. K., Mallet, A. I., Howell, S. A., and Thomas, P. A. 1994. An in- vitro study of the sterol content and toxin production of *Fusarium* isolates from mycotic keratitis. *Journal of Medical Microbiology.* **41**: 204-208.

Rebell, G. 1981. *Fusarium* infections in human and veterinary medicine. *In*: Nelson, P. E., Tousson, T. A. and Cook, R. J. (ed.). *Fusarium:* diseases, biology and taxonomy. Pennsylvania State University Press: University Park. pp. 212-219.

Reubel, G. H., Gareis, M., Amselgruber, N. M. 1987. Cytotoxicity evaluation of mycotoxins by an MTT- bioassay. *Mycotoxin Research.* **3**: 85-96.

Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W. Shephard, G. S. and Schalkwyk, D. J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology.* **82**: 353- 357.

Richardson, M. D. and Warnock, D. W. 1993. Fungal infection: diagnosis and management. Blackwell Scientific, Oxford. pp. 207.

Rimando, A. M. and Porter, J. K. 1997. Fusaric acid increases melatonin in serum of weanling rats. *Journal of Toxicology and Environmental Health.* **50**: 101-110.

Rosa, R. H. Jr., Miller, D. and Alfonso, E. C. 1994. The changing spectrum of fungal keratitis in South Florida. *Ophthalmology.* **101**: 1005.

Rotter, B. A, Thompson, B. K., Lessard, M., Trenholm, H. L and Tryphonas, H. 1994. Influence of low level exposure to *Fusarium* mycotoxins on selected immunological and haematological parameters in young swine. *Fundamental and Applied Toxicology.* **23**: 117-124.

Sandhu, D. K., Rattan, A. S. 1981. Keratomycosis: a review. *Mykosen.* **24**: 50.

Smith, T. K. and Sousadias, M. 1993. Fusaric acid content of swine feedstuffs. *Journal of Agriculture and Food Chemistry.* **41**: 2296-2298.

Smith, T. K., McMillan, E. G., and Castillo, J. B. 1997. Effects of feeding blends of *Fusarium* mycotoxin contaminated grains containing deoxynivalenol and fusaric acid on growth and feed consumption of immature swine. *Journal of Animal Science.* **75**: 2184-2191.

Snijders, C. H., Samson R. A., Hoekstra E. S., Ouellet T., Miller J. D., de Rooij-van der Goes P. C., Baar A. J., Dubois A. E. and Kauffman H. F. 1996. Analysis of *Fusarium* causing dermal toxicosis in marram grass planters. *Mycopathologia.* **135**: 119-128.

Srinivasan, M., Gonzales, C. A., George, C. Cerallos, V., Mascarenhas, J. M, Asokan, B., Wilkins, J., Smolin, G. and Whitcher, J. P. 1997. Epidemiology and etiological diagnosis of corneal ulceration in Madurai, South India. *Brazil Journal of Ophthalmology.* **81**: 965- 971.

Stack, M. E. and Eppley, R. M. 1992. Liquid chromatographic determination of fumonisins B_1 and B_2 in corn and corn- products. *Journal of AOAC International.* **75** (5): 834- 837.

Sugiura, Y., Barr, J. R., Barr, D. B., Brock, J. W., Elie, C. M., Ueno, Y., Patterson, D. G., Jr., Potter, E. M. and Reiss, E. 1999. Physiological characteristics and mycotoxins of human clinical isolates of *Fusarium* species. *Mycological Research.* **103** (11): 1462-1468.

Tanure, M. A., Cohen, E. J., Sudesh, S., Rapuano, C. J., Laibson, P. R. 2000. Spectrum of fungal keratitis at Wills Eye Hospital, Philadelphia, Pennsylvania. *Cornea.* **19** (3): 307-312.

Thalmann, A., Matzenauer, S. and Gruber- Schley, S. 1985. Survey on the occurrence of fusariotoxins in cereals. *Berlin Landwirtschaft.* **63**: 257-272.

Thiel, P. G. Meyer, C. J. Marasas, W. F. O. 1982. Natural occurrence of moniliformin together with deoxynivalenol and zearalenone in Transkein corn. *Journal of Agriculture and Food Chemistry.* **30:** 308- 312.

Thiel, P. G., Sydenham, E. W., Shephard, G. S. and van Schalkwyk, D. S. 1993. Study of the reproducibility characteristics of a liquid chromatographic method for the determination of fumonisins B_1 and B_2 in corn: IUPAC collaborative study. *Journal of AOAC International.* **76** (2): 361-363.

Thomas, P. A. 1994. Mycotic keratitis- an underestimated mycosis. *Journal of Medical and Veterinary Mycology*: **32** (4): 235-256.

Torres- Rodriguez, J. M. 1987. Infecciones oculares. Barcelona: Ediciones Doyma. pp. 28- 33.

Ueno, Y. and Shimada, N. 1974. Reconfirmation of the specific nature of reticulocytes bioassay system for trichothecene mycotoxins of *Fusarium* species. *Chemical and Pharmaceutical Bulletin.* **22** (11): 2744-2746.

Ueno, Y. 1984. Toxicological features of T-2 toxin and related trichothecenes. *Fundamental and Applied Toxicology.* **4**: 224-232.

Upadhayay, M. D., Karmacharya, P. C. D. and Koirala, S. 1991. Epidemiological characteristics, predisposing factors, and etiological diagnosis of corneal ulceration in Nepal. *American Journal of Ophthalmology.* **111**: 92-99.

Venter, S. L. and Steyn, P. J. 1998. Correlation between fusaric acid production and virulence of isolates of *Fusarium oxysporum* that causes potato dry rot in South Africa. *Potato Research.* **41**: 289-294.

Vieira, L. A. 1997. Ceratite micótica. *In*: Belfort, Jr. R., Kara-J. N. Córnea. Clínica-Cirúrgica. São Paulo: Roca; pp.189-203.

Whatman International Limited. 1991. Partisal SAX and high- performance liquid chromatography analytic columns. Technical Information. GHB/57. **95**: 2-6.

Wilhelmus, K. R., Robinson, N. M., Font, R. A., Hamil, M. B. and Jones, D. B. 1988. Fungal keratitis in contact lens wearers. *American Journal of Ophthalmology.* **106**: 708-714.

Zapater, R. C. De Arrechea, A. and Guevara, V. H. 1972. Keratomycosis due to *Fusarium dimerum. Sabouraudia.* **10** (3): 274-275.

APPENDICES

APPENDIX 1 *Fusarium* species culture media and mountant preparation

Sabouraud dextrose agar (SDA) (Sigma)- 32.5 g of Sabouraud dextrose agar powder was added to 500 ml distilled water in a 1000 ml conical flask. The contents were heated at 55°C on a hot plate to dissolve any particles. The flask was then placed in an autoclave and sterilized at 121°C for 15 min. After cooling to 45°C, 15 ml molten agar was poured aseptically into sterile petri dishes and allowed to solidify before storing the SDA plates at 4°C.

2% Water Agar- 2 g technical agar powder was added to 100 ml distilled water and sterilized in an autoclave at 121°C for 15 min.

Lactophenol- cotton blue mountant

phenol (pure crystals)	20.0 g
lactic acid	20.0 g
glycerol	40.0 g
water	20.0 ml
cotton blue	0.5 g/100ml water

The phenol crystals were dissolved in water heated to 60°C before adding lactic acid and glycerol. A small amount of cotton blue dye was finally added to the lactophenol mixture and thoroughly mixed before use.

APPENDIX 2 Synoptic key to sections and species of *Fusarium*

The 12 *Fusarium* sections as listed by Nelson *et al.* (1983)

No. of *Fusarium* Sections	Sections to *Fusarium* species
1	Eupionnotes
2	Spicarioides
3	Arachnites
4	Sporotrichiella
5	Roseum
6	Arthrosporella
7	Gibbosum
8	Discolor
9	Lateritium
10	Liseola
11	Elegans
12	Martiella-Ventricosum

The numbers used to identify each section in the synoptic key appear in front of the section name. Numbers printed in italics indicate sections included in more than one description under a given characteristic.

The synoptic key to the 12 *Fusarium* sections

Synoptic Key to Sections 1-12

Cultural characteristics

1. Rate of growth

a. Growth very slow, cultures less than 2 to 3 cm in diameter in 10 days. **1**

b. Growth moderately slow, cultures not more than 7 cm in diameter in 10 days. ***2***, **3**, **8** (*F. reticulatum*), **9**

c. Growth rapidly covering entire plate in 10 days. ***2***, **4**, **5**, **6**, **7**, ***8***, **10**, **11**, **12**

2. Aerial mycelium present or absent

a. Aerial mycelium present, sparse to felt- like or abundant, spore masses (sporodochia) present or absent. **2**, **3**, ***4***, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**

b. Aerial mycelium absent and the surface of the colony has a slimy yeast- like appearance. **1**

3. Colour of aerial mycelium

a. White. **2**, **3**, ***4***, **5**, **6**, ***7***, ***8***, **9**, ***10***, ***11***, **12**

b. Light purple. ***10***, ***11***

c. Tan. ***4***, ***7***, ***8***

4. Colour of colony from below.
Colour may diffuse into agar.

a. Colour absent or if present only very pale shades of orange, tan, brown, or light purple. **1**, **3**, ***4***, ***5*** *(F. graminum)*, ***6***, ***7***, ***8***, ***10***, ***11***, ***12***

b. Shades of carmine red. **2**, ***4***, ***5***, ***6***, ***7***, ***8***, **9**

c. Strong purple pigment diffusing into agar often in advance of colony. ***10***, ***11***, ***12***

5. **Colour of spore masses**
(sporodochia)

a. Cream. 11, 12

b. Orange to yellow to tan. **1**, **2**, **3**, ***4***, ***5***, **6**, **7**, ***8***, **9**, **10**, ***11***

Reddish- brown. ***4***, ***5***, ***8***

c. Blue- green to blue. ***12***

Macroconidia from sporodochia

1. Size

a. Short, generally 1-2 septate. ***1*** (occasionally), **3**

b. Medium long, generally 3-7 septate. ***1***, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**

c. Very long, generally 8-9 septate. **2**

2. Shape

marked dorsi- ventral curvature. The sides of the macroconidia are often unequally curved. ***1***, **3**, **4**, ***5***, ***6***, **7**, ***8***, ***11***

c. Macroconidia without marked dorsi- ventral curvature with the sides relatively straight and parallel for most of their spore length. **2**, ***6***, ***8*** , ***9***, ***10***, ***11***, **12**

d. Macroconidia very thin, needle- like, with thin walls. ***1***, ***5***, ***10***

e. Macroconidia relatively stout with a marked dorsi- ventral curvature. ***8***

f. Some macroconidia spindle- shaped. Spindle- shaped macroconidia are formed only in the aerial mycelium and may be produced on monophialides or polyphialides. ***5***, ***6***, ***9***

3. Shape of basal and apical cells

a. Basal cell not distinct or papillate. Not distinctly foot- shaped. **1**, **3**, ***4***

b. Basal cell distinctly foot- shaped or notched. **2**, ***4***, **5**, **6**, ***7***, **8**, **9**, **10**, **11**, **12**

c. Apical cell extended and whip- like. ***7***

Microconidia from aerial mycelium

1. Present or absent

a. Present and abundant. **2**, **4**, ***7*** (*F. scirpi*), ***8*** (*F. bactridioides*), ***9***, **10**, **11**, **12**

b. Absent or sparse (i.e. less than 1/10 of conidia present in low power compound microscope field). **1**, **3**, **5**, **6**, ***7***, ***8***, ***9***

2. In chains or false heads

a. In chains and false heads. **2**, **10**

b. In false heads only. **4**, **11**, **12**

3. Shape

a. Oval to egg- shaped (ovoid0 to kidney- shaped (reniform) to fusiform. **2**, ***4***, **7**, (*F. scirpi*), **8** (*F. bactridioides*), ***10***, **11**, **12**

b. Globose (napiform). ***4*** (*F. poae*), ***10*** (*F. anthophilum*)

Conidiophores

1. Type

a. Monophialides only (may produce either macroconidia or microconidia). **1**, **2**, **3**, ***4***, **5**, ***6***, ***7***, **8**, **9**, ***10***, **11**, **12**

b. Monophialides (may produce either macroconidia or microconidia) and polyphialides (produce only spindle- shaped macroconidia and microconidia). ***4***, ***6***, ***7*** (*F. scirpi*), ***10***

Chlamydospores

1. Present or absent

a. Present. ***1***, **4**, **6**, **7**, **8**, **9**, **11**, **12**

b. Absent. ***1***, **2**, **3**, **5**, **10**

2. Arrangement

a. Single or in pairs. **1**, ***4***, **9**, **11**, **12**

b. Long chains or large clumps of more than three cells. ***4***, **6**, **7**, **8**

Fusarium species and the 12 sections to which each species belongs.

Species in sections 1, 2, and 3 (Eupionnotes, Spicarioides and Arachnites)

Section Eupionnotes (1)
1. *F. aquaeductuum*
2. *F. merismoides*
3. *F. dimerum*

Section Spicarioides (2)
4. *F. decemcellulare*

Section Arachnites (3)
5. *F. nivale*

Species in the sections (4, 5, 6, 7 and 8) Sporotrichiella, Roseum, Arthrosporiella, Gibbosum and Discolor

Section Sporotrichiella (4)
6. *F. poae*
7. *F. tricinctum*
8. *F. sporotrichioides*
9. *F. chlamydosporum*

Section Roseum (5)
10. *F. graminum*
11. *F. avenaceum*

Section Arthrosporiella (6)
12. *F. semitectum*
13. *F. camptoceras*

Section Gibbosum (7)
14. *F. equiseti*
15. *F. scirpi*
16. *F. acuminatum*
17. *F. longipes*

Section Discolor (8)
18. *F. heterosporum*
19. *F. reticulatum*
20. *F. sambucinum*
21. *F. culmorum*
22. *F. graminearum*
23. *F. crookwellense*

Species in sections 9, 10, 11 and 12 (Lateritium, Liseola, Elegans, Martiella and Ventricosum)

Section Lateritium (9)
24. *F. lateritium*

Section Liseola (10)
25. *F. moniliforme*
26. *F. proliferatum*
27. *F. subglutinans*
28. *F. anthophilum*

Section Elegans (11)
29. *F. oxysporum*

Section Martiella and Ventricosum (12)
30. *F. solani*

Synoptic key to *Fusarium* species in sections 1- 3 (species 1-5)

Species in sections 1, 2, and 3 (Eupionnotes, Spicarioides and Arachnites)

A. Cultural characteristics

1. Rate of growth

a. Growth slow, less than 2 to 3 cm in diameter after 10 days. 1, 2, 3

b. Growth relatively slow, less than 7 cm in diameter after 10 days. 4, 5

2. Aerial mycelium present or absent

a. Present. 4, 5

b. Absent and colony surface slimy and yeast- like. 1, 2, 3

3. Colour of aerial mycelium

a. White. 4

b. White to light orange. 5

4. Colour of colony from below

Colour may diffuse into agar

a. Colourless to cream. 1, 2, 3

b. Tan to carmine red. 4

c. Cream to pale or bright orange. 5

5. Colour of spore masses (sporodochia)

a. Spore masses distinct, cream to yellow. 4

b. Spore masses distinct, pale to bright orange. 5

c. Spore masses making up entire colony surface, cream, tan or orange. 1, 2, 3

B. Macroconidia from sporodochia

1. Size

a. Small, 1-2 septate. 3, 5

b. Small to moderately large, 3- 7 septate. 1, 2, 3

c. Very large, 9 septate or more. 4

2. Shape

a. Spores straight with sides parallel for most of their length or slightly curved. 1, 3, 5

b. Spores curved. 1, 3, 5

c. Spores cylindrical to robust with the sides parallel for most of their length. 2, 4

d. Spores thin, needle- like. 1

3. Shape of basal and apical cells

a. Pointed, basal cell not distinctly notched. 1, 2, 3, 5

b. Blunt, or with distinctly shaped basal and apical cells. Basal cell distinctly notched. 1, 3, 4

C. Microconidia from aerial mycelium

1. Present or absent

a. Present. 4

b. Absent. 1, 2, 3, 5

2. In chains or false heads

a. In chains and false heads. 4

3. Shape

a. Oval and 0- 1 septate. 4

D. Conidiophores

1. Type

a. Monophialides only (may produce either macroconidia or microconidia). 1, 2, 3, 4, 5

E. Chlamydospores

1. Present or absent

a. Present. 2, 3

b. Absent. 1, 4, 5

Synoptic key to *Fusarium* species in sections 4- 8 (species 6-23)

Species in the sections (4, 5, 6, 7 and 8) Sporotrichiella, Roseum, Arthrosporiella, Gibbosum and Discolor

A. Cultural characteristics

1. Rate of growth

a. Relatively slow, less than 7 cm diameter after 10 to 14 days. **19**

b. Growth rapid, more than 7 cm diameter after 10 to 14 days. **6, 7, 8, 9, 10, 11, 12, 13, 14 ,15, 16, 17, 18, 20, 21, 22, 23**

2. Colour of aerial mycelium

a. White. **6, 7, *8, 9*, 10, *11, 14, 15*, 16 ,17, 18 ,19, *20*, 21, *22 ,23***

b. Tan, brown. ***8, 9, 11*, 12, 13, *14, 15, 20, 22, 23***

3. Colour of colony from below.

Colour may diffuse into agar

a. Spores in light orange to orange masses scattered over the surface of the colony. **7, 8, 12, 14, 15, *20***

b. Spores in reddish- brown masses concentrated in the center of the colony. **10, 11, 16, 17, 18, 19, *20*, 21, 22, 23**

B. Macroconidia from sporodochia

1. Shape

a. Thin, needle- like, with thin walls. **10, *11***

b. Stout, with thick walls. ***20 ,21, 22, 23***

c. Curved but with the walls mainly parallel through most of their length. **6, 7, 8, 9, 18, *20, 21, 22***

d. Strong, dorsi- ventral curvature (hunch- backed). **14, 15, 16, 17, 19, *23***

e. Spindle- shaped and produced on monophialides or polyphialides in the aerial mycelium. ***11*, 12, 13**

2. Shape of basal and apical cells

a. Basal cell distinctly notched or foot- shaped (pedicellate). **6, 7, 9, 10, 11, *14, 16, 16, 17*, 18, 19, *20, 21, 22, 23***

b. Basal cell not distinctly foot- shaped. **8, *21***

c. Apical cell cone- like, elongated or whip- like. ***14*, 15, *16, 17***

d. Apical cell nipple- like, sometimes strongly curved as a beak. ***20, 21, 22, 23***

C. Microconidia from aerial mycelium

1. Present or absent

a. Scarce to none. **10, 11, 12, 13, *14*, 16, 17, 18, 19, *20*, 21, 22, 23**

b. Fairly abundant. ***14*, 15, *20***

c. Abundant. **6, 7, 8, 9**

2. Shape

a. Oval, ellipsoidal, comma- shaped, and club- shaped. **14, 15, 20**

b. Globose (napiform). **6**

c. Lemon- shaped, pear- shaped, and spindle- shaped. **7, 8**

d. Mainly spindle- shaped. **9**

D. Conidiophores

1. Type

a. Monophialides only (may produce either macroconidia or microconidia). 6, 7, 10, 11, 14, 16, 17, 18, 19, 20, 21, 22, 23

b. Monophialides (may produce either macroconidia or microconidia) and polyphialides (produce only microconidia and spindle- shaped macroconidia in the aerial mycelium). 8, 9, 12, 13, 15

E. Chlamydospores

1. Present or absent

a. Present. 6, 7, 8, 9, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23

b. Absent. 10, 11

Synoptic key to *Fusarium* species in sections 9-12 (species 24-30)

Species in sections 9, 10, 11 and 12 (Lateritium, Liseola, Elegans, Martiella and Ventricosum)

A. Cultural characteristics

1. Rate of growth

a. Relatively slow, less than 7 cm in diameter after 10 to 14 days. **24**

b. Growth rapid, greater than 7 cm diameter after 10 to 14 days. **25, 26, 27, 28, 29, 30**

2. Colour of aerial mycelium

a. White. 24, 25, 26, 27, 28, 29, 30

b. Tan to carmine red. 24

c. Light purple. 25, 26, 27, 28, 29

3. Colour of colony from below.
Colour may diffuse into agar

a. Carmine red. ***24***

b. Tan to orange. ***24, 25, 26, 27, 28, 29, 30***

c. Light to dark purple pigment diffusing into agar. ***25, 26, 27, 28, 29, 30***

4. Colour of spore masses (sporodochia)

a. Tan to orange. **24, 25, 26, 27, 28**, ***29***

b. Cream. ***29, 30***

c. Blue- green to blue. ***30***

B. Macroconidia from sporodochia

1. Size

a. Long. ***25, 26, 27, 28, 30***

b. Short. ***25, 26, 27, 28***, **29**, ***30***

2. Shape

a. Thin, with thin walls.25, 26, 27, 28

b. Relatively thin, with thin walls. 25, 26, 27, 28, 29

c. Stout with thick walls, and walls parallel through most of their length. **24, 30**

3. Shape of basal and apical cells

a. Apical cell with distinct beak or sharply curved. **24**

b. Apical cell not distinctly shaped. **30**

C. Microconidia from aerial mycelium

1. Present or absent

a. Sparse. ***24***

b. Abundant. ***24*, 25, 26, 2, 28, 29, 30**

2. In chains or false heads

a. In chains and false heads. **25, 26**

b. In false heads only. **24, 27, 28, 29, 30**

3. Shape

a. Oval to kidney- shaped (reniform) and clavate. **24, 25, 26, 27, 29, 30**

b. Oval, pear- shaped or globose (napiform). **28**

D. Conidiophores

1. Type

a. Medium length monophialides producing microconidia. **24**

b. Short monophialides producing microconidia. **29**

c. Long monophialides and polyphialides producing microconidia. **26, 27, 28**

E. Chlamydospores

1. Present or absent

a. Absent. **25, 26, 27, 28**

b. Sparse. **24**

c. Abundant. **29, 30**

APPENDIX 3 Preparation of scanning electron microscopy (SEM) reagents

2% Glutaraldehyde solution- was prepared by diluting 2 ml glutaraldehyde with 100 ml distilled water.

Phosphate buffer (pH 7.0)- was prepared by dissolving 0.275 g di-sodium hydrogen orthophosphate anhydrous (Na_2 H_2PO_4) and 0.199 g sodium dihydrogen orthophosphate dihydrate (NaH_2PO_4: $2H_20$) in a beaker containing 200 ml Milli- Q water. The pH of the solution was adjusted with 0.1 M HCl to pH 7.0. The solution was transferred to a 250 ml volumetric flask and made up to mark.

0.5% Osmium tetroxide- 0.5 g of osmium tetroxide was diluted to 100 ml with water.

APPENDIX 4 Preparation of reagents and stock solutions for mycotoxin extraction and purification, TLC and HPLC

Methanol- 1% KH_2PO_4 (100:100 v/v) (pH 3.0)- 1% KH_2PO_4 was prepared by diluting 1 g potassium dihydrogen orthophosphate to 100 ml distilled water. An equal volume of methanol was added to the above solution and the pH adjusted to 3.0 using 0.1 M HCl.

1 M HCl- 10 ml HCl (99.9% purity) diluted to 100 ml with water.

1% methanolic HCl- 1 ml of concentrated HCl diluted to 100 ml with methanol

1% methanolic acetic acid- 1 ml if concentrated acetic acid diluted to 100 ml with methanol.

Fusaric acid, moniliformin and fumonisin B_1 stock solutions- 1 g each of pure toxin standard was dissolved in 1 ml methanol ($1g.ml^{-1}$). A volume of 100µl was transferred into each of 10 eppendorfs (1 ml). The methanol was evaporated under a stream of nitrogen so that a pure toxin residue of 100 µg remained. These stocks were stored in a refrigerator at 4°C until required.

***p*- anisaldehyde spray reagent-** 5 ml concentrated sulphuric acid was added drop-wise to 70 ml methanol and the mixture allowed to cool for 30 minutes. Glacial acetic acid (10

ml) and 0.5 ml *p*- anisaldehyde (4- methoxybenzaldehyde) were finally added. The mixture was gently swirled before use.

40% methanol: 60% aqueous 0.62 mM Na_2EDTA with 2% H_3PO_4 (40:60 v/v)- 0.62 mM Na_2EDTA was prepared by diluting 0.231 g Na_2EDTA to 880 ml of Milli- Q water. The solution was then made to mark in a 1000 ml volumetric flask with 20 ml *ortho*-phosphoric acid. Methanol (400 ml) was then added to 600 ml of the above solution.

10 mM phosphate buffer with 5 mM tetra butyl ammonium bromide (pH 7.0)- was prepared by dissolving 0.55 g disodium hydrogen orthophosphate anhydrous (Na_2HPO_4), 0.379 g sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_20$) and 5 mM tetra butyl ammonium- bromide (TBAB) salt (1.511 g) in a beaker containing 200 ml Milli- Q water. The pH of the buffer solution was adjusted with 0.1 M HCl to pH 7.0. The solution was transferred to a 250 ml volumetric flask and made up to mark.

0.1 M NaH_2PO_4 (pH 3.3)- 15.6 g sodium dihydrogen orthophosphate was dissolved in 800 ml Milli- Q water and the pH adjusted to 3.3 with *ortho*-phosphoric acid. The solution was made up to mark in a 1000 ml volumetric flask.

***ortho*- phthaldialdehyde (OPA) derivatizing reagent**- was prepared by dissolving 0.04 mg OPA in 1 ml methanol, and adding 5 ml 0.1 M sodium tetraborate (3.8 g in 100 ml distilled water) and 50 µl 2- mercaptoethanol.

1% KH_2PO_4- 1 g potassium dihydrogen orthophosphate was dissolved in 100 ml distilled water.

5% aqueous $NaHCO_3$- 5 g sodium hydrogen carbonate was dissolved in 100 ml distilled water.

APPENDIX 5 Preparation of reagents for tissue culture and determination of cell numbers.

PBS (pH 7.2)- phosphate buffered saline was prepared by diluting a single tablet (OXOID) to 100 ml distilled water. The pH of this solution was approximately 7.2. The solution was sterilized in an autoclave at 110°C for 15 minutes. PBS (pH 7.2) was used prior to the trypsinization procedure to wash off cellular debris and dead cells.

0.25% Trypsin- 2.5 g lyophilized trypsin was rehydrated with 20 ml sterile distilled water and made up to 100 ml with PBS. The mixture was dispensed into 2 ml aliquots and stored at -20°C until required.

1% Trypsin- 10 g lyophilized trypsin was rehydrated in 20 ml sterile distilled water and made up to 100 ml with PBS. The final mixture was dispensed in sterile Bijou bottles.

Mycotoxins

Stock solutions of fusaric acid, moniliformin and fumonisin B_1 were prepared by dissolving 1 mg of each toxin in 6 µl ethanol, 34 µl dimethylsulphoxide (DMSO) and 1.96 ml phosphate buffered saline to produce a final stock concentration of 0.5 mg/ml. The stock solutions were then filtered- sterilized through a 0.45 µ-filter (Millipore).

MTT (3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)

MTT (Sigma) was dissolved in PBS (pH 7.2) at 5 mg/ml and filtered through a 0.45 μ-filter (Millipore) to sterilize and remove insoluble residues of the yellow powder. The stock solution was stored in the dark at 4°C until required.

Freezing agent- 20 ml dimethylsulphoxide (DMSO) and 20 ml 5% FCS was added to 60 ml EMEM. This preservative was then filter- sterilized using a 0.22μ-filter.

Determination of cell numbers

Cell counts were done using a Neubar counting chamber. This consisted of 2 chambers, each of which is divided into nine squares of $1mm^2$ each. A cover glass is supported 0.1 mm above these squares so that the total volume over each square is 1.0 mm × 1.0 mm × 0.1 mm (or 0.1 mm^3). With a 10X objective, and a 10 ocular, one square (1 mm^2) will fill approximately the entire microscope field. According to convention, the cells touching the left and top margins of each square are included in the count.

Procedure:

After trypsinization, the cells were diluted in 20 ml 5% EMEM. A volume of 200 μl cells was added to 100 μl trypan blue so that a 1:2 dilution was obtained. A capillary tube was used to fill both chambers. Cells were counted with an inverted microscope at 100X magnification. The number of cells per ml in the original sample were counted as follows:

$$\text{cell/ml} = \text{average count per square} \times 10^4 \times \text{dilution factor}$$

APPENDIX 6A Percentage cell viability (%) of A549 (lung carcinoma) cells after 24 hours exposure to the mycotoxins fusaric acid, moniliformin and fumonisin B_1. Values given are the mean of 5 replicates with standard deviation shown in brackets.

	% Cell Viability in the MTT Cleavage Assay			% Cell Viability in the Crystal Violet Test		
Toxin concentration (ugh/ml)	**FA**	**MON**	**FB_1**	**FA**	**MON**	**FB_1**
0.5	2.7 (±0.05)	2.9 (±0.004)	24.3 (±0.01)	5.1 (±0.014)	1.26 (±0.008)	30.5 (±0.047)
0.25	12.7 (±0.018)	10 (±0.011)	47.1 (±0.019)	8.9 (±0.039)	20.6 (±0.044)	57.5 (±0.02)
0.125	26.7 (±0.023)	25.7 (±0.013)	58.6 (±0.022)	20 (±0.05)	45.9 (±0.019)	67.1 (±0.031)
0.063	35.7 (±0.013)	40 (±0.015)	67.1 (±0.028)	24.7 (±0.047)	57.9 (±0.042)	75.3 (±0.028)
0.031	48.7 (±0.026)	55.7 (±0.014)	78.6 (±0.016)	35.6 (±0.023)	73.6 (±0.039)	85.9 (±0.036)
0.015	64 (±0.03)	57.1 (±0.024)	82.4 (±0.012)	49.4 (±0.048)	86.6 (±0.024)	89.4 (±0.03)
0.007	82.7 (±0.044)	82.9 (±0.02)	85.7 (±0.024)	65.3 (±0.046)	90.7 (±0.03)	92.5 (0.039)
0.004	94.0 (±0.033)	94.3 (±0.03)	91.4 (±0.03)	94.7 (±0.039)	96.8 (±0.018)	95.2 (±0.049)

APPENDIX 6B Percentage cell viability (%) of VK (monkey kidney) cells after exposure to the mycotoxins fusaric acid, moniliformin and fumonisin B_1 in the MTT assay and the crystal violet test.

	% Cell Viability in the MTT Cleavage Assay			% Cell Viability in the Crystal Violet Test		
Toxin concentration (μg/ml)	**FA**	**MON**	**FB_1**	**FA**	**MON**	**FB_1**
0.5	30 (±0.047)	14.7 (±0.019)	11.9 (±0.035)	33.6 (±0.03)	21.1 (±0.042)	11.5 (±0.045)
0.25	42.3 (±0.041)	27.3 (±0.018)	23.5 (±0.05)	44.3 (±0.047)	29.7 (±0.041)	26.1 (±0.038)
0.125	59.3 (±0.023)	35.3 (±0.042)	34.8 (±0.046)	66.1 (±0.015)	35.6 (±0.037)	33.4 (±0.045)
0.063	63.7 (±0.037)	51.5 (±0.036)	46.8 (±0.019)	67.7 (±0.039)	54.2 (±0.046)	46.6 (±0.041)
0.031	85.3 (±0.035)	63.4 (±0.027)	54.5 (±0.018)	87.3 (±0.05)	65.5 (±0.037)	59.2 (±0.034)
0.015	90.7 (±0.039)	77.8 (±0.021)	66.1 (±0.048)	91.9 (±0.047)	84.7 (±0.047)	70.7 (±0.05)
0.007	94.1 (±0.018)	90.0 (±0.029)	82.7 (±0.022)	98 (±0.027)	92.7 (±0.02)	84.7 (±0.036)
0.004	96.1 (±0.014)	94.5 (±0.025)	96.7 (±0.048)	99.5 (±0.011)	94.7 (±0.02)	96.7 (±0.037)