

**PARAMETERS AFFECTING THE
PRODUCTION OF FUMONISIN B₁ BY
FUSARIUM VERTICILLIOIDES
IN CULTURE**

**Thesis submitted in the fulfilment of the requirements for the degree
MASTER OF SCIENCE
in the Department of Microbiology, University of the Western Cape**



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Dedicated to my parents,
whose love, support and encouragement
have made the realization of
my educational goals possible.

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Table of contents

	Page
Declaration	iii
Acknowledgements	iv
 CHAPTER 1	
General Introduction and Literature Review	2
 CHAPTER 2	
The production of fumonisin B₁ by <i>Fusarium verticillioides</i> strains in culture	
Abstract	52
Introduction	54
Materials and Methods	55
Results	62
Discussion	72
Acknowledgements	75
References	76
 CHAPTER 3	
The viability of lyophilised conidia of <i>Fusarium</i> and <i>Alternaria</i> species assayed with fluorescent and other stains	
Abstract	82
Introduction	84
Materials and Methods	86
Results	89
Discussion	94
Acknowledgements	96
References	97

CHAPTER 4

The antifungal effect of fumonisin B₁ on *Fusarium* and other fungal species

Abstract	101
Introduction	102
Materials and Methods	103
Results	105
Discussion	112
Acknowledgements	114
References	115

CHAPTER 5

The effect of fumonisin B₁ on the germination of conidia of *Fusarium* and other fungal species

Abstract	121
Introduction	122
Materials and Methods	123
Results	127
Discussion	141
Acknowledgements	143
References	143

SUMMARY	149
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Declaration

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW



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1. General Introduction and Literature Review

Fusarium species are widely distributed in soil and on agricultural commodities, both in temperate and tropical regions, and are known to be some of the most important plant pathogens (Booth, 1971). *Fusarium* species such as *F. verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), *F. graminearum* Scwabe, *F. avenaceum* (Fr.) Sacc. and *F. culmorum* (Smith WG) Sacc. are serious pathogens of grains causing several plant diseases. Although the predominant interest in the genus has been their role as plant pathogens, their involvement in human and animal diseases has been studied since the early 1900.

Fusarium species are frequently associated with diseases in animals including cattle, sheep, pigs and poultry (Wyllie and Morehouse, 1978; Marasas *et al.*, 1984). The toxicological effect of different *Fusarium* species in experimental animals are well documented and fully reviewed by Marasas *et al.* (1984). During the past 20 years *F. verticillioides* received special attention regarding its role in human and animal diseases. This fungus occurs on maize and other cereals throughout the world and its toxicity is being investigated extensively. Various strains of *F. verticillioides* were found to be highly toxic to experimental animals (Marasas *et al.*, 1984).

1.1 Fumonisin (Cawood *et al.*, 1991)

Many fungi commonly found on grain products have the capacity to form chemical substances that are toxic, mutagenic or carcinogenic when consumed by humans or animals. These substances are called mycotoxins, a term derived from “myco”, meaning fungus or mould, and “toxin”, meaning poison. The toxins can accumulate in maturing maize, cereals, soybeans, sorghum, peanuts, etc. and may remain in food and feed long after the fungus that

produced them has died. These toxins can therefore be present at potentially dangerous levels in products that are not visibly mouldy. They can also develop under storage conditions favourable for the growth of the toxin-producing fungus or fungi. Diseases caused by mycotoxins in humans and animals are called mycotoxicoses and are specific to the fungal species and the toxin present. Maize appears to be a common substrate for the natural occurrence of many mycotoxins.

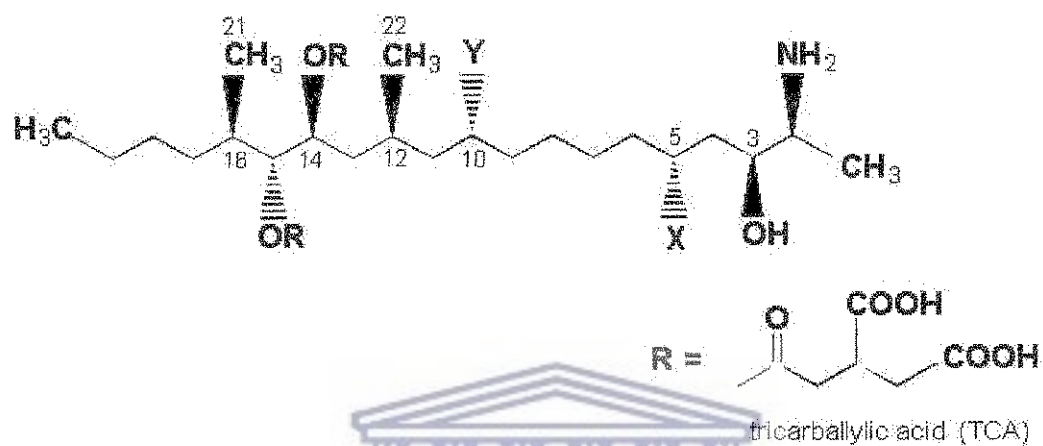
Among the various categories of identified mycotoxins, the fumonisins are assuming a growing significance since their discovery due to their diverse toxicological effects in humans and various animals, including horses, pigs and experimental animals (Gelderblom *et al.*, 1991; 1996; Marasas, 1996). Fumonisins are synthesized by fungal species belonging to the genus *Fusarium*, and are a group of structurally related mycotoxins that occur worldwide. The highest level of fumonisin B₁ recorded is 17900 µg/g, produced by *F. verticillioides* MRC 826, on sterilised maize (Alberts *et al.*, 1994). This is also the first known *Fusarium* species to synthesize fumonisins. *F. verticillioides* is one of the most prevalent seed-borne fungi associated with maize (*Zea mays*) intended for human and animal consumption throughout the world (Marasas *et al.*, 1984). This fungal species can be recovered from most maize kernels including those that appear healthy. There is also a positive correlation between the formation of fumonisins in maize in the field and the incidence of *F. verticillioides* (Rheeder *et al.*, 1992). *Fusarium* kernel rot of maize can be caused by *F. verticillioides* which is one of the most important ear diseases in warmer maize-growing areas. Fumonisins are also much more likely to be formed in maize in warm to hot, dry regions, conditions most common in the Transkei region of the Eastern Cape Province, South Africa as well as in China where the incidence of oesophageal cancer (OC) is also very high (Rheeder *et al.*, 1992).

Fumonisins were first isolated in 1988 from maize cultures of *F. verticillioides* strain

MRC 826 at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the South African Medical Research Council (MRC) by Gelderblom *et al.* (1988a). The structures of fumonisins were elucidated in 1988 in collaboration with the South African Council for Scientific and Industrial Research (CSIR) (Bezuidenhout *et al.*, 1988). One particular analogue turned out to be more frequently traceable, both in cultures of *F. verticillioides* on maize and in naturally contaminated substrates. This analogue was given the name fumonisin B₁ (FB₁). Characteristically, *F. verticillioides* strains are capable of producing this metabolite in higher concentrations than the other fumonisins (Marasas, 1996).

1.1.1 Characteristics

Fumonisin are a group of structurally related, polar metabolites (Bezuidenhout *et al.*, 1988). Their structures are based on a long hydroxylated hydrocarbon chain (pentahydroxyeicosane) containing methyl and either amino (B₁ and B₂) or acetyl amino groups (A₁ and A₂) (Dutton, 1996). FB₁ differs from FB₂ in that it has an extra hydroxyl at position 10 of the C-backbone of the fumonisin (Figure 1), the backbone of the FB₁ molecule can be chemically converted to FB₂ (Badria *et al.*, 1995). FB₃ was isolated later (Gelderblom *et al.*, 1992a; Plattner *et al.*, 1992b), and was found to differ from the other fumonisin analogues in the hydroxylation pattern (Figure 1).



GENERAL CHEMICAL STRUCTURE OF THE FUMONISINS

Fumonisin B₁ : X = OH, Y = OH

Fumonisin B₂ : X = OH, Y = H

Fumonisin B₃ : X = H, Y = OH

Figure 1. Chemical structure of fumonisins B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃).

1.2 Role of *F. verticillioides*

F. verticillioides (synonym = *F. moniliforme*), is frequently detectable in cereals, in particular as one of the fungi most frequently associated with maize (Marasas *et al.*, 1979; Bacon and Williamson, 1992; Marasas, 1996). *F. verticillioides* was also isolated from other substrates such as sorghum and millet (Gelderblom *et al.*, 1988a; Nelson *et al.*, 1991; Thiel *et al.*, 1991; Sydenham *et al.*, 1992). Due to the consumption of cereals, it was suspected that this species and its metabolites may be associated with disease in both humans and animals.

With the characterization of the fumonisins in 1988 by Gelderblom *et al.* (1988a), the toxic metabolites were initially detected in a culture of *F. verticillioides*. Most of the initial research concerning the fumonisins was related to the toxic substances produced by the strain of *F. verticillioides* known as 'MRC 826', originally isolated from maize originating from the Transkei region of the Eastern Cape Province, South Africa, where the inhabitants suffer from a high incidence of OC. In addition to its cancer-promoting activity in a short-term bioassay in rat liver (Gelderblom *et al.*, 1988a; 1988b; 1991), the role of pure FB₁ in leukoencephalomalacia (ELEM) in horses (Marasas *et al.*, 1988; Kellerman *et al.*, 1990) and pulmonary edema syndrome (PES) in pigs (Harrison *et al.*, 1990) has been confirmed.

Research conducted by Nelson *et al.* (1992), outlined the fungal species that produce fumonisins. The study involved 90 strains of *F. verticillioides* obtained from various geographical areas and isolated from different substrates. They were able to verify that only three of the *F. verticillioides* strains studied, did not demonstrate the capability of synthesizing FB₁, while the remaining 87 strains produced fumonisins in variable concentrations, ranging from minimal values of less than 10 µg/g to maximum values greater than 6000 µg/g. Concentrations of more than 1000 µg/g of FB₁ were demonstrated in 38 cultures out of the 90 *F. verticillioides* strains studied.

1.3 Other toxigenic species belonging to the genus *Fusarium*

Several studies in recent years have shown that the synthesis of fumonisins is not a unique characteristic of *F. verticillioides* strains, but that several other species of the genus *Fusarium* are involved. Eight *Fusarium* species other than *F. verticillioides* have been reported to produce fumonisins (Table 1). Within the section *Liseola*, five are identified; *F. proliferatum* (Matsushima) Nirenberg (Ross *et al.*, 1990; Thiel *et al.*, 1991), *F. anthophilum* (A. Braun) Wollenw. (Nelson *et al.*, 1992), *F. thapsinum* Klittich *et al.* (Klittich *et al.*, 1997), *F. globosum* Rheeder *et al.* (Sydenham *et al.*, 1997), three within the Section *Dlaminia*; *F. dlamini* Marasas *et al.* (Nelson *et al.*, 1992), *F. napiforme* Marasas *et al.* (Nelson *et al.*, 1992) and *F. nygamai* Burgess and Trimboli (Thiel *et al.*, 1991); one species in Section *Elegans*, *F. oxysporum* Schlecht. (Abbas and Ocamb, 1995); and one species in Section *Arthrosporiella*, *F. polyphialidicum* Marasas *et al.* (Abbas and Ocamb, 1995).

The relative production of FB₁, FB₂ and FB₃ for the species in the Section *Liseola* are summarized in Table 1. *F. verticillioides* and *F. proliferatum* are the most important producers of fumonisins which is noticeable in their overall higher levels of production and their association with several animal mycotoxicoses (Ross *et al.*, 1990; 1992).

F. globosum, a recently described *Fusarium* species classified in the section *Liseola*, was originally isolated from naturally infected maize kernels harvested in the Transkei region of the Eastern Cape Province, South Africa (Rheeder *et al.*, 1996).

Table 1. Fumonisin B₁, B₂ and B₃ production by *Fusarium* species.

<i>Fusarium</i> spp	Maximum fumonisin levels (μg/g)*		
	FB ₁	FB ₂	FB ₃
<i>F. verticillioides</i>	17 900	3 000	2 325
<i>F. proliferatum</i>	4 500	2 060	310
<i>F. anthophilum</i>	613	35	ND**
<i>F. globosum</i>	3 254	4	24
<i>F. dlamini</i>	82	ND	ND
<i>F. napiforme</i>	479	ND	ND
<i>F. nygamai</i>	7 162***	530	143
<i>F. thapsinum</i>	30	5	5

* = Summarised fumonisin levels from the literature. Respective FB₁₋₃ levels are not indicative of only one strain per species.

ND = No Data

*** = This high level of 7162 μg/g was recorded for only one strain. The next highest levels of FB₁ production by *F. nygamai* in the region of 3500 - 4000 μg/g.

2. Diseases caused by and effects of Fumonisin

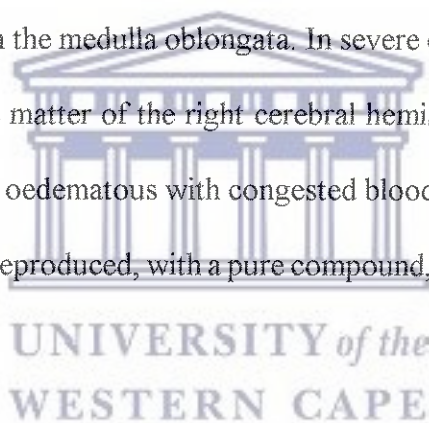
2.1 Horses

Diseases in animals and humans resulting from the consumption of mycotoxins are called mycotoxicoses. The effects in domestic animals include allergic reactions, reproductive failure, loss of appetite and suppression of the immune system. The involvement of PROMEC with the toxicology of *F. verticillioides* started in July 1970 when some horses in South Africa died of a neurotoxic disease known as equine leukoencephalomalacia (ELEM) (Kellerman *et al.*, 1972). It was also known that similar field outbreaks of ELEM in horses and donkeys occurred regularly in many countries (e.g., United States, Argentina, Egypt and China). Thousands of horses died of the disease in the United States during some seasons (Marasas *et al.*, 1984).

The predominant fungus isolated from the maize associated with field outbreak in South Africa during 1970 was *F. verticillioides*. In earlier diagnosed cases of ELEM (Wilson and Maronpot, 1971; Kellerman *et al.*, 1972; Marasas *et al.*, 1976; Pienaar *et al.*, 1981), common elements were observed in the affected animals. They were : (1) inappetence after a period of eating contaminated feed; (2) lethargy; and (3) as neurotoxic effects including uncoordinated movement and aimless walking with blindness. Death can also occur in some cases without any nervous symptoms and in others, liver-related symptoms are seen, such as swelling of the lips and nose and severe icterus (Dutton, 1996). When Prof BJ Wilson of the University of Tennessee reported that he had reproduced ELEM experimentally with an Egyptian isolate of *F. verticillioides* in a donkey, researchers from PROMEC started to dose pure cultures of *F. verticillioides* to horses at Onderstepoort (Wilson and Maronpot, 1971). The causative role of *F. verticillioides* in ELEM was confirmed with several South African

isolates of the fungus and the pathognomonic pathological changes were described in detail (Kellerman *et al.*, 1972; Marasas *et al.*, 1976; Pienaar *et al.*, 1981).

The mycotoxin which caused ELEM was at that time still unknown because intravenous injection of donkeys with metabolites of *F. verticillioides* (Fusarin C, moniliformin (MON), fusaric acid, and 2-methoxy-4-ethylphenol) failed to reproduce the disease (Buck *et al.*, 1979). This resulted in great confusion in the literature regarding mycotoxins produced by this species (Marasas *et al.*, 1984; Marasas *et al.*, 1986; Mirocha *et al.*, 1990). Marasas *et al.* (1988) was the first to describe the reproduction of ELEM by intravenous injection of a horse with FB₁. Clinical signs of neurotoxicosis appeared on day 8. Lesions included severe oedema of the brain and focal necrosis in the medulla oblongata. In severe cases, there may be a large liquified cavity within the white matter of the right cerebral hemisphere, with the cerebrum posterior to the cavity enlarged, oedematous with congested blood vessels (Kellerman *et al.*, 1972). ELEM was successfully reproduced, with a pure compound, by Kellerman *et al.* (1990) in two horses by oral dosing.



2.2 Pigs

ELEM is certainly not the only animal disease associated with consumption of feed contaminated with *F. verticillioides*. A 1981 study of the oral toxicity of *F. verticillioides* culture material to various animal species reported the deaths of two of three treated pigs (Kriek *et al.*, 1981). The principal lesions, however, were not in the brain, but in the lungs, where a fatal edema developed. An outbreak of PES in pigs occurred during the fall of 1989 when 34 mature pigs died on two farms in Georgia, USA (Harrison *et al.*, 1990). The animals had pulmonary oedema and hydrothorax, the thoracic cavities being filled with yellow liquid. This discovery led to the suspicion that a mycotoxicosis was involved. Abortions also occurred

on both farms and these stopped when the maize screenings were removed (Harrison *et al.*, 1990). The problem was traced to maize screenings from which *F. verticillioides* was isolated. The maize screenings were found to contain FB₁ at levels of up to 155 µg/g. Several studies linked PES with outbreaks of ELEM (Ross *et al.*, 1990), and it became accepted that the two diseases had a common cause, i.e., fumonisins.

2.3 Rats

Several experiments had been done where rats were dosed with maize infected with *F. verticillioides* related to outbreaks of ELEM (Wilson *et al.*, 1985; Voss *et al.*, 1989) or to OC (Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987b; Gelderblom *et al.*, 1988a). Rats were used as a model to explore the carcinogenic potential of fumonisins. Several studies on rats were done by researchers at PROMEC (Gelderblom *et al.*, 1991; 1992b; Shephard *et al.*, 1992a; 1992b; Gelderblom *et al.*, 1994; Shephard *et al.*, 1994a; 1994b). These studies were divided in two types, those exploring the absorption and excretion of fumonisins and those examining its effect on the animal. Voss *et al.* (1989) found that maize naturally contaminated with *F. verticillioides* associated with outbreaks of ELEM, was hepatotoxic when fed to rats. Wilson *et al.* (1985) reported that naturally contaminated maize, associated with ELEM, was hepatocarcinogenic when fed to rats for 123 to 176 days.

Gelderblom *et al.* (1988a) showed that FB₁ was hepatotoxic and induced glutamyltranspeptidase-positive GGT foci in the liver when fed to rats. The presence of GGT foci in the liver was taken as an indication of tumour promoting activity. A study conducted by Gelderblom and coworkers in South Africa (Gelderblom *et al.*, 1991) demonstrated that pure FB₁ can cause an increased incidence of hepatocellular carcinoma when fed to rats for 26 months at 50 µg/g feed. Subsequent studies demonstrated hepatocarcinogenesis of FB₁, FB₂ and FB₃ in short term (3 week) initiation-promotion bioassays in rat liver (Gelderblom *et al.*,

1993).

2.4 Poultry

Considering the economic importance of chickens and their dependence on maize-based feeds, which are often contaminated with *F. verticillioides* (Bragulat *et al.*, 1995a; 1995b), investigations using fumonisins only began in 1992, although earlier trials had been done with maize infected with *F. verticillioides* (Bryden *et al.*, 1987). Day-old broiler chicks were fed doses of FB₁ ranging from 0 to 400 µg/g 21 days and 300 µg/g for 2 weeks (Brown *et al.*, 1992; Ledoux *et al.*, 1992). Body weight gain was greatly reduced; hepatic necrosis, biliary hyperplasia, and thymic cortical atrophy were noted, along with diarrhoea and rickets.

Increased sphiganine and sphiganine:sphingosine ratios were shown in young chicks treated with culture material containing FB₁. Weibking *et al.* (1993) also found that day-old chicks fed ratios containing 199 and 200 µg/g of FB₁ for 21 days had lower body weight gains and feed efficiency when compared to the controls. There were also differences in organ weights.

Ducklings were used by Marasas (1982) and Vesonder *et al.* (1989) as a test animal to investigate material from an OC area and ELEM outbreak, respectively. Some of the isolates of *F. verticillioides*, when grown and incorporated into feed, caused 100 % mortality. These birds had slightly swollen and reddened livers, with low body fat and loss in weight compared to the controls.

3. Human Involvement

3.1 Human Oesophageal Cancer

Although many aetiological factors may play a role in OC, attention in this review will be focussed on only one factor, i.e. contamination of maize with fumonisins. Available evidence indicates that fumonisins are a risk factor for OC in different parts of the world. Very high incidence rates of OC (>50 per 100000 per annum) have been reported in three geographical areas in the world, e.g., Transkei region of the Eastern Cape Province of South Africa (Van Rensburg, 1985, Jaskiewicz *et al.*, 1987a; 1987b; Makaula *et al.*, 1996), Linxian County of Henan Province in northern China (Li *et al.*, 1980; 1989; Yang, 1980) and the Caspian littoral of Iran (Homozdiari *et al.*, 1975). The development of oesophageal brush cytological screening methods has presented real possibilities of the early diagnosis and treatment of OC in remote areas in Southern Africa (Jaskiewicz *et al.*, 1987b; Venter, 1995a; 1995b; 1998).

In South Africa, OC is the most common cause of cancer death in black males while in black females OC is the third most common cancer (Sitas *et al.*, 1997). The incidence rates of OC in South African whites are 3-4 times lower (Sitas *et al.*, 1997). Within South Africa, the highest incidence rates in both males and females are amongst the Xhosa-speaking population of rural Transkei, where age-standardised incidence rates (ASIR) as high as 55.6 per 100 000 per annum have been reported in males in the Kentani district during the period 1985-1990 (Makaula *et al.*, 1996).

In most rural communities in Southern Africa, the main staple diet is maize. Maize which is grown in these and other areas of the world, is highly susceptible to infection by several fungi, in particular *Fusarium* spp. and of these, *F. verticillioides* is the most prevalent

(Marasas *et al.*, 1979; 1980; 1982; 1988). Investigations into the high incidence rates of OC experienced in areas of the Transkei region of the Eastern Cape of South Africa and in Northern China, led to the discovery of this fungus in maize and the mycotoxins produced by them. Further investigations revealed very high levels of fumonisins in maize that was being used for human consumption in these regions. A significant difference in the levels of fumonisins in maize between high and low-risk OC areas in Transkei was found (Rheeder *et al.*, 1992; Marasas *et al.*, 1993). Higher levels of FB₁ and FB₂ in good as well as mouldy maize from these high-risk OC areas than those in corresponding maize from low rate areas.

In China it was also found that the incidence of fumonisins in high-risk areas was twice that in low risk areas (Chu and Li, 1994; Yoshizawa *et al.*, 1994). The FB₁ levels in some of these maize samples were very high, up to 118 µg/g in central China (Shephard *et al.*, 1996; Rheeder *et al.*, 1992). FB₁ and FB₂ were found in “good” homegrown maize in both low and high OC areas. FB₁ ranged from 0-550 µg/kg for low and 50-7900 µg/kg for high OC areas (Sydenham *et al.*, 1990b). In some cases in 1989, a 10-fold increase of FB₁ in the high OC areas (up to 117.52 µg/kg) over low (11.34 µg/kg) was reported. Maize meal from South Africa had levels of FB₁ ranging from 0-475 µg/kg, and that from Egypt, up to 2980 µg/kg (Sydenham *et al.*, 1991). High levels of FB₁ have also been reported from around the world (Sydenham *et al.*, 1993).

The role of fumonisins in human diseases and, most particularly, their carcinogenic potential in humans is very difficult to determine. Many research centers such as PROMEC, the International Agency for Research on Cancer (IARC), the U.S. Food and Drug Administration (FDA), etc. are in the process of evaluating the carcinogenic potential of fumonisins. These studies include the carcinogenicity in experimental animals, the mechanism of action of fumonisins in various animal models and in cell culture systems and

epidemiological studies in humans. Concern about the toxicological hazards posed to humans by the fumonisins, has led to the classification of these toxins derived from *F. verticillioides* as Group 2B carcinogens (i.e. possibly carcinogenic to humans) by the IARC in Lyons, France (IARC, 1993). This presentation reviews the literature since the characterization of fumonisins in 1988 until 1998, on fumonisin production by *Fusarium* species.

3.2 Control

3.2.1 Risk assessment

From the above mentioned information, it is certain that people who eat maize, are exposed to fumonisins. The evidence also indicates that certain rural populations are exposed to high levels of fumonisins. Thiel *et al.* (1992) estimated that people living in Transkei would have a daily intake of 0.014 $\mu\text{g/g}$ body weight from consuming so-called healthy maize, and this intake will increase to 0.44 $\mu\text{g/g}$ when mouldy maize is eaten. These risk assessments indicate a range of possibilities from very low risk in Switzerland, the Netherlands and USA (mean daily intake ranging from 0.02 - 0.09 $\mu\text{g/kg}$ body weight) to a very high risk in parts of rural South Africa. A number of estimates made for South Africans showed the considerable impact of differing maize consumption patterns by different population groups (Gelderblom *et al.*, 1996). Lower tolerance levels would be appropriate for countries where consumption of maize products is high, and higher levels for countries where consumption is low.

3.2.2 Limiting contamination

Mycotoxin production in the field is difficult to control. It is important to know and follow practices that minimise mycotoxin production levels after harvesting and production of foodstuffs. When weather conditions or hail predispose grain to infection by toxic fungi, it is

best to treat this grain with extreme caution. Storage of grain and feed at low moisture contents and temperatures will help prevent fungal growth. *F. verticillioides*, for example, will not grow in starchy seeds unless the moisture content is higher than 22 to 24 % (Kommedahl and Windels, 1981). When appropriate moisture and temperature conditions are maintained, fumonisin levels are believed not to increase during storage (Munkvold and Desjardins, 1997). Of great importance to the rural farmer is assistance with farming practices to limit mycotoxin formation during growing, harvesting and storage of maize. Methods such as crop rotation, sun drying of crops, and simple sealed storage facilities could minimise much of the spoilage that currently occurs (Dutton *et al.*, 1993).

A number of strategies for reducing fumonisin concentrations in maize are currently under development. Prevention of fumonisin production can be assisted by developing cultivars that are resistant to infection with toxigenic strains of *Fusarium* (Blackwell *et al.*, 1999). Genetic manipulation may provide solutions to the problem surrounding fumonisins in maize. Among the possibilities are genetically engineered resistance to *Fusarium* infection or detoxification of fumonisins in planta (Munkvold and Desjardins, 1997). Engineering plants to produce antifungal proteins is a possible approach to enhancing resistance to fungi (Munkvold and Desjardins, 1997).

4. Factors interfering with fumonisin synthesis

Although *F. verticillioides* occurs world-wide on a great variety of plant hosts and is one of the most prevalent fungi on maize, it does not produce equal amounts of fumonisins on all of them (Nelson *et al.*, 1991). Fungi may grow well under a given set of conditions but not necessarily produce mycotoxins. The quality of the grain and its suitability for storage are

adversely affected by a high moisture content, physical damage to the kernels, and the extent to which storage fungi have invaded the seed (Munkvold and Desjardins, 1997). In the field, during harvest and storage, maize grains are exposed to fungal contamination and the degree of mycotoxin contamination depends on environmental factors, the composition of the substrate, inoculum level and the length of incubation (Marin *et al.*, 1998).

4.1 Intrinsic parameters

Those parameters of plant and other substrates that are an inherent part of the tissues are referred to as intrinsic parameters. These parameters include pH, moisture content and nutrient content. It has been well established that fungi grow best at pH values below 4.0. It is a common observation that fruits undergo post-harvest fungal spoilage and this is due to the ability of these organisms to grow at pH values less than 3.5 (Mossel and Ingram, 1955; Juven, 1976).

Relative humidity (RH) of the incubation environment is important when controlling the water activity (a_w) within the substrate surfaces (Hattingh, 1995; James, 1996). When the a_w of the substrate is set at 0.96 a_w , it is important that the cultures should be incubated under conditions of relative humidity that do not allow the substrate to absorb moisture from the air or lose moisture when placed in an environment of low RH. There is a relationship between RH of the incubation chamber, the a_w of the substrate and the incubation temperature (James, 1996). In general, the higher the temperature, the lower the RH and *vice versa*.

The a_w of a substrate represents the activity with which the water content of the product makes itself noticeable externally (Rockland and Beuchat, 1987; Hattingh, 1995; James 1996). The a_w -value of a product depends on the composition, the water content and the temperature of the product. The water content would therefore express the origin whereas

a_w expresses the effect of moisture. Water activity is a critical environmental factor determining whether a substrate will support fungal growth and toxin production (Marin *et al.*, 1995).

It is important to note that the a_w is influenced by other environmental parameters such as pH, temperature and nutrition (Guthertz *et al.*, 1976). Firstly, at any temperature, fungal growth is reduced as the a_w is lowered. Secondly, the range of a_w over which growth occurs is greatest at the optimum temperature for growth. Thirdly, the presence of nutrients increases the range of a_w over which the fungi can survive. The general effect of lowering a_w below the optimum is to increase the length of the lag phase of growth and to decrease the growth rate.

Marin *et al.* (1995) regard the influencing a_w and temperature, on growth and FB₁ and FB₂ production by *F. verticillioides* on maize grain as the most critical environmental factors determining whether a substrate will support fungal growth and toxin production. Their objective was to determine the effect of a_w and temperature on colonization and production of FB₁ and FB₂ by Spanish isolates of *F. verticillioides* (25N) and *F. proliferatum* (73N and 131N) on maize kernels placed in petri dishes. Isolates examined in their study are representative of high fumonisin producers in Spain only, while strains from other parts of the world may differ in their ability to produce fumonisins in high levels. They showed in their study that there was an increase in fumonisin production with time. Maximum FB₁ and FB₂ was produced by isolates of both *F. verticillioides* (25N) and *F. proliferatum* (73N and 131N) at 0.956 and 0.968 a_w at 25°C and 30°C.

Cahagnier *et al.* (1995) studied the link between the growth of *F. verticillioides* and its production of fumonisin on maize grain, as a function of thermodynamic water activities. Experiments on maize grain at different a_w have demonstrated the influence of a_w on fumonisin biosynthesis, and on fungal growth defined by measurement of ergosterol levels. Fumonisin levels decreased threefold when a_w was lowered by 5%, while the growth rate of the fungus

was unchanged. The quantities of fumonisin produced by the same strain were 3000, 1100 and 11 $\mu\text{g/g}$, for respective a_w values of 1.00, 0.95 and 0.90.

It has been shown that moisture content and temperature are crucial in determining the extent of fumonisin production by *F. verticillioides* (Alberts *et al.*, 1990). The growth rate of *F. verticillioides*, as measured by ergosterol concentration, was higher at 25°C than at 20°C, reaching a stationary phase after four to six weeks in both cases. FB₁ production commenced after two weeks during the active growth phase, continued to increase during the stationary phase, and decreased after 13 weeks. The overall maximal yield of FB₁ (17.0 g/kg, dry weight) was obtained in maize cultures incubated at 20°C for 13 weeks, but it was not significantly higher than the maximum yield (16.6 g/kg, dry weight) obtained at 25°C after 11 weeks. However, a significantly higher mean yield was detected at 25°C (9.5 g/kg, dry weight) than at 20°C (8.7 g/kg, dry weight). Production reached a plateau after seven weeks of incubation at 25°C and after nine weeks of incubation at 20°C.

Another method involves medium consisting of 500 g yellow maize kernels and 500 ml distilled water added to a 30.5 x 61 cm autoclavable polyethylene bag (Nelson *et al.*, 1994). The maize was inoculated by drawing a suspension from a lyophilized culture into a sterile 5 ml syringe fitted with a 19 gauge needle and injecting 1 ml through the side of each polyethylene culture bag. Bags of inoculated maize were incubated in the dark at 20°C to 22°C for 4 weeks. Seven to 8 days after inoculation, holes were punched near the tops of the bags to promote aeration. Nelson *et al.* (1994) found that the most important factors in the production of fumonisins in bulk maize cultures were temperature control, moisture and aeration. Yields obtained in this study were consistent and ranged from 6 to 10 g/kg of FB₁.

In order to grow and produce mycotoxins the fungus of interest requires water, a source of energy, vitamins and related growth factors and minerals and or trace metals. The

influence of zinc, iron, cobalt, and manganese on submerged cultures of *F. verticillioides* NRRL 13616 was assessed by measuring dry weight accumulation, fusarin C biosynthesis, and ammonia assimilation (Jackson *et al.*, 1989). Zinc (26 to 3,200 ng/ml) inhibited fusarin C biosynthesis, increased dry weight accumulation, and increased ammonia assimilation. Maximum fusarin C levels, 32.3 $\mu\text{g}/\text{mg}$ (dry weight), were produced when cultures were supplied with manganese, whereas minimum fusarin C levels, 0.07 $\mu\text{g}/\text{mg}$, were produced when zinc, iron, cobalt, and manganese were supplied. These results suggest a multi-functional role for zinc in affecting *F. verticillioides* metabolism. Another study done by Scott *et al.* (1986) showed the effect of trace metal nutrition on the functioning of the patulin biosynthetic pathway in submerged cultures of *Penicillium griseofulvum* Dierckx [syn. *Penicillium urticae* (Bainier)] (NRRL 2159A). Of eight metal ions examined, only manganese strongly influenced secondary metabolite production. In control cultures or cultures deficient in calcium, iron, cobalt, copper, zinc, or molybdenum, pathway metabolites appeared in the medium at about 25 hr after inoculation. In manganese-deficient cultures, 6-methylsalicylic acid continued to accumulate, with only minor amounts of patulin being produced. Clear dose responses (patulin *versus* manganese) were found in different media, with no effect on growth yield. Addition of manganese to depleted cultures at 18, 26, or 36 hr resulted in increasing marker enzyme activity and patulin concentrations.

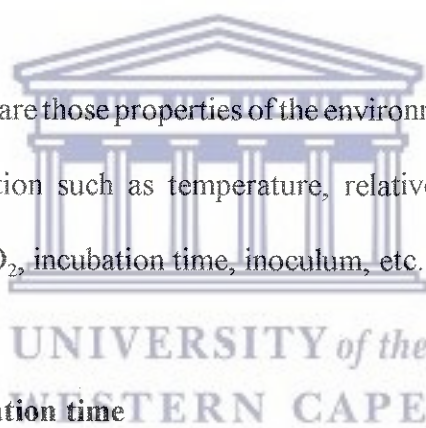
Certain metals added as salts to a defined basal culture medium influenced the level of aflatoxin production by *Aspergillus parasiticus* Speare in the low $\mu\text{g}/\text{ml}$ range of the added metal (Marsh *et al.*, 1975). In many cases no change or a relatively small change in mat weight and final pH of the medium, accompanied this effect. With zinc at added levels of 0 to 10 $\mu\text{g}/\text{ml}$ in the medium, aflatoxin increased 30-to 1000-fold with increasing of zinc, whereas mycelial (mat) weight increased less than threefold. At an added level of 25 μg or less of the

metal per ml, salts of iron, manganese, copper, cadmium, trivalent chromium, silver, and mercury partly or completely inhibited aflatoxin production, without influencing mat weight.

It can therefore, be concluded that trace metals exert a specific effect on mycotoxin biosynthesis. Several studies were done in MYRO liquid medium containing MgSO_4 to produce fumonisins (Blackwell *et al.*, 1994; Dantzer *et al.*, 1996). However, fumonisin production in MYRO medium, yielded very low levels when compared to those obtained in solid maize patties. Therefore, further studies need to be done in liquid medium, using trace metals, to increase FB_1 yields.

4.2 Extrinsic parameters

The extrinsic parameters are those properties of the environment that effect both fungal growth and mycotoxin production such as temperature, relative humidity, presence and concentration of gases such as O_2 , incubation time, inoculum, etc.



4.2.1 Temperature and incubation time

Just as fungi are able to grow over wide ranges of pH, osmotic pressure and nutrient content, they are also able to grow over a wide range of temperatures. Several studies indicated that the optimum incubation temperature for FB_1 production was at 25°C (Alberts *et al.* 1990; Marin *et al.* 1995; Melcion *et al.*, 1997). Alberts *et al.* (1990) studied the effect of temperature and incubation period on FB_1 production by *F. verticillioides* MRC 826 in maize cultures. The relationship between fungal growth and FB_1 production as well as heat stability of this mycotoxin were also investigated. MRC 826 was originally isolated from maize in Transkei, Southern Africa, and used in all experiments. The authors investigated the production of FB_1 at constant temperatures of 20, 25, and 30°C . Under the conditions used

the optimal incubation regimen for FB₁ production by *F. verticillioides* MRC 826 in cultures on maize in terms of cost effectiveness, is 7 weeks at 25°C producing 9.5 g/kg, dry weight. The effect of moisture levels on the production of FB₁ at different incubation temperatures, was not investigated at that time.

Le Bars *et al.* (1994) examined the toxigenic potential of *F. verticillioides* isolates from maize seeds compared to isolates from other plant seeds, to determine the effects of the main abiotic factors (temperature, aeration and moisture content) on FB₁ production on maize, and to evaluate the thermostability of this toxin. Maximum toxin production was found when the fungal strains were incubated at a temperature of 20°C. A notable decline in synthesis was observed with the same strains at an incubation temperature of 25°C. Progressively reduced concentrations of FB₁ were obtained in correspondence with the following temperatures, according to the order indicated: 15°C; 10°C; 30°C. Strains of *F. verticillioides* incubated at 35°C, in spite of considerable growth on both maize and potato dextrose agar, turned out to be incapable of synthesizing analytically significant amounts of toxin, even when the incubation period was protracted for as long as 10 weeks. Tests performed by advance preparation of the cultures in anaerobic conditions only showed minimal fungal development, while no FB₁ synthesis was found. The results from the study conducted by Le Bars *et al.* (1994) are, however, different from those found elsewhere in the literature (Alberts *et al.*, 1990; Marin *et al.*, 1995; Melcion *et al.*, 1997)

4.2.2 Presence and concentration of gases such as O₂

Differences in oxygenation are substantial in shake flask and fermenter-grown cultures and can greatly influence the level of FB₁ produced (Keller *et al.*, 1997). In previous studies, well-aerated conditions were linked to a higher FB₁ production. As a consequence considerably

more FB₁ can be produced in fermenters than in shake cultures (Miller *et al.*, 1994). From original reports of 74 mg/l of FB₁ (Jackson and Bennett, 1990), yields have now risen to over 500 mg/l of medium (Blackwell *et al.*, 1994).

4.2.3 Inoculum

The toxigenic ability of strains of *F. verticillioides* may depend on the geographic location from which they were isolated and the substrate or host of origin (Nelson *et al.*, 1991). The ability of an inoculum to produce fumonisins could possibly be genetic and therefore be lost during subculturing. Moreover, the concentration of viable conidia present in inocula is an important factor influencing production. Studies should therefore, investigate the effect of viable conidial concentration on fumonisin production in order to counteract variation between experimental runs.

Inoculum densities may be another factor affecting the production of fumonisin. The density of inoculum present could influence toxin production based on culture media standard. However there is little information about production regarding the effect of inoculum densities on FB₁ production. As with aflatoxin production it is important to determine which inoculum densities are optimal for FB₁ production. Garcia *et al.* (1995) reported that lower inoculum sizes may produce more aflatoxin yields than higher ones. Different factors may influence fumonisin production: strain, composition of growth medium, temperature, moisture level, oxygen tension and incubation time.

4.2.4 Inoculum viability

The determination of the viability of lyophilised fungal cultures used as an inoculum in experiments is very important. The standardisation of inocula includes the evaluation of

conidial viability for either quantitative and/or qualitative information.

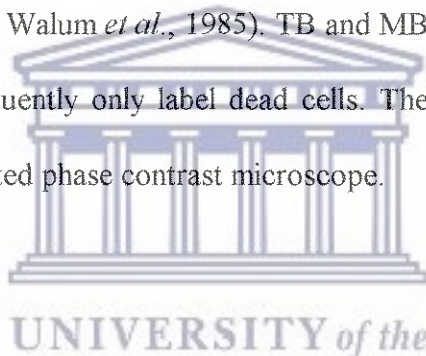
Plate count techniques have been used in most environmental introductions of microorganisms and for the detection of microorganisms (Lapage *et al.*, 1970; Sano *et al.*, 1993; Hjerstedt *et al.*, 1998). These techniques are well established, usually inexpensive, practical and rely on the ability of microorganisms to multiply and form colonies on agar media. It should, however, be recognised that fungi may have dormant stages, especially when subjected to environmental stresses and could in many cases be viable but not always culturable under certain set conditions. The plate count technique is reproducible, but variation between samples is dependant upon the source and the characteristics of the study organisms. Specificity can be determined by the use of selective media, unusual carbon sources, or the use of differential media. Quantitative evaluation is dependant upon the efficiency of a homogenous suspension of single cells or conidia. Poor dispersal and clumping of cells can lead to inaccurate quantification of colony counts.

A range of techniques can be applied for the enumeration and specific detection of microorganisms using both light and epifluorescence microscopy. Bacteria, yeast and fungi can be enumerated directly, while vital staining can distinguish viable cells (Parkkinen *et al.*, 1976; King *et al.*, 1981).

A new fluorescent staining technique for yeast cells (Millard *et al.*, 1997) utilizes the differential affinity of viable cells for FUN-1 cell stain (Molecular Probes, Inc., Eugene, Oregon). A mounting medium has been devised that includes the dye. This medium allows direct processing of cells from a broth and provides optimal conditions for fluorescence intensity. FUN-1 stain can be used to determine the metabolic activity of yeast cells. Only actively respiring cells are marked clearly with orange-red fluorescent structures, while dead cells exhibit diffuse, green-yellow fluorescence (Millard *et al.*, 1997).

Ethidium bromide (EB) is a powerful mutagen widely used in biochemical research laboratories for visualising nucleic acids (Sambrook *et al.*, 1989). The compound forms fluorescent complexes by intercalation and these compounds are readily visible under ultraviolet (UV) light. Singh and Kumar (1991) used EB to stain nuclei in mycelia and spores of different fungi. Nuclei show up bright red under green fluorescent excitation. This method proved to be very efficient, specific, reproducible and cost effective.

Trypan blue (TB) and methylene blue (MB) are two stains recommended for use in dye exclusion procedures for viable cell counting (Auger *et al.*, 1979). After exposure, the stain is taken up by cellular proteins within non-viable cells, while viable cells have the ability to exclude these dyes (Saijo, 1973; Walum *et al.*, 1985). TB and MB are unable to cross intact plasma membranes, and consequently only label dead cells. The viability of cells can be observed visually using an inverted phase contrast microscope.



4.3 Liquid Cultures

Most studies on the production of fumonisins have been conducted with solid maize-based media (Nelson *et al.*, 1994). Important factors related to production on solid media were reported to be temperature, with an optimal temperature range of 20-25°C, a_w and aeration (Alberts *et al.*, 1990; Le Bars *et al.*, 1994). Marin *et al.* (1995) found that increased a_w resulted in both increased growth and fumonisin production. Although Nelson *et al.* (1994) indicated good aeration to be an important factor in the production of FB₁, no data were provided concerning the effect of aeration. Liquid media would be ideal for the production of unlabeled and ¹⁴C-labeled fumonisins since the nutritional composition is less complex than maize and isolation procedures are simplified (Alberts *et al.*, 1994). Several complex- and chemically defined liquid media have been employed in the past to determine the environmental

and nutritional conditions required for optimal production of mycotoxins produced by *Fusarium* spp.

Alberts *et al.* (1994) evaluated a number of liquid media used previously (Jackson and Bennett, 1990) as well as vermiculite supplemented with the respective media for their ability to support FB₁ and FB₂ production by *F. verticillioides* MRC 826. In addition, fungal growth and FB₁ production under various physiological conditions were investigated in order to obtain a liquid medium which supports optimal fumonisin production. Differences in the trend of fumonisin production within the respective media was observed. From this it became clear that, apart from the genetic ability, certain physiological factors are determinative for fumonisin production. For a particular strain, the production in solid maize patty cultures may exceed production in liquid cultures 100 to 1000 fold.

The relationship between fungal growth and the production of fumonisin on maize grain by 25 strains of *F. verticillioides* of different origins has been investigated by Melcion *et al.* (1997). Although sporulation was essentially the same for all the strains, ergosterol assays revealed marked variations in fungal biomass. All strains studied produced highly variable amounts of FB₁, the highest levels being observed in strains of ergosterol content above 400 µg/l. However, no correlation could be established between the synthesised biomass and the quantity of fumonisins produced.

Branham and Plattner (1993) studied alanine as a precursor in the biosynthesis of FB₁ by *F. verticillioides* in liquid culture. Under static culture conditions, 20.8 µg/ml of FB₁ were produced. Under shake culture conditions, much higher levels of FB₁ was produced with levels reaching 159-240 µg/ml by 21 days after culture initiation. It is evident that alanine is incorporated intact into FB₁.

Keller *et al.* (1997) examined the effects of aeration and pH under conditions which

previously showed high levels of FB₁ production in liquid cultures. Both factors appear to have a profound effect on growth and the production of fumonisin. The effects of pH appear similar to that found by Blackwell *et al.* (1994) in that a pH lower than 4.0 is required for good fumonisin production. The best production was observed between pH 3.0 to 4.0. The effect of oxygen on fumonisin production supports observations made on solid media (Nelson *et al.*, 1994).

Since the nutritional composition of liquid media is less complex than maize and the isolation procedures for fumonisins are simplified, yielding a 89 % recovery, it is an advantage to produce fumonisins in such a medium (Miller *et al.*, 1994). The method developed for the purification of fumonisins from liquid culture was also applied to a maize culture, resulting in only 70.1 % recovery of fumonisins (Miller *et al.*, 1994). Several complex and chemically defined liquid media have been employed to determine the environmental and nutritional conditions required for optimal production of fumonisins produced by *Fusarium* species (Miller *et al.*, 1994; Dantzer *et al.*, 1996).

Dantzer *et al.* (1996) reported that *F. verticillioides* strain M5991 produced FB₁ concentrations of 619, 659, and 375 mg/l after 35, 47, and 52 days of incubation, respectively, in modified MYRO liquid medium. By analysis, a total yield of 20 g FB₁ was obtained from three serial batch fermentations.

Labelled fumonisins were readily produced in cultures of *F. verticillioides* using a defined liquid medium (Plattner and Shackelford, 1992a; Blackwell *et al.*, 1994). The formation of 3-acetyldeoxynivalenol (ADON) and other secondary metabolites of *Fusarium culmorum* in a stirred jar fermenter has been described in relation to changes in concentration of sugars, N, P and O₂, and in pH in the medium as well as in cellular parameters (Miller and Blackwell, 1986).

Currently, FB₁ is obtained primarily by using solid culture methods (Alberts *et al.*, 1990; Keller and Sullivan, 1996). Although FB₁ concentrations obtained in solid culture are typically quite high, subsequent extraction and purification present problems. In addition, current methods utilize complex media which makes analysis of biosynthetic pathways and control mechanisms difficult. Liquid culture methods of production could eliminate many problems associated with production in solid culture. Factors affecting the production of FB₁ from a shake flask scale of 100 ml to a fermenter scale of 100 liters were examined in their study. Best results were obtained by using a fed batch method that is nitrogen limited, with pH control. Keller *et al.* (1997) also concluded that within a pH range of 3.0 and 4.0, FB₁ production can exceed 1000 $\mu\text{g/g}$ provided there is sufficient aeration during the early growth period. However, these levels were not obtained.



5. Analytical methods for detecting fumonisins

The common occurrence of fumonisins in maize products and their implication for human health has driven the development of accurate analytical techniques for these mycotoxins.

5.1 Thin-layer chromatography (TLC)

TLC, which is qualitatively useful, is not accurate for quantifying fumonisin levels. Using TLC, Gelderblom *et al.* (1988a) first isolated fumonisins from culture extracts. TLC is the simplest but, like all the other analytical procedures, depends upon their efficient extraction and clean-up. The lack of a suitable chromophore in the molecule means that the metabolites must be derivatised with reagents to allow detection. Cawood *et al.* (1991) used silica gel G

plates with two developing solvents, chloroform/methanol/water/acetic acid (6:3:1). Fumonisin is being visualised with anisaldehyde spray reagent or ninhydrin (0.2 g in ethanol), whereafter it is heated at 120°C.

5.2 High-performance liquid chromatography (HPLC)

HPLC is the most commonly used method for the analysis of fluorescence derivatised fumonisins. Soon after the discovery of fumonisins, a method employing HPLC was reported from PROMEC (Sydenham *et al.*, 1990a; Alberts *et al.*, 1993). In this method, maleic anhydride was used to make the maleyl derivative that was detected by absorption at 250 nm. Although this method was adequate for determining the fumonisins in cultures of *F. verticillioides* and heavily contaminated feedstuffs, i.e. levels $>10 \mu\text{g/g}$ (Sydenham *et al.*, 1990a), it was not sensitive enough for the lower levels found in foods and physiological fluids and tissues. For this purpose, a HPLC method using precolumn derivatization with o-phthalaldehyde (OPA), isocratic elution, and fluorescence detection to analyze maize samples for FB₁ and FB₂, was developed (Shephard *et al.*, 1990) and the modified method has subsequently been used widely (Sydenham *et al.*, 1992; Sydenham *et al.*, 1996). This system allows levels as low as $5 \mu\text{g/kg}$ of FB₁, FB₂ and FB₃ to be detected and has been specifically modified for FB₂ measurements (Shephard *et al.*, 1995).

5.3 Gas chromatography/mass spectrometry (GC/MS)

GC/MS which is based on hydrolysis of the esterified side chain and derivatisation with trimethylsilyl or trifluoroacetate on the fumonisin backbone has been reported (Plattner *et al.*, 1990; 1991; Thiel *et al.*, 1991). Although sensitive and selective, the method involves expensive equipment and a hydrolytic pre-treatment (Shephard *et al.*, 1992b).

5.4 Enzyme-linked immunosorbent assays (ELISAs)

Competitive ELISAs, which are easy to perform and do not need extensive equipment, can be used only for qualitative screening of FB₁. An assessment of an ELISA based on polyclonal antibody (Pestka *et al.*, 1994) versus GC/MS and HPLC, indicated that it could assay fumonisins in food as effective as the other methods. Schneider *et al.* (1995) have developed a dipstick that can detect FB₁ down to levels of 40-60 ng/g of maize-based foods. Clearly this method will be useful where rapid tests are required as primary screens to check the safety of food and legislated tolerances.

5.5 Liquid Chromatographic-Mass Spectrometric methods (LC/MS)

The determination of fumonisins in naturally contaminated maize by HPLC generally requires the use of derivatives to provide the necessary sensitivity of detection (Shephard, 1998). However, the advances currently being made in the interfacing of MS to HPLC and the development of numerous commercial LC/MS systems has resulted in the application of this technique to the analysis of food and feed samples for fumonisins without prior derivatisation. The use of LC/MS also provides strong confirmation of the presence of the fumonisins (Shephard, 1998).

6. General aims and objectives

The general aims and objectives of this study are:

- (1) To optimise the yield parameters for the production of fumonisins in maize patty cultures, which could facilitate the bulk purification of these toxins needed for biological experiments on toxicity and carcinogenicity as well as commercial sales,
- (2) To develop an inoculum viability test to determine the percentage viable conidia in a specific culture to be used in subsequent experiments.
- (3) To determine the possible role of FB₁ as an antifungal agent and
- (4) To investigate the effect of FB₁ on the germination of freshly harvested conidia of *Fusarium* and other fungal species,



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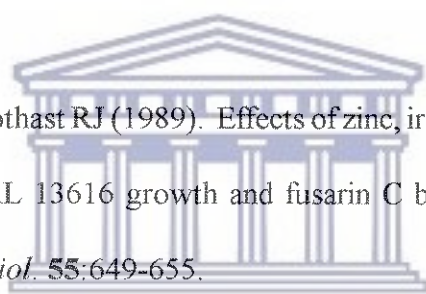
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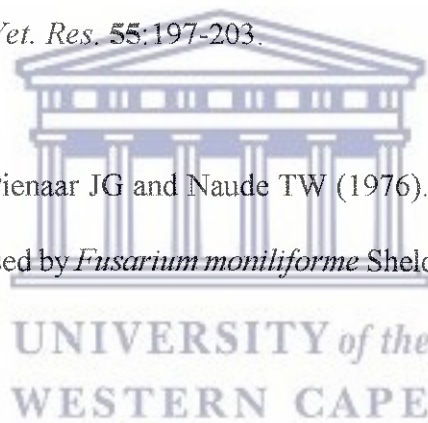


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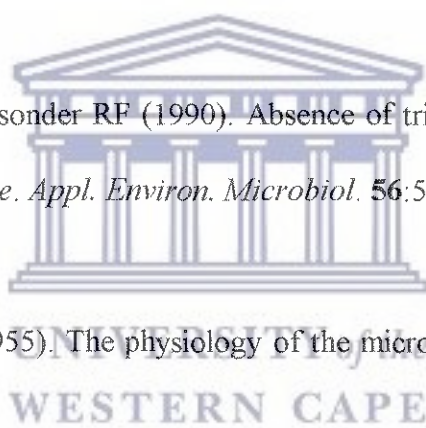
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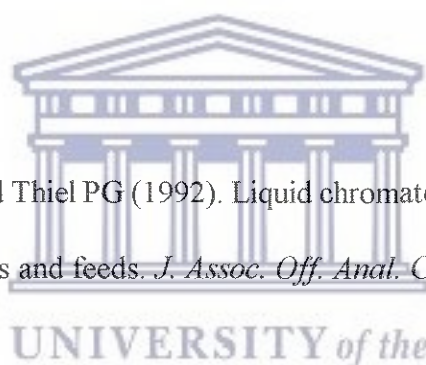
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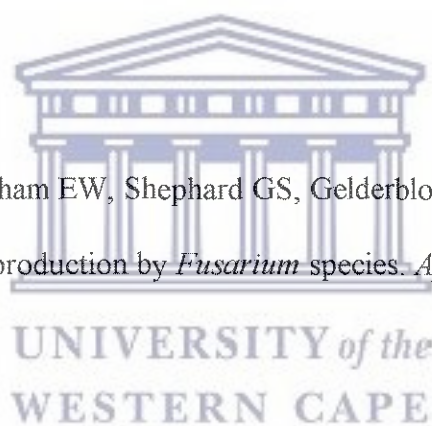


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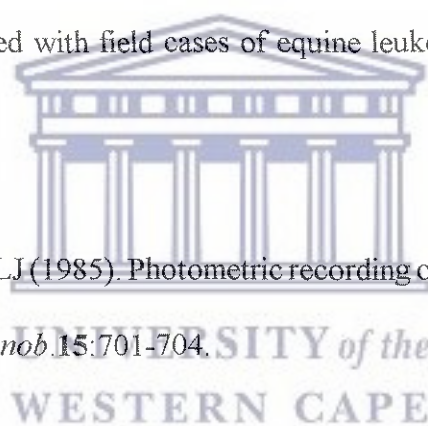
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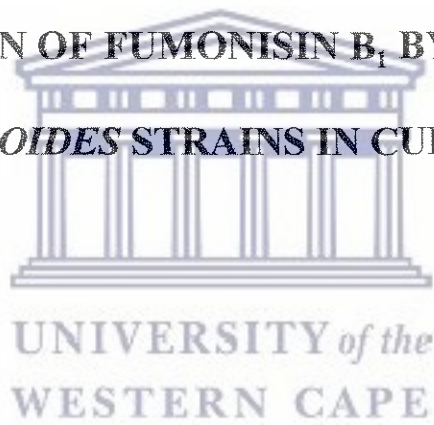
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CHAPTER 2

THE PRODUCTION OF FUMONISIN B₁ BY *FUSARIUM* *VERTICILLIOIDES* STRAINS IN CULTURE



Abstract

The production of fumonisin B₁ by *Fusarium verticillioides* strains were studied in maize patty cultures and liquid media. The effects of different parameters on the production of fumonisin were investigated compared to a standardized set of parameters. In the case of maize patty cultures a moisture content of 30 g maize to 30 ml water and incubation at 25°C was used. The parameters that were varied included initial moisture content of maize patty cultures as well as temperature, initial pH and the addition of the fumonisin precursors, L-methionine and L-alanine. Preliminary investigations were done in modified maize patties and MYRO liquid medium at two concentrations of L-methionine (0.1% and 0.3%), inoculated with *F. verticillioides* MRC 4316 and incubated at 20°C and 30°C, respectively. It was found that the production of FB₁ was increased in maize patty cultures by the addition of 0.3 % L-methionine, and that cultures incubated at 25°C gave a significantly higher yield of FB₁ (1614 µg/g) ($P < 0.05$) than the cultures incubated at 30°C (888 µg/g). In liquid cultures however, the addition of 0.1 % L-methionine slightly enhanced FB₁ production ($P > 0.05$), but a higher concentration of 0.3 % L-methionine, greatly suppressed FB₁ production at both 25°C and 30°C ($P < 0.05$). The results obtained in the three-way interactions of initial maize patty moisture content, L-methionine and temperature, showed that the highest yield of FB₁ (5777.26 µg/g) was produced by MRC 4316 at an initial moisture content of 30 ml water to 30g of maize in the patty, 0.3 % L-methionine at 25°C. MRC 826 was negatively affected by these parameters, producing lower levels of FB₁ (3492.24 µg/g), compared to MRC 4316 at an initial moisture content (20 ml water to 30 g maize), L-methionine (0.3 %) and 25 °C. Among the single factors tested, the addition of 0.3 % L-methionine had the greatest effect on FB₁ production. Optimum initial pH (2.0) of maize patties resulted in high FB₁ yields

(6445.58 $\mu\text{g/g}$), but with no statistical differences between the pH levels tested. The addition of 0.3 % L-alanine yielded 7359.73 $\mu\text{g/g}$ FB_1 at initial patty moisture content (30 ml water to 30 g maize). However, further increase in the L-alanine concentration (0.5%) suppressed FB_1 production. *F. verticillioides* MRC 7424 (= NRRL 13616), produced the highest levels of FB_1 (116 $\mu\text{g/ml}$), while South African isolates, *F. verticillioides* MRC 4316 and MRC 826, produced lower FB_1 levels (93 $\mu\text{g/ml}$ and 62 $\mu\text{g/ml}$, respectively) in MYRO liquid medium. In general, FB_1 production in maize patty cultures far exceeded levels obtained in liquid shake cultures. It is concluded that not only the genetic ability of a particular strain of *F. verticillioides* to produce FB_1 , but the interaction of a variety of physiological and nutritional factors and the culture medium, are important in the production of FB_1 .



Introduction

Mycotoxins, toxic secondary metabolites of fungi, have plagued humans since the commencement of agriculture. The mass production of food and its storage allow for the colonisation or infection of the stored produce by fungi. *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), an ubiquitous fungus belonging to the section Liseola, is one of the principal fungi found in maize worldwide (Marasas *et al.*, 1984). The organism has been associated with human oesophageal cancer (Marasas *et al.*, 1979, 1981) and several animal mycotoxicoses (Marasas *et al.*, 1984). *F. verticillioides* produces a group of mycotoxins known as fumonisins under suitable environmental conditions. Fumonisin B₁ (FB₁) is of interest because of its biological activities when administered to or consumed by animals. The mycotoxin is present in maize infested by *F. verticillioides* and in feed prepared from such maize. Culture material of *F. verticillioides* MRC 826, which was originally isolated from maize in a high oesophageal cancer rate area in Transkei, was shown to be hepatocarcinogenic in rats (Marasas *et al.*, 1984). Liver cancer developed in rats fed a 50 mg pure FB₁/kg diet for 26 months (Gelderblom *et al.*, 1991). Pulmonary edema was induced in weanling pigs fed 92 mg FB₁/kg diet under experimental conditions within 6 days (Osweiler *et al.*, 1992).

Fusarium species are often isolated from maize and yet, even in visibly mouldy maize, fumonisins are not always present. Conversely, visibly healthy maize may contain high levels of fumonisins (Bacon and Williamson, 1992). Most studies on the production of fumonisins have been conducted with solid maize-based media (Nelson *et al.*, 1994). Important factors related to production in solid media were reported to be temperature, moisture and aeration (Alberts *et al.*, 1990; Le Bars *et al.*, 1994). Marin *et al.* (1995) found that increased water activity resulted in both increased fungal growth and fumonisin production. Blackwell *et al.*

(1994) indicated that a low oxygen tension was required for optimal FB₁ production in liquid culture since higher levels of FB₁ were produced by increasing inoculum size or volume of media in flasks. Keller and Sullivan (1996) examined factors affecting the production of fumonisin B₁ from a shake flask scale of 100 ml to a fermenter scale of 100 liters. Best results were obtained by using a fed batch method that is nitrogen limited, with pH control. Shim and Woloshuk (1999) reported on the production of FB₁ as early as 18 hours in a defined medium containing 1.25 mM or 2.5 mM ammonium phosphate. Although total FB₁ production was greater in resuspension cultures grown in higher concentrations of ammonium phosphate, the accumulation was independent of the inoculum size and carbon/nitrogen ratio. Keller *et al.* (1997) examined the effects of aeration and pH under conditions which previously showed high levels of FB₁ production in liquid cultures. Both factors appear to have a profound effect on growth and the production of fumonisin.

The purpose of this report is to describe FB₁ biosynthesis regarding (1) the interaction of moisture content, incubation temperature and L-methionine, (2) the effect of pH, (3) L-alanine and moisture content in maize patty cultures. (4) The production of fumonisins in MYRO liquid medium, comparing three strains of *F. verticillioides*, was also investigated.

Materials and Methods

Fungal strains. Two of the three strains of single-spored lyophilized *Fusarium verticillioides* cultures (MRC 826 and MRC 4316) used in all experiments were originally isolated from maize in the Transkei region of the Eastern Cape, South Africa, during 1975 and 1985, respectively, and deposited in the culture collection of Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) at the Medical Research Council (MRC) of South

Africa. *F. verticillioides* MRC 826 used in the present study has previously been shown to be a high fumonisin producer on maize grain (Alberts *et al.*, 1990). *F. verticillioides* MRC 7424 (= *F. verticillioides* NRRL 13616) (Miller *et al.*, 1994) was obtained from USDA, Peoria, USA.

Lyophilization. One freeze-dried vial of each strain was resuspended in 2ml sterile distilled water and inoculated onto carnation leaf agar (CLA) (Nelson *et al.*, 1983) slants. The slants were incubated at 22°C under a mixture of white and black fluorescent light (300-380 nm) with a 12 h photo period for 14-48 days. Freeze-dry buffer (10ml) were added to each slant and superficially scratched with a sterile bent platinum wire to dislodge the conidia in the buffer. A homogenous conidial suspension was prepared for each strain by adding suspensions obtained from the CLA slants together and stirred for 15 minutes with a magnetic stirrer. Two ml aliquots from these suspensions were aseptically added to 8 ml vaccine vials and stoppered with a sterile fluted butyl rubber stopper. These vials were freeze-dried for 72 hrs (Freeze-drier model : SSE-FD-05/3-V, SS Engineering, SA). Vials were sealed under vacuum, then double sealed with an aluminium cap, numbered, dated and stored at 4°C.

Inoculations. All inoculations were prepared from standardized conidial suspensions of lyophilized conidia adjusted to 1×10^6 conidia/ml with the aid of a Neubauer hemacytometer.

Media. All the chemicals and media, unless stated differently, were analytical grade and purchased from NT Laboratory Supplies, Eppindust, South Africa.

Influence of L-methionine and incubation temperature on the production of FB_1 by *F. verticillioides* in liquid media and maize patty cultures. Maize patty cultures were prepared by weighing 30 g quantities of ground yellow maize kernels out in 90 mm diameter Pyrex petri dishes. Petri-dishes were autoclaved at 110°C, 150 kPa (for 1 hr) on two consecutive days. The liquid medium used was MYRO medium as described by Blackwell *et*

al. (1994): 1 g $(\text{NH}_4)_2\text{HPO}_4$; 3 g KH_2PO_4 ; 0,2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 5 g NaCl; 40 g sucrose; 10 g glycerol per litre of ultrapure water. Aliquots of 50 ml modified MYRO medium in 250 ml Erlenmeyer flasks were prepared and autoclaved at 110°C, 150 kPa for 15 minutes.

FB_1 was produced in liquid shake cultures, using MYRO medium, and maize patty cultures as described above. Both media were modified by adding L-methionine at 0.1 % or 0.3 % and using 0 % as a control. The rationale for this approach was that methionine is a substrate utilized by many fungal methyl transferases, and that it is a pre-cursor of FB_1 (Cahagnier *et al.*, 1994). Triplicate sets of 50 ml liquid cultures were amended with 0.1 %, 0.3 % and control L-methionine, respectively. Duplicate sets of maize patty cultures were also prepared at these concentrations. Both sets of cultures were inoculated with *F. verticillioides* MRC 4316 and incubated at 25 °C and 30 °C in the dark for 21 days. The fumonisin B_1 concentrations in these triplicate cultures were determined by HPLC (see below).

The effect of L-alanine and moisture content on the production of FB_1 . To determine the effect of L-alanine on fumonisin production (Branham and Plattner, 1993), the addition of three concentrations (0.1 %, 0.3 % and 0.5 %) of L-alanine (Sigma Chemical Co, St Louis, MO 63178, USA) to the medium was investigated. Two initial moisture content levels (46 % and 50 %, prior to sterilisation) were used for each treatment. The experiment was set up as for L-methionine, except that incubation was done at only one temperature (25°C). Patties were each inoculated with 1 ml of a standardized spore suspension (1×10^6) of *F. verticillioides* MRC 4316.

Effect of pH on FB_1 production in maize patty cultures. Three Erlenmeyer flasks, each containing 600 ml water, at 3 different pH values (2, 4 and 7 = control) were prepared. The pH was adjusted with 1M HCl prior to sterilization. L-methionine was added at 0.3 % and 0.0 % for the control to the respective flasks. This water was used to prepare the maize patty

cultures. Only one moisture content (46 %) was used for each treatment. Patties were inoculated with 1 ml of a standardized spore suspension (1×10^6) of *F. verticillioides* MRC 4316. Cultures were incubated at 25 °C for 21 days.

Interaction of moisture content, temperature and L-methionine on FB_1 production in maize patty cultures. The selected fumonisin-producing strains were used to study the influence of temperature, moisture content of the medium and the addition of L-methionine on FB_1 production. The substrate used was prepared as described by Alberts *et al.* (1993). Sterilized distilled water (30 ml or 20 ml) containing 0.15 % and 0.3 % L-methionine, respectively, was added to 30 g ground maize to achieve the initial moisture content of the maize patties. All experiments were done in duplicate. Thus, for each L-methionine concentration, i.e. 0.1 and 0.3 %, 12 petri dishes were prepared. Patties were inoculated with 1 ml of the standardized spore suspensions of (1×10^6) of *F. verticillioides* MRC 826 and MRC 4316, respectively. The control was prepared at only one moisture content (50 %). Culture conditions were therefore at three L-methionine concentrations (0.1 %, 0.3 % and control) to two moisture contents (46 % and 50 %) to two temperatures (25 °C and 30 °C). All cultures were incubated stationary for 21 days.

FB_1 production by three *F. verticillioides* strains in liquid shake cultures. Aliquots of 50 ml sterilized modified MYRO medium per 250 ml Erlenmeyer flask were prepared and inoculated with 1ml of the freshly prepared standardized spore suspension (1×10^6) of MRC 826, MRC 4316 and MRC 7424, as described above. The flasks were incubated in the dark at 25 °C on a New Brunswick Scientific rotary shaker, 100 rpm, 3.81 cm throw for 21 days.

Statistical analyses. Statistical analyses were performed by means of the NCSS 2000 (Hintze, 1998) statistical analysis package. The General Linear Model analysis of variance, that can cope with unequal sample sizes, was generally used. The multiple comparison test used

was the Newman-Keuls test. The factorial design analyses on many of the experiments were performed twice, namely, (1) with the “Control” included as a one-way analysis of variance to test whether the “Control” was significantly different from the other treatment combinations; and (2) with the “Control” excluded in a proper factorial analysis to test for treatment factor main effect and interactions.

Fumonisin standards. FB₁ was isolated and purified as described previously by Cawood *et al.* (1991). For use as an analytical standard, FB₁ was further subjected to two successive column separations on silica gel and reverse phase (C18) columns. The purity of FB₁ (> 95 %) was verified by Nuclear Magnetic Resonance (NMR) spectroscopy and Atomic Absorption at the Department of Physics and High-Performance Liquid Chromatography (HPLC) at the Department of Chemistry, University of Stellenbosch, Stellenbosch.

High-performance liquid chromatography (HPLC) analytical technique for the quantification of FB₁. (1) *Sampling of maize patty cultures.* All other reagents, unless stated otherwise, were analytical grade purchased from Merck (Darmstadt, Germany). Patties were dried overnight at 50°C. Each patty was separately ground in a commercial coffee grinder. The coffee grinder was thoroughly cleaned between each sample. A 5 g sub-sample was weighed out into a 250 ml centrifuge bottle and extracted by adding 50 ml of methanol/water (3:1) and homogenizing for 5 minutes with a Polytron homogeniser. This was followed by centrifugation at 10 000 rpm for 10 minutes at 4°C. The supernatant was filtered (Whatman No 1) and the filtrate collected for analysis. The pH of the filtrate was adjusted to 5.8-6.3 with 0.1M KOH. 1-5 ml (amounts varying according to concentration) of a sample was applied to pre-conditioned (5 ml methanol and 5 ml 75 % methanol-water) Bond-Elute SAX cartridge (3 cc capacity; Varian, Harbor City, CA 90710, USA) on a Solid-phase extraction tube manifold (Supelco, Bellefonte, PA 16823, USA), as described by Shephard *et al.* (1990). The cartridge

was rinsed with 8 ml 75 % methanol-water and 3 ml methanol, the fumonisins eluted with 10 ml of 1 % acetic acid in methanol and collected in vials under the influence of gravity. The contents of each vial was transferred to a WISP vial and evaporated to dryness under a stream of nitrogen at 60°C on a Silli-therm (Pierce, Rockford, IL 61105, USA). The collection vial was rinsed with methanol and added to the WISP vial for drying. The latter was sealed and stored at 4°C. The filtrate was derivatised for fluorescence detection according to the maleyl derivatisation technique and analysed by HPLC (Alberts *et al.*, 1993).

(2) Liquid Cultures. The pH of the culture filtrate was adjusted to 6 with 0.1M KOH. Purification was done by filtering 1ml of the culture filtrate through a 0.22 μ m pore size cameo syringe filter (MSI). The filtrate was derivatised for fluorescence detection according to the maleyl derivatisation technique and analysed by HPLC (Alberts *et al.*, 1993).

The method described is suitable for the determination of FB₁ in extracts of maize and liquid cultures of *F. verticillioides* MRC 4316 as a result of the high levels found to be produced by this strain in culture. Several complex and chemically defined liquid media have been employed in the past to determine the environmental and nutritional conditions required for optimal production of fumonisins produced by *Fusarium* spp. (Miller *et al.*, 1994; Dantzer *et al.*, 1996). The analytical technique used for the quantification of FB₁ in liquid cultures of *F. verticillioides* MRC 4316 had a recovery of approximately 95 % for FB₁ compared to a recovery of 70 % in maize patty cultures. The high recoveries obtained from liquid cultures could mainly be ascribed to the fact that liquid cultures contain less impurities which could interfere with purification and derivatisation procedure than maize cultures (Alberts *et al.*, 1993; 1994). This analytical technique allows levels as low as 10 μ g/kg of FB₁ to be detected (Sydenham *et al.*, 1996)

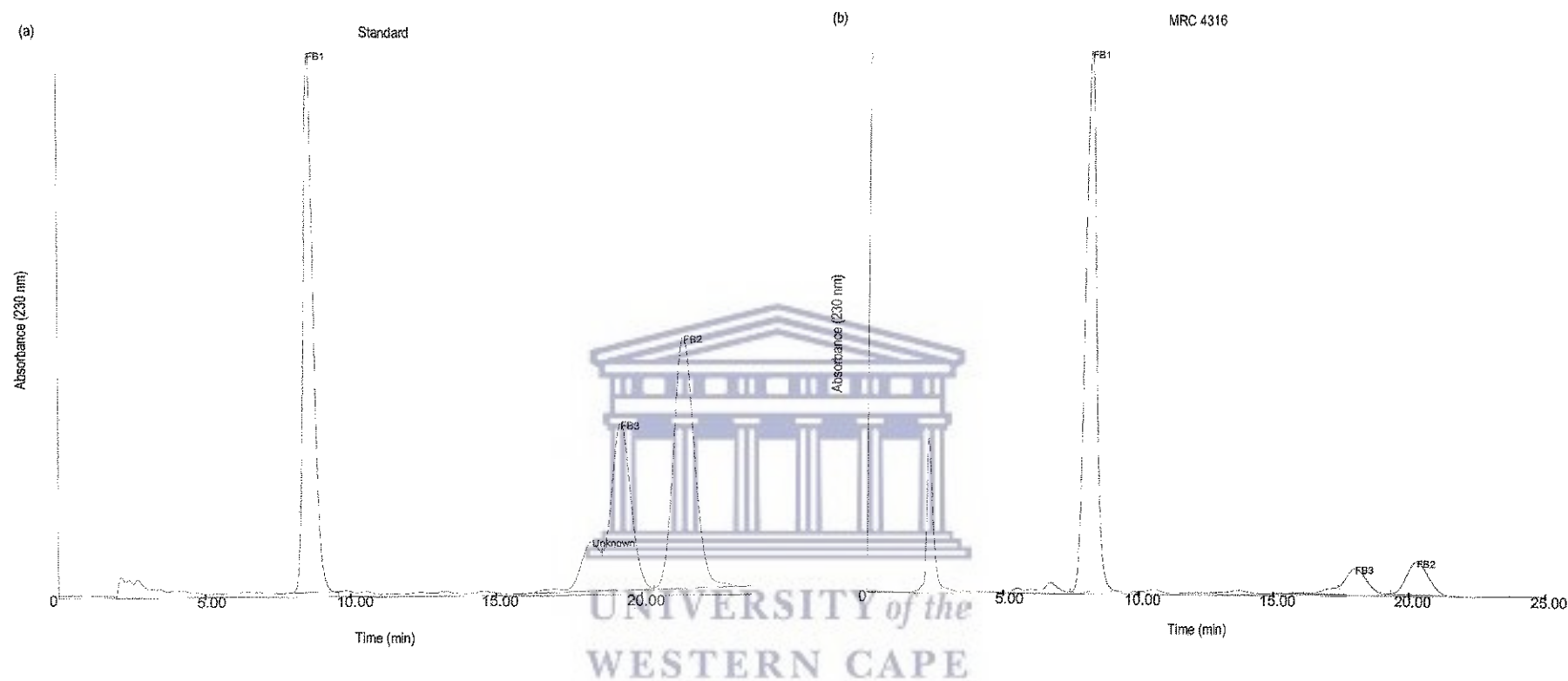


Figure 1. HPLC-eluting chromatograms of FB₁ standard (Std) and a purified extract of culture material of *Fusarium verticillioides* MRC 4316.

Results

HPLC chromatograms demonstrating the eluting position of FB₁ standard and a purified extract of a *F. verticillioides* MRC 4316 maize culture are illustrated in Figure 1.

The relationship of L-methionine and incubation temperatures on FB₁ production in liquid and maize patty cultures. Fumonisin B₁ production in maize patty and liquid shake cultures were compared under the same physiological conditions in order to find a medium which will support the optimal production of FB₁. The FB₁ concentrations obtained on modified maize and liquid MYRO medium are illustrated in Figure 2. A slight increase in FB₁ production is seen with an increased addition of L-methionine (0.1%) in maize patty cultures at 20°C. By adding more L-methionine (0.3%), the FB₁ production was further increased, but it was not statistically significant ($P > 0.05$). By comparing the results from the two incubation temperatures, it was found that at both temperatures, production was increased by an increased addition of L-methionine, and that cultures incubated at 25°C gave a significantly higher yield ($P < 0.05$) of FB₁ than the cultures incubated at 30°C.

The addition of 0.1% L-methionine slightly increased FB₁ production in liquid medium but this was not statistically significant ($P > 0.05$). A further increase in L-methionine concentration (0.3%) significantly suppressed FB₁ production in liquid cultures at both temperatures ($P < 0.05$). Incubation temperature did not have a significant effect on FB₁ production ($P > 0.05$) although FB₁ production was numerically higher at 25 °C than at 30°C. From this can be concluded that FB₁ production in liquid cultures in this experiment was more sensitive to L-methionine concentration than incubation temperature.

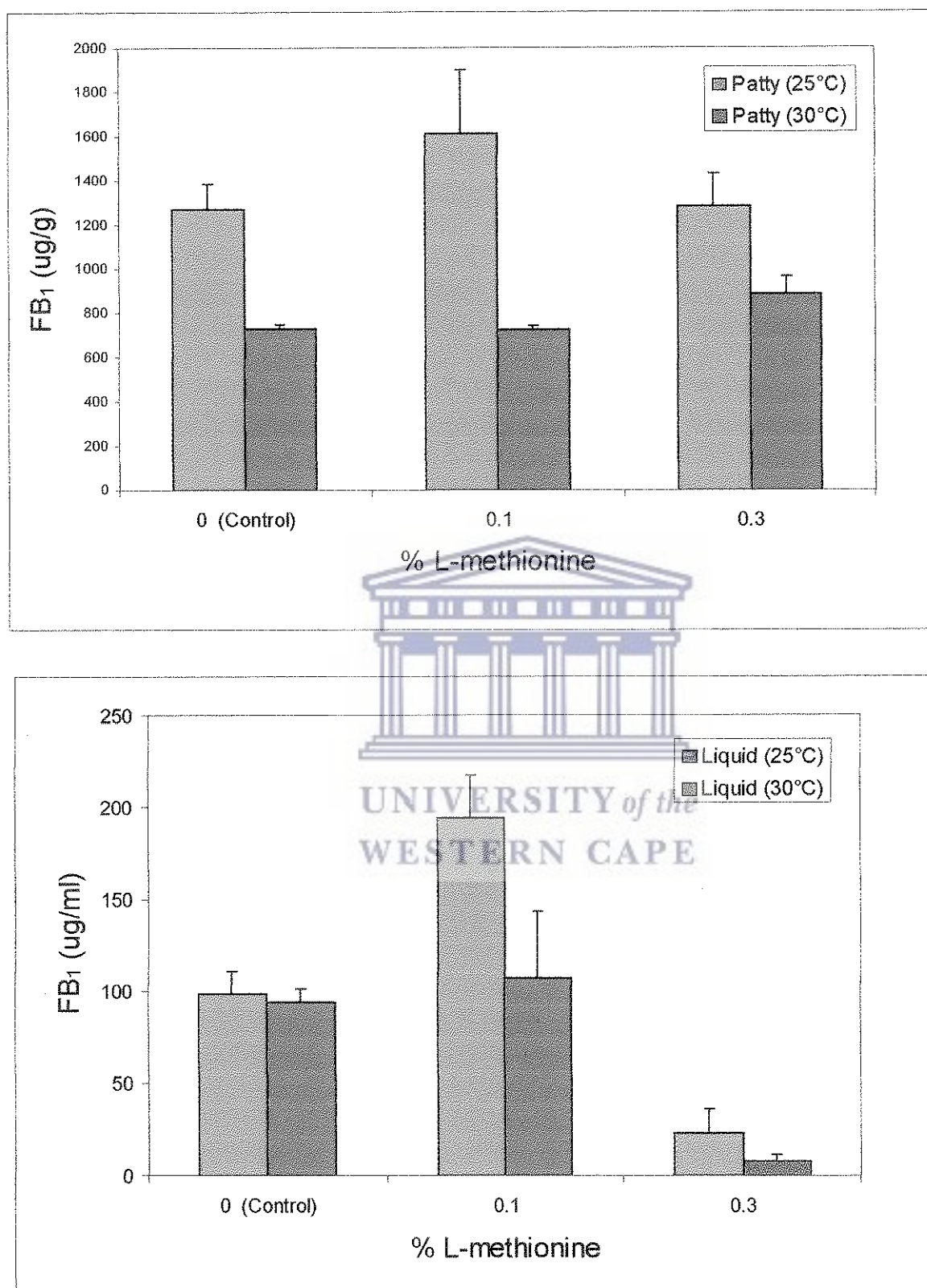


Figure 2. The effects of L-methionine and temperature on the production of fumonisin B₁ in maize patty and liquid shake cultures by *Fusarium verticillioides* MRC 4316.

The interaction between moisture content, temperature and L-methionine on fumonisin B₁ production. Table 1 summarises the mean concentrations of FB₁ produced by two strains of *F. verticillioides* on maize patties when considering the moisture content, temperature and the addition of L-methionine to the medium. In general, comparing the results obtained for the two isolates used, FB₁ production was higher for MRC 4316 than for MRC 826 (Table 1 and Figure 3). Under standard (control) incubation conditions, MRC 826 produced higher levels of FB₁ (3241 $\mu\text{g/g}$) than MRC 4316 (2524.1 $\mu\text{g/g}$). These isolates showed different responses to FB₁ production when growth conditions were manipulated.



Table 1. The interaction between moisture content, temperature and L-methionine on fumonisin B₁ production by *F. verticillioides* MRC 4316 and MRC 826 in maize patties.

Treatments	Moisture Content (%)*	Temperature (°C)	L-methionine (%)	MRC 4316**	MRC 826**
				FB ₁ (µg/g)***	
Z ₁	46	25	0.1	4499.90	2655.20
Z ₂	46	25	0.3	4369.50	3242.40
Z ₃	46	20	0.1	3906.10	3212.80
Z ₄	46	20	0.3	5410.4 [#]	3492.30
Z ₅	50	25	0.1	3841.70	2856.40
Z ₆	50	25	0.3	5777.3 [#]	2771.30
Z ₇	50	20	0.1	4264.20	3088.30
Z ₈	50	20	0.3	3708.40	2990.00
Control	50	25	0.0	2524.10	3241.00

* Moisture content prior to sterilisation

** Standardised inoculum of 1x10⁶ CFU / ml

*** Mean of three cultures

Significant ($P < 0.01$) - Compared to controls.

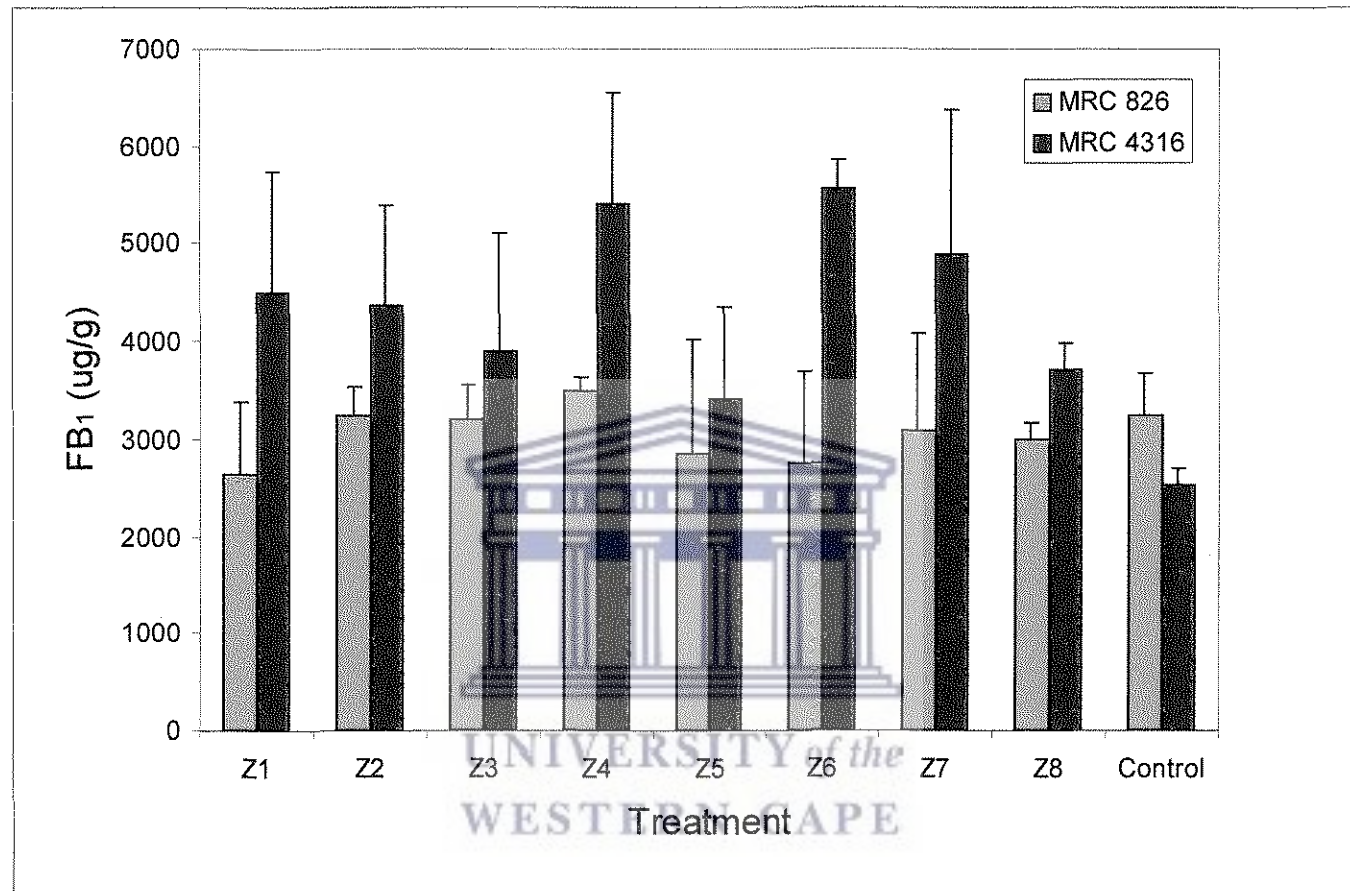


Figure 3. Comparison of fumonisin FB₁ production of two strains of *Fusarium verticillioides*, i.e. MRC 4316 and MRC 826, with regard to the interaction of moisture content, temperature and L-methionine Z1 - Z8 culture conditions as indicated in Table 1.

The highest levels of FB₁ (5410.42 µg/g and 5777.26 µg/g) were produced by MRC 4316, at treatments Z4 and Z6, respectively, (Z4 = 20 ml/30 g × 20°C × 0.3 % L-meth. and Z6 = 30 ml/30 g × 25°C × 0.3 % L-meth.). FB₁ production by MRC 4316 on maize patties at these specific culture conditions was significantly higher ($P < 0.01$) than control samples. Statistically, the ANOVA for FB₁ production showed that only the three-factor interaction was significant at $P < 0.012$, in affecting FB₁ production by this strain. Among the single factors tested, L-methionine had the greatest effect ($P > 0.05$) on FB₁ production. The lowest levels of FB₁, almost similar to controls, were produced by MRC 4316 under culture conditions Z5 and Z8, with even lower levels being produced at Z5. MRC 826 reacted completely differently to the treatment conditions than MRC 4316. At almost all the culture conditions, except for Z2, Z3 and Z4, FB₁ production by MRC 826 was reduced. The lowest FB₁ levels measured were with Z1 (2655.16 µg/g), and the highest being Z4 (3492.24 µg/g). Therefore, different strains of *F. verticillioides* have different inherent abilities to produce FB₁ under similar culture conditions.

Effect of initial pH. The effect of initial pH on FB₁ production was examined in maize patty cultures at three different pH levels (2.0, 4.0 and 7.0 = control), one moisture content (20 ml to 30 g maize) and 0.3 % L-methionine, using *F. verticillioides* MRC 4316 (Figure 4). The highest FB₁ yield (6445.6 µg/g) was obtained at a low pH of 2.0, but with no statistical differences ($P > 0.05$) between individual treatments.

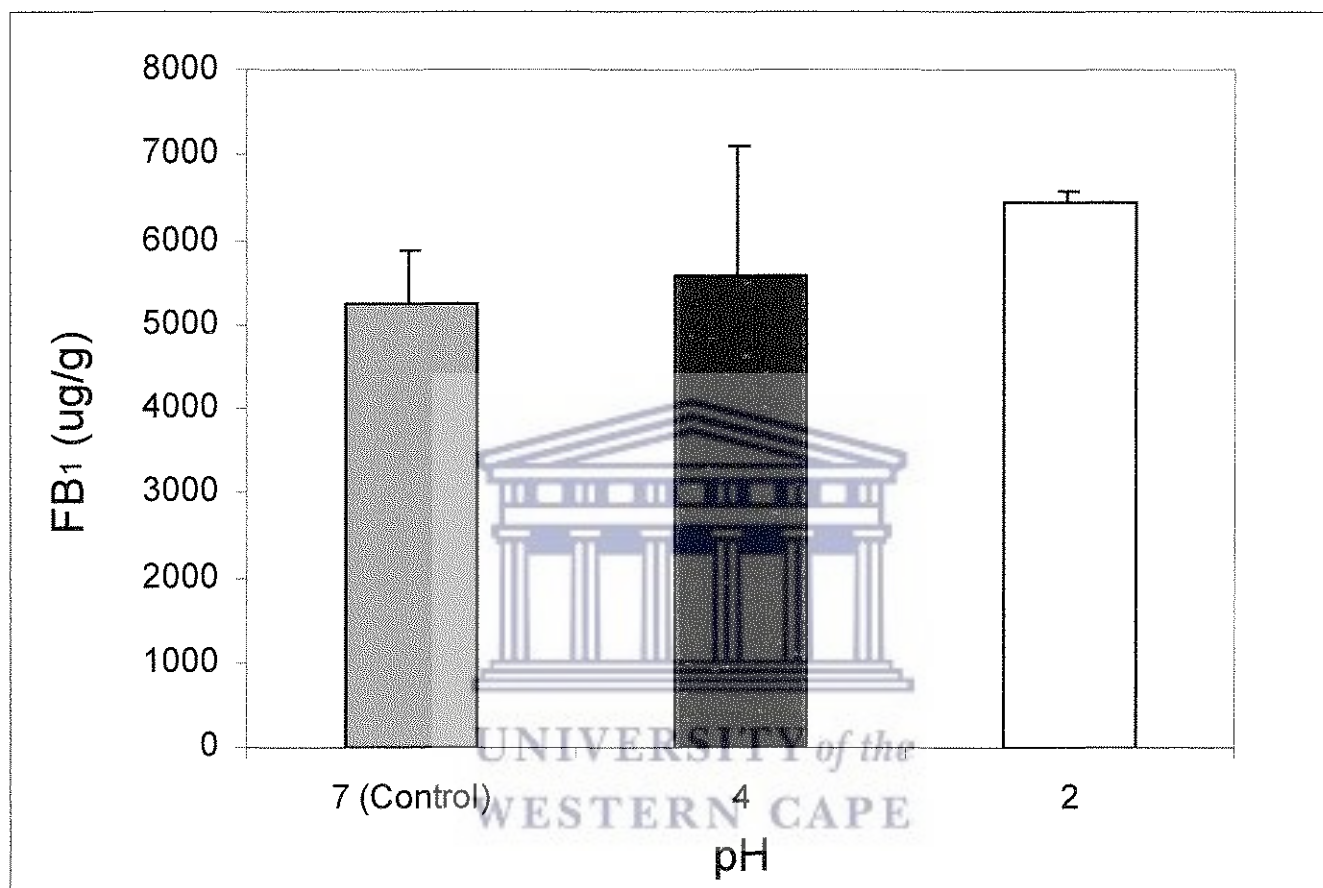


Figure 4. The effect of initial pH of maize patties on the production of fumonisin B₁ by *Fusarium verticillioides* MRC 4316.

L-alanine. The combined effect of L-alanine and initial moisture on FB₁ production in maize patty cultures is shown in Figure 5. The addition of 0.1 % L-alanine slightly enhanced FB₁ production in maize patties at the higher moisture level (30 ml to 30 g maize), but had the opposite effect at the lower moisture level (20 ml to 30 g maize). An increase in L-alanine concentration to 0.3 %, further stimulated FB₁ production at both moisture levels. The maximum FB₁ level (7359.7 µg/g) was reached with the L-alanine concentration at 0.3 % and moisture level at 30 ml water to 30 g maize in the patties. When the L-alanine concentration was raised to 0.5 %, FB₁ production was decreased. A lower moisture level resulted in lower FB₁ production, when compared with the higher moisture levels. Although numerically higher yields of FB₁ were obtained under the conditions mentioned, no significant differences were noted ($P > 0.05$).



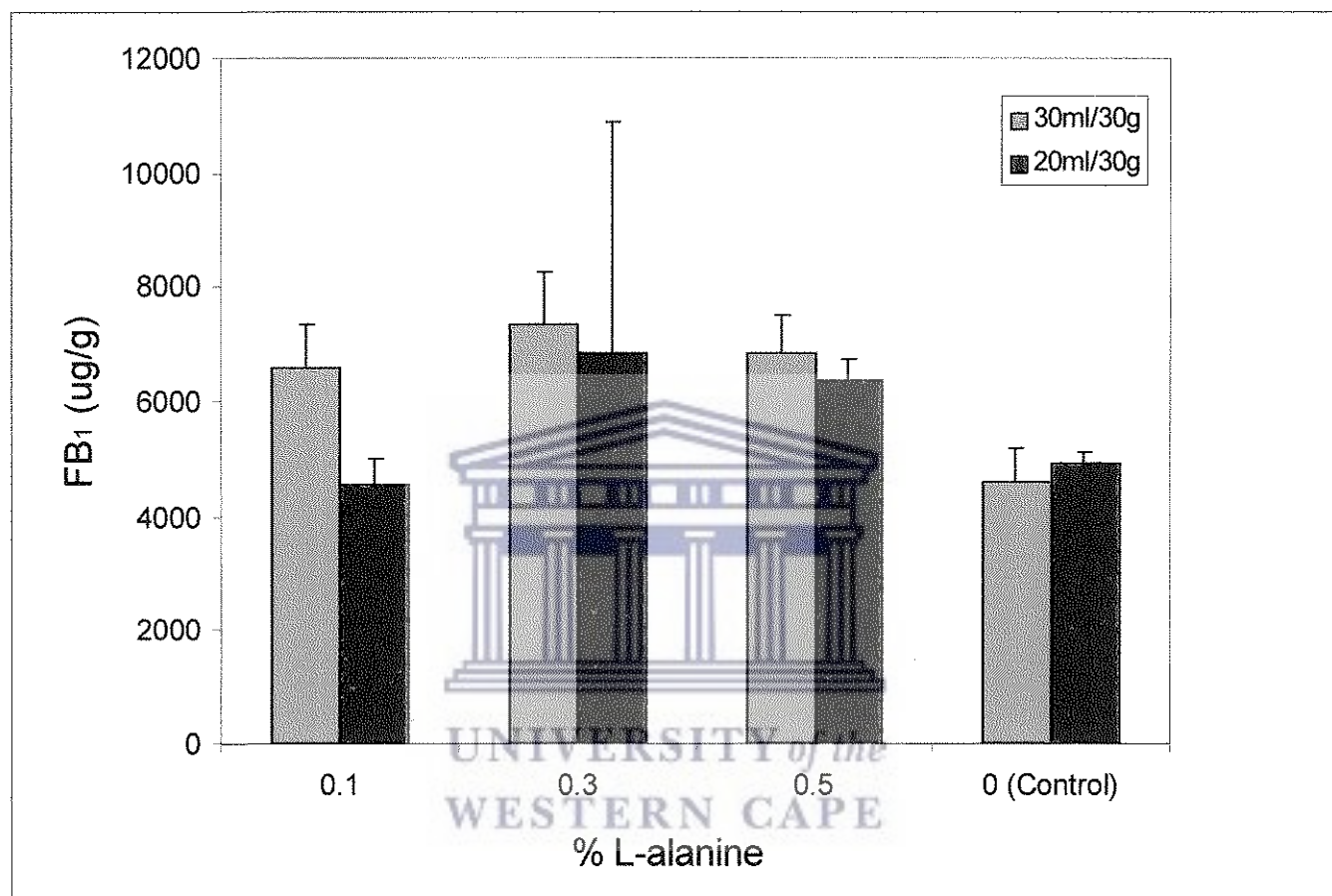


Figure 5. The effect of L-alanine on the production of fumonisin B₁ in maize patty cultures by *Fusarium verticillioides* MRC 4316.

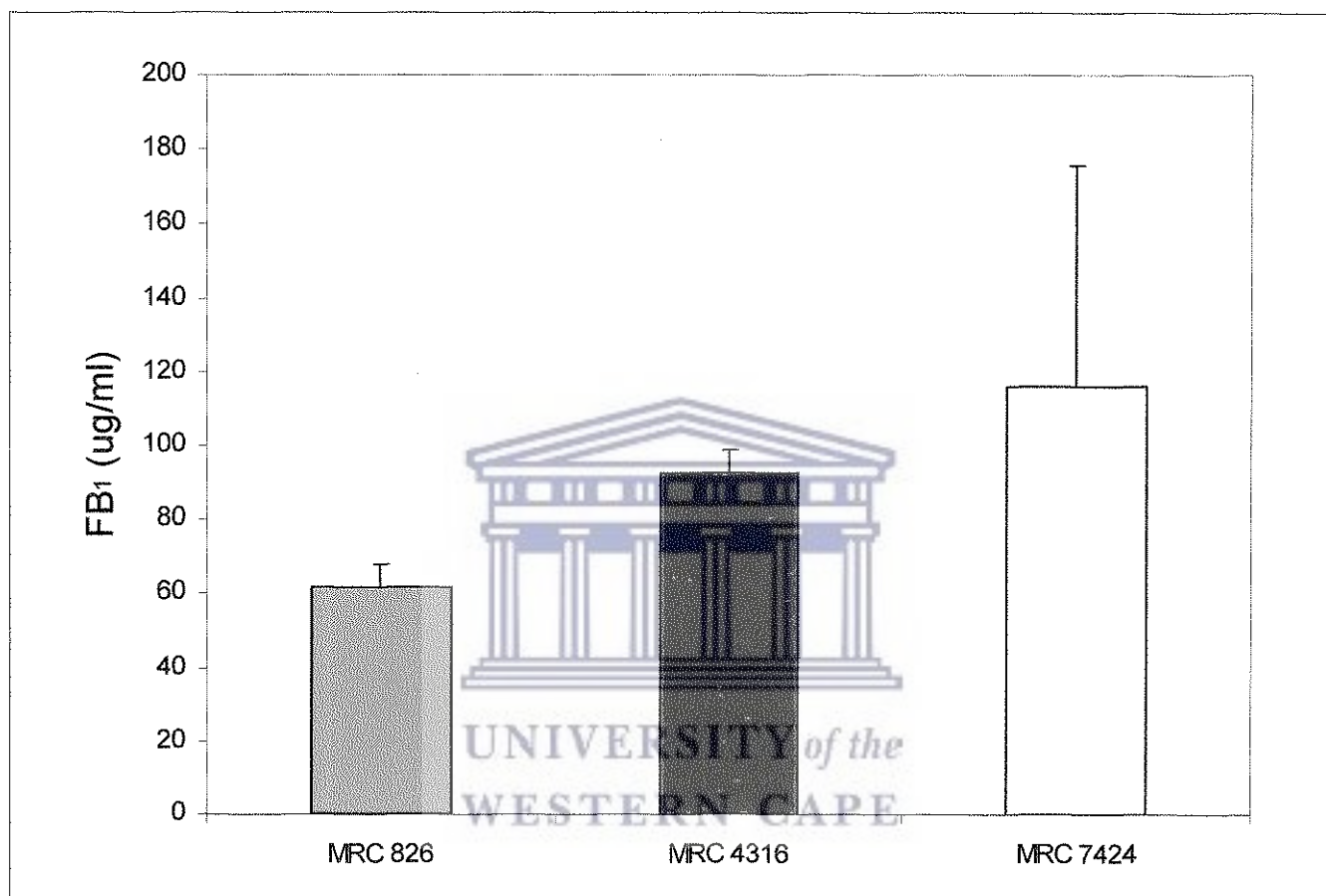
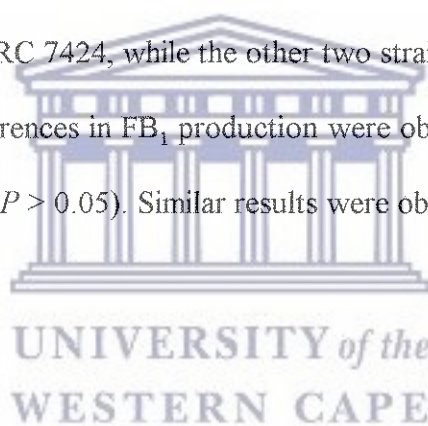


Figure 6. Fumonisin production by *Fusarium verticillioides* MRC 826, MRC 4316 and MRC 7424 in liquid shake cultures, using MYRO medium.

Fumonisin B₁ production by Fusarium verticillioides strains in liquid cultures.

Fumonisin B₁ was produced in liquid shake cultures, using the defined MYRO medium (Blackwell *et al.*, 1994). Figure 6 represents the mean FB₁ concentrations (µg/ml) produced by three strains of *F. verticillioides* under the treatment conditions used. Some differences in FB₁ yields were observed between the strains used, viz. *F. verticillioides* MRC 7424, produced the highest amount of FB₁ (116 µg/ml). In contrast, the South African isolates, *F. verticillioides* MRC 4316 and MRC 826, produced lower FB₁ levels (93 and 62 µg/ml, respectively). *F. verticillioides* MRC 826 produced considerably smaller amounts of FB₁ compared to MRC 7424. Interestingly, there was a high variability in FB₁ production by replicates of *F. verticillioides* MRC 7424, while the other two strains were quite consistent. However, although marked differences in FB₁ production were observed, these differences were not statistically significant ($P > 0.05$). Similar results were obtained for FB₂ and FB₃.



Discussion

Preliminary research has shown that the *F. verticillioides* strains which produced relatively high levels of FB₁ in maize patties did not necessarily do so in MYRO medium, irrespective of the addition of the fumonisin precursor, L-methionine. While low FB₁ yields were obtained in liquid medium, FB₁ production in maize patty cultures exceeded production in liquid cultures, implying that a solid substrate medium enhances fumonisin production in the present study. The addition of L-methionine suppressed production of FB₁ in liquid cultures to a great extent and FB₁ production in liquid cultures was more sensitive to L-methionine than incubation temperature.

This study investigates the interaction of the initial moisture content of the maize substrate, the L-methionine addition and the incubation temperatures on FB₁ yields. It is

important to note that the production of FB₁ can be either negatively or positively influenced when a single growth parameter is altered. Apart from these interacting physiological conditions, the FB₁ production is also linked to the inoculum factor. The amount of FB₁ produced by an individual *F. verticillioides* strain depend on many complex environmental factors and genetic characteristics of the isolate (Leslie *et al.*, 1992). This was confirmed when the control cultures of MRC 826 were compared to MRC 4316 for their abilities to produce FB₁. When the physiological conditions were manipulated, the better producer under standard (control) conditions (MRC 826), was negatively influenced with respect to FB₁ production, whereas MRC 4316 was stimulated to produce higher levels of FB₁.

Under the conditions (moisture content, temperature and L-methionine) described above, a stimulation in FB₁ production at all interactions were observed compared to controlled conditions for *F. verticillioides* MRC 4316. Significantly higher FB₁ levels than controls were observed for culture conditions Z4 and Z6. The optimum regimen for the production of FB₁ by *F. verticilliosis* MRC 4316 and MRC 826 was found to be 0.3 % × 25°C × 30 ml water and 0.3 % × 20°C × 20 ml water, respectively. Between the three parameters used, L-methionine and temperature were the most important factors in the production of FB₁ by *F. verticillioides* MRC 826 and MRC 4316.

Blackwell *et al.* (1994) reported that a pH lower than 4.0 appears to be required for good fumonisin production and this is similar to our findings, in that the highest level of FB₁ was produced at pH 2. This is also in agreement with investigations regarding other mycotoxins produced by *F. verticillioides* (Faber and Sanders, 1986) and *F. graminearum* (Vasavasa and Hsieh, 1987), where mycotoxin production was stimulated at low pH values in liquid cultures. Here, we have determined the best pH for FB₁ production is at pH 2.0. We also showed that even in a medium such as maize, with a complex nutritional composition

(Kruger *et al.*, 1992), including high concentrations of phosphates and amino acids buffering the medium (Vasavasa and Hsieh, 1987), pH influenced the production of fumonisin in maize patty cultures, although not significantly.

A previous study on the effect of L-alanine on FB₁ production (Branham and Plattner, 1993) was carried out in liquid media, while our assay was done on natural maize. The findings of Branham and Plattner (1993) provided evidence that L-alanine was incorporated intact into FB₁, indicating that alanine is a precursor in the biosynthesis of FB₁. They also found that alanine, although successfully incorporated, reduced the production of FB₁ in liquid cultures. Plattner and Shackelford (1992) observed that added L-methionine, also a known fumonisin precursor, sharply reduced FB₁ production under similar conditions. However, in our study, the addition of L-alanine resulted in the stimulation in the FB₁ production in maize patty cultures. The present experiment showed that the optimal incubation regimen for FB₁ production by *F. verticillioides* MRC 4316 in maize patty cultures is a moisture content of 30 ml water to 30 g maize and the addition of 0.3 % L-alanine at 25°C. It is important to note that both moisture and L-alanine played an important role in this stimulatory effect on FB₁ production.

Comparing the results obtained for the three *Fusarium* strains used, MRC 7424 was the best FB₁ producer in liquid shake cultures followed by MRC 4316. Conversely, the best producer on maize patties (MRC 826), produced the lowest yields of FB₁ in liquid media. A possible explanation may include the area of origin (Nelson *et al.*, 1991) or mating status (Leslie *et al.*, 1992). Although the areas of origin were similar for two of the strains, i.e. MRC 826 and MRC 4316, a numerical difference in FB₁ production was still observed. The origin and genetic ability of a particular strain are therefore important factors that influence FB₁ production in different types of media.

Our investigation together with previous studies have detailed the important influence of several parameters on the ability of *F. verticillioides* strains to produce fumonisins. To understand why these strains are able to produce fumonisins, a knowledge of the complex interaction that occurs between biotic and abiotic parameters and their impact on toxin production. Our results again reflect the interacting factors and the intraspecific differences between strains, which may also be present in field conditions. Variation of a single factor such as temperature in field conditions due to seasonal change, can therefore have a major effect on fumonisin production. A chain reaction may occur when changes in moisture, pH, etc. also take place which will influence fumonisin production further.

It is concluded that not only the genetic ability of a particular strain of *F. verticillioides* to produce FB₁, but the interaction of a variety of physiological and nutritional factors and the culture medium, are important in the production of FB₁. However, for a more closely monitored physiological environment, experiments should be aimed at a continuous fermentation system for FB₁ production (Miller and Blackwell, 1986; Miller *et al.*, 1994).

Acknowledgments

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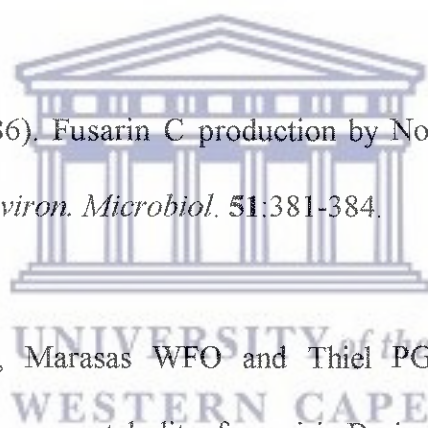
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CHAPTER 3

**THE VIABILITY OF LYOPHILISED CONIDIA OF *FUSARIUM*
AND *ALTERNARIA* SPECIES ASSAYED WITH FLUORESCENT
AND OTHER STAINS**

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Abstract

Lyophilisation of fungal cultures proves to be an excellent method to preserve a wide range of fungi over long periods of time. It is, however, necessary to determine the viability of conidia stored in lyophilised vials at 4°C on a regular basis. Membrane-permeant nucleic acid-binding dyes (FUN-1) are viability stains that are relatively new fluorescent probes for assessing the viability of metabolically active yeast cells. The purpose of the present study was to microscopically determine the viability of lyophilised conidia of *Fusarium* and *Alternaria* species, including *F. verticillioides* (= *F. moniliforme*) strains MRC 826, 4316 and 7424, *F. subglutinans* MRC 1077, *F. graminearum* MRC 1785 and *A. alternata* MRC 1843, using the yeast *Saccharomyces cerevisiae* as a control. FUN-1 was compared to two other staining methods, i.e. ethidium bromide (EB) and methylene blue (MB) and the viability of the conidia was compared to colony-forming units (CFU) on solid media as a control. FUN-1 stained the cylindrical intra-vascular structures (CIVS) orange-red and the nucleus with a green fluorescence in viable conidia. Non-viable conidia exhibited a diffuse, green-yellow fluorescence throughout the cell. EB stained the nuclei of viable conidia and yeast cells bright red. Although the viability of yeast cells could be determined with MB, this method was not suitable for conidial cells because it did not distinguish between viable and non-viable conidia. The fluorescence of EB was enhanced by using green fluorescent excitation (465-550 nm), and showed advantages over the FUN-1 stain due to its simplicity, reproducibility, cost and time-effectiveness when used on conidia. FUN-1 and EB stains gave good results when the microscopic viability of metabolically active yeast cells were compared to their CFU's. Even though these stains differentiated between viable and non-viable lyophilized conidia microscopically, no correlation between the CFU's and the microscopic viability of the

lyophilised conidia of *F. verticillioides*, *F. subglutinans*, *F. graminearum* and *A. alternaria* was found (FUN-1: $r = 0.303$, $P > 0.05$; EB: $r = 0.257$, $P > 0.05$). Neither FUN-1 nor EB can therefore be recommended to accurately determine the viability of lyophilized conidia by microscopy only.



Introduction

Lyophilisation of fungal cultures proves to be an excellent method to preserve a wide range of fungi over long periods of time (Smith and Onions, 1994). It is, however, necessary to determine the viability of conidia stored in lyophilised vials at 4°C on a regular basis. The standardisation of inocula includes the evaluation of conidial viability and, therefore, could play an important role in experimental studies concerning mycotoxin production. Viability of conidia can be measured by methods such as plate counts, germ tube formation and staining with vital dyes. The determination of viability by plate counts may take several days, or sometimes up to one week. Furthermore, Goiman-Yahr *et al.* (1980) reported on the poor plating efficiencies and Restrepo *et al.* (1982) on the unreliability and prolonged incubation of the plate count method. The observation of germ tube formation needs several hours depending on the fungal species to be tested (Restrepo *et al.*, 1982; Sano *et al.*, 1991).

Living cells have a semipermeable membrane, i.e. different membranes allow different amounts of an aqueous solution to pass through. Semipermeability can be determined through dye exclusion techniques, which involve using basic dyes, composed of salts of the particular dye and organic acids. Trypan blue (TB) and methylene blue (MB) are two stains recommended for use in dye exclusion procedures for viable cell counting (Auger *et al.*, 1979). After exposure, the stain is taken up by cellular proteins within non-viable cells, while viable cells have the ability to exclude these dyes (Saijo, 1973; Walum *et al.*, 1985; Denyer *et al.* 1993). The stains TB and MB are unable to cross intact plasma membranes, and therefore only label dead cells. The viability of cells can be observed visually using an inverted phase contrast microscope.

Ethidium bromide (EB) is a powerful mutagen widely used in biochemical research laboratories for visualising nucleic acids (Sambrook *et al.*, 1989). The compound forms fluorescent complexes by intercalation, which are readily visible under ultraviolet (UV) light. Singh and Kumar (1991) used EB to stain nuclei in mycelia and spores of different fungi, and this stain proved to be highly efficient, nucleus-specific, stable in solution and less expensive than other fluorescent stains. A relatively new family of fluorescent probes has been developed for assessing the viability and metabolic activity of yeast cells. This class of halogenated unsymmetric cyanine dyes is exemplified by the FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] stain, a membrane-permeant nucleic acid-binding dye that has been found to give rise to cylindrical intravacuolar structures (CIVS) in viable *Saccharomyces cerevisiae* Hansen cells. Only actively respiring cells, i.e. metabolically active cells, are marked clearly with orange-red CIVS, while dead cells exhibit diffuse, green-yellow fluorescence (Millard *et al.*, 1997).

In order to evaluate fungal cell viability, the choice of method depends on the need for quantitative and/or qualitative information. Enumeration of viable cells by colony counting is time-consuming and does not reliably report on the metabolic activity of slow growing or non-dividing cells. Conventional direct count methods, which typically involve vital staining with indicators such as MB (Parkkinen *et al.*, 1976), are used to assess the activity of cellular oxidoreductases in yeasts (Millard *et al.*, 1997). The objective of the present study was to microscopically determine the viability of lyophilised conidia of *Fusarium* and *Alternaria* species with fluorescent and other stains.

Materials and Methods

The fungal strains used in this study were obtained from the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) of South Africa. FUN-1 (Molecular Probes, Inc.) viability stain was compared to two other staining methods, i.e. EB and MB. The viability of lyophilised conidia of *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) strains MRC 826, 4316 and 7424, *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (MRC 1077), *F. graminearum* Schwabe (MRC 1785) and *Alternaria alternata* (Fr.) Keissler (MRC 1843) and freshly harvested conidia of *F. verticillioides* MRC 826 were investigated. Lyophilised and actively growing cultures of *Saccharomyces cerevisiae* (MRC 7872) were used as controls following the staining method described by Millard *et al.* (1997). Heat killed yeast cells and conidia were included as negative controls. Conidium viability was compared to the colony-forming units (CFU's) on solid media, using potato dextrose agar (PDA) and yeast peptone dextrose agar (YPD).

Culture conditions. Yeasts were cultured in YPD broth medium, containing 10 % yeast extract, 10 % peptone and 20 % glucose. Cultures were derived from single colonies on culture plates grown for 24 to 48 hrs at 30°C. Overnight yeast broth cultures shaken at 200 rpm in a 30°C incubator were used for the positive staining procedure. Cultures of *F. verticillioides* MRC 826 were prepared by growing the fungus on carnation leaf agar (CLA) (Nelson *et al.*, 1983). CLA slants were incubated at 22°C under a mixture of white and black fluorescent light (300-380 nm), with a 12:12 hr light:dark photo period for 14-48 days. Five ml sterile Nonidet-P40 water (to prevent clumping of cells) was added aseptically to the CLA slant and the colony superficially scraped with a sterile bent platinum wire to dislodge the

conidia into the water. Mycelial growth was removed by filtering suspensions through two layers of sterile muslin cloth (Keyser *et al.*, 1999). Conidial suspensions were standardized by microscopically adjusting densities [to approximately 1×10^6 conidia/ml] with the aid of a Neubauer hemacytometer.

Staining with fluorescent and other dyes. One ml of the overnight yeast culture was centrifuged at 3000 rpm for 7 min in a microcentrifuge and the pellet resuspended in a GH staining solution, consisting of 20 % glucose and 10 mM Na-HEPES at pH 7.2. The GH medium was sterilised by filtration through a 2 μ m-pore-size syringe filter (Corning Inc, Germany). The yeast cell suspension was also adjusted to a density of approximately 10^6 cell/ml with the aid of a hemacytometer. This was necessary to avoid too highly concentrated cell suspensions that may be difficult to count, while suspensions that are too diluted may give erroneous results.

FUN-1 dye stock solution was prepared in DMSO at a final concentration of 1 - 10 mM. For staining, FUN-1 stock solutions, a 1:10 dilution EB [98 % stock solution (20 μ g/ml), ICN Biomedicals Inc, Ohio] and MB (1 % in saline) were added in equal 100 μ l volumes to both yeast and conidial suspensions (both freshly harvested conidia and lyophilized cultures), respectively, such that the final DMSO concentration was below 0.2 % and FUN-1 cell stain between 5 - 20 μ M. Each tube was gently mixed. Yeast cells stained with FUN-1 were incubated for 60 min at 30°C in the dark, while the fungal cultures were incubated for 60 min at 30°C. Both yeast and fungi stained with EB were incubated for 10 min at room temperature. Staining with MB required 5 min at room temperature for both yeast and fungal cultures. The yeast cells and conidia remained in the presence of the dyes for observation under the microscope.

Microscopy. Microscopy was carried out with a Olympus light microscope and a

fluorescent microscope (Zeiss Axioskop), which was equipped with a digital camera linked to a computer to capture images by means of a Vysis software programme. Epifluorescence illumination was provided by a 100 W mercury arc lamp. The fluorescent filter sets included the following: for the FUN-1 dye, a fluorescing iso thycyanide (FITC) filter set, 485 - 500 nm excitation (EX) and 490 - 550 nm emission (EM), while for EB green fluorescence filter, EX 465 - 550 nm, were used. The full experiment was repeated three times in duplicate.

Enumeration of yeast cells and conidia. Yeast and fungal CFU's were counted by using a hemacytometer. With FUN-1 and EB (fluorescent stains), conidia and yeast cells were initially viewed under the microscope using visible light, using a 40x objective. The diaphragm was adjusted to reduce light to keep the hemacytometer grid visible. While keeping the visible light on, the microscope was switched to fluorescence and the cells and conidia were observed, counted and viability calculated. Staining with MB required only visible light with no fluorescence.

Plate count. One ml each from the lyophilised and fresh cultures were serially diluted ($8\times$) and agar plates of each dilution were poured by adding 25 ml potato dextrose agar (PDA) to 1ml conidial suspension or 25 ml YPD agar to 1ml yeast suspension in a 90 mm diameter petri dish. Fungal cultures were incubated at 25°C for 7 days in the dark, while yeast cultures were incubated at 30°C for approximately 2 days in the dark. Colonies were counted in each petri dish and the mean was obtained from triplicate determinations. Viability counts were expressed as colony forming units (CFU's) per ml.

Statistical analysis. All analyses were performed by using the general linear model analysis of variance procedure (ANOVA) of the SYSTAT 8.0 for Windows, SPSS Inc., Chicago, USA.

Results

Viability counts of fluorescent staining with FUN-1 and EB, dye exclusion test with MB and CFU's/ml on PDA and YPD are summarized in Table 1. FUN-1, EB and MB stains gave good comparative results when the microscopic viability of metabolically active yeast cells were compared with their CFU's. Some variations between the dyes and the colony count methods, except in the case of *S. cerevisiae*, showed very similar results for the methods used. Results obtained from all the stains show clearly that the yeast strain did not survive the freeze drying process. This was confirmed by the plate count technique.

The results obtained for fluorescent staining with EB and FUN-1, were very similar for each of the fungi tested. However, the conidial counts obtained by CFU's on PDA for lyophilised *F. verticillioides* strains and *A. alternata* were somewhat lower. The FUN-1 and EB determinations for viability compared fairly well for each strain tested, but viabilities obtained using the CFU method were lower. *F. subglutinans*, however, was the only test fungus which showed markedly lower CFU counts compared to the different staining methods. Although viable conidia were observed with the two staining methods under the microscope, no CFU's were found on PDA for *F. graminearum*.

Table 1. Comparison of conidium viability between the number of colony forming units (CFU's) and fluorescent and other stains.#

Fungal Cultures	Viability counts (conidia/ ml ×10 ⁶)						CFU's
	FUN-1		EB		MB		
	Total	Viable ##	Total	Viable ##	Total	Viable	
<u>Lyophilised cultures</u>							
<i>F. verticillioides</i> MRC826	11.60	10.20	11.60	10.30	9.00	as	1.6×10 ⁴
<i>F. verticillioides</i> MRC 4316	9.90	7.30	10.00	7.90	7.90	as	6.7×10 ⁵
<i>F. verticillioides</i> MRC 7424	5.50	4.80	6.00	5.20	5.80	as	1.4×10 ⁴
<i>F. subglutinans</i> MRC 1077	4.40	3.10	7.20	4.30	3.20	as	3×10 ²
<i>F. graminearum</i> MRC 1785	2.70	1.50	2.20	1.10	1.90	as	0
<i>A. alternaria</i> MRC 1843	0.36	0.28	0.31	0.31	0.33	as	5×10 ⁴
<i>S. cerevisiae</i> MRC 7872	5.00	0	4.20	0	4.00	0	0
<u>Conidial culture*</u>							
<i>F. verticillioides</i> MRC826	7.50	7.20	7.10	as	7.00	7.00	3.7×10 ⁶
<u>Yeast culture**</u>							
<i>S. cerevisiae</i> MRC 7872	14.10	8.70	11.10	9.20	12.50	7.60	5.6×10 ⁶

= All tests were done in duplicate and repeated three times.

= $P > 0.05$, compared to the CFU,s (control)

as = All viable and non-viable conidia stained blue with MB

* = Conidia harvested from CLA slants

** = Yeast cells incubated overnight in YPD broth

FUN-1 = FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)- methylidene)-1-phenylquinolinium iodide]

EB = Ethidium bromide

MB = Methylene blue



Figure 1. *Saccharomyces cerevisiae* labelled with FUN-1 dye for approximately 30 min at 30°C. (a) Viable cells gave rise to orange red cylindrical intravacuolar structures (CIVS) within the yeast vacuoles. (b) Dead cells are stained uniformly with bright yellow-green fluorescence.



Figure 2. (a) Fungal nuclei stained with Ethidium bromide (EB) and observed under fluorescent light (green fluorescent filter, excitation 465 - 550nm). EB stained the nuclei of viable conidia and yeast cells bright red. (b) Conidia stained with FUN-1 and observed under fluorescing iso thycyanide (FITC) filter set (excitation 485 - 500 nm and emission 490 - 550 nm). Within viable conidia the nuclei stained prominently green while the red cylindrical intravacuolar structures (CIVS) was not always visible. Dead conidia stained uniformly with a faint yellow-green fluorescence (yellow arrow).

When lyophilised and freshly harvested conidia were stained with FUN-1 dye and observed by epifluorescence microscopy, orange red cylindrical intravacuolar structures (CIVS) could be observed in viable cells (Figs. 1 & 2b). Cells which contained no CIVS were stained uniformly with a bright yellow green fluorescence (Fig. 2b, yellow arrow), and were considered to be non-viable because heat-killed cells were found to stain with similar fluorescence. Optimal loading with FUN-1 was achieved when yeast cells and conidia were exposed to limiting levels of the dye (12.5 μM), which was then processed by the cells into CIVS. Excessive loading ($>12.5 \mu\text{M}$) with FUN-1 stain increased the amount of green fluorescence, whereas suboptimal loading typically limited the number of CIVS formed. Optimal FUN-1 concentration for maximal CIVS formation was 12.5 μM for yeast cell staining and 50 μM for conidial staining.

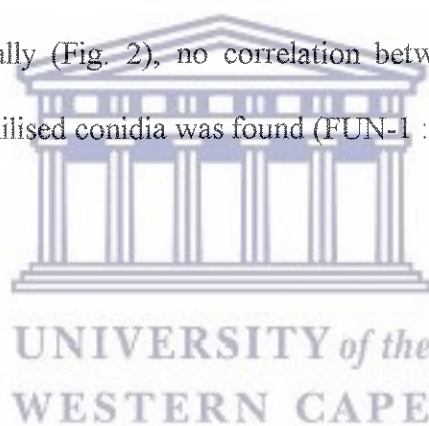
Excellent nuclear staining of lyophilised cultures was obtained with EB in all the test fungi and control yeast (Fig. 2a). The nuclei were stained brick red under green excitation (EX) at 465-550 nm. Green EX was preferred because of bright nuclear fluorescence, poor to no fluorescence of other cell component, and absence of background fluorescence. To visualize septa, cell walls and the grids of the hemacytometer, normal transmitted light was switched on while viewing with fluorescent light. Nuclear fluorescence with EB was stable as there was no visible fading even after 24 hrs.

EB reacted differently when tested on freshly harvested conidial cultures. No distinction could be made between viable and non-viable freshly harvested conidia as no nuclear staining was visible. This was however, not the case with actively growing yeast cultures.

When performing viability counts with MB on lyophilised cultures of the fungal strains used and yeast control, all cells stained dark blue with no differentiation between viable and

non-viable lyophilised conidia and yeast cells. The fact that no viable counts were obtained with *S. cerevisiae*, was probably because the yeast cells did not survive the lyophilisation process. These results were confirmed by the CFU's, as no growth was found on YPD agar plates. MB differentiated between viable and non-viable cells when tested with freshly harvested conidia and yeast cells. After exposure to treatments with MB, the stain was taken up by non-viable cells and stained dark blue. The viable cells did not take up the dye and were phase bright. Yeast cell buds emerging from mother cells were counted as a separate cell when the bud was at least one-half the size of the mother cell.

Even though only FUN-1 and EB stains differentiated between viable and non-viable lyophilised conidia microscopically (Fig. 2), no correlation between the CFU's and the microscopic viability of the lyophilised conidia was found (FUN-1 : $r = 0.303$, $P > 0.05$; EB : $r = 0.257$, $P > 0.05$).



Discussion

The stains tested have several advantages over the plate count technique because they are rapid, highly sensitive and simple to use. Colony forming units on PDA and YPD agar showed different efficiencies for different isolates tested. *F. verticillioides*, *A. alternata* and *S. cerevisiae* strains showed higher CFU's than the other fungal cultures tested, whenever measured. The results were consistent when these tests were repeated. PDA was found to be more useful for detecting live fungal units of *F. verticillioides* and *A. alternata*, while YPD plates were used with *S. cerevisiae*. The absence of correlation between the results of CFU's on agar and the fluorescent and dye exclusion techniques may be due to the fact that these tests measure different parameters. While colony counting on plates could reflect on the growth rate

of cells, the staining assays reflects the internal enzyme content of the cell and the permeability of the cells (Calich *et al.*, 1978). The agar used might not be the optimal growth medium for the lyophilised cultures with very little or no CFU's formed, i.e. *F. subglutinans* and *F. graminearum*, respectively.

The present investigation shows that all three staining methods can be successfully employed to assay viability of actively growing yeast cells. Numerically, no marked differences in results between the stains were observed. CIVS derived from FUN-1 stain have been used to specifically identify the vacuolar compartment in *S. cerevisiae* (Burgess *et al.*, 1994) and is used to distinguish viable from non-viable cells.

The CIVS was also visible in conidia of all the fungi when treated with FUN-1. No CIVS were formed when heat killed conidia were treated with FUN-1 stain. Dead conidia stained uniformly with a faint yellow-green fluorescence. This is due to the fact that the transport of FUN-1 stain from the cytosol to the vacuole does not occur in dead cells.

EB and MB were the only stains that reacted differently when tested on lyophilised conidia and fresh conidia harvested from slants. MB did not differentiate between viable and non-viable lyophilised conidia, but did so with freshly harvested conidia. Non-viable cells do not have the metabolic capability to expel the intruding MB dye. EB reacted conversely when tested with freshly harvested conidia of *F. verticillioides*. EB successfully distinguished between viable and non-viable lyophilised conidia but not freshly harvested conidia. It is evident that the metabolic activity of lyophilised conidia are lower than that of freshly harvested conidia (Smith and Onions, 1994). The cell wall permeability of dead cells is greater than that of viable cells. Lyophilised conidia with a lower metabolic activity than that of freshly harvested conidia may also have a higher permeability. This may explain why these dyes reacted differently when tested on conidia with different metabolic activity.

Neither FUN-1, EB nor MB can therefore, be recommended to accurately determine the viability of lyophilised conidia. At present, plate count methods remain the most valid technique for the detection of the viability of lyophilised conidia. However, for the purpose of determining or screening for percentage viability in a specific inoculum it is recommended that EB is used in the case of lyophilised conidia, and MB in the case of freshly harvested conidia.

Although viable fluorescent stains are recommended as a good way to determine the cell viability of a fungus, it needs relatively complicated procedures and has a time limit in which the stain can be used. In this study, fluorescent and other cell viability staining of lyophilised conidia depended on isolate differences and technical procedure as reproducible results were obtained with all the isolates tested. The result of this study emphasize that the use of dyes to determine viability of lyophilised conidia requires a critical definition of protocols for a specific fungal species, and that a good correlation with colony counts needs to be demonstrated. Plating efficiencies could be increased by altering the nutritional content of the media and cultural conditions (Goihman-Yahr *et al.*, 1980). Many conidia may not have the ability to germinate under specific growth conditions which may influence CFU counts. The findings of this study could contribute useful applications in various studies on living and dead conidial populations.

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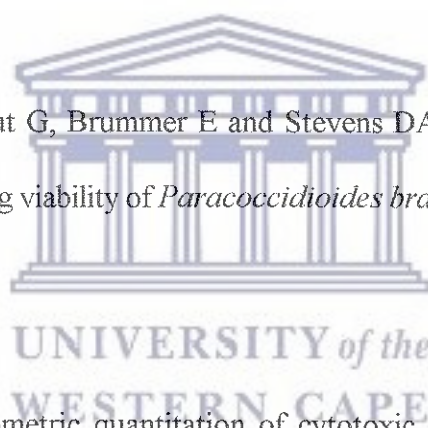
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CHAPTER 4

THE ANTIFUNGAL EFFECT OF FUMONISIN B₁ ON

FUSARIUM

AND OTHER FUNGAL SPECIES

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Abstract

Fumonisin is a mycotoxin produced by several *Fusarium* species that are commonly found on maize and maize products. Fumonisin has diverse toxicological effects in animals and is associated with oesophageal cancer in humans, but its function in nature is obscure. To determine the antifungal effect of fumonisin B₁ (FB₁) on *Fusarium verticillioides* (= *F. moniliforme*), *F. proliferatum*, *F. globosum*, *F. subglutinans*, *F. graminearum*, *Penicillium expansum*, *Aspergillus flavus*, *Alternaria alternata* and *Botrytis cinerea*, the sensitivity of these fungi was tested by an agar-diffusion method on PDA plates at FB₁ concentrations of 40-0.05 mM at pH 5.45. Fumonisin B₁ inhibited mycelial growth of five of the nine fungi tested. The minimum inhibitory concentration of FB₁ ranged from 0.25-0.5 mM for *A. alternata*, 1-5 mM for *P. expansum* and *B. cinerea*, and 5-10 mM for *F. graminearum*, whereas the other fungi tested showed no sensitivity to the mycotoxin. A small inhibition zone was visible with *F. proliferatum*, a FB₁-producing species, at 40 mM. The mycelial growth of the other two FB₁-producing species, *F. verticillioides* and *F. globosum*, was not affected by the toxin. This is the first report on the antifungal activity of FB₁.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), an ubiquitous fungus belonging to the section Liseola, is one of the principal fungi found in maize worldwide (Marasas *et al.*, 1979; 1981). Under suitable environmental conditions, *F. verticillioides* produces a group of toxins known as fumonisins (Gelderblom *et al.*, 1988). Toxigenic *Fusarium* species in South African maize are associated with human oesophageal cancer (OC) and several animal mycotoxicoses (Marasas *et al.*, 1979; 1981). Mycological comparisons of home-grown maize from different areas in the Transkei region of the Eastern Cape province of South Africa revealed a statistically highly significant correlation between the incidence of *F. verticillioides* in maize and OC rate (Marasas *et al.*, 1981; Rheeder *et al.*, 1992). Fumonisins are involved in the neurotoxic disease, leukoencephalomalacia, in horses (Kellerman *et al.*, 1990). Pathognomonic brain lesions were reproduced after 20 doses containing 1-4 mg of 95 % pure fumonisin B₁ (FB₁)/kg diet (total dose 8.4 g FB₁) for 29 days (Kellerman *et al.*, 1990). Culture material of *F. verticillioides* MRC 826, originally isolated from maize in a high OC area in Transkei, was hepatocarcinogenic in rats (Marasas *et al.*, 1984). Liver cancer developed in rats fed a diet containing 50 mg pure FB₁/kg for 26 months (Gelderblom *et al.*, 1991). Pulmonary oedema was induced in weanling piglets fed 92 mg FB₁/kg diet under experimental conditions within 6 days (Osweiler *et al.*, 1992).

FB₁ caused a dose-dependent inhibition of the growth rate of yeast (*Saccharomyces cerevisiae* Hanson) cells (Wu *et al.*, 1995). Although FB₁ is phytotoxic at concentrations as low as 0.1 μ M, no inhibition of the growth of several species of Gram-positive and Gram-negative bacteria was observed at concentrations as high as 1000 μ M (Lamprecht *et al.*, 1994; Becker *et al.*, 1997). The effects of FB₁ on filamentous fungi are unknown. Fumonisins are of

interest because of their physiological action when administered to or consumed by animals, but their function in nature is obscure. The diverse toxicological effects of fumonisins in animals and plants raise the possibility that these toxins may also inhibit the growth of fungi. This study investigated the antifungal effect of FB₁ on some *Fusarium* and other fungal species.

Materials and Methods

The fungi used in this study were from the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) of South Africa: *Fusarium verticillioides*, *Fusarium proliferatum* (Matsushima) Nirenberg (MRC 7431), *Fusarium globosum* Rheeder, Marasas & Nelson (MRC 6647), *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (MRC 1077) and *Fusarium graminearum* Schwabe (MRC 1785), *Penicillium expansum* Link (MRC 7200), *Aspergillus flavus* Link ex Fries (MRC 3791), *Alternaria alternata* (Fr.) Keissler (MRC 1843) and *Botrytis cinerea* Pers. ex Fr. (MRC 1364). Standardized conidial suspensions were prepared by growing the *Fusarium* spp. on Carnation Leaf Agar (CLA) and the other fungi on Potato Dextrose Agar (PDA) slants (Nelson *et al.*, 1983). The PDA slants were incubated at 25°C in the dark whereas the CLA slants were incubated at 22°C under a white fluorescent light (300 - 380 nm) with a 12:12h light:dark photo period for 14-48 days. Sterile water (5ml) was added aseptically to each CLA and PDA slant and the colonies were superficially scraped with a sterile bent platinum wire to dislodge the conidia into the water. Large pieces of mycelium were removed by filtering the suspensions through two layers of sterile muslin cloth. Conidial densities were microscopically adjusted with the aid of a Neubauer hemacytometer to approximately 1×10^6 conidia/ml.

The sensitivity of the respective strains of fungi to FB₁ was tested by an agar-diffusion method on PDA plates. All experiments were carried out in triplicate (3 petri plates for each concentration of each fungus). FB₁ was extracted, isolated and purified as previously described (Cawood *et al.*, 1991). Briefly, maize cultures of *F. verticillioides* MRC 826 were prepared as described by Alberts *et al.* (1990) incubated in the dark at 25°C for six weeks, oven-dried at 50°C, ground and stored at 4°C. Culture material (500 g) was extracted twice with ethyl acetate and the fumonisins extracted three times with MeOH:H₂O in a ratio 3:1. The filtrate was evaporated to dryness under vacuum at 50°C. The aqueous methanol extract was fractionated on an Amberlite XAD-2 column using MeOH:H₂O (1:3) and MeOH:H₂O (1:1) as eluting solvents, while FB₁ was eluted with 100 % MeOH. Two Silica columns (Silica Gel 60) were used for isolation and subsequent purification of FB₁ using CHCl₃:MeOH:CH₃COOH (6:3:1) and CHCl₃:MeOH:H₂O:CH₃COOH (55:36:8:1) as eluants respectively. Final purification was carried out on a low pressure Reverse Phase C₁₈ column using a linear gradient of MeOH:H₂O (1:1-4:1). The fractions containing FB₁ were pooled and evaporated to dryness under vacuum at 50°C. The FB₁ had a purity of 90-95% as determined by high-performance liquid chromatography, atomic absorption spectroscopy and nuclear magnetic resonance (Cawood *et al.*, 1991).

Solutions of FB₁ at concentrations of 40, 20, 10, 5, and 1 mM were prepared in sterile water at pH 5.45 (adjusted with 0.1M NaOH). Petri dishes (90 mm) containing PDA (30 ml agar each) were surface-inoculated with 120 µl of the prepared standardised conidial suspension of each fungus and evenly spread across the agar surface and allowed to dry. Five 1-cm-diameter wells were aseptically made in each of the inoculated PDA plates using a pre-sterilized cork borer. Each well was loaded with 200 µl of the respective FB₁ solutions. Fumonisin solutions were filter-sterilized (pore-size 0.22 µm) before being added to the wells

of the plates. Amphotericin B (Amp B) E-test strips (Davies Diagnostics) were used as a positive control due to the efficacy to a wide range of fungi at fairly constant levels (Holeman and Einstein, 1963; Hildick-Smith, 1968). Negative controls were included for each test by substituting FB₁ with sterile, distilled water and pH adjusted to 5.45 with 0.1 M NaOH. Plates were incubated at 25°C for 2 days and the inhibition zones (clear zones) were measured in triplicate, from the edge of the well and means determined. Once the sensitivity range for each fungus was known, the minimum inhibitory concentration (MIC) was determined by following the above procedure, and by using 0.5, 0.25 and 0.05 mM concentrations of FB₁ to which the strain was sensitive. The reproducibility of the method was assessed by repeating the experiment twice.

Results



Growth inhibition zones were observed for five, i.e. *A. alternata* (Fig. 1a), *P. expansum* (Fig. 1b), *B. cinerea*, *F. graminearum* (Fig. 1c) and *F. proliferatum*, of the nine fungi tested. The standardised inoculum resulted in confluent growth for all the strains tested. A result was defined as positive when triplicate samples showed inhibition (clear) zones at all concentrations of FB₁. Inhibition zones were proportionate to the concentrations used for the respective sensitive fungi, and differed for each fungus tested according to their sensitivity to FB₁. In the case of *B. cinerea*, resistant colonies grew within the clear inhibition zones. Fully resistant fungi, i.e. *F. verticillioides*, (Fig 2), *F. globosum*, *F. subglutinans* and *A. flavus*, grew up to the edge of the well showing no sensitivity to FB₁.

Macroscopic changes in cultural characteristics were noted in FB₁-sensitive fungi such as *P. expansum* and *A. alternata* in which pigment production was inhibited directly adjacent

to the inhibition zone. Stereo-microscopy demonstrated that conidia did not form in the non-pigmented areas of these cultures. It was also observed that in all FB₁-sensitive strains, poor germination occurred or that only short germ tubes developed within the zones of inhibition. Hyphal stunting and abnormal growth continued during the observation period. At lower concentrations of FB₁, germ tubes appeared longer, developing into short, distorted hyphal growth which showed swollen and necrotic tips. This was seen in *P. expansum*, *A. alternata* and *B. cinerea*. Control plates for each strain, for which FB₁ was replaced with water, displayed normal macroscopic and microscopic growth with normal hyphal growth and conidial production.



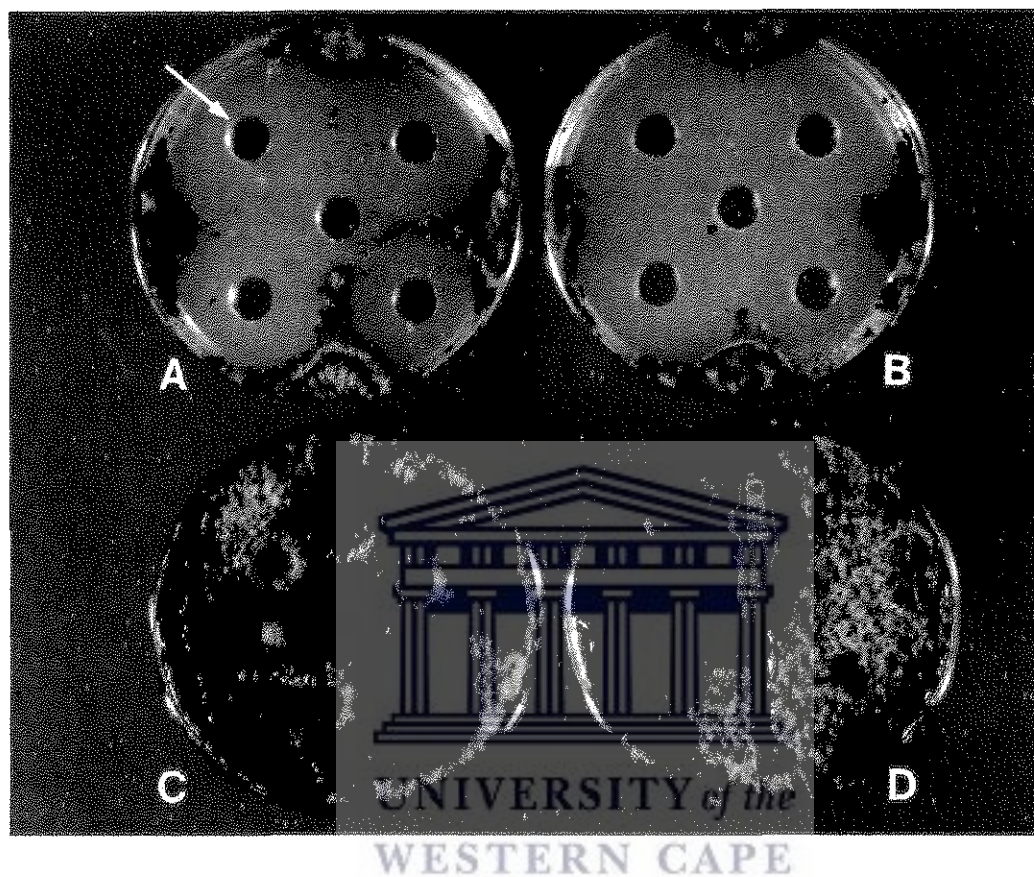


Figure 1a. Sensitivity of *Alternaria alternata* MRC 1843 to different concentrations of FB_1 using an agar-diffusion method. The respective FB_1 concentrations are clockwise from the top (arrow): 40, 20, 5, and 10 mM, and 1mM in the centre (A). Duplicate result (B). Negative controls (C): Wells filled with sterile water. Positive control (D): Amphotericin B E-test (An inert and non-porous plastic strip with a predefined and exponential gradient of the dried and stabilized Amp B on the surface. Concentration range of 0.002 to 32 $\mu\text{g/ml}$).

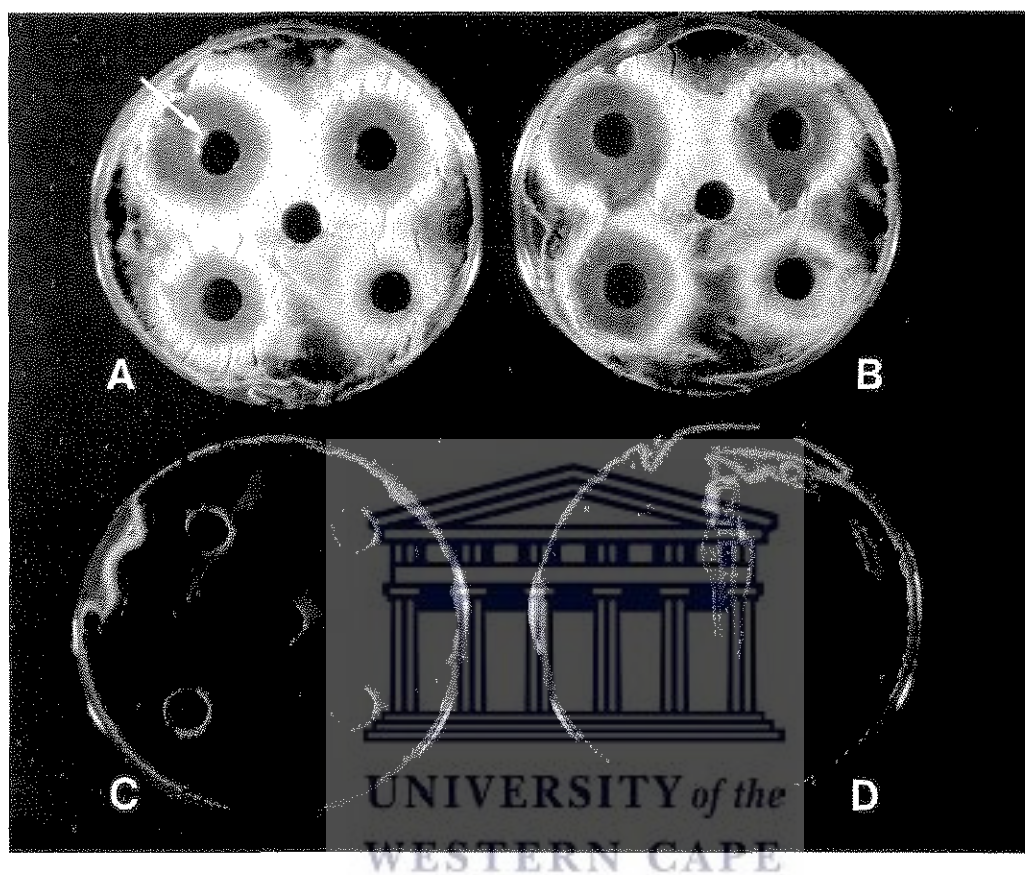


Figure 1b. Sensitivity of *Penicillium expansum* MRC 7200 to different concentrations of FB_1 using an agar-diffusion method. The respective FB_1 concentrations are clockwise from the top (arrow): 40, 20, 5, and 10 mM, and 1 mM in the centre (A). Duplicate result (B). Negative controls (C): Wells filled with sterile water. Positive control (D): Amphotericin B E-test (An inert and non-porous plastic strip with a predefined and exponential gradient of the dried and stabilized Amp B on the surface. Concentration range of 0.002 to 32 $\mu\text{g/ml}$).

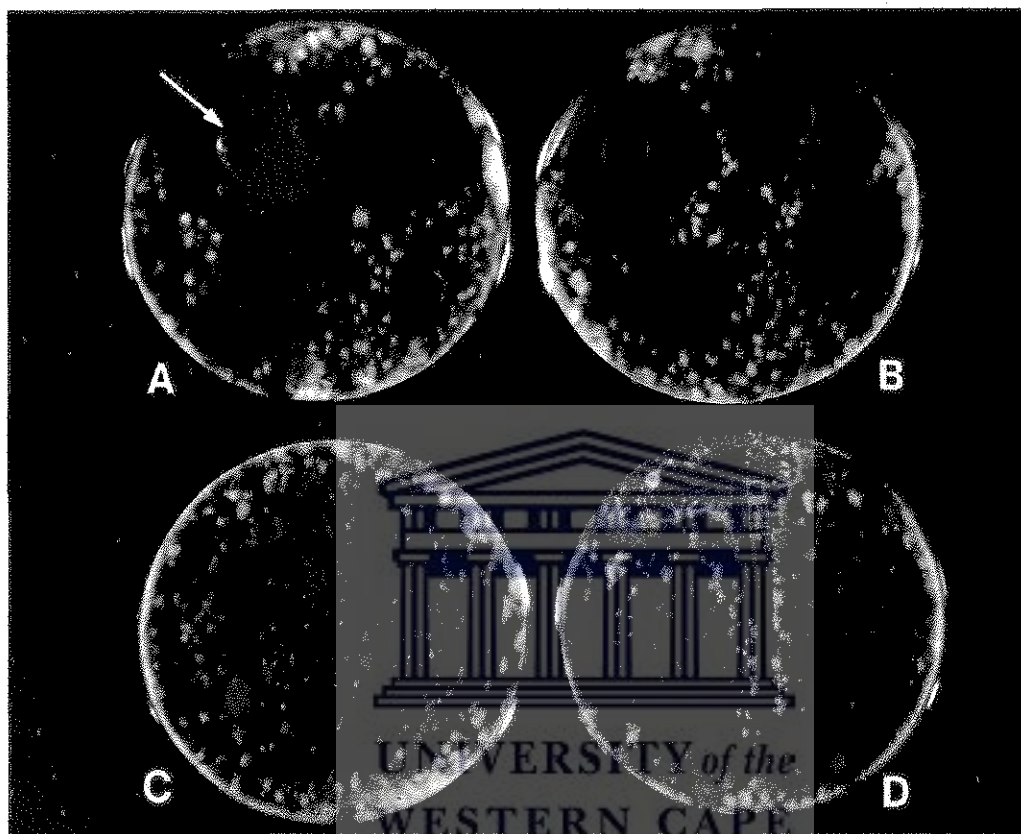


Figure 1c. Sensitivity of *Fusarium graminearum* MRC 1785 to different concentrations of FB_1 using an agar-diffusion method. The respective FB_1 concentrations are clockwise from the top (arrow): 40, 20, 5, and 10 mM, and 1 mM in the centre (A). Duplicate result (B). Negative controls (C): Wells filled with sterile water. Positive control (D): Amphotericin B E-test (An inert and non-porous plastic strip with a predefined and exponential gradient of the dried and stabilized Amp B on the surface. Concentration range of 0.002 to 32 $\mu\text{g/ml}$).

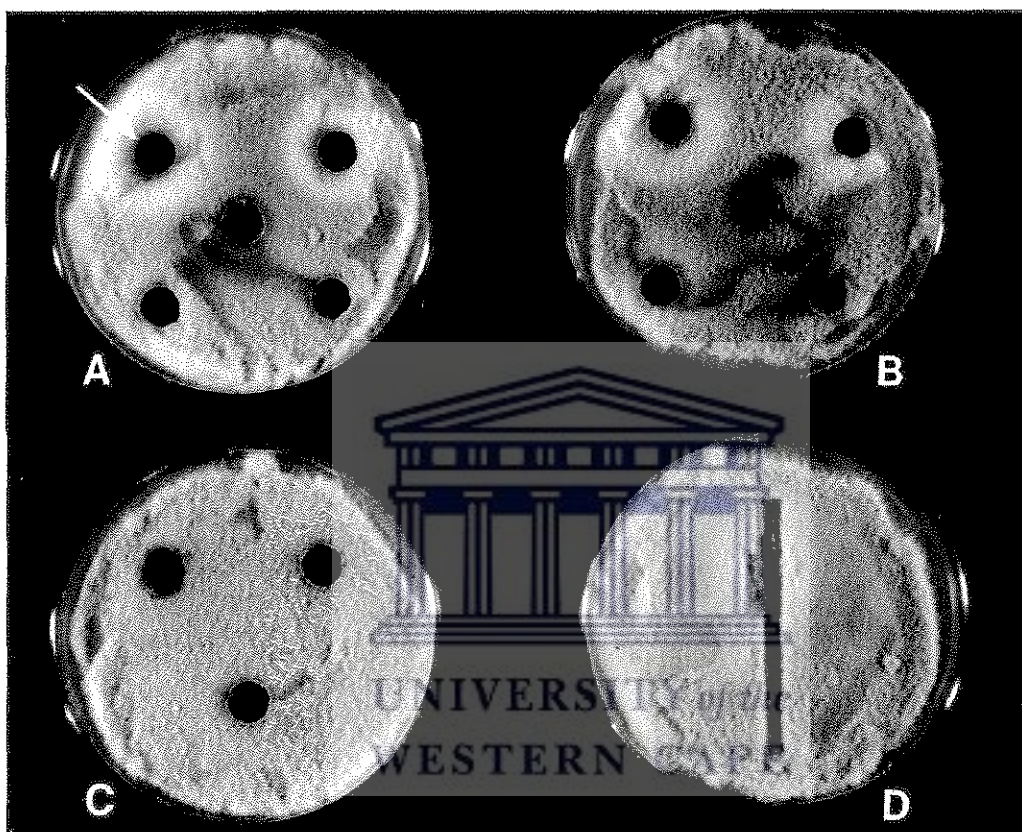


Fig. 2. Fully resistant fungus, fumonsin B₁-producing *Fusarium verticillioides*.

The MIC values for FB₁ sensitive strains are shown in Table 1. They ranged from 0.25-0.5 mM for *A. alternata*, 1-5 mM for *P. expansum* and *B. cinerea*, and 5-10 mM for *F. graminearum*. At the highest concentration of 40 mM FB₁, a small inhibition zone was visible in the case of *F. proliferatum*, a fumonisin producing species, but this fungus was considered to be resistant to FB₁. The MIC for fungi sensitive to Amp-B was 4 µg/ml for *A. alternata*, 4 µg/ml for *P. expansum* and 12 µg/ml for *F. graminearum*.

Table 1. Minimum inhibitory concentration (MIC) of fumonisin B₁ for different fungi

Fungi	MRC* number	MIC (mM)
<i>Alternaria alternata</i>	1843	0.25 - 0.5
<i>Penicillium expansum</i>	7200	1 - 5
<i>Botrytis cinerea</i>	1364	1 - 5
<i>Fusarium graminearum</i>	1785	5 - 10
<i>Fusarium proliferatum</i>	7431	> 40
<i>Fusarium verticillioides</i>	826	> 40
<i>Fusarium globosum</i>	6647	> 40
<i>Fusarium subglutinans</i>	1077	> 40
<i>Aspergillus flavus</i>	3791	> 40

* Accession numbers in the culture collection of the Programme on Mycotoxins and Experimental Carcinogens, Medical Research Council, Tygerberg, South Africa.

Discussion

The FB₁-producing *Fusarium* species isolated from maize, *F. verticillioides*, *F. globosum* and *F. proliferatum*, were resistant to FB₁ even though a small inhibition zone at the highest FB₁ concentration of 40 mM was noted in the case of *F. proliferatum* (Ross *et al.*, 1990; Sydenham *et al.*, 1997). However, amongst two non-producing *Fusarium* spp. also isolated from maize, *F. subglutinans* was resistant and *F. graminearum* was sensitive. The most sensitive fungi tested were non-producing species not isolated from maize, i.e. *A. alternata*, *B. cinerea* and *P. expansum*. This is the first report on the antifungal activity of FB₁.

The high concentrations of fumonisins used in our experiments are based on the evidence that maize samples from the Kentani district in the high oesophageal cancer area of Transkei, that are naturally contaminated with *Fusarium* species that produce FB₁, contained levels of fumonisin up to 117.5 µg/g (Rheeder *et al.*, 1992). Thus, even at unnaturally high levels of the toxin, some of the test organisms remained resistant to FB₁.

It has been reported that *F. verticillioides* can inhibit maize infection by *A. flavus* (Wicklow *et al.*, 1988; Zummo and Scott, 1992). However, present study showed no inhibiting effect of FB₁ in relation to *A. flavus*. It is possible that the interactions between the two fungi in maize kernels is influenced by other mechanisms or metabolites produced by them within the kernels.

A negative correlation between the isolation frequencies of *F. graminearum* and *F. verticillioides* from maize kernels was found by Blaney *et al.* (1986) while Van Wyk *et al.* (1988) reported that the pre-inoculation of maize germlings with *F. verticillioides*, protected the seedlings from subsequent infection by *F. graminearum*. The fact that FB₁ inhibited the

growth of *F. graminearum* in our study may offer some explanation as to why there was a negative correlation in the isolation frequencies of the two fungi. However, *F. graminearum* was the only fungus that appeared to adapt to the presence of FB₁, because it resumed growth after two days in the presence of the mycotoxin. The inhibition zones of the other fungi tested remained clear after two days.

The MIC is a quantitative indicator to derive the lowest concentration of FB₁ that prevents any visible growth of the test fungus under a specific set of laboratory conditions. It is a relative measurement because it is influenced by change in the conditions (pH, temperature, inoculum density, incubation time and media) under which the MIC is determined (Gehrt *et al.*, 1995; Pujol *et al.*, 1997). The inability of the fungus to grow in the presence of FB₁, the reason for the inhibition zone, is used as a measurement of sensitivity to FB₁. Large zones of inhibition indicate that the organism is more sensitive, while small or no zones of inhibition indicate resistance to FB₁. *Alternaria alternata* proved to be the most sensitive followed by *B. cinerea*, *P. expansum*, *F. graminearum* and *F. proliferatum*. Moreover, there was a dose-dependent increase in zone diameter with FB₁ concentration among the FB₁-sensitive fungi.

Although some species of *Alternaria*, e.g. *A. alternata* f. sp. *lycopersici*, produce host-specific phytotoxins (AAL toxins), which are structurally closely related to the fumonisins, the particular *A. alternata* strain, MRC 1843 used in our experiment showed remarkable sensitivity to FB₁ (Bezuidenhout *et al.*, 1988; Shephard *et al.*, 1993). Although this strain was not tested for its ability to produce AAL toxin, toxin formation in liquid medium by *A. alternata* f. sp. *lycopersici* normally begins after 48 hours at low concentrations (Shephard *et al.*, 1993). It is therefore most unlikely that AAL toxin, if produced in PDA by MRC 1843, contributed to its sensitivity. Furthermore, no growth inhibition was visible on the control plates after 72hrs.

It was of interest to observe that single colonies grew within the inhibition zones in the

case of *B. cinerea*. This may represent a mycotoxin resistance mechanism expressed by conidial variants in the inoculum used. Analyses of field isolates of *B. cinerea* showed that their DNA content per nucleus varied considerably, indicating that aneuploidy/heteroploidy is a widespread phenomenon in this species (Büttner *et al.*, 1994). Spontaneous colony variants in fungal cultures are characteristic of heteroploid shifts to new types of aneuploid variants (Tolmsoff, 1983). However, heteroploidy as a source of resistance to mycotoxins has not been studied in fungi.

Amp B acts on the ergosterol pathway and interferes with selective membrane functions that may result in stasis or death of most fungi (Gale *et al.*, 1981; Rippon, 1988). Interestingly, in this study three of the fungi showing inhibition zones with FB₁, namely, *A. alternata*, *P. expansum* and *F. graminearum*, were sensitive to Amp B, while *B. cinerea* and *F. proliferatum* demonstrated resistance. The Amp B resistance of *B. cinerea* could also be due to the resistant conidia present in the inoculum. FB₁ and Amp B may therefore have similar modes of action on the ergosterol pathway of fungi, but further study is necessary to elaborate this. FB₁ inhibited both the growth of the fungi tested and their spore formation. The specific mode of action of FB₁ on fungi remains unknown.

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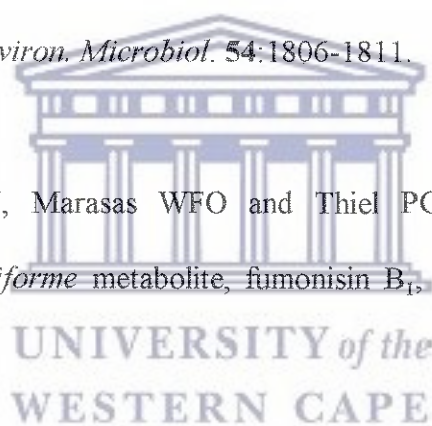
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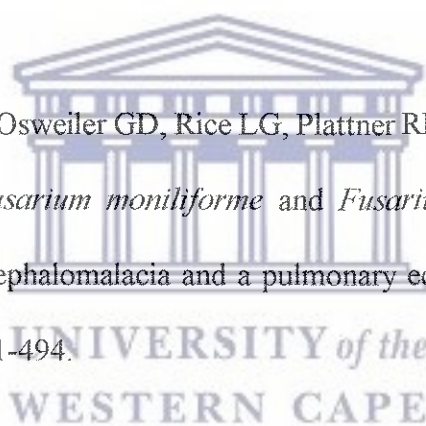
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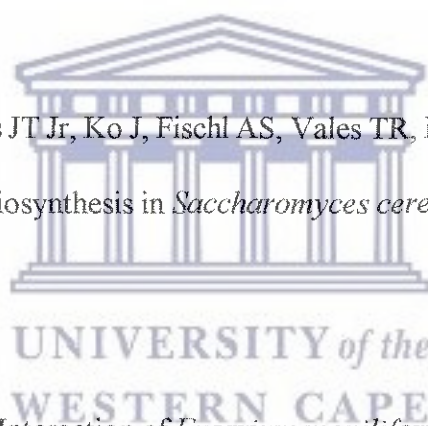
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CHAPTER 5

THE EFFECT OF FUMONISIN B₁ ON THE GERMINATION OF CONIDIA OF *FUSARIUM* AND SOME OTHER FUNGAL SPECIES

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Abstract

Fumonisin is a group of structurally related mycotoxins produced by a number of *Fusarium* species that occur worldwide some of which can produce these toxins at levels up to several g/kg on sterilized maize. Fumonisin has been shown to cause equine leucoencephalomalacia in horses, to be carcinogenic to laboratory animals (rats and mice), and has been reported to be associated with pulmonary edema in swine. Nine fungal species, i.e. *Fusarium verticillioides* MRC 826, *F. proliferatum* MRC 7431, *F. globosum* MRC 6647, *F. subglutinans* MRC 1077, *F. graminearum* MRC 1785, *Penicillium expansum* MRC 7200, *Aspergillus flavus* MRC 3791, *Alternaria alternata* MRC 1843 and *Botrytis cinerea* MRC 1364, were used for the germination assay in PDA at different fumonisin B₁ concentrations, i.e. 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 20 and 40 mM. Germ tube lengths were reduced at increasing concentrations of FB₁ for all the fungal species examined, except for *F. subglutinans*, in which case germ tube lengths were increased. Statistical analyses showed that the inhibiting effect of FB₁ was highly significant ($P < 0.001$). *P. expansum*, *B. cinerea* and *A. alternata* were the only test fungi completely inhibited by FB₁. The conidia of *F. subglutinans* germinated faster than the controls at increased concentrations of FB₁. This stimulatory effect on conidium germination diminished with time, as swellings on the tips appeared and stunted germ tubes were formed.

Introduction

Fusarium verticillioides (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) is associated with ear-rot of maize world-wide (Booth, 1971; Marasas *et al.*, 1979; Marasas, 1982) and is regarded as the most common seed-borne fungus of maize in South Africa (Rheeder *et al.*, 1993). The fungus often causes symptomless infections in maize kernels (King and Scott, 1981) and is associated with several human and animal diseases (Thiel *et al.*, 1992). Fumonisin B₁ (FB₁) and B₂ (FB₂), produced by *F. verticillioides*, causes leukoencephalomalacia in horses (Kellerman *et al.*, 1990) and pulmonary oedema syndrome in pigs (Harrison *et al.*, 1990). FB₁ is also hepatotoxic and hepatocarcinogenic in rats (Gelderblom *et al.*, 1991; Gelderblom *et al.*, 1993). Fumonisins have also been statistically associated with high risk of oesophageal cancer in humans who consume contaminated home-grown maize in South Africa (Transkei) (Rheeder *et al.*, 1992) and the People's Republic of China (Linxian County) (Chu and Li, 1994).

Keyser *et al.* (1999) reported on the antifungal activity of FB₁ to mycelial growth of *Fusarium* and other fungal species. Growth inhibition zones were observed for *Alternaria alternata* (Fr.) Keissler (MRC 1843), *Penicillium expansum* Link (MRC 7200), *Botrytis cinerea* Pers. ex Fr. (MRC 1364) and *F. graminearum* Schwabe (MRC 1785) of nine fungi tested. The FB₁-producing *Fusarium* spp. isolated from maize, i.e. *F. verticillioides*, *F. globosum* Rheeder (MRC 6647) and *F. proliferatum* (Matsushima) Nirenberg (Ross *et al.*, 1992; Sydenham *et al.*, 1997) were resistant to FB₁ while two non-producing *Fusarium* spp. isolated from maize, *F. subglutinans* (Wollenw. & Reinking) Nelson, Toussoun & Marasas (MRC 1077) and *F. graminearum*, were resistant and sensitive, respectively.

Several different methods exist to test the susceptibility of fungi against antimycotic

drugs and mycotoxins. These include broth dilution (Sandhu *et al.*, 1979), agar-diffusion (Keyser *et al.*, 1999), disk diffusion (Utz and Shacomy, 1976; Holt, 1978) and microtiter bioassays (Fisher and Armstrong, 1977). Morphological deformations observed in mycelia of certain filamentous fungi induced by antibiotics and mycotoxins have been reported (Richmond, 1975; Gunji *et al.*, 1983; Keyser *et al.*, 1999). These bioassay methods were found to be useful in detecting proper minimum inhibitory concentration (MIC) zones and also different deformations such as curling, swelling and hyphal stunting caused by the specific antifungal agents.

Results obtained with MIC's are defined on the basis of visible mycelial growth. Conidia should germinate and produce germ tubes for monitoring the growth inhibition or stimulation and the fungicidal activity of the compound, compared to a control. If a compound is capable of inhibiting the germination of conidia, while either affecting or not affecting the growth of the organism, the MIC is considered the concentration of the compound required for the inhibition of conidial germination, but not necessarily the concentration required for inhibition of the growth of the organism. The use of conidial germination has resulted in excellent reproducibility of germ tube inhibition results obtained in different laboratories (Manavathu *et al.*, 1996; Denning *et al.*, 1997; Pujol *et al.*, 1997). The objective of the study was to investigate the effect of FB₁ on the germination of freshly harvested conidia of *Fusarium* and some other fungal species.

Materials and Methods

Fungal isolates. *Fusarium verticillioides* MRC 826, *F. proliferatum* MRC 7431, *F. globosum* MRC 6647, *F. subglutinans* MRC 1077, *F. graminearum* MRC 1785,

P. expansum MRC 7200, *A. flavus* MRC 3791, *A. alternata* MRC 1843 and *B. cinerea* MRC 1364 were obtained from the culture collection of the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) of South Africa for this study. Freshly harvested conidia (FHC) were collected as described previously (Keyser *et al.*, 1999). For the germination assay for each fungal species, filtered suspensions of FHC were suspended in 5 ml sterile distilled Nonidet P40 water to prevent clumping of conidia, and the density adjusted microscopically to 1×10^6 conidia/ml with a Neubauer hemacytometer.

Fumonisin B₁. FB₁ was obtained as a powder (96 % pure) from PROMEC. The FB₁ was extracted, isolated and purified as previously described (Cawood *et al.*, 1991).

Standardization of FB₁ solutions. A 21 ml stock solution of FB₁ at a concentration of 40 mM was prepared by dissolving 0.433 g FB₁ in 0.5 ml 1 M NaOH and 2.5 ml H₂O (pH adjusted to 7). The fumonisin solutions were filter-sterilized by using millipore filters (pore-size 0.22 μ m, Corning Inc, Germany) and made up to 21 ml with 18 ml autoclaved potato dextrose agar (PDA) previously cooled to 50°C. A serial dilution of the stock solution was made with PDA, to yield FB₁ concentrations of 0.25, 0.5, 1, 2.5, 5, 7.5, 10, 20 and 40 mM, respectively. These FB₁-amended PDA solutions were kept in a water bath at 50°C to prevent them from solidifying. FB₁ is thermostable and could therefore be kept at 50°C.

For each fungal species, 200 μ l of the respective conidium suspension, was separately transferred to a 1.5 ml centrifuge tube by means of a sterile micro-pipette (9 concentrations of FB₁ were done in triplicate). Three controls, containing no FB₁, were also prepared. The conidia were harvested for each treatment by centrifuging the conidial suspension for 5 minutes at 3000 rpm. The pellets in each of the tubes, were resuspended in 200 μ l of respective FB₁-amended PDA stock solutions and kept on a hot block (Pierce Reacti-Therm III Heating

module) at 50°C.



Figure 1. Incubation chamber consisting of a pre-sterilized 90 mm diameter Pyrex petri dish, lined with Whatman No.1 filter paper (a); a taped microscope slide (b); with three round 12 mm diameter cover slips on top (c), placed on a bent glass rod.

Three 200 μ l aliquots of the standardized, evenly dispersed conidia in PDA containing a specific FB_1 concentration, were aseptically transferred onto the three round cover slips on top of the microscope slide in the incubation chamber as illustrated in Figure 1. Five ml sterile water was used to saturate the Whatman filter paper, which then served as moisture chamber for the incubation period at 25°C in the dark. Conidia were allowed to germinate in the presence of FB_1 and the slides were examined under the microscope every hour. A control slide, containing no FB_1 in the PDA conidial suspension, was prepared for each fungus examined. All experiments were stopped when the conidia in control slides reached 100 % germination. The percentage conidium germination was based on the random sampling of 100 conidia per FB_1 treatment. For each FB_1 treatment the germ tube length of 40 randomly selected germinating conidia was measured. In the case of fungi with multi-celled conidia, a conidium was considered germinated if a germ tube was visible from at least one of the cells. Germ tube length of control and FB_1 -treated conidia were measured by using a image analysis system which consisted of a Leica conventional light microscope linked to the PC running HLImage 97++ image analysis software, a Pulnix video camera and a frame grabber. The image capturing device was calibrated for each objective used, so that all measurements were actual measurements expressed in μ m. The germ tube length data given in the results represent the means for all tests done.

Data analysis. Statistical analyses were performed by means of the NCSS 2000 statistical analysis package (Hintze, 1998). The General Linear Model analysis of variance, that can cope with unequal sample sizes, was generally used. The multiple comparison test used was the Newman-Keuls test. The factorial design analyses on many of the experiments were performed twice, namely: (1) with the "Control" included as a one-way analysis of variance to test whether the "Control" was significantly different from the other treatment combinations;

and (2) with the “Control” excluded in a proper factorial analysis to test for treatment factor main effect and interactions.

The test for parallel regression lines in the germination experiment was performed as follows: (1) by performing a covariance analysis with treatment level as covariate and “Strain” as factor to estimate the residual sum of squares when fitting a common linear regression slope; (2) performing a regression analysis on the data for each strain separately to get the residual sum of squares when fitting a best linear regression line to that strain; and (3) the difference between the residual arrived at in step one and the sum of the residuals arrived at in step two, yields a test to ascertain whether the assumption of a common regression slope is valid.

Results



The effect of nine FB_1 concentrations, ranging from 0.25 to 40 mM, on the conidium germination of nine test fungi was determined. The inhibiting effect of FB_1 was determined by means of percentage germination of conidia and germ tube or hyphal length measurements (Table 1).

Germ tube lengths were reduced at increasing concentrations of FB_1 for *A. alternata*, *P. expansum*, *B. cinerea*, *F. graminearum* and *F. proliferatum*, *F. verticillioides*, *A. flavus* and *F. globosum* (Table 1, Fig. 2) compared to the control cultures. Statistical analyses showed that the inhibiting effect of FB_1 was highly significant ($P < 0.001$) for the fungal strains tested, and that significant intra-strain differences occurred ($P < 0.001$). *P. expansum*, *B. cinerea* and *A. alternata* were the only test fungi that were completely inhibited by FB_1 . Concentrations of FB_1 higher than 1 mM prevented the germination of *P. expansum* completely. The germination of both *B. cinerea* and *A. alternata* was completely inhibited at a FB_1 concentration of 40 mM

(Table 1).

The percentage germination of all test fungi was influenced when grown in the presence of FB₁ compared to their control cultures (Figs. 3-7). The mean percentage germination over time for *F. verticillioides*, *F. graminearum*, *F. proliferatum*, *P. expansum* and *A. alternata* was numerically faster in the control cultures on PDA than in FB₁ amended PDA. The germination rate in control cultures for *F. globosum* (Figure 4a), *A. flavus* (Figure 6a) and *B. cinerea* (Figure 7b), were similar to the corresponding FB₁ amended cultures. The conidia of *F. subglutinans* germinated faster than the controls at increased concentrations of FB₁.

Of the nine fungi tested, *F. subglutinans* was the only fungus which germinated faster in the presence of high concentrations of FB₁ compared to the controls (Fig. 4b). Although a complete inhibition of germination was found at intermediate concentrations of FB₁ at 0.5, 1, and 5 mM (Figure 8a), higher concentrations between 7.5 and 40 mM stimulated growth and longer germ tubes than in the control cultures were formed (Figure 8). FB₁ amended cultures reached 100 % germination one hour earlier than control cultures. However, the tips of the germ tubes became swollen and stunted soon after germination (Figure 8b). The controls formed normal germ tubes with several septa (Figure 8c).

The initial microscopic evaluations of the conidial germination and percentage germination of the conidia of *F. verticillioides*, *F. globosum* and *A. flavus*, indicated that these fungi were slightly resistant to FB₁ with no obvious differences in germ tube lengths between control and test cultures. However, statistical analyses indicated that germ tubes were significantly inhibited compared to the controls at 100 % germination ($P < 0.001$) (Table 1, Fig. 8). Comparison of images of *A. flavus* at different FB₁ concentrations revealed that the germ tubes of the control cultures were not only thicker, but that the conidia were also slightly bigger than conidia germinating in the presence of high concentrations of FB₁ (Images not

included).

At FB_1 concentrations of 0.25 mM, *A. alternata*, *P. expansum*, *B. cinerea* and *F. graminearum* gave reduced germ tube lengths, while at 1mM and higher concentrations, all except *F. subglutinans*, produced shorter germ tubes (Table 1). Thus, distinct differences between *A. alternata*, *P. expansum*, *B. cinerea* and the remaining six fungi were observed, with *A. alternata* being very sensitive to FB_1 .

Control cultures of *A. alternata*, *B. cinerea* and *P. expansum* showed normal germinating conidia with relatively long and thick germ tubes (Figs. 9-11), and the germ tubes of *A. alternata* developed septa soon after germination (Figure 9a-c). In the presence of FB_1 at concentrations ranging between 2.5 and 20 mM, the germ tubes became swollen at their tips for all three these fungi. Stunting occurred before any septa were formed in *B. cinerea* and *P. expansum*. At concentrations higher than 20 mM, conidia of *A. alternata*, *B. cinerea* and *P. expansum*, initially produced very short germ tubes, which did not develop any further at longer incubation.

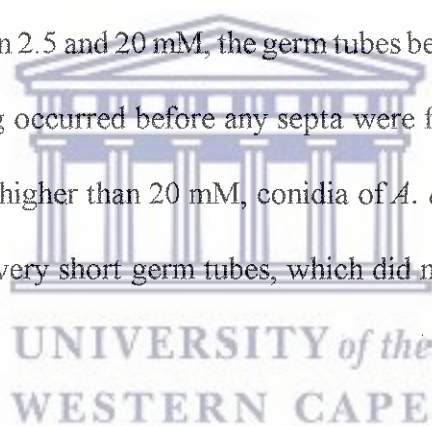


Table 1. Mean germ tube length (μm) of nine fungi at different concentrations of fumonisin B₁ after 100% germination of control conidia in PDA at 25°C.

Fungi Tested	Fumonisin concentrations (mM)					
	Control	0.25	1	5	10	40
	Germtube length (μm)					
<i>Fusarium verticillioides</i> MRC 826	12.09	11.67	10.50	-	8.93	8.26
<i>Fusarium proliferatum</i> MRC 7431	20.69	19.53	17.95	-	14.36	12.98
<i>Fusarium subglutinans</i> MRC 1077	18.66	NM	ng	-	NM	NM
<i>Fusarium graminearum</i> MRC 1785	18.38	16.85	13.38	-	11.07	8.70
<i>Fusarium globosum</i> MRC 6647	29.73	29.14	25.15	-	26.83	21.01
<i>Botrytis cinerea</i> MRC 1364	27.11	26.45	19.11	15.31	13.21	ng
<i>Alternaria alternata</i> MRC 1843	68.72	40.46	20.96	11.55	9.49	ng
<i>Aspergillus flavus</i> MRC 3791	18.19	17.82	16.90	16.51	16.46	14.53
<i>Penicillium expansum</i> MRC 7200	13.97	10.73	ng	ng	ng	ng

- = Germ tubes not measured
 NM = Germ tubes not measurable due to swellings
 ng = No germination

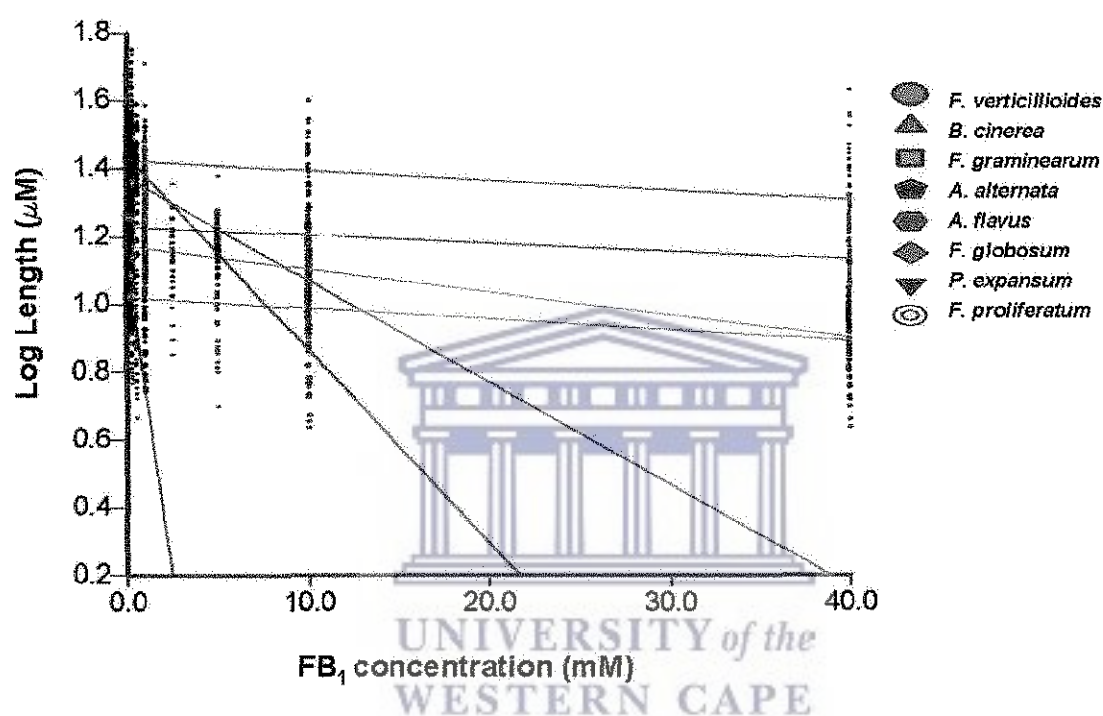


Figure 2. Effect of FB_1 at different concentrations on the germ tube length of *Fusarium verticillioides*, *F. proliferatum*, *F. globosum*, *F. graminearum*, *P. expansum*, *A. flavus*, *A. alternata* and *B. cinerea*. The Scatter plot graph represents the mean Log germ tube length (>40 determinations per fungus) at different FB_1 concentrations. *F. subglutinans* not included in graph - germ tubes not measurable due to swellings.

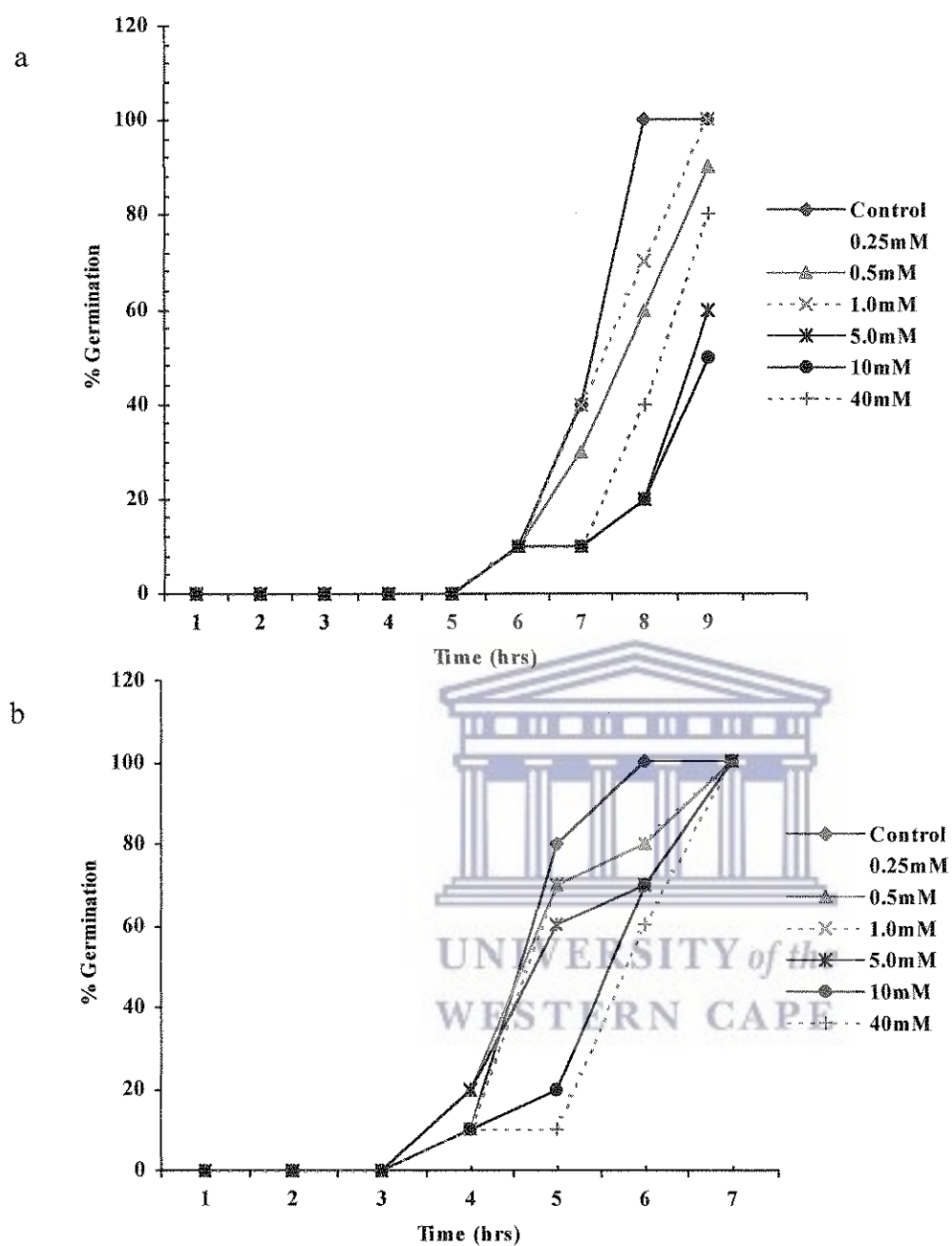


Figure 3. Mean percentage germination of (a) *Fusarium verticillioides* (MRC 826) and (b) *F. proliferatum* (MRC 7431) at different concentrations of FB₁ after incubation on PDA at 25°C, compared to 100 % germination of control cultures.

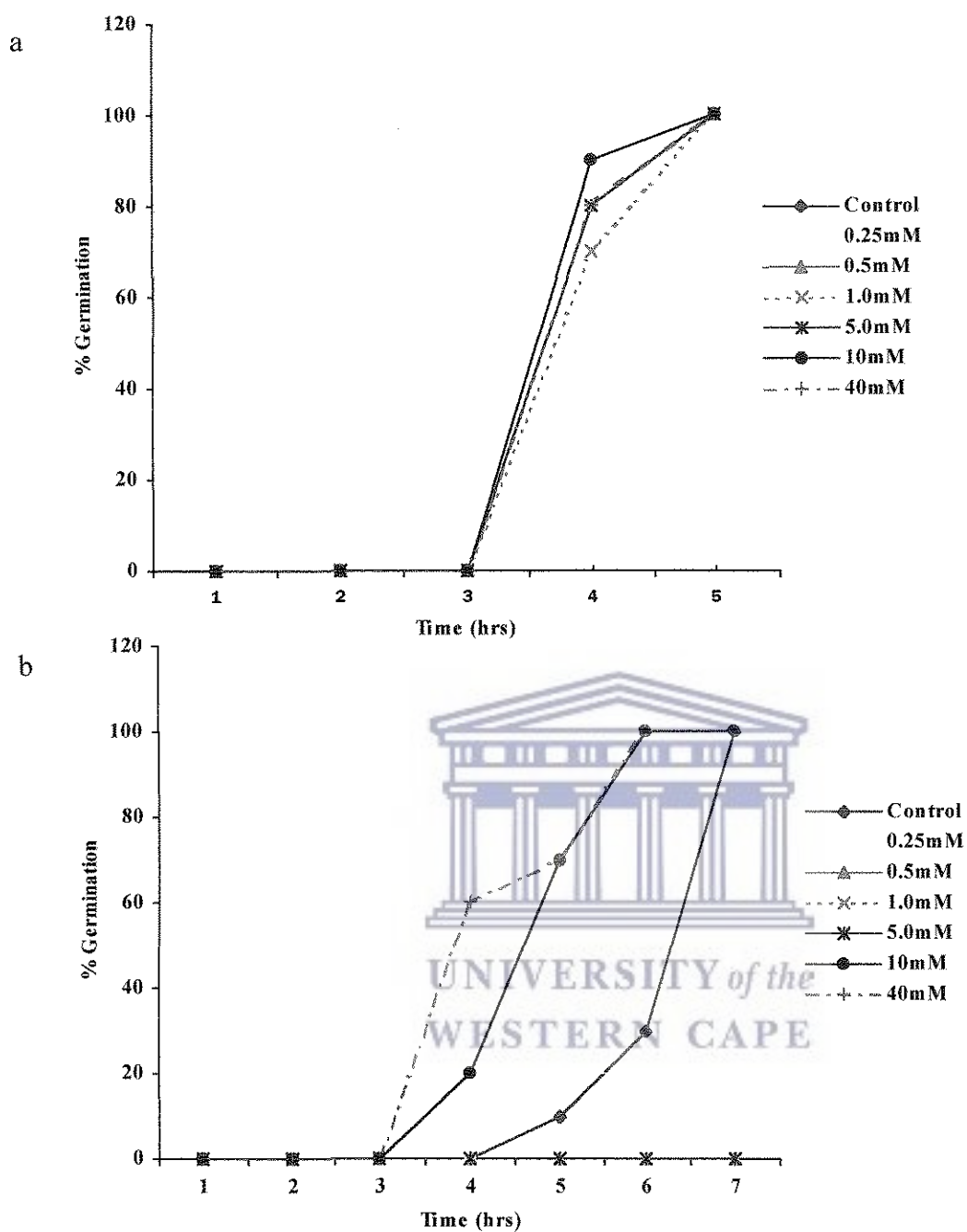


Figure 4. Mean percentage germination of (a) *Fusarium globosum* (MRC 6647) and (b) *F. subglutinans* (MRC 1077) at different concentrations of FB₁ after incubation on PDA at 25°C, compared to 100 % germination of control cultures.

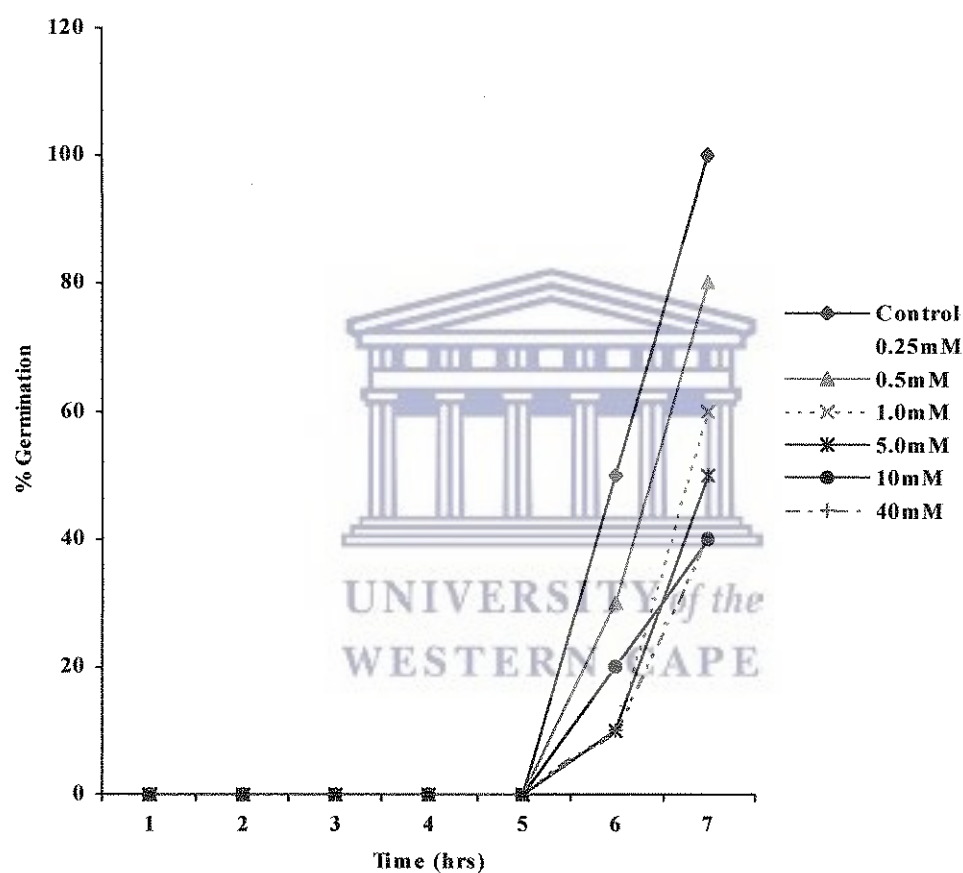


Figure 5. Mean percentage germination of *Fusarium graminearum* (MRC 1785) at different concentrations of FB_1 after incubation on PDA at 25°C, compared to 100 % germination of control cultures.

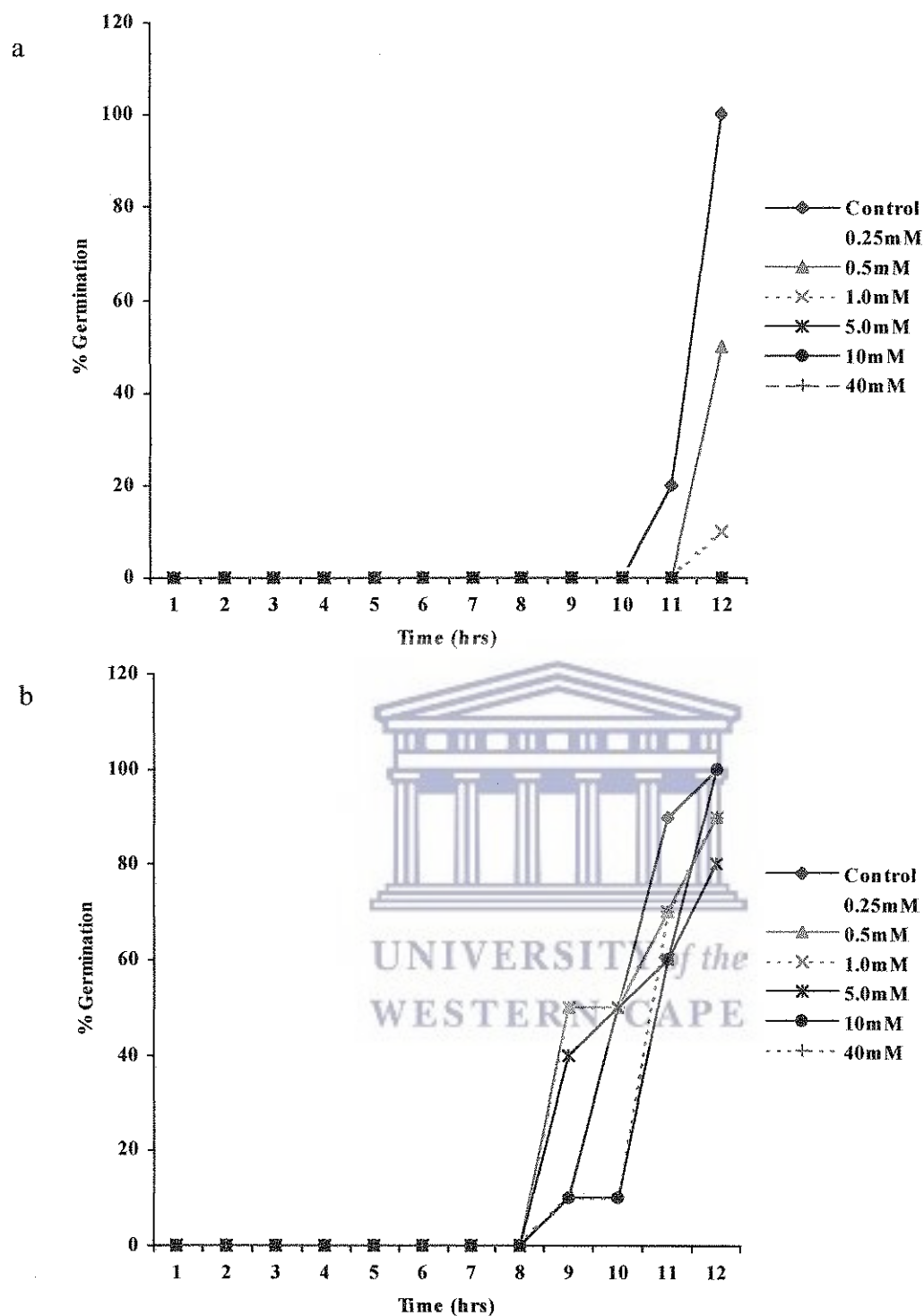


Figure 6. Mean percentage germination of (a) *Penicillium expansum* (MRC 7200) and (b) *Aspergillus flavus* (MRC 3791) at different concentrations of FB_1 after incubation on PDA at 25°C, compared to 100 % germination of control cultures.

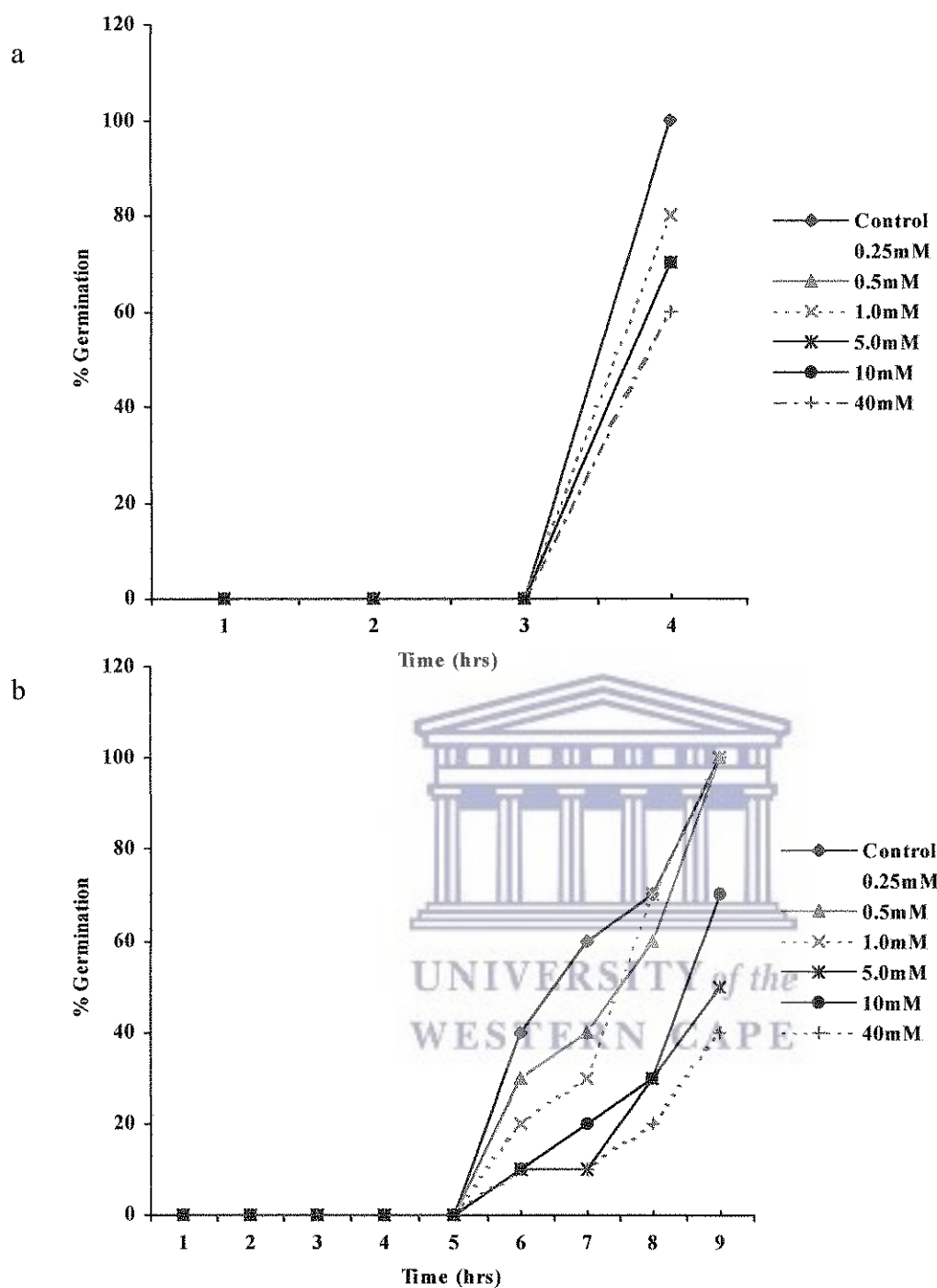


Figure 7. Mean percentage germination of (a) *Alternaria alternata* (MRC 1843) and (b) *Botrytis cinerea* (MRC 1364) at different concentrations of FB_1 after incubation on PDA at 25°C, compared to 100 % germination of control cultures.

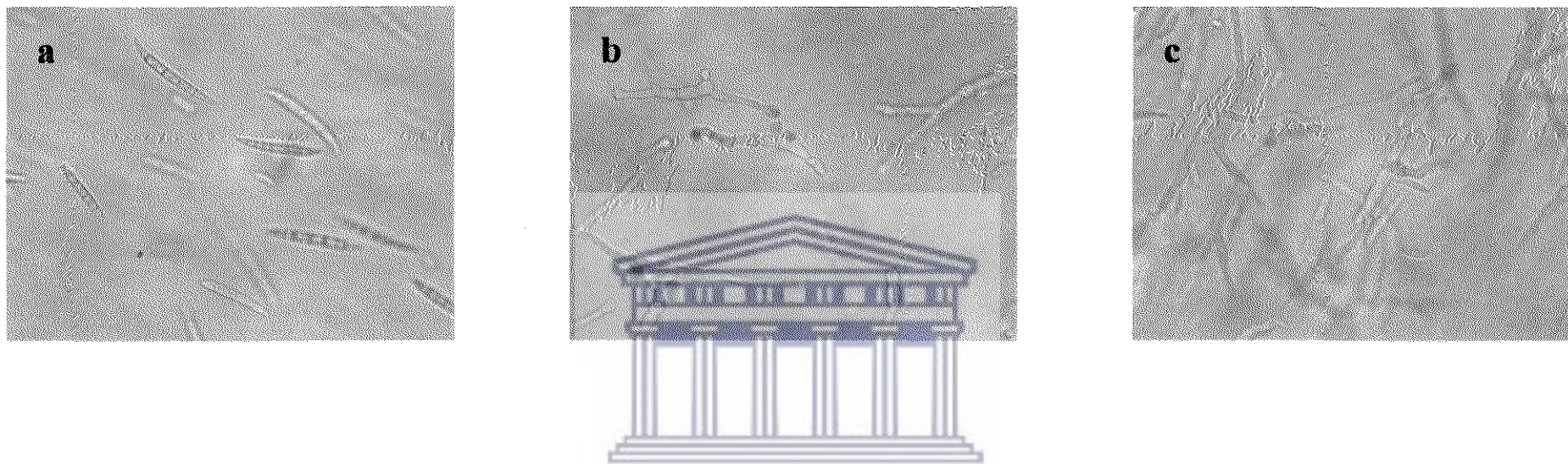


Figure 8. Stimulatory effects on the germination of *Fusarium subglutinans* conidia at increasing concentrations of FB_1 in PDA at 25°C. (a) Conidia did not form germ tubes after 7hrs incubation and exposure to intermediate concentrations of 0.5 to 5mM FB_1 . (b) Swollen germ tube tips developed after 7hrs incubation exposed to higher FB_1 concentrations (7.5 - 40mM). (c) Germ tubes rapidly developed into hyphal growth after 6hrs incubation in control cultures.

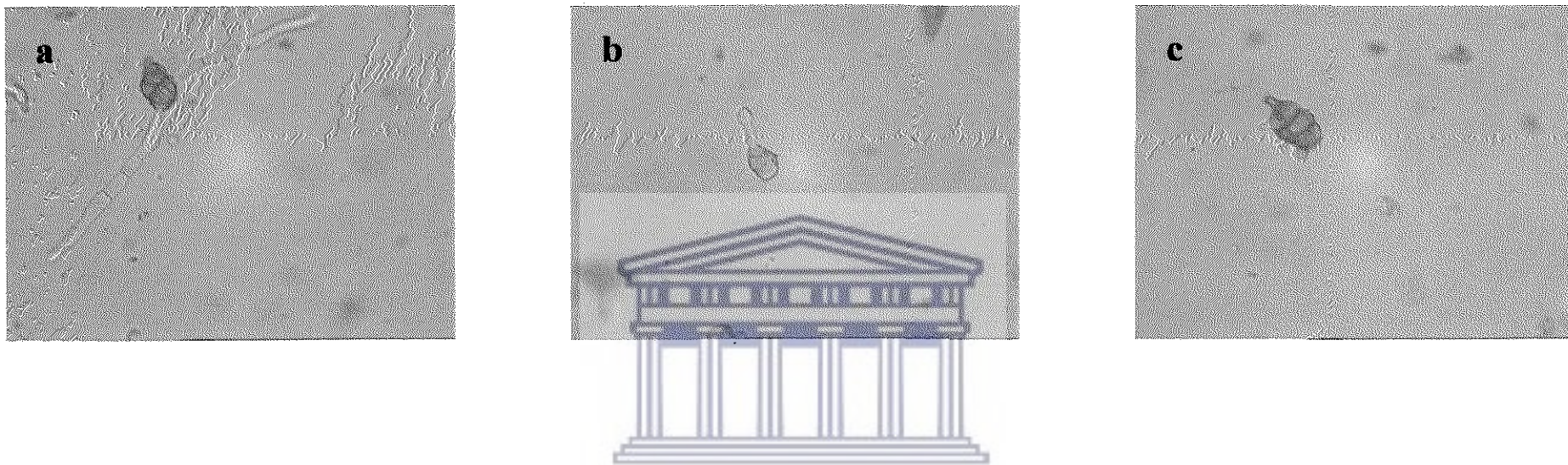


Figure 9. Inhibitory effect of increasing concentrations of FB_1 on the germination of *Alternaria alternata*. (a) Control (b) 2.5-20mM FB_1 and (c) 40mM FB_1 in PDA after 4hrs incubation at 25°C.

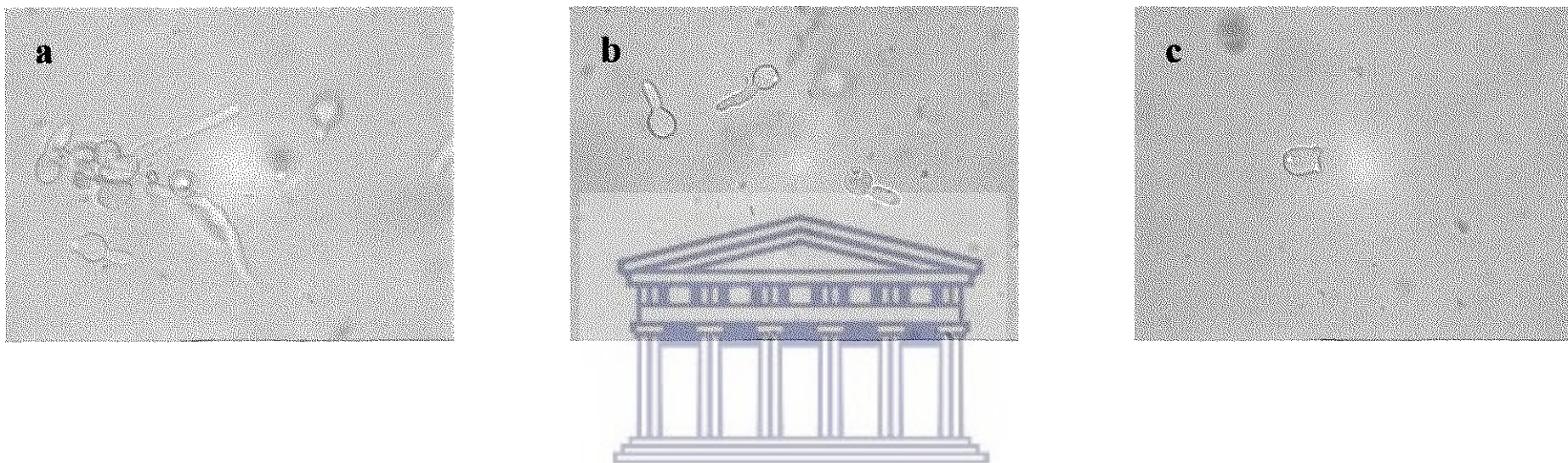


Figure 10. Inhibitory effect of increasing concentrations of FB_1 on the germination of *Botrytis cineria*. (a) Control (b) 2.5-20mM FB_1 and (c) 40mM FB_1 in PDA after 7hrs incubation at 25°C.

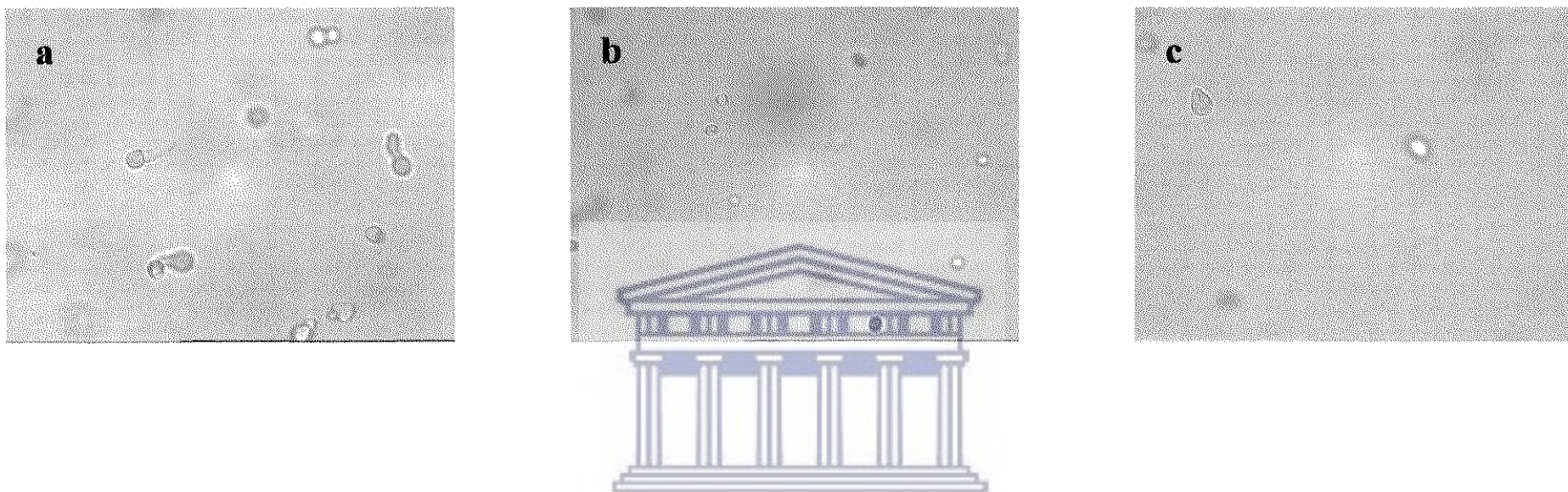


Figure 11. Inhibitory effect of increasing concentrations of FB_1 on the germination of *Penicillium expansum*. (a) Control (b) 2.5-20mM FB_1 and (c) 40mM FB_1 in PDA after 7hrs incubation at 25°C.

Discussion

The FB₁-producing *Fusarium* species isolated from maize, i.e. *F. verticillioides*, *F. globosum* and *F. proliferatum* showed a decrease in germ tube length with an increase in FB₁ concentrations, compared to the control cultures. Thus, indicating that these fungi can only tolerate their own toxic metabolite to a certain extent. However, amongst two non-producing *Fusarium* spp., i.e. *F. subglutinans* and *F. graminearum* also isolated from maize, the former was induced to germinate faster in the presence of FB₁ but soon developed stunted germ tubes, and the latter one developed shorter germ tubes than control cultures. The most sensitive fungi tested were species not isolated from maize, i.e. *A. alternata*, *B. cinerea* and *P. expansum*, which did not germinate at higher FB₁ concentrations.

The conidial germination bioassay was more sensitive in the detection of the antifungal activity of FB₁ than the Petri dish bioassay. Since the MIC's of FB₁ to various fungi was defined on the basis of visible mycelial growth (Keyser *et al.*, 1999), this technique allowed the conidia to germinate and produce germ tubes for monitoring of the growth inhibition and fungicidal activity of FB₁. The MIC's of FB₁ for visible mycelial growth were closely comparable to those obtained from conidial germination.

It is important to note that FB₁ did not completely inhibit germination of *F. verticillioides*, *F. globosum*, *F. proliferatum*, *F. graminearum* and *A. flavus* at concentration higher than 0.25 mM, but only reduced germ tube length. FB₁ at these concentrations was equally effective in suppressing the germination rate of all but *F. subglutinans*, but still allowed some conidia to germinate, only slower. This investigation therefore has demonstrated that FB₁ has a dose-dependent effect on the rate of germination and the subsequent germ tube elongation on the fungi tested except for *F. subglutinans*.

There are reports that *F. verticillioides* can inhibit maize kernel infection by *A. flavus* in inoculated maize ears and lead to reduced aflatoxin contamination in kernels (Wicklow *et al.*, 1988; Zummo and Scott, 1992). The results reveal a significant reduction in germ tube length of *A. flavus* with increasing FB₁ concentrations.

The conidia of *F. subglutinans* were induced to germinate faster than the controls with increased concentrations of added FB₁. Apart from this stimulatory effect on the % germination, these germ tubes showed swellings on their tips while the controls formed septa and branches. This stimulatory effect on conidium germination diminished with time, as swellings on the tips appeared and stunted germ tubes were formed. No measurable germ tube lengths could be determined for *F. subglutinans* at these stimulatory conditions, due to stunting of germ tubes. No tests were performed on the viability of ungerminated conidia.

The results of the germination studies clearly show strong antifungal activity for FB₁ towards *B. cinerea*, *P. expansum*, *A. alternata* and *F. graminearum*, confirming the findings of Keyser *et al.* (1999) that FB₁ at similar concentrations completely inhibited mycelial growth of these four fungi. This antifungal assay also allowed conidia to germinate almost synchronously, within short periods of time of incubation, to obtain reproducible results. Longer incubation could result in rapid growth of germ tubes, and hyphal or mycelial mass may increase rapidly.

One of the concerns in the development of a standard method for the in vitro susceptibility testing of filamentous fungi is the nature of the inoculum. It has been shown that the MIC's of antifungal agents for filamentous fungi are dependent on the nature and the size of the inoculum (Manavathu *et al.*, 1996; Guarro *et al.*, 1997; Keyser *et al.*, 1999). There are no universally accepted procedures for the determination of fungal susceptibilities. Variabilities in the test results are related to inoculum size, medium composition, medium pH, incubation

temperature, incubation time and end point criteria (National Committee for Clinical Laboratory Standards, 1985). The use of conidia is therefore an attractive option in a study like this. This technique make use of freshly harvested conidia allowing them to germinate in the presence of the specific antifungal agent (FB₁). This method demonstrated that the germination process was also affected by FB₁.

The mechanism by which FB₁ enters fungal cells has not been elucidated. It has been speculated by Keyser *et al.* (1999) that FB₁ and Amphotericin B may have similar modes of action on the ergosterol pathway of fungi. If so, increases in membrane permeability, which could be caused by high FB₁ concentrations, could also enhance penetration of FB₁ to the fungal cell interior, and this might account for the increased inhibition of germination, stunting and swollen hyphal tips.



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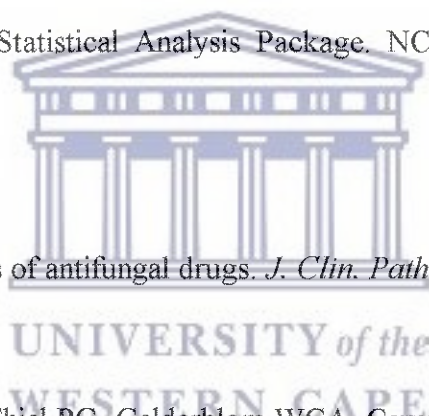
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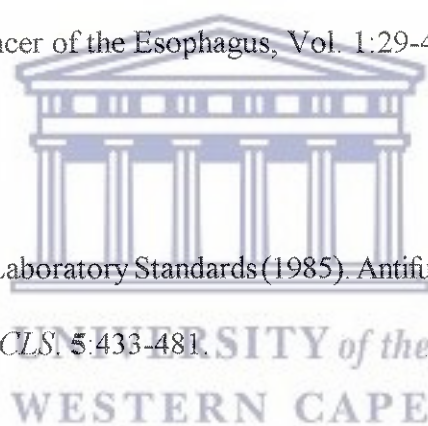
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SUMMARY

Fusarium verticillioides is a very important mycotoxin-producing fungus associated with maize. *F. verticillioides* produces a group of mycotoxins known as fumonisins under suitable environmental conditions. A series of studies was designed to provide information regarding some of the factors associated with the production of fumonisin B₁ (FB₁) in maize patties and MYRO liquid medium. Our investigation together with previous studies have detailed the important influence of several factors on the production of fumonisins by *F. verticillioides* strains. To understand why these strains are able to produce these toxins, an investigation into the complex interaction that occurs between biotic and abiotic parameters and their impact on fumonisin production was necessary. The results reflect the interacting factors and the intraspecific differences between strains, which may also be present in field conditions.

The parameters that were varied under a predetermined set of culture conditions, included initial moisture content of maize patty cultures, temperature, initial pH and the addition of the fumonisin precursors, L-alanine and L-methionine to the cultures. Investigations into the three-way interactions of initial maize patty moisture content (30 ml water to 30g of maize), L-methionine (0.3 %) and temperature (25°C), resulted in the highest yield of FB₁ (5777.26 µg/g) produced by MRC 4316. In contrast, MRC 826 was negatively affected, producing lower levels of FB₁ (3492.24 µg/g), compared to MRC 4316 at an initial moisture content (20 ml water to 30 g maize), L-methionine (0.3 %) and 25 °C. An American strain of *F. verticillioides* MRC 7424 (= NRRL 13616), produced the highest levels of FB₁ (116 µg/ml), while the South African isolates, MRC 4316 and MRC 826, produced lower FB₁ levels (93 and 62 µg/ml, respectively) in MYRO liquid medium.

In general, FB₁ production in maize patty cultures far exceeded levels obtained in liquid

shake cultures. It appears that not only the ability of a particular strain of *F. verticillioides*, but the interaction of a variety of physiological and nutritional factors and the culture medium, are important in the production of FB₁. Thus, variation of a single factor such as temperature under field conditions due to seasonal change, may therefore have a major effect on fumonisin production. A chain reaction may occur when changes in moisture, pH, etc. take place, which may influence fumonisin production further.

Lyophilisation of fungal cultures proves to be an excellent method to preserve a wide range of fungi over long periods of time. It is, however, necessary to determine the viability of conidia stored in lyophilised vials at 4°C on a regular basis. At present, plate count methods remain the most valid technique for the detection of the viability of lyophilised conidia. Membrane-permeant nucleic acid-binding dyes (FUN-1) are viability stains that are relatively new fluorescent probes for assessing the viability of metabolically active yeast cells. The purpose of this study was to microscopically determine the viability of lyophilised conidia of *Fusarium* and *Alternaria* species, using the yeast, *Saccharomyces cerevisiae*, as a control. FUN-1 viability stain was compared to two other staining methods, i.e. ethidium bromide (EB) and methylene blue (MB) and the viability of the conidia was compared to colony-forming units (CFU) on solid media as a control. For the purpose of determining or screening for percentage viability in a specific inoculum, results indicate that EB can be used in the case of lyophilised conidia, and MB in the case of freshly harvested conidia. Although FUN-1 are recommended as a good way to determine the cell viability of a fungus, it needs relatively complicated procedures and has a time limit in which the stain can be used. The result of this study emphasize that the use of dyes to determine viability of lyophilised conidia require a critical definition of protocols for a specific fungal species, and that a good correlation with CFU needs to be demonstrated. The findings of this study could find useful applications in

various studies on living and dead conidial populations.

The diverse toxicological effects of fumonisins in animals and plants raised the possibility that fumonisins may also inhibit the growth of filamentous fungi. This study investigated the antifungal activity of FB₁ to some *Fusarium* and other fungal species. The sensitivity of these fungi was tested by an agar-diffusion method on PDA plates. FB₁ inhibited the mycelial growth of five of the nine fungi tested. The FB₁-producing *Fusarium* species isolated from maize, i.e. *F. verticillioides*, *F. globosum* and *F. proliferatum* were resistant to FB₁ even though a small inhibition zone at the highest FB₁ concentration of 40mM was noted in the case of *F. proliferatum*. However, amongst two non-producing *Fusarium* spp. also isolated from maize, one (*F. subglutinans*) was resistant and one (*F. graminearum*) was sensitive. The most sensitive fungi tested were non-producing species not isolated from maize, i.e. *Alternaria alternata*, *Botrytis cinerea* and *Penicillium expansum*. The minimum inhibitory concentration of FB₁ ranged between 0.25-0.5mM for *A. alternata*, 1-5mM for *P. expansum* and *B. cinerea* and 5-10mM for *F. graminearum*, while the other fungi tested showed no sensitivity to FB₁. This is the first report on the antifungal activity of FB₁ to filamentous fungi.

Another study investigated the effect of FB₁ on the germination of freshly harvested conidia of *Fusarium* and some other fungal species. The FB₁-producing *Fusarium* species isolated from maize, i.e. *F. verticillioides*, *F. globosum* and *F. proliferatum* showed a decrease in germ tube length with an increase in FB₁ concentrations. This indicated that these fungi can tolerate their own toxic metabolite to a certain extent. However, amongst the two non-fumonisin producing *Fusarium* spp. examined, i.e. *F. subglutinans* and *F. graminearum*, isolated from maize, *F. subglutinans* was induced to germinate faster in the presence of FB₁ but soon developed stunted germ tubes, while *F. graminearum* developed shorter germ tubes compared to the control cultures. The most sensitive fungi tested were species not isolated

from maize, i.e. *A. alternata*, *B. cinerea* and *P. expansum*, which did not germinate at higher FB₁ concentrations at all. Statistical analyses showed that the inhibiting effect of FB₁ was highly significant ($P < 0.001$). The conidial germination bioassay was more sensitive in the detection of the antifungal activity of FB₁ than the petri dish bioassay. The minimum inhibitory concentrations of FB₁ for visible mycelial growth were closely comparable to those obtained from conidial germination.

Results of these studies provide considerable information on the parameters affecting the production of FB₁, and will be of great benefit in further studies focussing on fumonisin production.

