Characterisation, Isolation, Purification and Toxigenicity of Diplodiatoxin produced by *Stenocarpella maydis* in Maize

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A dissertation submitted for the degree of PhD at the University of Western Cape, Cape Town, South Africa, 2002.

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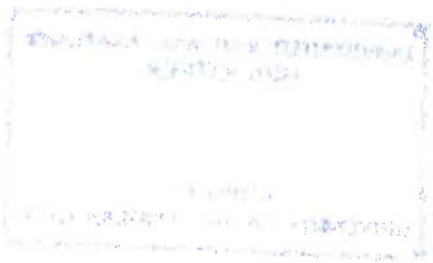

DECLARATION

I declare that "Characterisation, Isolation, Purification and Toxigenicity of Diplodiatoxin produced by *Stenocarpella maydis* in Maize" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

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Abstract

Characterisation, Isolation, Purification and Toxigenicity of Diplodiatoxin produced by *Stenocarpella maydis* in Maize

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PhD Thesis, Department of Biotechnology, University of Western Cape.

Stenocarpella maydis is an important pathogen causing stalk rot, ear rot and seedling blight in maize. *S. maydis* infection causes severe losses in the crop productivity and yield. When the infected crop is consumed by animals it causes mycotoxicoses known as diplodiosis. Diplodiosis is characterised by ataxia, paralysis, neurological disturbances and severe liver damage. *S. maydis* produces diplodiatoxin which is a bicyclic β-γ unsaturated carboxylic acid containing a β-keto side chain. To study the effects of pure diplodiatoxin, *S. maydis* isolates from different maize growing regions in South Africa were collected and screened for the presence of diplodiatoxin. The presence of diplodiatoxin in these isolates was detected by thin layer chromatography and mass spectrometry. Isolates from the Potchefstroom region produced the highest concentration of diplodiatoxin, whereas some of the isolates from the Potchefstroom and Cedara regions did not produce the toxin. Experiments were conducted to optimise the *in vitro* production of diplodiatoxin using the isolate MC43. The diplodiatoxin was produced in detectable quantities in the media in cultures older than 6-8 weeks. A protocol was developed for the bulk production of diplodiatoxin from the isolate MC43 for further experiments.

The effect of diplodiatoxin on the growth of various bacterial species was undertaken. The bacterial species showed a range of susceptibility to diplodiatoxin. The increasing order of susceptibility to diplodiatoxin was *S. aureus* < *B. cereus* < *B. subtilis* < *P. fluorescence* < *E. coli.* The effect of different concentrations of diplodiatoxin on the growth of *S. aureus* in liquid media was studied and it was shown that diplodiatoxin was bacteriostatic.

The toxicity of diplodiatoxin on mammalian cell lines was undertaken by testing its toxicity on the Chinese hamster ovary cells. The activity was observed even at low

concentrations of diplodiatoxin, experiments were conducted to analyse whether diplodiatoxin was causing necrosis or apoptosis and the results confirmed that diplodiatoxin was inducing apoptosis in the mammalian cells.

The evaluation of the toxicity of diplodiatoxin was undertaken in rats. The acute and sub-acute effects of diplodiatoxin were monitored in male and female rats with special reference to biochemical target enzymes aspartate aminotrasferase (ASAT), alanine aminotrasferase (ALAT), acid phosphatase (AcP), alkaline phophatase (AkP) and acetlycholinesterase (AChE). Diplodiatoxin causes loss of body weight and reduced feed intake along with other clinical symptoms. The acute and sub-acute treatment of diplodiatoxin resulted in significant increases in the liver and serum ASAT and ALAT. Decreases in the serum AcP, AkP and liver AkP were observed, whereas liver AcP increased in both male and female treated rats. In addition significant inhibition of brain AChE was seen in acute and sub-acute treated animals indicating the effect of diplodiatoxin on nerve synapsis. These results clearly suggest that diplodiatoxin is a toxic compound and that it is hepatotoxic and neurotoxic.

A phylogenetic analysis was undertaken to determine the genetic relationship among the various isolates of *S. maydis* using the ITS sequence, as some of the isolates were not producing diplodiatoxin. The ITS sequence data suggested that the isolates could be separated into two groups, one containing the high toxin producing isolates and the other with non-toxin, moderate and high toxin producing isolates. When they were compared with other ascomycete species to see the appropriate phylogenetic placement they grouped into two clusters and were close to other ascomycete members other than *Diplodia spp*. The toxin producing isolates were close to *Diaporthe spp* and *Phomopsis spp* and the non-toxin producing isolates along with moderate and high toxin producing isolates were found to be close to *Fusarium spp* and *Nectria spp.* The data suggests that isolates identified as *S. maydis* may have been misidentified and in fact belong to one of the two species clusters. Further, *Fusarium*, *Nectria, Diaporthe* and *Phomopsis* species have been identified as the pathogens of a wide variety of different plant hosts suggesting that the ear rot in maize is a part of a set of diseases of plants caused by this group of fungi. This may have implications for pathogen reservoirs and disease control strategies.

Dedicated to

My

Beloved Parents

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Rao SK and Jasper.G.Rees. 2003. Genetic variation among the various toxin and non toxin producing isolates of *Stenocarpella maydis* assessed by ITS sequencing. [In preparation]

LIST OF CONFERENCES ATTENDED

- National Academy of Science, Hyderabad, India, January, 1998
- BioY2K Combined Millenium, Rhodes University, South Africa, January, 2000
- International Education Association of South Africa, Natal University, South Africa, September, 2000
- Pharmacy in the New Millenium, University of Rhodes, South Africa, October, 2000
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LIST OF ABBREVIATIONS

AB_1	Aflatoxin B_1
AB_2	Aflatoxin B_2
AG_1	Aflatoxin G_1
AG_2	Aflatoxin G_2
AM_1	Aflatoxin M_1
AM_2	Aflatoxin M_2
AP_1	Aflatoxin P_1
AQ_1	Aflatoxin Q_1
AChE	Acetlycholinesterase
ASAT	Aspartate aminotransferase
ALAT	Alanine aminotransferase
AkP	Alkaline phosphatase
AcP	Acid phosphatase
ATP	Adenosine tri-phosphate
AFLP	Amplified length polimorphic DNA
BRC	Biomedical Resource Center
BSA	Bovine serum albumin
bp	Base pairs
$CdCl_3$	Cadmium Chloride
CD3OD	Deuterated Methanol
$CHCl_3$	Chloroform
CH_3OH	Methanol
CMA	Corn meal agar
CO	Carbon monoxide
CZM	Cepex medium
CSIR	Council for scientific and industrial research
DNA	Deoxyribonucleic acid
dNTP	2' –deoxynucleotide 5' -triphosphate
2,4-DNPH	Dinitrophenylhydrazine
DTNB	4. 5, 5-dithiobis (2-benzoic acid)
EDTA	Ethylenediaminetetra-acetate
FA_1	Fumonisin A_1
FA_2	Fumonisin A_2
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FC_1	Fumonisin C_1
FC_2	Fumonisin C_2
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid

HBV	Hepatitis B virus
H_2SO_4	Sulphuric acid
IARC	International agency of research on cancer
IGS	Intergenic spacer
ITS	Internal transcribed spacers
KCL	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
LDH	Lethal dosage
$MgCl_2$	Magnesium chloride
s	Second
S	Ribosomal sub-unit
SDS	Sodium dodecyl sulphate
MeOH	Methanol
MRC	Medical Research Council
MS	Mass Spectrometry
MSV	Mosaic virus
MDMV	Maize dwarf mosaic virus
MSM	Minimal salts medium
$MgSO_4$	Magnesium sulphate
NMR	Nuclear magnetic resonance
NaOH	Sodium hydroxide
$NaNO_3$	Sodium nitrogen trioxide
NaCl	Sodium chloride
OA	Ochratoxin A
OH	Hydroxide
OD	Optical density
P.citrinin	Penicillium Citrinin
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RBC	Red blood cells
RAPD	Random polymorphic amplified DNA
RFLP	Restriction fragment length polymorphism
Rf	Migration value
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
T	Trichothecene
TE	Tris EDTA
TLC	Thin layered Chromatography
Tris	2-amino-2-hydroxymethylpropane-1, 3-diol
Tris HCl	HCl – buffered Tris
UV	Ultraviolet rays.

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

1. MYCOTOXINS

1.1 Introduction

Mycotoxins attract worldwide attention because of the significant economic losses associated with their impact on human health, animal productivity, domestic and international trade. Over 300 mycotoxins have been discovered, of which a few are of serious concern (Smith and Moss 1985; Rheeder *et al.*, 1994). Exposure to these mycotoxins can produce both acute and chronic effects ranging from death to effects upon the central nervous, cardiovascular, pulmonary systems and upon the alimentary tract. Mycotoxins may be carcinogenic, mutagenic, teratogenic and immunosuppressive (Ferrante *et al.*, 2002). Mycotoxins are currently considered as a major problem in developing countries (Miller, 1994).

Infection caused by fungus is called mycosis. A toxin produced by mould or mildew is termed as mycotoxin, *myco* meaning 'fungus', *toxin* 'poison'. These mycotoxins are toxic secondary metabolites of low molecular weight and are found to have a wide variety of structures. Mycotoxicosis is the disease caused by the mycotoxin after being consumed by the animals or humans. Mycotoxicosis is characterised by ataxia, paralysis, neurological disturbances and severe liver damage. The impact of mycotoxins on health depends upon amount consumed, toxicity of the compound, the body weight of the individual, the presence of other mycotoxins and other dietary effects (Kuiper-Goodman, 1991). Major problems with mycotoxin exposure came to light with the outbreak of the Turkey X disease in 1960's when thousands of turkeys died in England. A careful survey of the early outbreaks showed that they were all associated with contaminated feed and the infecting fungus was identified as *Aspergillus flavus* and the toxin was given the name aflatoxin by virtue of its origin. This

discovery has led to a growing awareness of the potential hazards of these substances as contaminants of food and feed causing illness, damage to the immune system (Berek *et al.*, 2001) and also leading to death in humans and animals.

1.2 Common mycotoxins produced by fungi

The mycotoxigenic fungi involved with the human food chain belong mainly to three genera viz., *Aspergillus, Fusarium* and *Penicillium*. While *Fusarium* species are destructive plant pathogens producing mycotoxins before and after harvesting (Coker, 1979), *Penicillium* and *Aspergillus* species are commonly found as contaminants of food and feed during drying and subsequent storage. The mycotoxins produced by these pathogens are discussed below.

1.2.1 Aflatoxins

Aflatoxins are mainly produced by *Aspergillus flavus, A. nominus* and *A. parasiticus.* They are difuranocoumarin derivatives. The four main naturally produced aflatoxins are B_1, B_2, G_1 and G_2 with B_1 usually being the aflatoxin found at the highest concentration in the contaminated food and feed (Pitt, 1996). The nomenclature of B and G is derived from the blue and green fluorescent colours produced under UV light on thin layer chromatography plates, with the subscript members indicating major and minor compounds (Sweeney and Dobson 1998). Aflatoxin M_1 and M_2 are monohydroxylated derivatives of aflatoxin B_1 and aflatoxin B_2. Aflatoxin M_1 has been widely found in a number of food products including infant formula, dried milk, cheese and yoghurt (Galvano *et al.*, 1996; Aksit *et al.*, 1997). Aflatoxin producing moulds occur throughout the world, in subtropical and tropical climates, especially in oilseeds, edible nuts and cereals (Coker, 1979). Aflatoxins are also found in a wide variety of other food stuffs (Candlish *et al.*, 2001; Kim *et al.*, 2001). Recently in South Africa there was aflatoxin poisoning and the highest contamination of aflatoxins was found in peanuts (MRC, 2002).

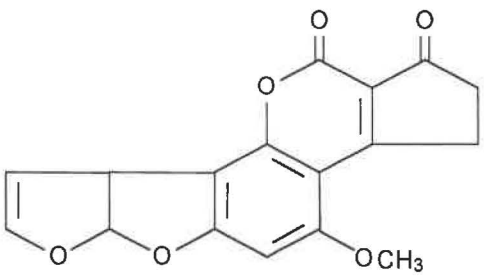


Fig 1.1 Aflatoxin B_1

1.2.2 Fumonisins

Fumonisins are primarily produced by *Fusarium moniliforme*, however *F. proliferatum* (Keller and Sullivan, 1996), *F. napiforme* (Nelson *et al.*, 1992) and *F. nygamai* (Smith *et al.*, 1994), together with *Gibberella fujikuroi* (Desjardins *et al.*, 1997) are also known to produce these toxins. Their basic chemical structure is a C-20, diester of propane-1, 2, 3-tricarboxylic acid and pentahydroxycosane containing a primary amino group (Savard and Blackwell, 1994). They have a similar structure to sphingosine, which forms the backbone of sphinogolipids. Six fumonisin structures have been identified to date and they are FB_1, FB_2, FB_3, FB_4, FA_1 and FA_2. However, of these only the B-series have been confirmed as natural products, with FB_1 usually being the most abundant (Marasas, 1996; Pitt and Hocking, 1997). Two new fumonisins, hydroxylated fumonisins C_1 and fumonisin C_3 have been isolated from culture of *F. oxysporum* (Seo-Jeong *et al.*, 1996). Both compounds are structurally similar except for an additional hydroxyl group at C_3 of hydroxylated fumonisin C_1. *F. graminearum* produces fumonisins like deoxynivalenol, nivalenol, zearalenone (Marasas *et al.*, 1984). Fumonisins are usually found in grains and cereals. FB_1 has also been reported in maize from a variety of agroclimate regions including North, South America and Africa. The toxin especially occurs when maize is grown under warm and dry conditions. It was also found that considerable variation exists in fumonisins produced in maize varieties grown in different geographical locations (Prathap Kumar *et al.*, 1997).

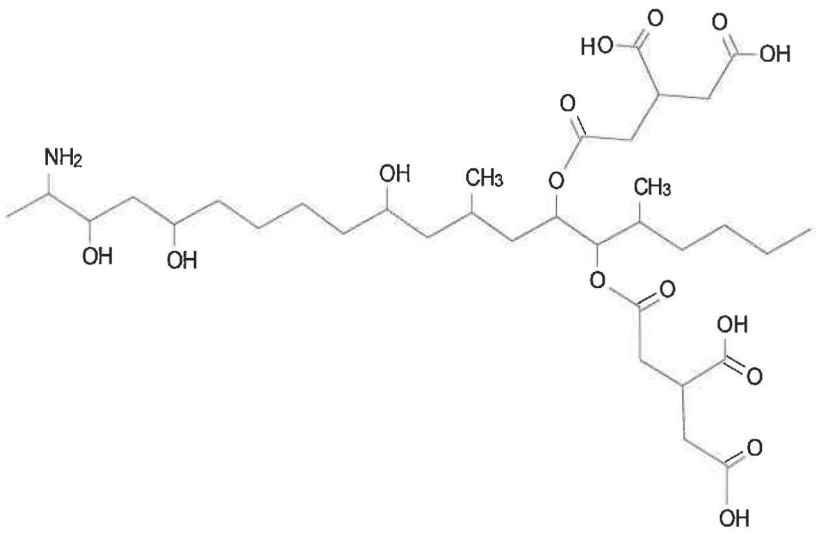


Fig 1.2 Fumonisin B_1

1.2.3 The Penicillium mycotoxins

The genus *Penicillium* contains many toxigenic species (approximately one hundred), and the range of mycotoxin classes produced is much broader than that of any other genus. *Penicillium* toxins are placed in two broad groups based on the effect, they are hepatotoxins and neurotoxins (Pitt and hocking, 1997). The three most important *Penicillium* mycotoxins are ochratoxins, patulin and citrinin. Recently, similar penicillium mycotoxin was isolated from strain *Penicillium vulpinum* by (Kozlovskii *et al.*, 2000) and from *Penicillium citrinum* by (Malstrom *et al.*, 2000).

1.2.3.1 Ochratoxins

This group of toxins are mainly produced by *Penicillium viridicatum* and *Aspergillus ochraceous*. Ochratoxin A is produced by *A. ochraceous* and related species. It is a derivative of isocoumarin linked to L-phenylalanine (Moss, 1996) and is the most toxic of the isocoumarin compounds. Ochratoxin appears to occur mainly in wheat and barley in the temperate zones of Northern Hemisphere (IARC, 1993). It also occurs in maize, rice, peas,

beans, cowpeas. Ochratoxin was also produced by *Aspergillus carbonaries* in coffee cherries (Joosten *et al.*, 2001).

1.2.3.2 Patulin

Patulin is produced mainly by *P. expansum*, a fruit pathogen that causes apple rot. It usually occurs in rotten apples, apple juice and other apple products (Roach *et al.*, 2002). It is used as an antibiotic.

1.2.3.3 Citrinin

Citrinin is produced mainly by *P. citrinin*. It was reported that *P. expansun* and *P. verrucosum* were also producers (El-Banna *et al.*, 1987). Citrinin is quinone methide with two intramolecular hydrogen bonds. It is used as an antibacterial agent against Gram-positive bacteria.

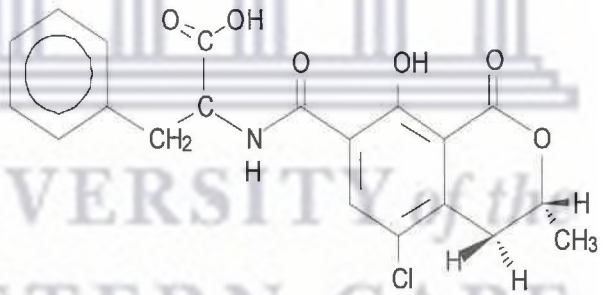


Fig 1.3 Ochratoxin A, (*Penicillium verrucosum*)

1.2.4 Trichothecenes

The trichothecenes are a group of mycotoxins that are produced by a variety of different *Fusarium* species. Numerous other fungal genera such as *Cephalosporium, Cylindrocarpon, Myrothecium, Trichoderma, Trichothecium, Stachybotrys* and *Verticimonosporium* also produce trichothecenes (Smith *et al.,* 1994). The trichothecenes are chemically the most diverse of all the mycotoxins. They are all tricyclic sesquiterpenes with a basic 12, 13-epoxy-trichothec-9-ene ring system. They can be designated into four subclasses. Type A, having a functional group other than a ketone at position C-8; Type B having a ketone at position C-8; Type C having a second epoxyl group at C7, 8 or C9, 10 and Type-D containing a macrocyclic ring between C4 and C5 with two ester linkages (Smith *et al.,* 1994). Trichothecenes are found in cereals, maize, wheat and bewered alcoholic beverages (Odhav and Naicker, 2002). Trichothecene contamination has been a major problem in developing countries (IARC, 1993). Africa, America and Europe are the continents were trichothecene contamination was found highest in the recent years (Miller, 1991, 1994).

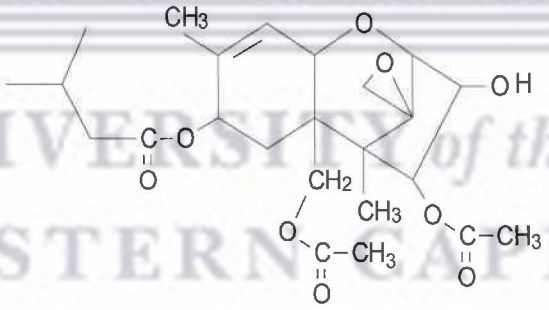


Fig 1.4 T-2 Toxin

1.3 Characterization of mycotoxins

1.3.1 Production of mycotoxins

Mycotoxins are secondary metabolites and have no obvious function in the cell growth unlike primary metabolites. Fungal growth and mycotoxin contamination are the consequences of interactions between the fungus, the host and the environment. The appropriate combinations of these factors determine the infestation and colonisation of the substrate and the type and amount of mycotoxin produced. However, a suitable substrate is required for fungal growth and subsequent toxin production, although the precise factors which initiate toxin formation are complex.

Similarly, specific crop growth stages, poor soil fertility, high crop densities and weed competition have been associated with the increased mould growth and toxin production. Mycotoxin formation is also affected by associated growth of other moulds or microbes. For example, pre-harvest aflatoxin contamination of peanuts and corn are favoured by high temperatures, prolonged drought conditions and high insect activity, while post-harvest production is favoured by warm temperatures and high humidity.

The production of mycotoxin is not only influenced by the physiochemical, environmental or cultural conditions in which the fungus is growing, but also the genotype of the organism. It is important to know that the production of any particular mycotoxin depends on a particular strains/isolate and not on the species. For example, although aflatoxins are known to be produced by *Aspergillus flavus* and *A. parasiticus*, there are strains of these species which are non-aflatoxigenic. Studies conducted on the association of morphology and toxin production with vegetative compatibility groups of *A. flavus*, *A. parasiticus* and *A. tamarri* showed significant differences among the vegetative compatibility groups and the toxin production (Horn *et al.*, 1996).

1.3.2 Extraction and Detection of mycotoxins

The mycotoxins are extracted in complex mixtures from the plants infected with fungi. Generally mycotoxins are soluble in polar solvents and insoluble in non-polar solvents. The polarity and the degree of solubility varies from the toxin to toxin. Usually the extraction is done by specific methods like solvent extraction methods. After extraction the compound is ready for further analysis. The detection of mycotoxin is usually difficult due to the presence of large number of other secondary metabolites produced by the fungus. Ultraviolet indicators such as zinc silicate may be added to enhance the detection (Musonik, 1996). Usually bioassays are used for the detection of unknown mycotoxins and analytical methods are preferred for known mycotoxins. Therefore it is important to use methods that are sensitive and efficient. The Thin Layer Chromatography is used as a presumptive test and Column Chromatography is used for the purification of mycotoxins in bulk amounts. Currently more sensitive testing methods such as High Performance Liquid Chromatography and Mass Spectrometry are being utilised. The analytical procedure involves the process of extraction, cleanup, detection, isolation, purification, quantification and final confirmation (Smith *et al.*, 1985).

1.3.3 Analytical methods

Chemical methods of detection are preferred to biological assays for known mycotoxins as they are easily quantifiable, interference by non-fungal co-extracts is less and they are more sensitive than biological assays (Ramawat and Suri, 1999). Basically, various types of chromatography techniques are being used to separate compounds of interest from a crude extract. Compounds are separated on the basis of polarity, affinity with the solvent and adsorption properties onto the matrix. Also most mycotoxins are detected by analytical chromatography (Roach *et al.*, 2002). Various analytical techniques are discussed below.

1.3.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) has been the most widely used analytical method for separation and identification of mycotoxins because it is quick, easy and economic. The technique involves the separation of compounds on a thin layer of silica using an appropriate solvent system and the characterisation of the resultant separated compounds by developing the TLC plate with a specific detection reagent. With every combination of solvents each mycotoxin will have a characteristic migration and separating pattern, known as the Rf value. The Rf value is affected by the solvent system, and therefore different solvent systems provide a variety of methods of separation of compounds. If the compounds are not properly resolved, resolution can be achieved by using 2-dimensional TLC. Also mycotoxins are detected by physio-chemical properties of molecules often involving a combination of absorption and fluorescence properties or the use of chromogenic reagents. Some mycotoxins absorb UV and emit visible light. TLC is only a presumptive test and further confirmation is done by HPLC. Compounds of interest can also be separated by eluting from the silica and can be re-dissolved in a suitable solvent for further analysis.

1.3.3.2 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is widely used separation technique and is one of the best and most sensitive methods used for detection and quantification of mycotoxins (Solfrizzo *et al.*, 2001). HPLC coupled with sensitive detection and sophisticated data retrieval systems has facilitated the identification of selected mycotoxins and their quantification. HPLC is much faster and has high resolution. Currently HPLC is also coupled with other techniques like fluorescence for more accurate results for the detection of mycotoxins (Llorens *et al.*, 2002).

1.3.3.3 Mass Spectrometry

The generally used method for the structural analysis of organic compounds is Mass Spectrometry. It is a technique used for characterising molecules according to the manner in which they fragment when bombarded with high-energy electrons. It is not strictly a spectrometric method as electromagnetic radiation is neither absorbed nor emitted. However, the data obtained are in a spectral form and the relative abundance of mass fragments from a sample is recorded as a series of lines or peaks. The bombardment procedure produces many fragments, each of which carry a charge, which facilitate their separation and detection by electrical means. The spectra are always recorded under the conditions of high vacuum to prevent loss of the charged fragments by collision with the molecules of atmospheric gases. The components of the mass spectrometry include a sample inlet system, an ionisation source and an accelerator chamber where the molecules of the samples are ionised, fragmented, accelerated into an analyser and ion detection and recording system. Used in conjunction with UV, infrared and visible spectral data, MS is highly sensitive and an extremely valuable aid in the identification and structural analysis of organic compound. The spectroscopy is a widely used technique for validation of structure of mycotoxins (Musser *et al.*, 2002).

1.3.3.4 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) is one of the most important techniques used for the structural analysis of compounds (Harper *et al.*, 2001). Certain atomic nuclei have a spin associated with them, which causes them to behave like tiny magnets since nuclei are charged. When the nuclei are placed between the poles of a powerful magnet, they align themselves either with or against the field of the magnet. Nuclei that align with the field have a slightly lower energy than those that aligned against the field. Hydrogen nuclei (protons) are the most common nuclei used for determining organic structures. A sample of the compound under study is placed (usually in $CDCl_3$ or CD_3COCD_3) in the centre of radio frequency coil between the poles of a powerful magnet. The magnetic field must be extremely homogenous. The spinning protons align with or against the field. A continuously

increasing amount of energy is supplied to the nuclei by the coil. When this energy corresponds exactly to the energy difference between the aligned and non-aligned nuclei, it is absorbed by the aligned nuclei (lower energy state) and they are transferred to the non-aligned (higher energy state). The nuclei are said to be in resonance. A plot of the energy absorbed by the sample against the frequency of the coil gives the NMR spectrum.

1.4 Effect and Mechanism of action of mycotoxins

1.4.1 Effect of mycotoxins on animals

Mycotoxins have devastating effects on animal husbandry and it has been a severe problem to mankind since the beginning of organised crop production. Mycotoxins have been associated with various diseases in livestock, domestic animals and humans throughout the world. The effect of mycotoxins differs from species to species, For example fumonisins cause leukoencephalomalacia in horses (Marasas *et al.*, 1998; Kellerman *et al.*, 1990), pulmonary oedema and hydrothorax in pigs (Harrison *et al.,* 1990), and hepatotoxicity and liver cancer in rats (Gelderblom *et al.,* 1996). They inhibit sphingolipid biosynthesis, which has been linked as a contributing factor in both toxicity and carcinogenicity (Merrill *et al.,* 1993). Fumonisin produced by *Fusarium verticillioides* strain MRC 836 was reported to have effect in non-human primates (Gelderblom *et al.,* 2001). Fumonisin has a significant effect on the enzyme levels aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) in rabbits (Conkova *et al.*, 2001) and a remarkable increase in the level of enzyme alkaline phosphatase was observed in rats when treated with fumonisin (Theumer *et al.,* 2002).

Aflatoxin is regarded as the most potent liver carcinogen known for a wide range of animal species. Aflatoxin B_1 acts as a potent carcinogen in some animals, there are measurable effects on long-term exposures to low levels of this mycotoxin on animals, including humans (Dragon and Pitot, 1994; Eaton and Groopman, 1994; Costantini *et al.*, 2000). The toxin appears to act by modifying the structure of DNA in the liver cells. This requires activation of aflatoxins by oxidation into the epoxide form (Harris, 1991). Recently it was reported that

aflatoxin affects the reproductive system in the sub-acute studies done on rats (Ibeh *et al.,* 2000).

Trichothecenes, which are also known to be potent mycotoxins cause immunotoxicity and inhibit protein synthesis. They pre-dispose animals to other diseases and toxicoses (Prelusky *et al.,* 1994). Most animals, including humans, appear to display the greatest susceptibility to these toxins. Trichothecenes causes neurogenic symptoms in adults (Rao *et al.*, 2000). Also trichothecene has a measurable effect on mammalian reproductive system (Rao *et al.*, 2000).

Ochratoxin A is a potent nephrotoxin, teratogen and carcinogen (Krogh, 1987) and inhalation can lead to renal failure (Dipaulo *et al.*, 1994). It has a significant affect on epithelial cells which play a vital role in kidney physiology (Horvath *et al.*, 2002). It was also recently reported that Patulin has adverse effects on rodent foetuses, together with immunological, neurological and gastrointestinal effects (Smith *et al.*, 1994; Canas and Aramda, 1996). Citrinin (Ditrinin) is a well-established renal toxin affecting monogastric domestic animals such as pigs and dogs. The effect on humans is not yet known.

1.4.2 Effect of mycotoxins on humans

Mycotoxins cause significant damage to the human immune system (Berek *et al.*, 2001). Usually humans are exposed to mycotoxins by consuming food contaminated with fungi or food products derived from infected animals and plants (Linsell, 1977; Kuiper-Goodman, 1991). Such exposure is difficult to avoid because fungal growth in food is not easy to prevent. Even though in developed countries heavily contaminated food supplies are not permitted in the market, concern still remains for possible adverse affects resulting from long-term exposure to low level of toxins in the food supply. Mycotoxins are generally stable at low temperatures but degrade at higher temperatures and during chemical treatment, but still the food and feed might remain toxic (Jackson *et al.,* 1996). Evidence of acute mycotoxicosis has been reported from many parts of the world, mainly the third world countries and the symptoms are characterised by vomiting, abdominal pain, convulsions, coma and death with cerebral edema and failure of the liver, kidney and heart. Conditions

increasing the likelihood of acute mycotoxicosis in humans include limited availability of food, environmental conditions that favour fungal development in crops and commodities, and lack of regulatory systems for mycotoxin monitoring and control.

In 1998, the International Agency for Research on Cancer (IARC) placed aflatoxin B1 on the list of human carcinogens (IARC, 1993). This was supported by a number of epidemiological studies done in Asia and Africa that have demonstrated a positive association between dietary aflatoxin and liver cancer. Additionally, the expression of aflatoxin-related diseases in humans may be influenced by factors like age, sex, nutritional status, and/or concurrent exposure to other causative agents such as viral hepatitis (HBV) or parasitic infestations. Aflatoxins and cyclosporin are also known to cause breast cancer in humans (Eaton and Groopman, 1994; Costantini *et al.,* 2000). In addition, the high incidence of human cancer in certain part of the world such as South Africa (Rheeder *et al.,* 1992) and China (Luo *et al.*, 1990) has been epidemiologically correlated with fumonisins. Fumonisin was found to cause cancer in humans (Sydenhan *et al.*, 1990; Chu and Li, 1994), especially oesophageal cancer (Seegers *et al.*, 2000; Chelule *et al.*, 2001).

1.4.3 Effect of mycotoxins on specific enzymes

Mycotoxicoses leads to the changes in the levels of specific target enzymes. The biochemical parameters are a sensitive index of the changes due to specific toxicant and can constitute an important diagnostic tool in toxicological studies. Most mycotoxins are neurotoxic and hepatotoxic so they significantly affect the target enzymes. AChE a target enzyme, predicts the early toxicity. Aflatoxin B changes the AChE turnover and hence the cholinergic transmission in the brain and adenohypophysis. This inturn results in behavioural deficits and / or performance decrements via a disturbance of hypothalamo-hypophysial axis (Egbunike and Ikegwuonu, 1984). Zearalenone had a measurable effect on the enzymatic parameters like ASAT, ALAT and LD in rabbits (Conkova *et al.,* 2001). Ochratoxin A had a significant effect on ASAT and ALAT, also the levels of these enzymes was much higher in older female rats than the younger ones (Dortant *et al.,* 2001). An investigation on the broiler chicks fed with contaminated Fumonisin B_1 resulted in elevated levels of ASAT and ALAT

(Henry *et al.,* 2000). Exposure to certain toxic organic metabolites in liver with reference to these enzymes was observed by (Verplanke *et al.,* 2000). These enzymes are also used to predict the early toxicity caused by certain pesticides like phosphorothinoate (RPR-II) (Rahman *et al.,* 1999). AkP and AcP are excellent indicators of early toxicity of organophosphoric compounds (Rahman *et al.*, 2000). The mechanism of activity of these enzymes is described below.

1.4.3.1 Acetylcholinesterase

Acetyl Cholinesterase (AChE) is a target enzyme for some toxic metabolites. AChE plays a key role in many organisms, including humans. These enzymes are useful in predicting early toxicity of the toxic compounds. The primary biological function of AChE enzyme in the nervous system is to break down the neurotransmitter acetylcholine (ACh) within the cholinergic nervous system (Mason *et al.,* 2000). When AChE is altered by organic compounds, it can not perform the break down function and thus acetylcholine accumulates. Acetylcholine accumulation increases nerve impulse transmission and leads to nerve exhaustion and failure of the nervous system. When the nervous system fails, muscles do not receive the electrical input they require to move. The respiratory muscles are the most critical muscle group affected, and respiratory paralysis is often the immediate cause of death.

The enzyme AChE is a serine esterase and is functionally related to other enzymes in this family. The active site contains a catalytic triad composed of aspartate, histidine and serine residue functional groups (i.e., the carboxyl, imidazole and alcohol, respectively). The serine hydroxyl is nucleophilic and attacks the acetylcholine ester function. This enzyme also contains a cation binding site recognises and binds the choline quaternary ammonium group. Thus, the activity of AChE is inhibited in response to the toxin, thereby blocking transmission of nerve impulse. Thus AChE is an excellent indicator of affect of toxin on nerve synapsis.

1.4.3.2 Aspartate amino transferase (ASAT) and Alanine amino transferase (ALAT)

Enzymes such as aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) also play a vital role in predicting the toxicity of the compound. ASAT and ALAT are the membrane enzymes, found in heart, liver, skeletal muscle, kidney, pancreas, spleen, lungs, red blood cells and brain and any injury to these organs will result in the release of these enzymes. These enzymes are used in diagnostic centres for the prediction of early toxicity. ASAT and ALAT are enzymes produced in the liver cells and are detected in the blood streams. The normal range for ASAT is 0-40 U/L, when liver cells are damaged, these enzymes are released and elevated levels are detected in serum. The level of ASAT and ALAT are generally taken to be an indicator of the damage that is caused to liver cells. Liver cells are damaged even with a little increase in the level of these enzymes. Early toxicosis can be predicted by monitoring the levels of these enzymes (Kaur *et al.*, 2000).

1.4.3.3 Acid phosphatase and Alkaline phosphatase

Phosphatases are important critical enzymes in the biological systems, responsible for detoxification, metabolism and biosynthesis of energetic macromolecules for different essential functions. Interference with these enzymes leads to biochemical impairment, lesions of tissues and loss of cellular functions (Enan *et al.*, 1982). Phosphatases are lysosomal markers. These enzymes have been extensively studied for significant physiological and pathological processes. Phosphatases are lysosomal enzymes that catalyse the splitting of phosphoric acid from certain phosphoric esters. They are usually located on absorptive or secretary surface of cells as membrane bound enzymes and these enzymes are biomarkers of membrane damage or tissue necrosis (Rahman *et al.*, 2000). Acid Phosphatase (AcP) is a lysosomal enzyme, and increase in the level of this enzyme is a sensitive indicator of activated macrophages and polymorphonuclear leukocytes (Henderson, 1984). AcP has a heterogeneous and ubiquitous distribution. It is present in prostate gland, erythrocytes, leukocytes, platelets, liver, spleen and other tissues. Alkaline Phosphatase (AkP) is found in the cell membranes and is a sensitive indicator of cell damage (Henderson, 1984). This enzyme is a glycoprotein that has been found to originate mainly from bone and liver

(Righetti and Kaplan, 1971). Mammalian AkP comprises of a group of membrane glycoproteins that catalyse the hydrolysis of different monophosphate esters (McKenna *et al.,* 1979). The biological function of AkP at a molecular level has been proposed which suggested that AkP, together with other membrane phosphohydrolases and transferases, may act in concert to regulate the dimensions of the membrane. Multiple molecular studies which are characteristics of selected tissue types and cell types have been documented and serum concentrations correlated with certain diseases, such as prostate cancer (Fisherman, 1974). Also AcP is used for the detection and monitoring of prostrate and breast carcinoma (Varley *et al.*, 1980)

1.4.4 General mechanism of action of mycotoxins

The mechanism of action of a mycotoxin can be studied at different levels. The *in vivo* effects of mycotoxins and other xenobiotics depend primarily on the activities of enzyme systems regarded as "drug–metabolising enzymes" responsible for their detoxification. However, in some instances, certain steps of the process results in the production of a compound more toxic than the initial molecule. The extent of the biochemical effects thus depend on the balance between the rates of the various pathways that metabolise the toxin, i.e., those proceeding via the formation of active metabolites and those leading to inactive derivatives.

The major areas of biochemical effects of mycotoxins in the cells are as follows:

1 Interaction with the cell membranes
2 Interference with energy metabolism
3 Interaction with the DNA or protein molecules
4 Inhibition of the replication of DNA molecules
5 Inhibition of transcription (RNA synthesis)
6 Inhibition of translation (protein synthesis)
7 Interference with the metabolism of purines

According to Kiessling (1986) the primary mechanism of action of mycotoxin may be to modify the DNA. In certain cases the mycotoxin reacts directly with the enzyme protein or co-enzyme. All these primary events may lead to secondary effects in the terms of modified enzyme activities and, hence, changes in metabolic activity and regulation. A number of mycotoxins interact with DNA either directly or after metabolic activation. Aflatoxin B_1 is transformed into several kinds of metabolites by cytosolic and microsomal enzyme systems. Among the resultant metabolites, aflatoxin M_1, Q_1 and P_1 are far less mutagenic and carcinogenic than aflatoxin B_1. A diagrammatic representation of mode of action of aflatoxin B_1 is described below in (Fig. 5.1).

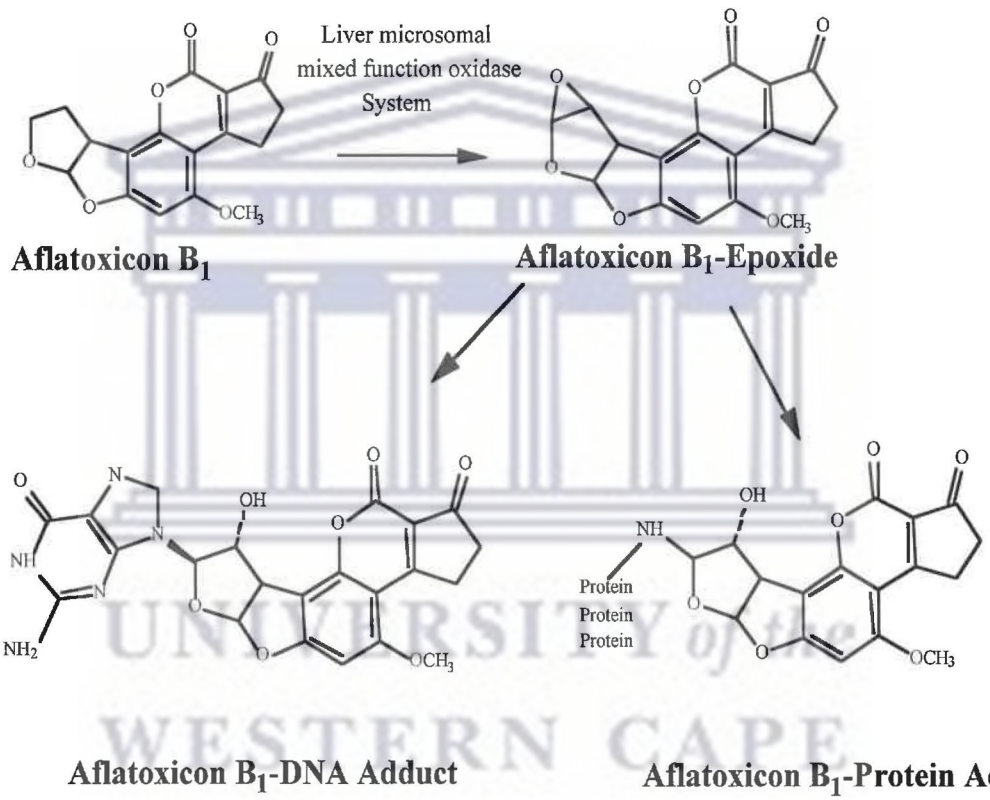


Fig 1.5 Mode of action of Aflatoxin B_1

- Fumonisins mainly act on cell membrane (ceramides/spingolipids) (Ferrante *et al.*, 2002)
- Penicillium inhibits protein synthesis by acting on 30s ribosomal unit.
- Ochratoxin A is reported to be able to cause break down in the DNA-strand i*n vitro* and *in vivo*.

1.5 Genetic analysis of mycotoxin producing fungi

Genetic analysis of mycotoxin producing fungi is important as it reveals the variability within fungal population for a better understanding of disease outbreak and prediction of disease (Huff, Bunting and Plumley, 1994). Molecular techniques represent a powerful tool for the identification and understanding the genetic variation among the various organisms, and to confirm the proper taxonomic placements, which resolve the taxonomic controversies. These techniques are helpful in understanding the mechanism involved in the pathogenicity. Molecular markers are also used for detecting variation within population of phytopathogens (Crowhurst *et al.,* 1991) and other organisms (Badhuri and Cottrel, 1998). A number of molecular techniques are currently available to study genetic relationships among the fungal populations (Bostock *et al.*, 1993). Random amplified polymorphic DNA (RAPD) (Pavanen-Huhtala *et al,* 1999; Calvo-Bado *et al.,* 2001), Restriction fragment length polymorphism (RFLP) (Viji *et al.,* 2000; Aradhya *et al.,* 2001) and Amplified fragment length polymorphism (AFLP) (Pei and Ruiz, 2000; Purwantara *et al.,* 2000) are the various tools for screening which are been used to screen isolates of a species, and to study intra- and inter- specific relationships between the fungi. A genetic and physical map has also been developed in toxin and nontoxin producing strains of *Leptosphaeria maculans* using RAPD, RFLP and AFLP (Cozijsen *et al.*, 2000).

Further, ribosomal DNA genes of fungal genomes are very useful for genetic diversity and phylogenetic studies. The region is highly conserved (White *et al.,* 1990; Henson and French 1993) and is easily accessible for investigation by PCR amplification. The 18S, 5.8S and 28S genes do evolve at a slow rate and are used for studying the relationship among distantly related organisms. Sequence variation in the rDNA genes are used for classifying organisms at the level of classes or families (Burns *et al.,* 1991). Variations in ITS sequences could be most informative for closely related organisms (Hyun and Clark, 1998). Also the internal transcribed spacer (ITS) region is a powerful tool to study relatedness as it is highly conserved and changes in the sequences of this region could be used to explore variation within/between species in fungi (Aradhya *et al.*, 2001; Jensen and Eilenberg, 2001; Angeles *et al.*, 2001).

1.6 Fungal pathogens affecting maize

Maize (*Zea mays* L.) is an economically important crop all over the world and is an important stable food in South Africa particularly for those living in rural areas. Its true potential as a high yielding crop is mainly hindered by several fungal, viral and bacterial infections. The major fungal pathogens causing ear rot in South Africa are *Stenocarpella maydis* (Berk.) Sutton (*Diplodia maydis*), *S. macrospora* Earle Sutton (*D. macrospora*), *Fusarium moniliforme* Sheldon (*Gibberella fujikuroi*), *F. subglutinans* Woolen W. and Reinking (*G. fujikuroi* var. *subglutinans*), *F. graminearum* Schwabe (*G. zeae*) and *Aspergillus flavus* (Flett and Wehner, 1991; Rheeder *et al.,* 1993). Other than these ear rot pathogens, maize encounters the infections of *Exserohilium turcicum* causing leaf blight. Maize streak virus (MSV) and Maize dwarf mosaic virus (MDMV). Plant breeders and pathologists working with maize pathogens have observed that the incidence in South Africa is on the increase (Rheeder *et al.,* 1990). The increased incidence over recent years has been attributed to difficulty in controlling the disease, use of susceptible germplasm, agricultural procedures currently practised and environmental conditions aggravate the disease.

1.7 *Stenocarpella maydis*

1.7.1 History

Stenocarpella ear rot was first reported in South Africa in 1907 by Smith and Hedges (1909). *S. maydis* ear rot is known since 1909 in USA (Heald *et al.,* 1909). In 1912 Webb found the fungus on samples of maize stubble from a field in Natal where cattle had shown symptoms of toxicosis (Van der Bijl, 1914). During the 1950s and early 1960s disease was most common in major corn belts all over the world. However, the disease has gained attention since the severe nation wide outbreak of *S. maydis* stalk rot in the 1980s (Chambers, 1986). These epidemics resulted in severe losses in yield and grain quality as well as toxicity to livestock fed with infected grains (Rabie *et al.,* 1987). In South Africa the estimated loss due to *S. maydis* in 1987/88 was approximately 200 million Rands (Flett and Mc Laren, 1994; Nowell, 1997) and the severity of the disease is increasing every year

(Chelule *et al.*, 2001). Apart from South Africa and America *Stenocarpella maydis* was also found in China (Henan) recently (Hamilton, 2000).

1.7.2 Epidemiology

The distribution and severity of ear rots are dependent on the climatic conditions. The outbreak of the disease (Diplodiosis) is favoured by dry weather early in the season followed by wet conditions just before and after silking (Shurtleff, 1980). Fungal ramification decreases as the ears become dried (Clayton, 1927). Maize is the only crop infected by this pathogen known to date (Flett, 1991), but the present study revealed that *S. maydis* is related to other fungi which are plant pathogens and that it is very closely related to other *fusarium* species.

1.7.3 Identification

S. maydis is a member of Ascomycota and belongs to the family Botryosphaeriaceae. Earlier *S. maydis* was known as *Diplodia maydis* (Sutton, 1980). Another closely related species is *S. macrospora* but, this species is less common and more aggressive than *S. maydis* and can cause severe losses (Latterell and Rossi, 1983). The fungus can be identified as white cottony mycelial growth on the infected husk and kernels with black fruiting bodies called as pycnidia. The pycnidia are immersed, spherical to globose, dark brown to black in structure. The pycnidial wall is multicellular and darker around the circular protruding papillate ostiole, which is 40 µm in diameter. No telomorph is known in pycnidia (Shurtleff, 1980).

1.7.4 Symptoms

The diseases caused by *S. maydis* infection in maize are referred to as mould corn mildew, ear rot and dry rot. Early infection may result in small shrunken ears of no value while less severe attacks produce ears of poor feeding quality. In severe attacks embryos are destroyed or viability is lost, so that the maize is of no use as seed. Ear rot appears as a white, cottony fungal growth between kernels, almost always progressing from the ear shank upwards and

from the cob outward (Vincelli, 2001). Occasionally, infections progress downwards from the tip of the cob. Several affected ears can be obvious from a distance as the husk turns brown and are dry as compared to the rest of the plant. In most cases, no symptoms appear on the leaves of infected plants. Infection of the kernels with a relatively low moisture content cause darkening of the embryos.

1.7.5 Disease Cycle

S. maydis survives between seasons in the residue of corn stalk, cobs and fallen kernels. Spores of the fungus are produced in fruiting structures called pycnidia, which are produced on infested corn residues. During wet weather, the spores ooze out of these fruiting structures and are spread by rainsplash. When plants are silking, spores are splashed up to the ear and then deposited by rainwater around the ear shank and have an opportunity to infect. These spores can germinate and penetrate the ear shank, growing up into the cob and outward into the kernels. Ears are most susceptible to infection within a week or two of mild-silk. Research to date indicates that susceptibility of ears steadily decline after that, although some ears can still be infected as long as four weeks after midsilk. Wet weather and moderate temperatures during silking allow the infection to occur if spores are present. Corns being the host of S. maydis, the inoculum levels are usually highest in fields of continuous corn, but eventually decline when rotated to other crops (Vincelli, 2001).

1.7.6 Control

The most effective means of controlling crop disease is by breeding for disease resistance (Flett and McLaren, 1994). The environmental and agricultural factors on the epidemic have posed problems to breeders and researchers (Nowell, 1997). The increased incidence of *S. maydis* infection over recent years has been attributed to difficulties in controlling the disease, several agricultural practices such as crop rotation are being used to lower the incidence of the disease (Flett, 1991). The use of fungicides e.g., benzomyl and triazole group do control the disease to a certain extent. However, several factors influence the control efficacy and economic viability of the crop (Nowell, 1997). Since the use of

fungicides is becoming a health hazard and an environmental problem, fungicide application is limited to seed crops only, and other alternative measures such as biological control against *S. maydis* have been suggested (Nowell, 1997). Moreover, integrated disease control strategies are being developed to pursue a maximal control efficacy of the disease.

1.7.7 Diplodiatoxin

The toxin produced by *S. maydis* is called diplodiatoxin, *S. maydis* cultures cause diplodiosis, which came into light in South Africa during 1918 when a neurotoxic disease was diagnosed in cattle. Since then interest developed and research on diplodiatoxin has been undertaken in several laboratories.

The structure of diplodiatoxin is unique among fungal metabolites in containing a β-keto side chain and the β-γ-unsaturated acid unit (Fig. 6) Diplodiatoxin crystallised from chloroform has a melting point 186-187 °C and $[\propto]^{28}_{D}$ +101°. Elemental analysis and high-resolution mass spectroscopy showed it to have a molecular composition of $C_{18}H_{28}O_4$. The UV spectrum of diplodiatoxin showed ketone absorption [λ^{EtOH}_{max}293nm (ε50)]. The molecular weight of diplodiatoxin is 308.19gram. Its IR-spectrum exhibits strong OH absorption between 2500 and 2800cm^{-1} indicating a carboxyl group, and a sharp band at 1700cm^{-1} attributed to the CO group (Steyn *et al.,* 1972). Stereo structure of diplodiatoxin was confirmed by the synthesis using highly stereo controlled strategy, in which the intramolecular Diels-Alder reaction of a (E, E, E)-triene is involved (Ichihara *et al.,* 1986).

A closely related species, *Diplodia macrospora*, is known to produce diplodiosporin and diplodiol (Chalmers *et al.*, 1978; Cutler *et al.,* 1980). A detailed biosynthetic pathway for the biosynthesis of diplodiosporin was proposed by (Chambers *et al.,* 1979; Gorst-Allman and Vleggaar, 1986).The toxicity of diplodiosporin and diplodiol is known but the severity is not as high as Diplodiatoxin.

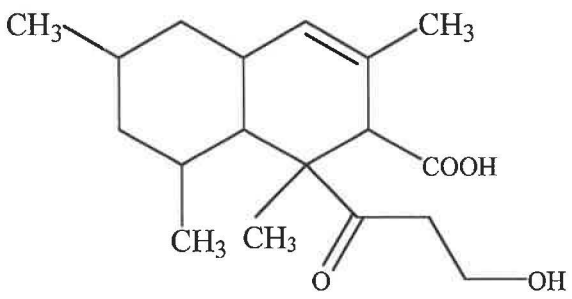


Fig 1.6 Structure of diplodiatoxin

1.7.8 Effect of Diplodiatoxin

Diplodiatoxin is a toxic compound and when consumed in higher quantities may even lead to death. *S. maydis* causes field outbreaks of mycotoxicosis known as Diplodiosis, which is characterised by ataxia, paralysis, lacrimation, neurological disturbances and severe liver damage. Diplodiatoxin affects the animals that feed on the infected crop. The effect of crude extract of *S. maydis* on various animals is discussed below.

1.7.9 Effect on Poultry

The toxicity of *S. maydis* to ducklings, chicken broilers and laying hens was investigated by (Rabie *et al.,* 1987). Among the isolates used, those obtained from South African Medical Research Council (MRC) were most toxic. Most *S. maydis* isolates showed acute toxicity in ducklings. Ducklings fed with 50%, 20% and 10% of diet containing *S. maydis* died within five days. Even at dietary level of 5% the death was reported within seven days. Administration of contaminated diet also resulted in loss of body weight in chicken broilers (Rabie *et al.,* 1987). Isolates MRC 2829 and 2407 resulted poor weight gains and higher mortality. Even at 2% and 1% dietary levels, both isolates resulted in significantly poorer weight gains. *S. maydis* also resulted in reduction of egg production and loss in weight of the eggs in laying hens. Of the two isolates of *S. maydis* tested, MRC 2407 was less toxic than MRC 2829. The fact that the least toxic isolate (MRC 2407) still resulted in a highly significant reduction (17%) in egg production when fed with a dietary level of 0.5%,

indicates that *S. maydis* is of economic importance in poultry industry. It is, however, important that additional isolates, randomly selected from commercial corn, should be evaluated before predicting the level of toxicity (Boasch *et al.,* 1989; Still *et al.,* 1992; Singh *et al.,* 1994).

1.7.10 Effect on Cattle

Diplodiosis occur in cattle eating infected maize ears on harvested maize farms a few days to two weeks of post-harvation (Marasas, 1977). Early symptoms are lacrimation and salivation, accompanied by quivering of the shoulder and flank muscles and ataxia. These symptoms become more conspicuous with time, the back is arched, muscular tremors become general, and marked ataxia develops. Infected animals walk only when compelled to and then signs of uncoordinated movements are shown by high stepping movement of the legs, lateral swaying, and a tendency to walk with hindquarters bent to one side. They knuckle over at the fetlocks after walking a short distance and are unable to rise. There is virtually no increase in body temperature throughout the toxicosis. If the ingestion of infected maize is continued at this stage, symptoms become more pronounced. Complete muscular paralysis sets in and is followed by death. On the other hand, a fairly rapid recovery is usually made if the feeding is discontinued when clinical symptoms become evident. The signs of paralysis disappear within a few days but stiffness may persist for sometime. Some cattle developed permanent involuntary high-stepping gait of the hind limbs (Kellerman *et al.,* 1985).

1.7.11 Effect on Sheep

The first clinical sign of diplodiosis in sheep appears two to sixteen days after consumption of infected maize. The syndrome is characterised by marked ataxia, first evident as stiffness in movements and unsteadiness on the feet. The sheep are weak and stand with arched back and legs spread out. When attempting to walk, the hindquarters collapse, and the animal falls to the ground and has difficulty in rising. These signs continue for one to three days without any rise in body temperature. Recovery resumes if feeding with intoxicated food is stopped,

if not then it leads to complete paralysis and death (Kellerman *et al.,* 1985). *S. maydis* culture also causes perinatal mortality in lambs. The infected lambs and calves were either stillborn or died soon after birth. Histopathological examination revealed a consistent, permanent status spongiosus on the white matter on their brain (Kellerman *et al.,* 1991). Almost all lambs born from ewes that had been exposed to *S. maydis* were dead or died soon after birth. Histopathological examination of affected lambs revealed spongy degeneration (status spongiosus) of varied degree in the central nervous system. Spongy lesions were present throughout the white matter in the brain in several affected cases and in some of these focal areas of leukoencephalomacia were evident (Aucock *et al.,* 1980; Kellerman *et al.,* 1985, 1991). In lambs that were mildly affected, the lesions were locally distributed with predilation for the white matter of the cerebral and cerebellar gyri. Preliminary transmission electron microscopical studies showed that the spongy changes were attributed to widespread intramyelinic vacuoles and expansion of extracellular spaces. In mildly affected lambs, the lesions were limited more or less to the matter of cerebral and cerebellar hemisphere and central cerebrum and cerebellum (Prozesky *et al.,* 1994).

1.7.12 Effect on Vervet Monkeys

Vervet monkeys of different age, sex and weight have also been used to determine the toxigenicity of *S. maydis*. It has been reported to cause mycotoxic peripheral myelinopathy and hepatitis in monkeys (Fincham, 1991). In some treated cases, peripheral motor and sensory functions were impaired resulting in a loss of fine motor control in hands and feet. Toxication causes effects on various serum enzyme levels viz., aspartate transaminase (ASAT), alanine transaminase (ALAT), lactate dehydrogenase (LD), gammaglutamyl transferase, alkaline phosphatase (AkP) and creatinine phosphokinase (Smith *et al.,* 1994).

1.7.13 Genetic aspect of *S. maydis*

Information regarding the variability within fungal populations is important for better understanding of disease outbreaks and the prediction of disease development (Huff, Brunting and Plumley, 1994). Molecular markers are useful tools for the detection of genetic

relatedness among various populations of phytopathogens (Crowhurst *et al.,* 1991). Traditionally *S. maydis* pathogens have been differentiated from each other on the basis of morphological structures, growth and colour development of fungus in the medium, but these methods are unsatisfactory because morphological and cultural characteristics are unstable and can alter frequently under different environmental conditions. Also *S. maydis* was found to be similar to the other fungal strains like *S. macrospora* with respect to size of spores and additional requirement for biotin *in vitro* etc (Stevens and Chapman, 1942; Shurleff, 1980, Sutton, 1980; Latterell and Rossi, 1993). The genetic relationship among various isolates of *S. maydis* and with other group of fungi is not yet known.

1.8 Research needs and Proposed plan

Mycotoxigenic fungi are responsible for extensive financial losses encompassing a broad spectrum of crop and animal husbandry and pass along the food chain to the consumers. Farmers are impacted by both fungal contamination during cultivation and subsequent storage, resulting in destruction and downgrading of grains. Livestock in tropical countries are especially affected by acute toxicity and the consumers are vulnerable to the long term mutagenic and immunosuppressive effects of mycotoxins.

The effect of crude extract of *S. maydis* has been studied in cattle, sheep, goat, poultry and monkeys. It is difficult to conclude whether the toxicity is due to diplodiatoxin or due to synergistic action of the other secondary metabolites present in the extract. The effect of pure form of diplodiatoxin has not been studied previously. Hence, the present study is aimed to evaluate the exact nature of activity of diplodiatoxin, by screening various isolates for the production of diplodiatoxin, undertaking isolation and purification of diplodiatoxin, studying the toxicity of diplodiatoxin and analysing the genetic relationship (using ITS sequencing) among the toxin and non-toxin producing isolates.

The objectives of the present study were as follows:

1. To screen the various isolates of *S. maydis* collected from different geographical regions of South Africa for the production of diplodiatoxin.
2. To optimise the conditions for *in vitro* production of diplodiatoxin.
3. To isolate and purify the diplodiatoxin.
4. To test anti-bacterial activity of diplodiatoxin.
5. To test the toxicity of diplodiatoxin on mammalian cell lines.
6. To test acute toxicity of diplodiatoxin on male and female rats.
7. To test sub-acute toxicity of diplodiatoxin on male and female rats
8. To sequence the ITS region of toxin and non-toxin producing isolates of *S. maydis* to study their genetic relationship.

Chapter 2

SCREENING AND *IN VITRO* PRODUCTION OF DIPLODIATOXIN

2.1 Introduction

Stenocarpella maydis is a significant pathogen which causes stalk rot and ear rot in maize. It produces a toxin known as diplodiatoxin. *S. maydis* is known to have measurable affect on animals. So cultures of *S. maydis* or infected maize have been used to study the toxicity of crude extract in experimental animals but the toxicity may be due one principle compound or due to the synergistic effect of several compounds present in the extract. Thus it is important to know the exact nature of the active principle compound in crude extract, for which a pure culture of *S. maydis* and optimised conditions for the toxin production are a prerequisite.

Mostly, mycotoxins are produced during the stationary growth phase of mycelium and are released into the media or are accumulated in the hyphal cells. Requirements for growth and production of mycotoxins are entirely different. It is only when the nutritional factors run out and limits the growth, then the toxin biosynthesis occurs rapidly (Smith and Moss, 1985). Water stress, high temperature stress and salt induce toxin production (Sweeney and Dobson, 1998). The presence of various carbohydrates and nitrogen sources, phosphates and trace elements in the growth media also affects mycotoxin production (Luchese and Harrison, 1993). The *in vitro* growth of the mycelium and production of mycotoxin also dependents upon pH of the media (Murray and Walter, 1991; Marvin *et al.*, 1995), the type of media and the incubation conditions such as temperature (Marvin *et al.*, 1995; Velluti *et al.*, 2000), humidity, type of cultures and light. Addition of a particular precursor for mycotoxin biosynthesis into the media also induces mycotoxin production due to a phenomenon called feedback mechanism. For example isotope labelled alanine, which is a precursor of fumonisin B_1 biosynthesis when added in the media increased production of mycotoxin (Branham and Plattner, 1993). Cultural conditions also help in enhancing the production of mycotoxins and other secondary metabolites, like batch fermentation enhances the production of fumonisin

(Maia *et al.*, 2001). The use of liquid medium has been promising as this greatly reduces the number of contaminants and has been found to increase the yield of mycotoxins (Musonik, 1996). Liquid cultures are recommended even for the growth of *Stenocarpella maydis* (Bussard and Larson, 1978). Usually, mycotoxins are produced during the stress conditions, either accumulated in the mycelium or released into the medium.

As some isolates do not produce toxin, screening and detecting the toxin producing isolates becomes important (Leslie *et al.,* 1992). Modern techniques, which are simpler and efficient, are employed for the detection of mycotoxins (Machado and Kemmelmeier, 2001). Detection and screening of known mycotoxins are done by TLC, HPLC and Mass Spectrometry. The most simple and effective method is Thin Layered Chromatography, It has been recently used for the screening of some unknown compounds (Laserson *et al.*, 2001). It has been used for a long time for quick detection of mycotoxins. The TLC is considered as the presumptive test and further confirmatory tests are required.

Confirmation may include analysing the sample by Mass Spectrometer along with a known standard. Mass spectrometry is highly sensitive and is extremely valuable aid in the identification and structural analysis of organic compounds. Fungal metabolites are usually detected by Mass Spectrometry (Prasain *et al.*, 2002).

The present study is aimed to screen the various isolates of *S. maydis* from the infected maize fields in South Africa for the presence of diplodiatoxin and to study various factors governing its *in vitro* production.

2.2 Materials

Potato Dextrose Agar (PDA)

Infusion from potatoes (See below)	1000ml
Glucose	2% (w/v)
Agar	15% (w/v)

Potato Infusion:

Boil 200g scrubbed and sliced potatoes in 1000 ml water for 1 hour. Pass through a fine sieve.

Corn Meal Agar (CMA)

Corm meal	5% (w/v)
Agar	1% (w/v)
Distilled water	800ml

Czepex Medium (CzM)

Sucrose	3% (w/v)
Sodium nitrate	3% (w/v)
Magnesium sulphate	0.5M (5ml)
Potassium chloride	0.5% (w/v)
Iron (III) sulphate	0.01% (w/v)
Di-potassium hydrogen phosphate	1 % (w/v)
Agar-agar	1.3% (w/v)
Suspended in distilled water	48g/litre

Minimal Salts Medium (MSM)

$NaNO_3$ (Sodium nitrate)	12% (w/v)
KCl (Potassium chloride)	1.04% (w/v)
KH_2PO_4 (Potassium phosphate monobasic)	1.63% (w/v)
K_2HPO_4 (Potassium phosphate dibasic)	2.09% (w/v)
$MgSO_4$:	0.5M (5ml)

For 1000ml, dissolve the listed salts in 800ml of distilled water in the order indicated. Bring the final volume to 1000ml with distilled water.

Potato Dextrose Broth (PDB)

Potato Extract	20% (w/v)
Dextrose	2% (w/v)
Distilled water	1000ml

.

Chemicals and Reagents

Methanol, Ethanol, Toluene, Ethylacetate, Benzene, Chloroform, Formic acid, Hydrochloric acid, Sodium hydroxide, Sulphuric acid.

Spraying reagent

Vanillin: Vanillin 0.1% is dissolved in 50% H_2SO_4 diluted in 50% ethanol at –80 ° C.

Thin Layered Chromatography plates (TLC)

The TLC plates are coated with silica gel – G, 0.5mm thickness

The chemicals were from Sigma or Merck laboratories in South Africa

2.3 Methods

2.3.1 Collection and maintenance of *S. maydis* isolates

Twenty different isolates of *Stenocarpella maydis* viz., D79, D72, D80, D74, D78, AX30, 8Y, 6Y, 2Y, C1A, C3C, CH3, U3H, MC34, MC35, MC43, MC50, U3, U5 and U2, were collected by Professor Achar's group from maize cultivars grown in different maize growing areas in South Africa viz., Potchefstroom, Transkei and Cedara. Single spores were isolated and sub cultured on Potato Dextrose Agar PDA to obtain pure cultures for further experiments. Cultures were maintained on PDA at 26 °C in the dark and 55% to 60% relative humidity and were sub-cultured at week intervals. Simultaneously, healthy maize plants were grown as control in the green house (Fig. 2.1) parallel to the infected ones.

2.3.2 Screening of isolates of *S. maydis* for the presence of diplodiatoxin

2.3.2.1 Suspension cultures

The fungal isolates were grown in Potato Dextrose Broth with pH 4.5 in a 3l Erlenmeyer flask, each containing 2l of the medium. Culture were agitated at 80rpm on a rotary shaker for the first 6 weeks of the incubation and then kept under static conditions for another 2 weeks.

Fig 2.1 Healthy maize plants grown in green house

1. Healthy maize plants grown in the green house parallel to the infected maize plants
2. The healthy maize cobs were grown and the extract from the healthy cob was spotted on TLC along with the extract of infected cob for the detection of the diplodiatoxin and for analysing the common compounds present

2.3.2.2 Extraction and Thin Layer Chromatography

After 6 weeks of growth in suspension cultures the mycelium was homogenised along with the supernatant in a food mixer and extracted overnight with chloroform: methanol (1:1, v/v) to yield a crude extract. The extract was vacuum filtered through Whatman Filter paper No. 1 to remove debris. The solvent was evaporated at 70 °C under vacuum and the residue was re-dissolved in 2ml of ethyl acetate. All the extracts were stored at 4 °C. For the chromatographic separation of compounds, extracts from different isolates were loaded on a thin layer chromatographic (TLC) plate (silica gel-G; 0.5mm thickness) and the following solvent systems were used for the separation of compounds.

1. Toluene: Ethyl acetate: Formic acid (4:5:1, v/v)
2. Methanol: Chloroform (4:1 v/v)
3. Benzene: Chloroform: Acetic acid (5:4:1, v/v)
4. Ethyl acetate (100%)

TLC plates were viewed under UV light and developed with 0.1% vanillin followed by heating the TLC plate at 120 °C for 2 minutes for the detection of diplodiatoxin. The diplodiatoxin reacts with vanillin to give an orange spot on TLC plate when heated at 100 °C for 2 minutes.

2.3.2.3 Atomic Pressure Chemical Ionisation Mass Spectrometry

The presence of diplodiatoxin in these isolates was further confirmed by VG Quattro Mass Spectrometer with electrospray ionisation (ApcI-MS) in ESMS unit (University of Stellenbosch). The samples were diluted to (1:10) with ethyl acetate and 50µl of the dilution was loaded in the instrument. The carrier solvent was ethyl acetate delivered to the ionisation source at the flow rate of 50µl/minute. The mass spectrum was scanned from m/z = 100-1000 at a scan rate of 5seconds/scan.

2.4 Optimisation of *in vitro* growth of *S. maydis* and production of diplodiatoxin

2.4.1 Cultural conditions

For the optimisation of mycelial growth and the production of diplodiatoxin, The MC43 isolate of *S. maydis* was used. The fungal inoculums for the below experiments were 500mg (fresh weight) per each flask. Cultures were grown in 1Litre Erlenmeyer flasks, each containing 500ml of the media. For the first 6 weeks cultures were agitated at 80rpm on a rotary shaker, followed by static conditions for another 2 weeks under a periodic cycle of 16hours light and 8hours at 26 ± 1 °C and 60 ± 5% relative humidity.

2.4.2 Effect of various media compositions

Mycelium of MC43 isolate was inoculated in the various media like potato dextrose broth (PDB), Corn Meal Extract (CME), Czepex Liquid Medium (CLM) and Minimal Salts Medium (MSM) with pH 4.5. The *in vitro* growth of the mycelium and production of the diplodiatoxin (on the basis of TLC) was evaluated in 2 to 8 week-old cultures.

2.4.3 Effect of pH

The MC43 isolate of *S. maydis* was inoculated in potato dextrose broth and the pH of PDB media was adjusted ranging from 3.0 to 5.0. The growth of mycelium and the production of diplodiatoxin were evaluated in 2 to 8 week-old cultures.

2.4.4 Effect of incubation temperature

MC43 isolate grown in PDB media was incubated under a varied range of temperature (22 to 32 °C) to study the effect of temperature stress on the growth of mycelium and production of diplodiatoxin.

2.5 Results

2.5.1 *Stenocarpella maydis* isolates

Early infection of *Stenocarpella maydis* on maize appears as development of white powdery mass on the cob (Fig. 2.2) and the stalk causing severe ear rot and seedling blight. Various isolates collected from different geographical regions of South Africa exhibited differential growth and colony colour when grown *in vitro* on PDA media (Fig. 2.3). During the initial stage of growth, mycelium grew as white filaments but the colour change after 7 to 8 days of growth. Most of the isolates were yellow, brown or white in colour but the U3H isolate was green. The pycnidia were immersed, spherical to globose, dark brown in colour and conidia were either straight or slightly curved, septate, smooth walled and pale brown in colour. But, in suspension culture mycelium was brown in colour in all the isolates.

2.5.2 Screening of isolates for the presence of diplodiatoxin

2.5.2.1 Thin Layer Chromatography

Various isolates of *S. maydis* viz., D79, D72, D80, D74, D78, AX30, 8Y, 6Y, 2Y, C1A. C3C, CH3, U3H, MC34, MC35, MC43, MC50, U2, U3 and U5 collected from different regions in South Africa were screened for the presence of diplodiatoxin (Table 2.1). Thin Layer Chromatographic separation of compounds from the crude extract (Section 2.3.2.2) of 20 different isolates collected from Potchefstroom, Transkei and Cedara showed considerable differences in the presence or absence of diplodiatoxin. Diplodiatoxin reacted with 1% of vanillin to give an orange spot on the TLC plate, which clearly indicated the presence of toxin in the isolates. Diplodiatoxin could not be viewed under UV light. It was also observed that MC34, MC35, MC43 and MC50 isolates of Porchefstroom region produced the maximum quantity of diplodiatoxin whereas D79, D72, D74, D80 and D78 isolates did not show the presence of diplodiatoxin. All isolates collected from Transkei showed the presence of very low quantities of diplodiatoxin (Fig. 2.4). C1A and C3C isolates of Cedara showed moderate quantity of diplodiatoxin and CH3 and U3H isolates of this region did not produce diplodiatoxin.

1 2

Fig 2.2 Infected and healthy maize cobs

1. The Infected maize cob showing the infection caused by *Stenocarpella maydis*
2. Healthy maize cob

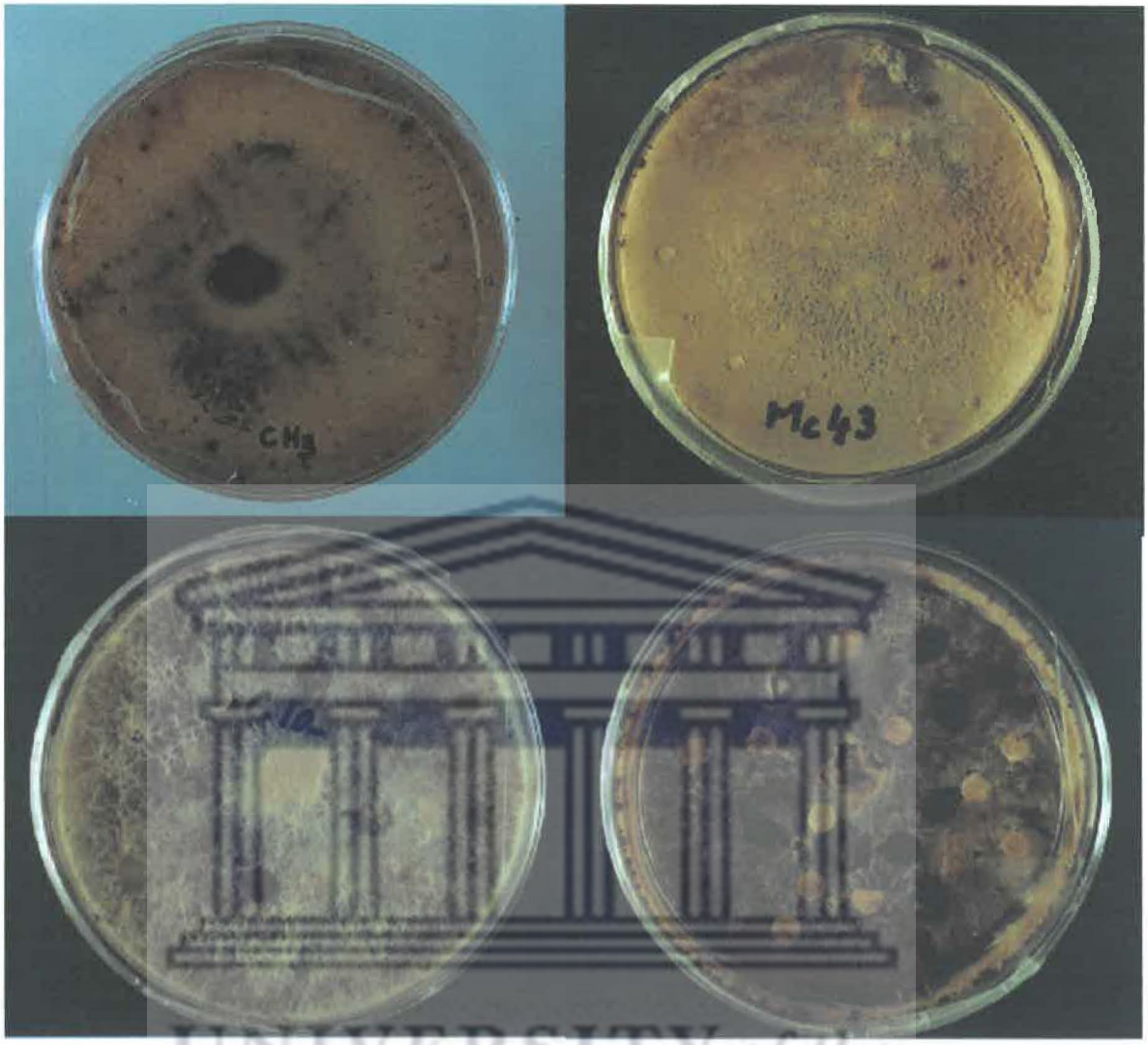


Fig 2.3 CH3, MC43, C1A and D74 isolates of *Stenocarpella maydis* grown on PDB

1. The isolates of *Stenocarpella maydis* sub-cultured on to potato dextrose agar plates from the infected maize cobs
2. The temperature at which they are grown is 26 ± 1 °C
3. The isolates are CH3, MC43, C1A and D74
4. The fungus grows as thick mycellial mat and produces spores after 3-4 weeks of incubation

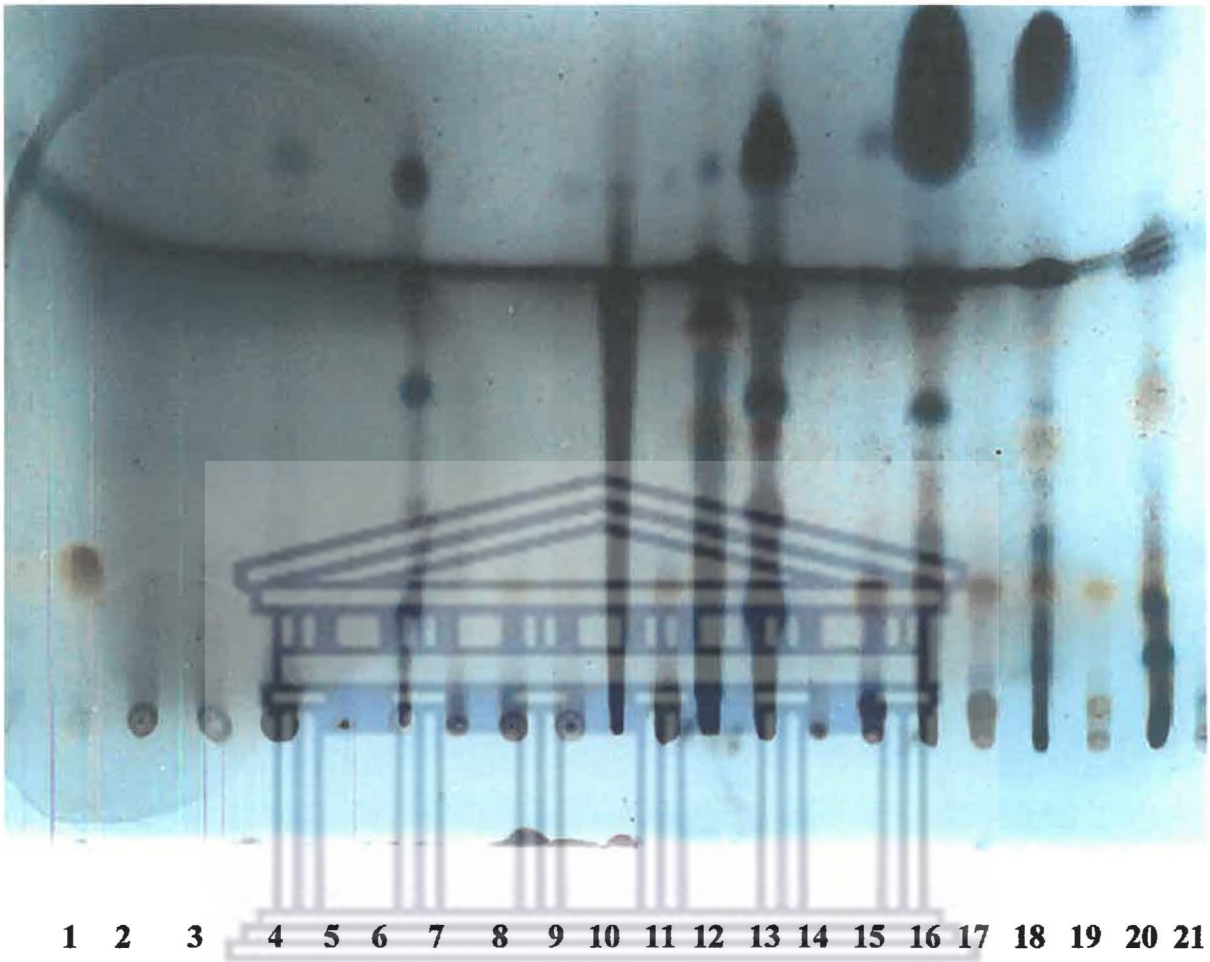


Fig 2.4 Thin Layered Chromatography of various isolates of *Stenocarpella maydis*

1. Thin Layer Chromatography of the 20 isolates *of Stenocarpella maydis*
   From lane one onwards
   Diplodiatoxin=1, D79=2, D72=3, D80=4, D74=5, D78=6, 6Y=7, 8Y=8, AX30=9, CH3=10, 2Y=11, U2=12, U3=13, U3H=14, C1A=15, MC34=16, MC43=17, MC50=18, C3C =19, MC35=20 and U5=21 respectively
2. The TLC plates are developed with 1% Vanillin
3. Diplodiatoxin reacts with Vanillin to give an orange spot

Origin	Name of the isolate	Colour on PDB	Presence of Diplodiatoxin
Potchefstroom	D 79	Yellow	-
	D 72	Brown	-
	D 80	White	-
	D 74	Brown	-
	D 78	White	-
	AX 30	Brown	+
Transkei	8 Y	Brown	+
	6 Y	Yellow	+
	2 Y	Brown	+
Cedara	C1A	Yellow	++
	C3C	Yellow	+++
	CH3	Yellow	-
	U3H	Green	-
Potchefstrom	MC 34	Yellow	+++
	MC 35	Brown	+++
	MC 43	Brown	+++
	MC 50	Yellow	+++
	U 2	White	++
	U 5	White	+++
	U 3	Brown	++

Table 2.1 Screening of various isolates of *Stenocarpella maydis* for the presence of diplodiatoxin

1. The table illustrates various *Stenocarpella maydis* isolates with their origin, colour of mycelium presence of diplodiatoxin and absence of diplodiatoxin
2. The table shows the concentration of diplodiatoxin present in various isolates of *Stenocarpella maydis*
   a) '+++' High concentration of diplodiatoxin
   b) '++' Moderate concentration of diplodiatoxin
   c) '+' Low concentration of diplodiatoxin
   d) '-'Absence of diplodiatoxin
3. The presence of the diplodiatoxin was observed on the basis of visual intensity of the compound on Thin Layered Chromatography

2.5.2.2 Atomic Pressure Chemical Ionization Mass Spectrometry

TLC was done as a preliminary test to know the presence of diplodiatoxin. The presence of diplodiatoxin in these isolates was further confirmed by Atomic Pressure Chemical Ionization Mass Spectrometry (APcI-MS). The mass spectra through the range m/z = 100 to 500, no high mass peaks were observed in the samples (Fig. 2.5). Samples containing diplodiatoxin showed a strong negative ion at m/z = 307. The mechanism of ionization for negative atomic pressure chemical ionisation is de-protonation, therefore, the ion would be [m-h]. The molecular ion would then have a mass of 308. The peak at m/z = 233 was shown to be a fragment of m/z = 307 because the ions at m/z = 307 when isolated and fragmented, showed that the peak at m/z = 233 as a derivative of compound 307 and probably not a contamination. Other peaks in spectra indicate other compounds present in the crude extract of the fungus.

In addition to diplodiatoxin, all the isolates also contained a compound with a dominant peak at m/z = 233 and an additional compound with a dominant peak at m/z = 216 was found in 6Y and C1A isolates. Since, the target study was mainly to see the effect of diplodiatoxin, other compounds in the extract were not taken into consideration. The spectrum obtained by ApcI-MS also indicated a number of other compounds present in the crude extract and thus the requirement for bulk-purification using column chromatography was necessary. No other compounds with a mass/charge ratio greater than m/z = 500 were observed in any of the isolates used in the study.

Thus, the absence of diplodiatoxin was confirmed in D79, D72, D78, D80 and D74 isolates of *S. maydis*. And the presence of diplodiatoxin was confirmed in MC43, which was used for the further subsequent work.

2.6 Optimisation of *in vitro* growth of *S. maydis* and production of diplodiatoxin

2.6.1 Effect of media composition

The effect of various liquid media viz., PDB, CME, CLM and MSM was studied on the growth of *S. maydis* and production of diplodiatoxin (Table 2.2). After 6-weeks of incubation, growth of the mycelium was maximum (30.8g fresh weight) in PDB and least

(22.0g/l fresh weight) in CME media. Therefore, the growth of mycelia in PDB was much higher than in CME. The rate of increase in the growth was rapid up to the fourth week of incubation and thereafter the cultures attained a stationary phase. On the other hand, PDB was optimal for the production of diplodiatoxin but the production of diplodiatoxin in detectable quantity was observed only after 6-weeks of growth. Thus, PDB was found to be the optimal media for the maximum growth of *S. maydis* and production of diplodiatoxin.

2.6.2 Effect of pH

The pH of PDB media played an important role in the growth of *S. maydis* and production of diplodiatoxin. Increasing the pH of PDB media from 3.0 to 4.5 increased growth of mycelia and production of diplodiatoxin (Table 2.3). Further increase in the pH to 5.0 inhibits the growth. Production of diplodiatoxin in detectable quantities was observed only after 6 weeks of growth *in vitro* at pH 4.0 and 4.5. However, at all the other pH tested, the production was delayed even after 8 weeks of incubation, thus indicating that pH 4.5 was optimal for the production of diplodiatoxin. Maximum diplodiatoxin production was favoured by pH 4.5 and growth of mycelium in this media was 1.5 times over the growth after 6 weeks at pH 3.0. Thus, it was concluded that pH 4.5 was optimal for the maximum growth of mycelium and production of diplodiatoxin.

2.6.3 Effect of incubation temperature

Incubation temperature had a marked effect on *in vitro* growth of *S. maydis* and the production of diplodiatoxin (Table 2.4). The incubation temperature was ranging from 22 °C to 34 °C. The maximum growth of mycelium and production of diplodiatoxin was observed at 28 °C. A further increase in temperature reduced growth and production of diplodiatoxin and the production of diplodiatoxin also delayed to seven weeks of growth. Similarly, lower temperature also drastically reduced the growth of fungus, and diplodiatoxin was produced only after 8 weeks of growth under these conditions. Thus, the optimal incubation temperature for the maximum growth of the fungus and production of diplodiatoxin was 28 °C. Under these conditions, diplodiatoxin production was favoured and was found in detectable quantity after 5 weeks of growth.

Fig 2.5 Mass Spectrometry of *Stenocarpella maydis* isolates

1. The Mass Spectrometry is VG Quattro with electrospray ionisation (ApcI-MS) in ESMS unit (University of Stellenbosch)
2. The solvent used was ethylacetate
3. The mass spectrum was scanned from m/z = 100-1000 at a scan rate of 5sec/scan
4. The graph shows the presence of diplodiatoxin in some of the isolates and absence of diplodiatoxin in the other isolates
5. The presence of diplodiatoxin is indicated by a strong negative ion at m/z = 30
6. The isolates showing mass spectrum are Fig a= MC50, Fig b= C3C, Fig c= 6Y, Fig d= D72, Fig e= U2, Fig f= U3H, Fig g= MC34, Fig h= C1A

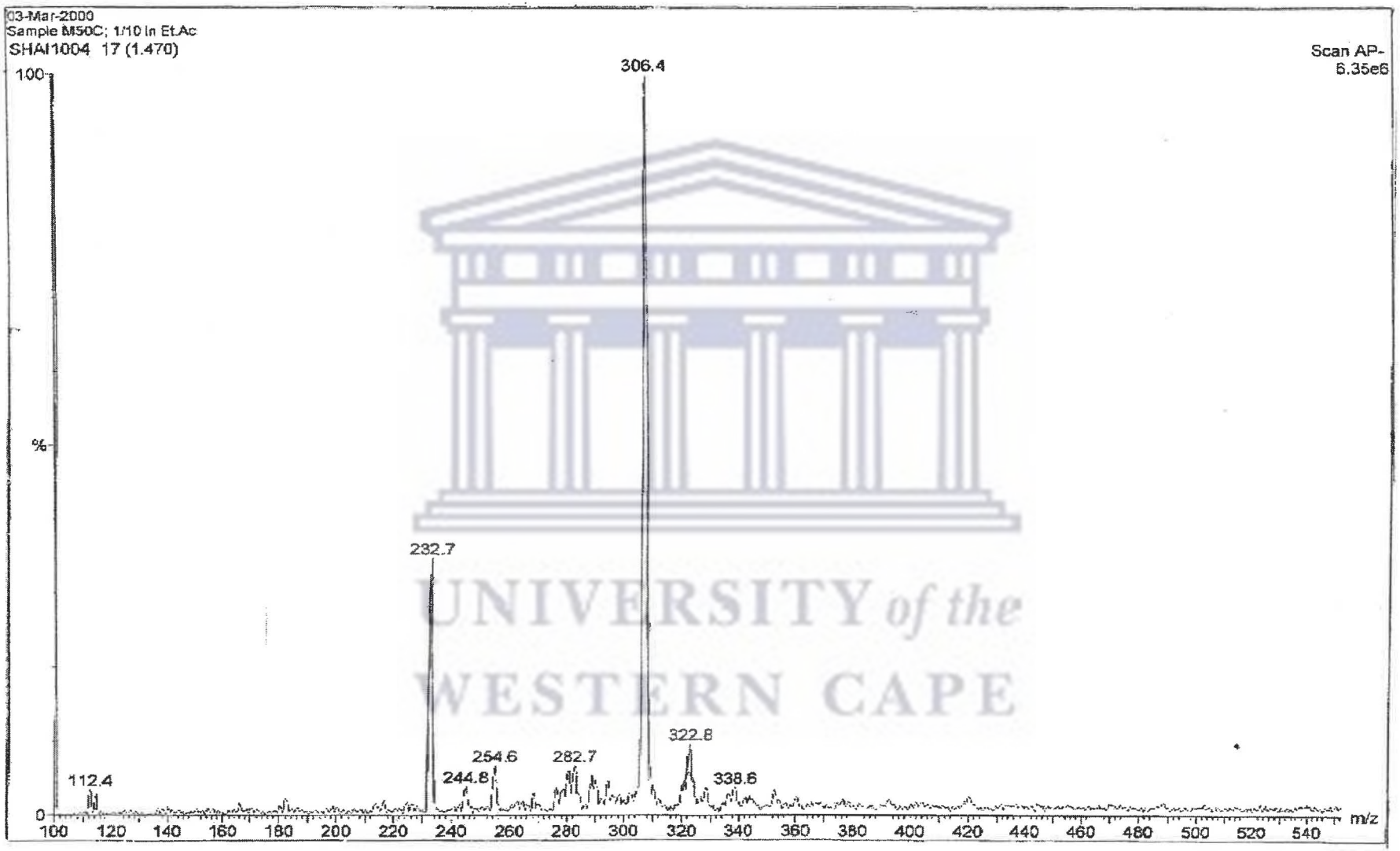


Figure a. (MC50)

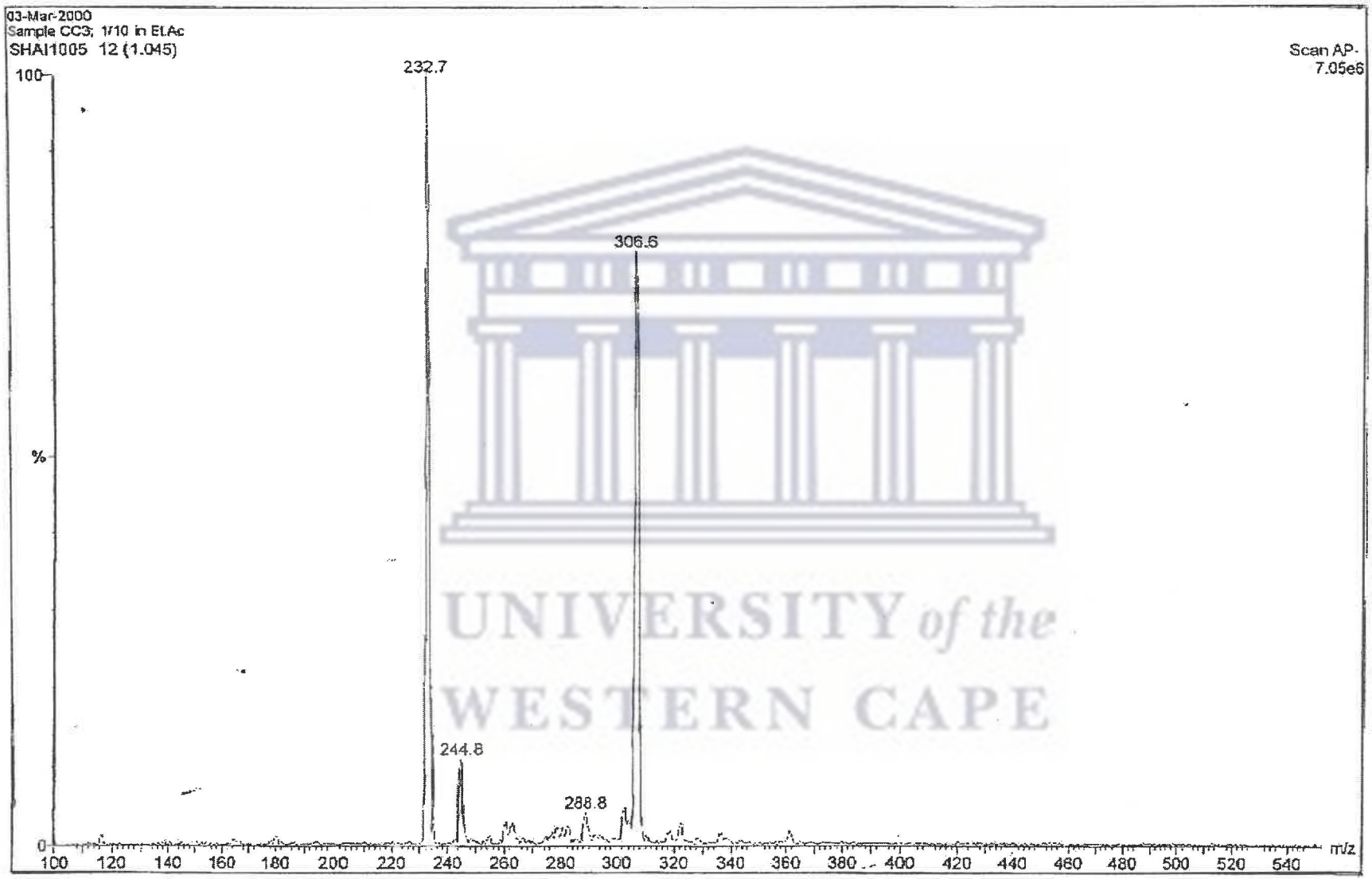


Figure b. (C3C)

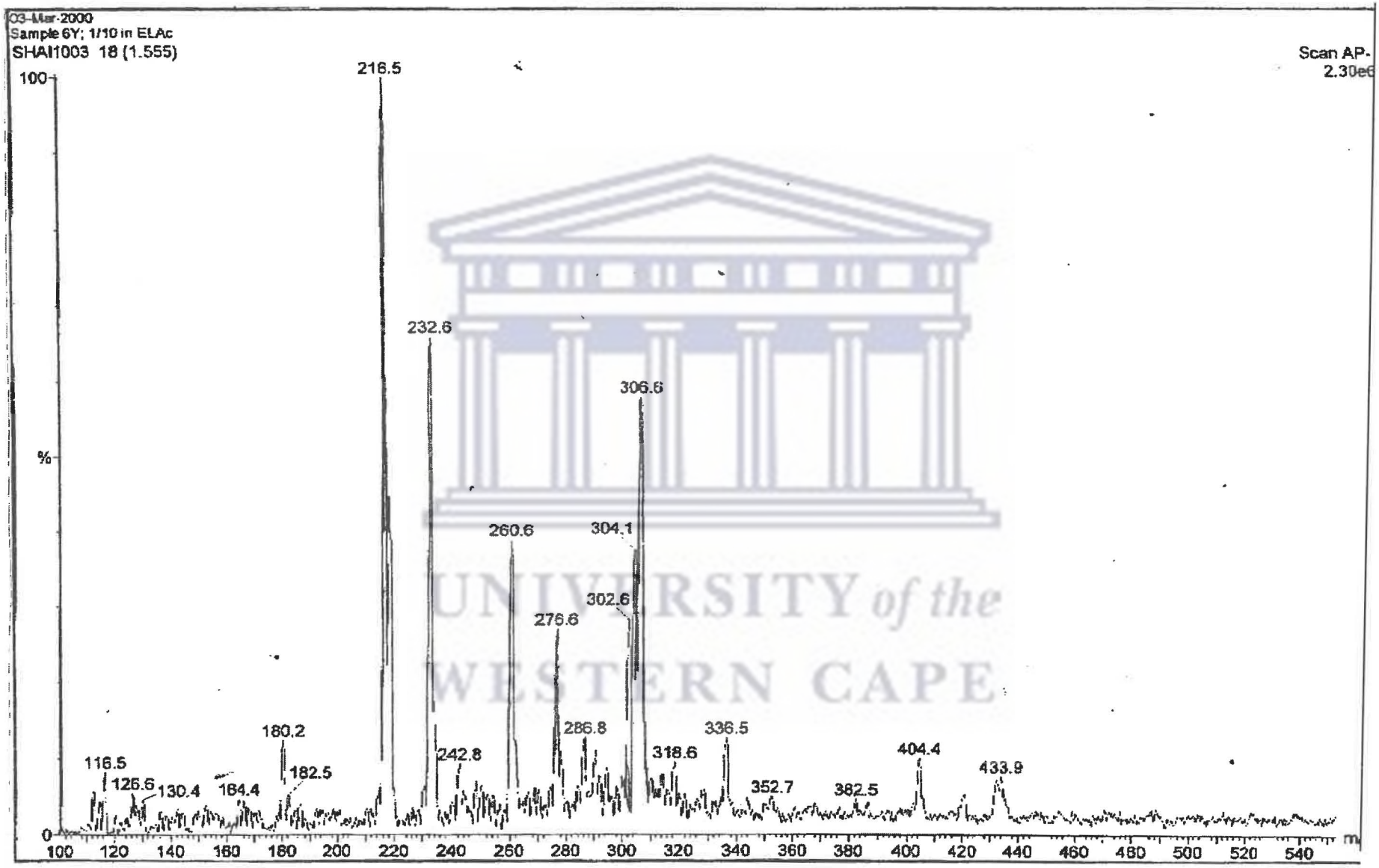


Figure c. (6Y)

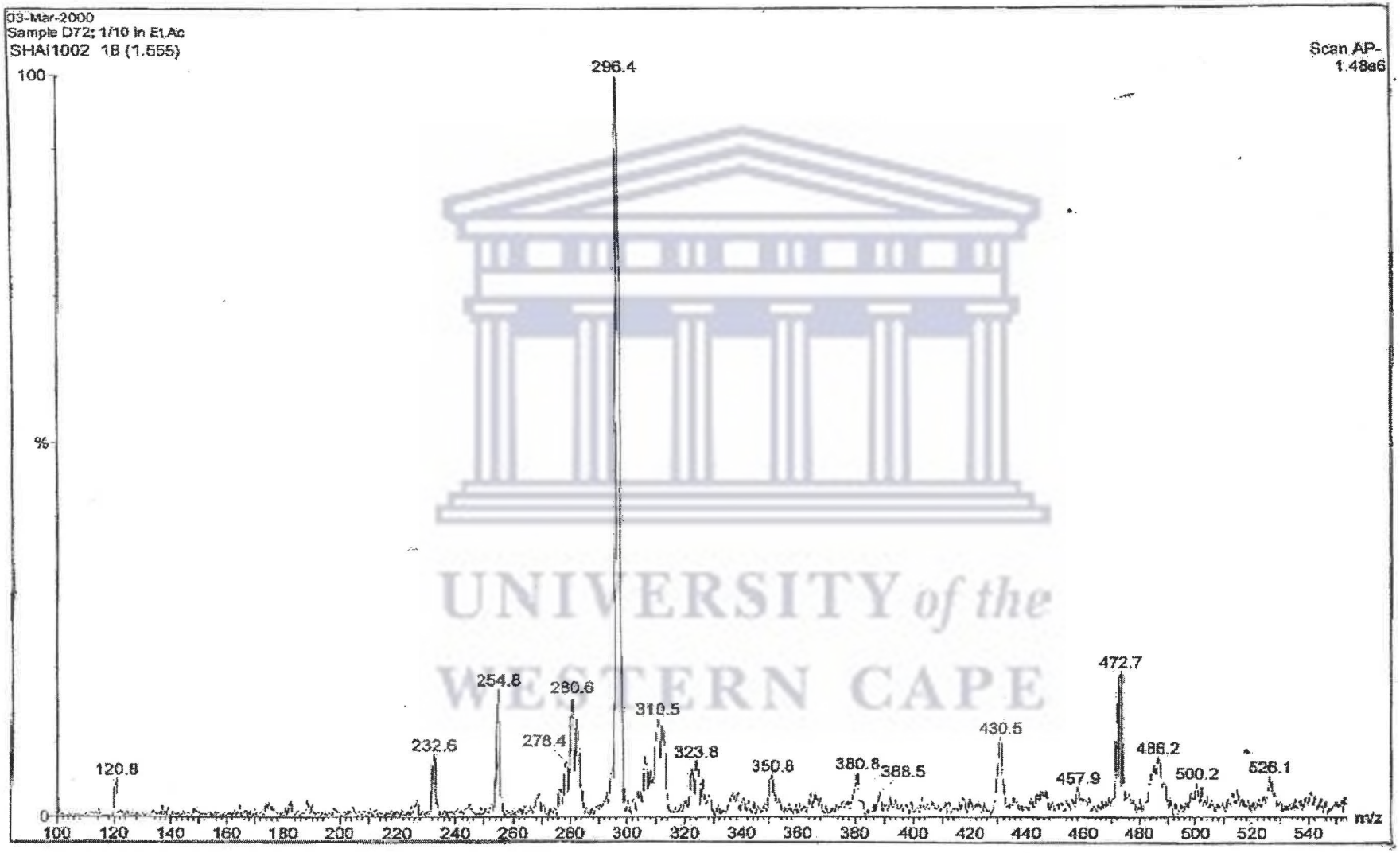


Figure d. (D72)

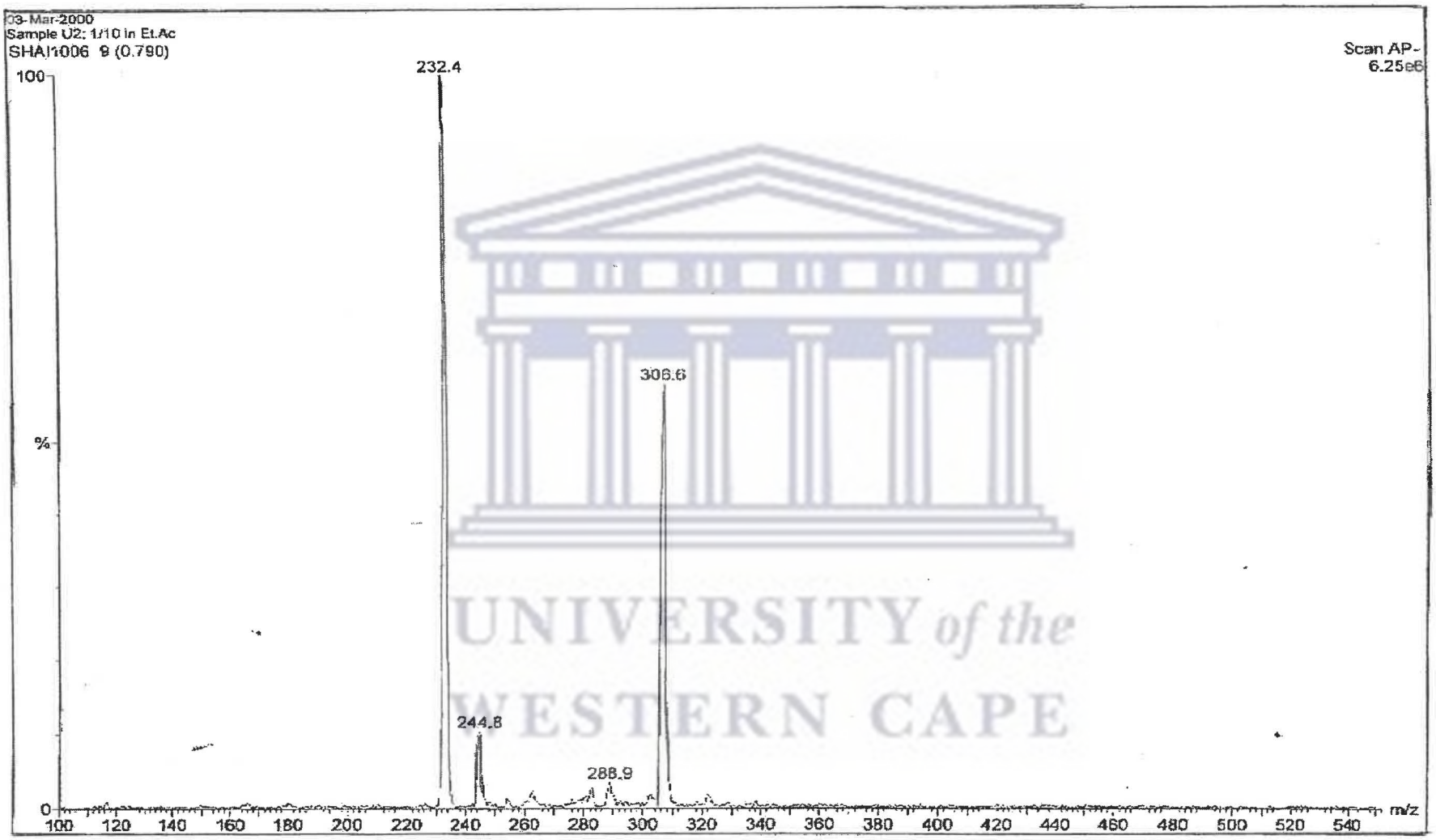


Figure e. (U2)

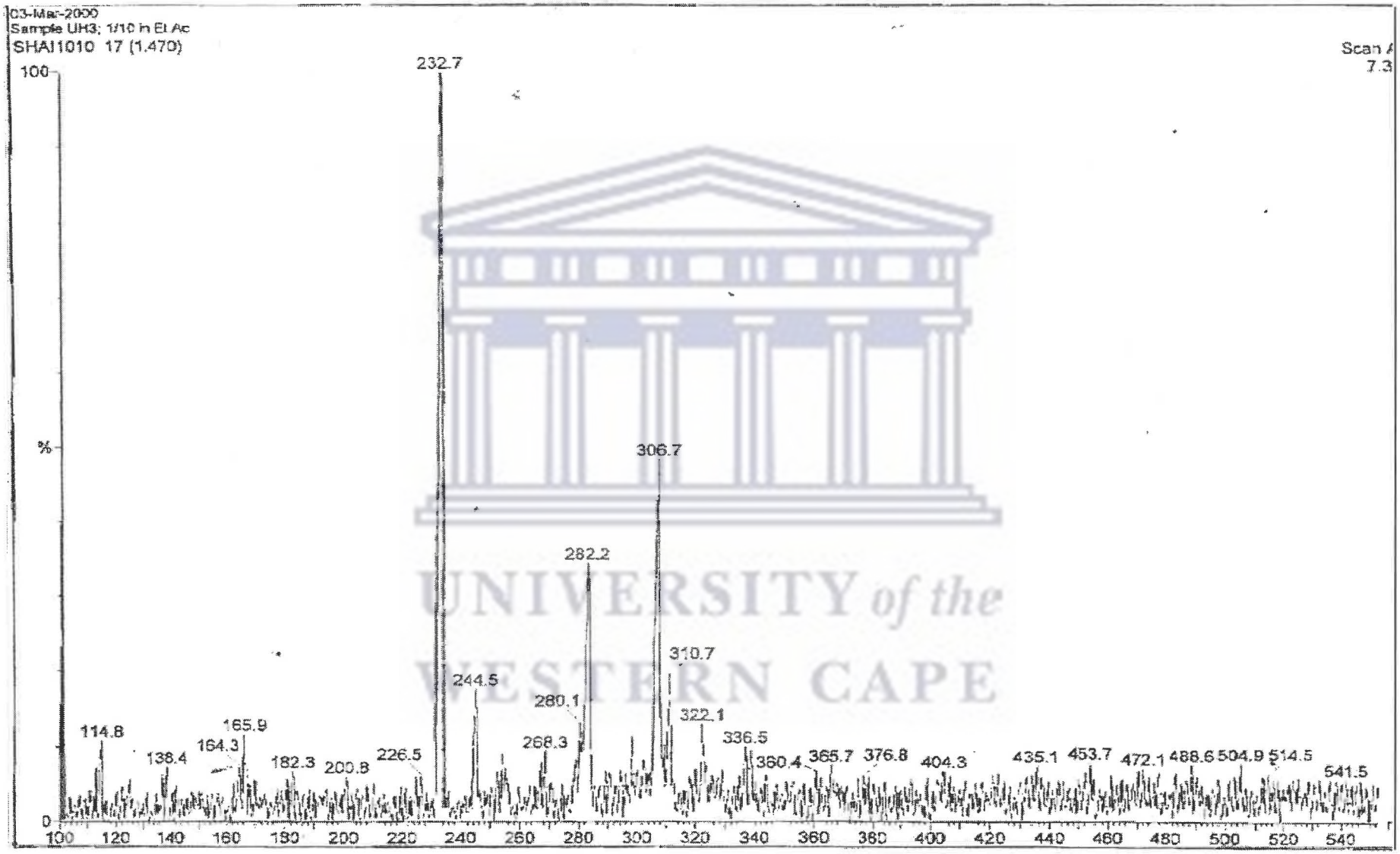


Figure f. (U3H)

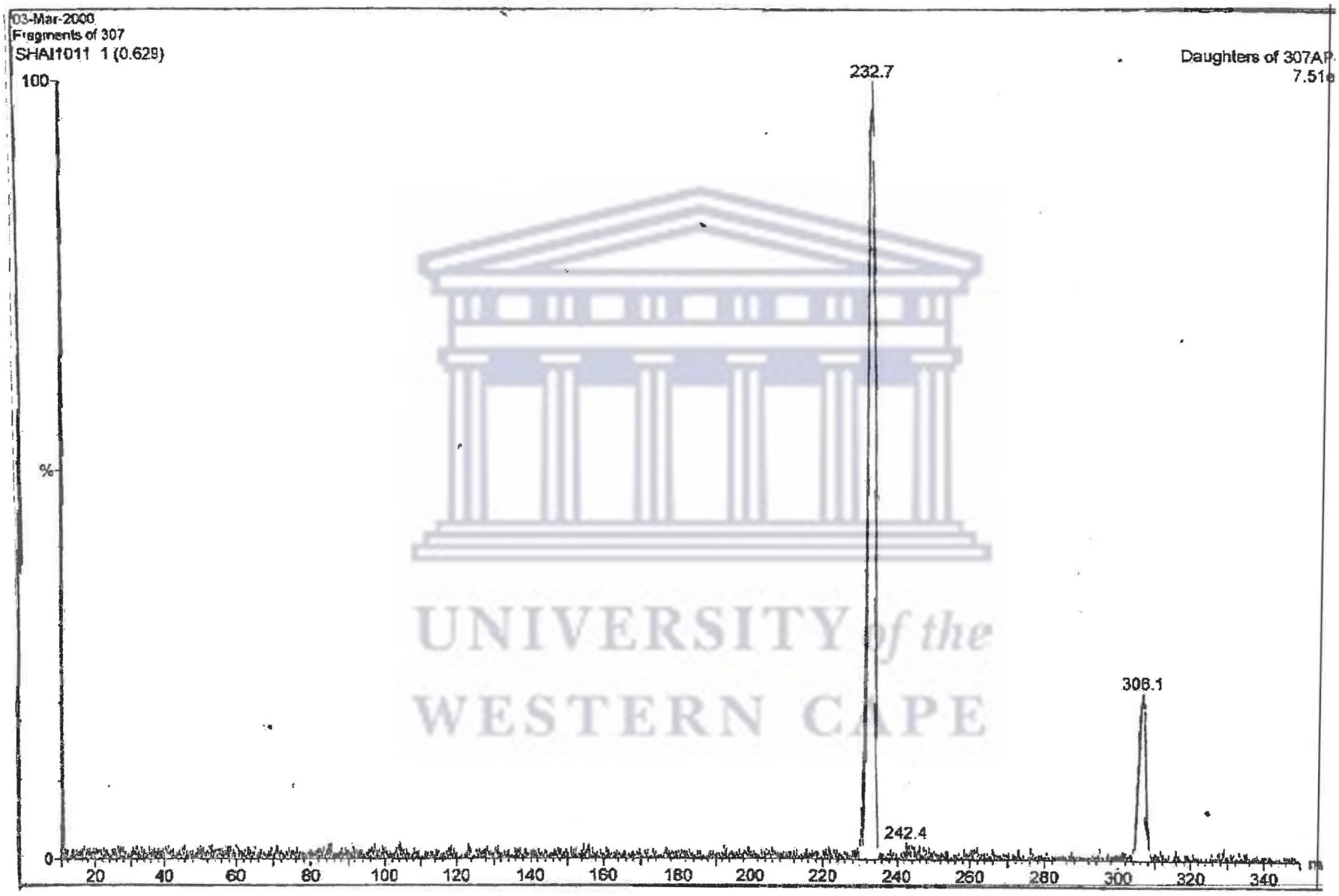


Figure g. (MC34)

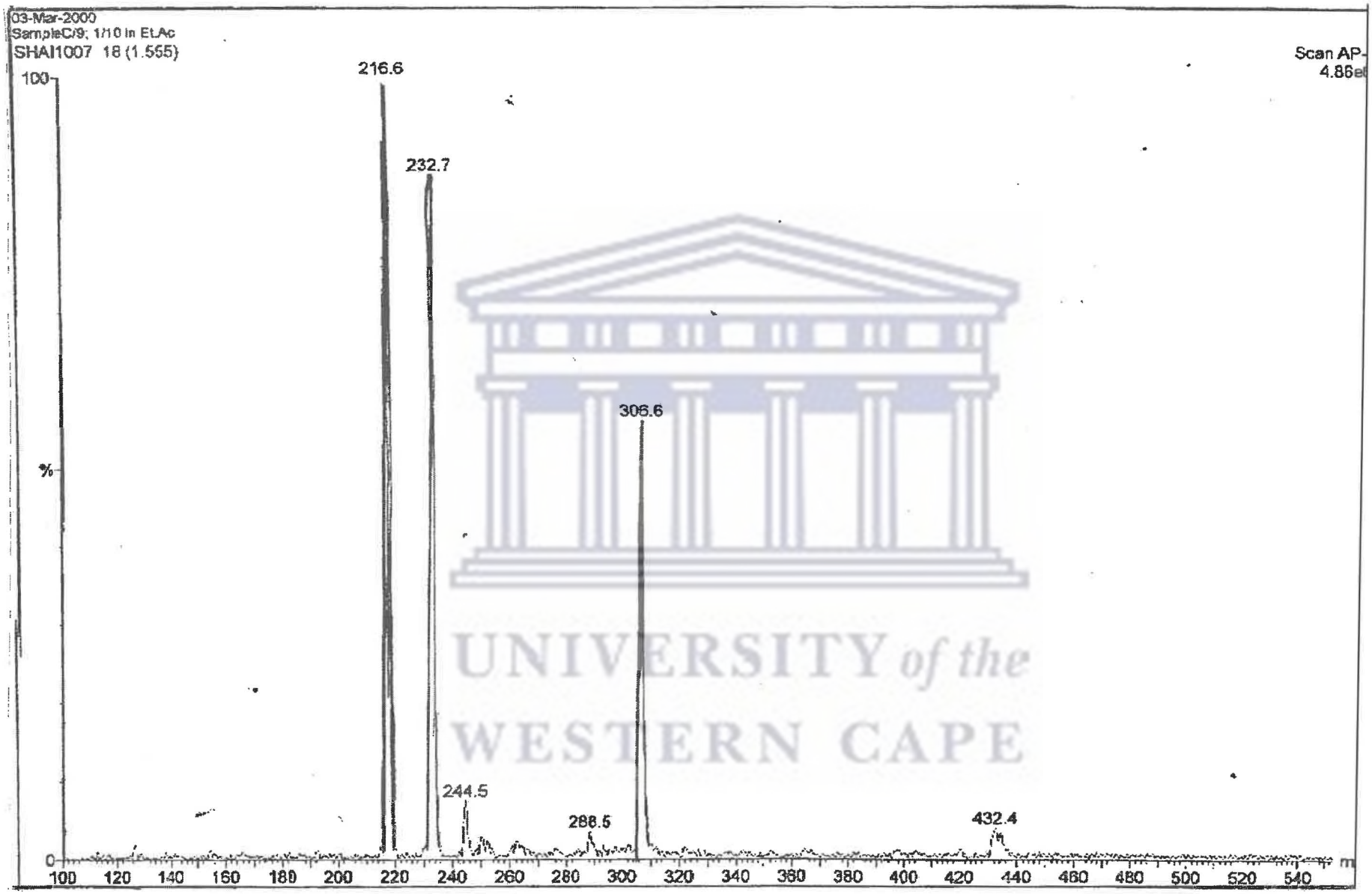


Figure h. (C1A)

Media	Growth (g fresh weight/litre)					Diplodiatoxin			
	2	3	4	5	6 weeks	2 to 5	6	7	8 weeks
PDB	18.0	20.8	29.0	30.8	30.8	-	+	++	++
CME	17.6	10.8 *	20.0	21.5	22.0	-	+	+	+
CLM	12.8	15.2	21.8	25.4	25.6	-	+	+	+
MSM	19.8	22.3	24.2	28.1	30.0	-	-	+	+

Table 2.2 The effect of various media on the growth of *Stenocarpella maydis* and the production of diplodiatoxin

1. The table illustrates various medium and growth pattern of *Stenocarpella maydis* ranging from 2-6weeks
2. The growth of the mycelium, production and concentration of diplodaitoxin in various media ranging from 2-8 weeks
3. Detection of toxin
   a) '+++' High concentration of diplodiatoxin
   b) '++' Moderate concentration of diplodiatoxin
   c) '+' Low concentration of diplodiatoxin
   d) '-'Absence of diplodiatoxin
4. The presence of the diplodaitoxin was observed on the basis of visual intensity of the compound on Thin Layered Chromatography

pH	Growth (g fresh weight/litre)					Diplodiatoxin			
	2	3	4	5	6 weeks	2 to 5	6	7	8 weeks
3.0	11.2	13.8	18.6	20.8	22.1	-	-	+	+
3.5	12.2	14.8	20.1	23.5	24.7	-	-	+	+
4.0	14.1	19.9	24.2	27.9	28.3	-	+	+	+
4.5	15.5	18.9	27.5	30.2	30.2	-	+	++	++
5.0	15.1	19.7	25.6	29.3	29.3	-	-	+	+

Table 2.3 The effect of pH on growth of *Stenocarpella maydis* and the production of diplodiatoxin in PDB

1. The table illustrates various ranges of pH and growth pattern of *S. maydis* incubated for 2-6 weeks in potato dextrose broth
2. The growth of the mycelium and production of diplodiatoxin at various pH levels incubated from 2-8 weeks in potato dextrose broth
3. Detection of toxin
   a) '+++' High concentration of diplodiatoxin
   b) '++' Moderate concentration of diplodiatoxin
   c) '+' Low concentration of diplodiatoxin
   d) '-'Absence of diplodiatoxin.
4. The presence of the diplodaitoxin was observed on the basis of visual intensity of the compound on Thin Layered Chromatography

Temp (°C)	Growth (g fresh weight/litre)					Diplodiatoxin				
	2	3	4	5	6 weeks	2 to 4	5	6	7	8 weeks
22	12.8	15.1	17.7	19.1	20.6	-	-	-	+	+
26	18.1	21.5	26.1	27.3	29.7	-	-	+	+	++
28	19.6	22.0	27.2	28.8	31.9	-	+	++	++	+++
30	17.7	20.9	26.6	27.7	29.1	-	-	+	++	++
32	18.19	20.93	25.98	28.01	30.12	-	-	+	+	++

Table 2.4 The effect of incubation temperature on the growth of *Stenocarpella maydis* and the production of diplodiatoxin in PDB

1. The table illustrates various temperatures and their effect on the growth of *Stenocarpella maydis* from 2-6 weeks incubated in potato dextrose broth
2. The growth of the mycelium and production of diplodiatoxin at various temperatures incubated from 2-8 weeks in potato dextrose broth
3. Detection of toxin
   a) '+++' High concentration of diplodiatoxin
   b) '++' Moderate concentration of diplodiatoxin
   c) '+' Low concentration of diplodiatoxin
   d) '-'Absence of diplodiatoxin
4. The presence of the diplodaitoxin was observed on the basis of visual intensity of the compound on the Thin Layered Chromatography

2.7 Discussion

Stenocarpella maydis isolates were collected from the different geographical regions in South Africa and were screened for the production of diplodiatoxin. Production of mycotoxin depends upon the genetic makeup of the isolates and not all the isolates will necessarily produce mycotoxin (Leslie *et al.,* 1992). For the screening purposes, the TLC and ApcI-MS were used, as MS is currently being used for the detection of most mycotoxins (Lombaert, 2002).

Thin Layer Chromatographic-based preliminary screening of isolates of *S. maydis* showed a considerable variation among the isolates for the production of diplodiatoxin. It was concluded that maximum amount of diplodiatoxin was produced by MC43, C1A and MC50 isolates of Potchefstroom region. However some of the isolates from this region did not produce diplodiatoxin. The presence or absence of diplodiatoxin was further confirmed in strains C1A, MC43, MC50 by ApcI-MS. The presence of a strong negative ion at m/z = 307 confirmed the molecular weight of 307 which is supported by the available literature (Steyn *et al.,* 1972; Ichihara *et al.,* 1986). Absence of diplodiatoxin was observed in D79, D72 and D80 and isolates collected form Potchefstroom region.

Thus, it may be suggested that these isolates differ in their genetic makeup and studies on the molecular aspects of *S. maydis* offers an excellent opportunity to identify the genetic relationship among the toxin and non-toxin producing isolates.

The toxic effect of crude extract of *S. maydis* on animals has been reported but the toxicity may be due to the presence of an unknown toxic metabolite(s) or due to the synergistic effect of several compounds present in the extract. Thus, it is important to know the exact nature of the toxic metabolites in the crude extract, for which optimisation of *in vitro* conditions for the production of diplodiatoxin is a pre-requisite.

In the present chapter, *in vitro* growth of *S. maydis* and production of diplodiatoxin was standardised by manipulating the pH of the media, media composition and incubation temperature. The pH 4.5 was found to be optimum for the production of diplodiatoxin and growth of the mycelium. In addition it was found that lower range of pH inhibited the growth of *S. maydis* and production of diplodiatoxin. The production of diplodiatoxin

in detectable quantity requires six weeks of incubation. This is a deviation from the general trend of the increased production of secondary metabolites with the factors limiting the growth of mycelium.

Among the various media compositions tested in the present investigation, PDB was found to be optimal for both growth of the mycelium and production of diplodiatoxin. It was also observed that Corn Meal Extract stimulated the growth of *S. maydis* but the production of diplodiatoxin was not high may be due to the reason that maize being the natural host of *S. maydis* and thus stimulates its growth. Similar results were also obtained by (Chen and Strange, 1994). They used chickpea cell sap to grow *Ascophyta rabiei*, which causes blight in chickpea, to produce phytotoxins. This natural media stimulated the growth of fungus drastically due to the presence of certain stimulatory compounds in the cell sap and also as chickpea is the host plant for *A. rabiei*.

Production of mycotoxins and other secondary metabolites in response to stress is well known and high incubation temperature is the best way of creating stress environment. But, there are examples of deviation from this general rule and the present investigation also showed a deviation from this general trend of high temperature induced production of mycotoxins. When the cultures were incubated with continuous agitation, the growth of mycelia and the production of diplodiatoxin were very low. Thus, attempts of providing aeration stress like incubating the cultures in a stationary condition for 2 weeks after 6 weeks of continuous aeration resulted in a kind of aeration stress resulted in increased production of diplodiatoxin. So, results followed the trend of aeration stress – induced production of mycotoxins.

Unlike other reports of high temperature stress for the increased production of mycotoxins (Sweeney and Dobson, 1998; Romas *et al.,* 1998, Marvin *et al.,* 1995), diplodiatoxin was produced at 28 °C, the temperature found to be optimal for the growth of the mycelium. And a minimum of 5-6 weeks of incubation is required for the production of diplodiatoxin. (Alberts *et al.,* 1990) also reported that the incubation period and temperature are critical factors for the production of mycotoxins.

The temperature, pH, the type of the medium and incubation conditions play a vital role in the production of diplodiatoxin, It is concluded that *in vitro* production of diplodiatoxin is favoured by the factors enhancing the growth of *S. maydis* and that stress conditions like aeration stress increased the production of diplodiatoxin.

Chapter 3

ISOLATION AND PURIFICATION OF DIPLODIATOXIN FROM *STENOCARPELLA MAYDIS*

3.1 Introduction

Mycotoxins are a particular problem in developing countries (Coker, 1979) as they are considered to be involved in the aetiology of certain human and animal diseases. It is important to trace the toxigenic fungi. Thus developing good analytical methods for the detection of certain mycotoxins become necessary. As mycotoxins display a wide diversity of structure, chemical and physical properties there is a need to develop a specific method to isolate mycotoxins. However, most mycotoxins are now readily identified qualitatively and quantitatively.

Production of a pure culture of the fungus of interest is a pre-requisite for the extraction and purification of mycotoxins. In general, mycotoxins are found in high concentration at the site of infection. The fungus from the infected site has to be identified and has to be sub-cultured in an appropriate medium. The use of liquid media has been promising as this greatly reduces the number of contaminants and has been found to increase mycotoxin yields. Extraction and isolation is done by using different solvent systems. Usually, the analysis and detection of mycotoxins follows a pattern of extraction, clean up, separation, detection, isolation, purification, structural confirmation and quantification.

Most mycotoxins are slightly soluble in polar solvents and usually insoluble in non-polar solvents. They may exhibit different solubility with various organic solvents and water. In practice, mycotoxins are extracted using mixtures of organic solvents such as chloroform, acetonitrile, methanol, acetone, ethylacetate, or dichloroethane, often in combination with small proportion of water or acid. With the correct proportions of water to solvent the toxins are often more readily extracted into the solvent. The presence of pigment, fats and lipids in extracts will reduce the efficiency of the ensuing separation techniques. By adding solvents

such as hexane to extraction solvents, many of the fats and lipids can be partitioned into the hexane portion of the solvent and discarded (Smith and Moss, 1985).

Many interfering compounds may be partially removed during extraction, but further clean up of the extract is normally necessary. The ultimate aim of the fractional purification is to remove the greater part of the co-extracted substances, thus, reducing the chemical complexity of the final extracts, which can then proceed to the detection and quantification phase of the analytical method. Column Chromatographic techniques are widely practised for purifying the compound in bulk amounts. Some mycotoxins are purified from food products by Column Chromatography (Lombaert, 2002). Chromatography is generally defined as a physical method of separation, in which components to be separated are distributed between two phases, one is stationary while the other is mobile. Choice of the column and the material depends upon the characteristics of the specific compounds to be isolated. The sample extracts are applied to the clean up columns, and after elution of the column with suitable solvents that do not elute the mycotoxins, appropriate elution solvents can be applied to the column and the mycotoxin eluted and collected.

The final stage of the preparation of the sample for analysis is volume reduction, which is either by evaporation of the solvents in a rotary evaporator under reduced pressure or in a steam bath. The dried sample can then be re-dissolved in a known volume of solvent to be used for analysis (Smith and Moss, 1985). In the developments of analytical methods for mycotoxins the use of chemical detection has been preferred to biological assays for known mycotoxins (Smith and Moss, 1985). Chemicals are more easily quantifiable, less subject to interference by non-fungal co-extracts and normally more sensitive than biological assays. After the purification by Column Chromatography or HPLC the compound must be sent for further analysis and the final confirmation of the eluted sample may be done using Nuclear Magnetic Resonance or Mass Spectrometry.

Diplodiatoxin have been synthesised artificially (Steyn *et al.*, 1972) but, the synthesised compound was a mixture of two different isomers, thus was a racemic mixture, affecting its activity. Isolation of mycotoxin in its natural form is always advantageous. Thus, the aim of the present study was to isolate and purify the diplodiatoxin from the toxin producing

isolates of *Stenocarpella maydis* using various analytical methods and also further confirmation of the toxin by Nuclear Magnetic Resonance (NMR).

3.2 Materials

Potato Dextrose Agar (PDA)

Infusion from potatoes (See below)	1000ml (v/v)
Glucose	2% (w/v)
Agar	1.5% (w/v)

Potato Infusion:

Boil 200 g scrubbed and sliced potatoes in 1000 ml water for 1 hour. Pass through a fine sieve.

Potato Dextrose Broth (PDB)

Potato Extract	20% (w/v)
Dextrose	2% (w/v)
Distilled water	1000ml (v/v)

Chemicals and Reagents

Methanol, Ethanol, Toluene, Ethyl actetate, Benzene, Chloroform, Formic acid, Hydrochloric acid, Sodium hydroxide, Dueterated Methanol (CD3OD)

Spraying reagent

Vanillin: Vanillin 0.1% was dissolved in 50 % H_2SO_4 diluted in 50 % ethanol at –80 ° C

Thin Layered Chromatography plates

TLC plates coated with silica gel–G, 0.5mm thickness

Silica Gel: Silica gel for Column Chromatography pore size (0.040-0.063mm)

Standard diplodiatoxin compound

Standard diplodiatoxin was obtained from Professor P. S. Steyn, CSIR, South Africa

All the chemicals were obtained from Sigma and Merck laboratories in South Africa

3.3 Methods

3.3.1 Inoculation and incubation of fungal isolate

The fungal isolate MC43 was grown on a sterile filter paper disc of 2mm in diameter on potato dextrose agar (PDA) plate for two weeks at 26±1 °C and then the filter paper disc along with the fungus was transferred to a conical 5l flask containing 2l of potato dextrose broth (PDB). The liquid cultures were incubated for 6 + 2 weeks in 16-hour light and 8 hour dark period at 26±1 °C and 55-60% relative humidity for the production of diplodiatoxin. Cultures were agitated at 80rpm for the initial 6 weeks of growth and then were incubated at static conditions for 2 weeks.

3.3.2 Extraction

The 8 weeks old fungal culture, along with the supernatant, was homogenised and hexane was added to the homogenate to remove the fatty acids. Then the mixture was extracted overnight with equal volume of methanol and chloroform (1:1 v/v), this step was repeated 3-4 times to facilitate complete extraction of diplodiatoxin. The $CHCl_3$/MEOH aqueous extract was then vacuum dried at 70 °C in a rotary evaporator and the residue was re-dissolved in 50ml of ethylacetate.

3.3.3 Isolation of diplodiatoxin by Column Chromatography

The isolation of diplodiatoxin was undertaken using Column Chromatography. The residue re-dissolved in 50ml of ethylacetate was chromatographed over a 50cms column, silica gel 60 (230-400 mesh; Pore size 40–60µm). The column was eluted with 300ml of ethylacetate delivered at a flow rate of 5ml/minute and 10ml fractions were collected. The presence of

diplodiatoxin in each fraction was detected by the TLC (Section 2.3.2.2). Fractions containing diplodiatoxin were pooled together and the solvent was evaporated at 50 °C under vacuum using a rotary evaporator and white minute crystals were dissolved in 8ml of ethanol.

3.3.4 Confirmation of diplodiatoxin

The confirmation of the isolation of diplodiatoxin by Column Chromatography was performed by TLC and the solvent system used was ethylacetate. Plates were sprayed with 1% vanillin in 50% sulphuric acid diluted with ethanol at –80 ^{0}C and then the plate was heated at 100 ^{0}C for 2 minutes until the spot developed. The purity and confirmation of the toxin was done by Nuclear Magnetic Resonance (NMR-Varian Gemini 300 MHz) (University of Natal, S.A) and compared with the standard using CD_3OD as a solvent.

3.3.5 Quantification using a standard curve

Diplodiatoxin was quantified by using a standard curve of synthetic diplodiatoxin at 293nm. For a standard curve, diplodiatoxin was dissolved in ethanol to yield a stock concentration of 1mg/ml and dilution range varying from 0.056 to 0.80mg/ml was made. Optical density was recorded at 293nm (Steyn *et al.*, 1972) against ethanol as a blank. Standard curve was drawn between the absorbance at 293nm and the concentration of diplodiatoxin in mg/ml. Further, diplodiatoxin extracted from MC43 isolate of *S. maydis* was diluted 4 times with ethanol and optical density was recorded at 293nm and concentration and yield of diplodiatoxin was calculated

3.4 Results

3.4.1 Cultural conditions

Optimisation of conditions for the maximum production of diplodiatoxin has been done in chapter 2. The *S. maydis* isolate MC43 showed the highest toxin production so this isolate was used to extract, isolate and purify diplodiatoxin. When fungus was grown on a PDA

plate with filter paper discs, it spreads evenly as brown filamentous mass over the filter paper yielding a uniform inoculum for inoculation (Fig 3.1). After incubation at 80rpm for 6 weeks, when cultures were kept at static conditions (Fig 3.2) that generated a kind of aeration stress, which favoured *in vitro* production of diplodiatoxin. Standardisations of the conditions like temperature, medium, pH have been discussed in detail in chapter 2.

3.4.2 Extraction, isolation and purification of diplodiatoxin

Eight-week-old cultures were used to isolate diplodiatoxin. The crude cultures contained other secondary metabolites. Thus, attempted to purify to its maximum extent using a wide range of solvent systems was necessary. Overnight extraction with equal volume of chloroform and methanol yielded number of compounds along with the diplodiatoxin. Fatty acids were removed from the extract by extracting three times with equal volume of hexane. Further purification by Column Chromatoghraphy yielded a pure diplodiatoxin, which was detected by spraying TLC plates with 1% vanillin. It appeared as an orange spot when heated at 100 °C for 2 minutes. The protocol developed (Fig 3.4) was efficient enough to isolate diplodiatoxin in the pure form. Diplodiatoxin was not visible under the UV and the other compounds visible under UV light after chloroform/methanol extraction are shown in the (Fig 3.3).

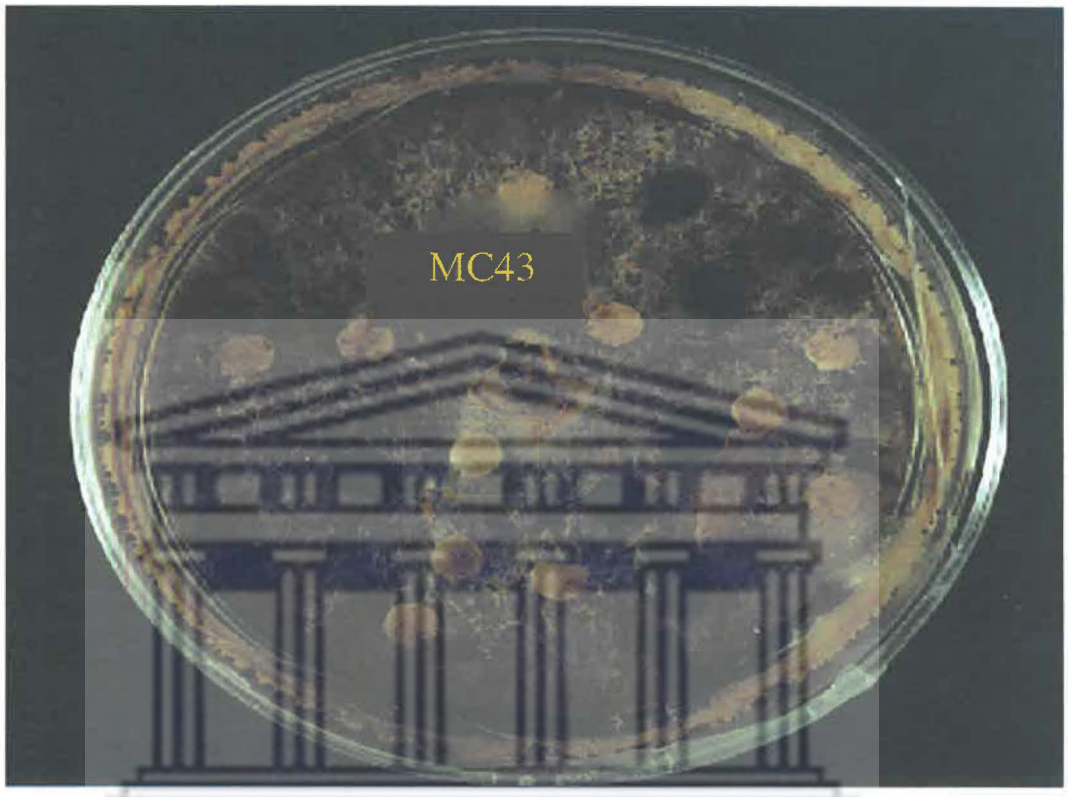


Fig 3.1 MC43 isolate of *S. maydis* grown over filter paper discs on PDA media

1. The isolate MC43 is grown in potato dextrose agar medium
2. The temperature at which the isolate is grown is 26±1 °C
3. The filter paper disc on the petri-plate is 2mm in length, which facilitates equal volume of fungus to grow for subsequent experiments

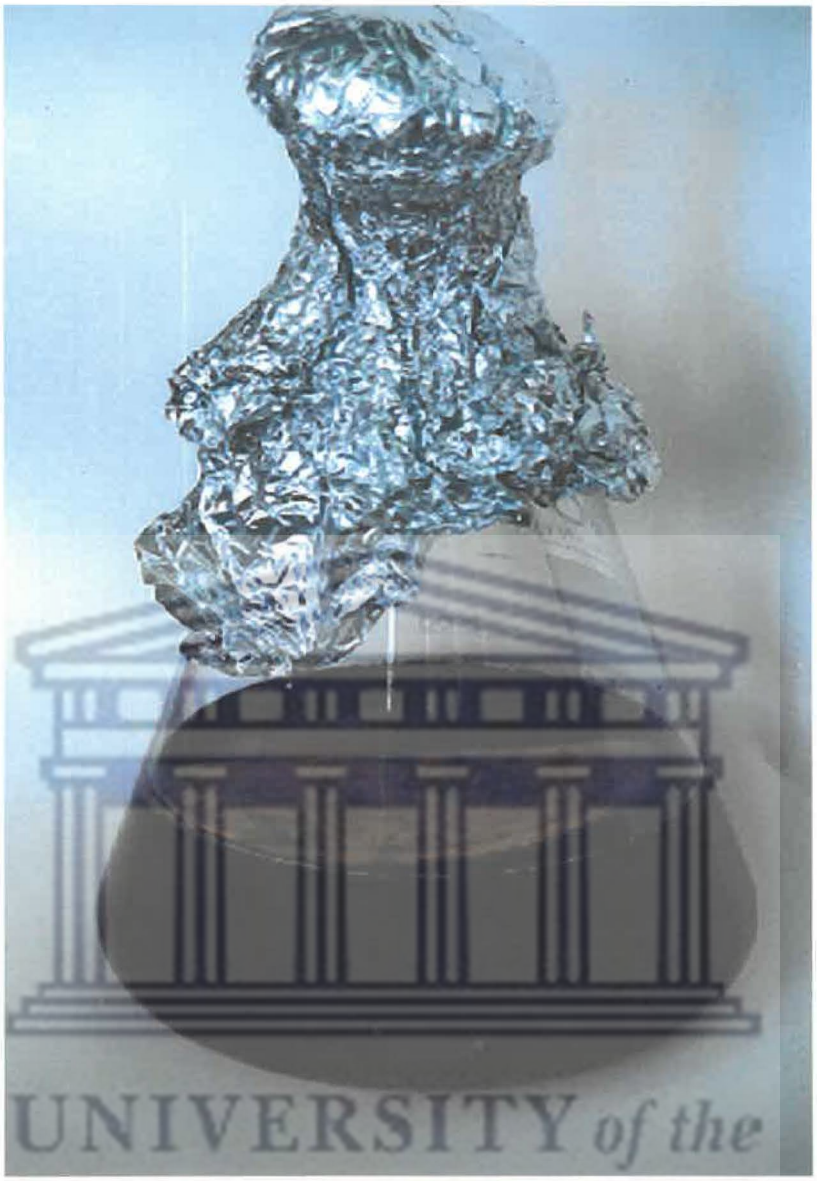


Fig 3.2 The MC43 isolate of *S. maydis* in PDB under static conditions formed a mat of fungal mycelium

1. The MC43 isolate is grown in potato dextrose broth (Liquid medium)
2. The temperature is 26±1 °C and relative humidity is 55-60%
3. A thick mycelial mat is formed when the culture is incubated under static conditions, after 6 weeks of agitation (80rpm)

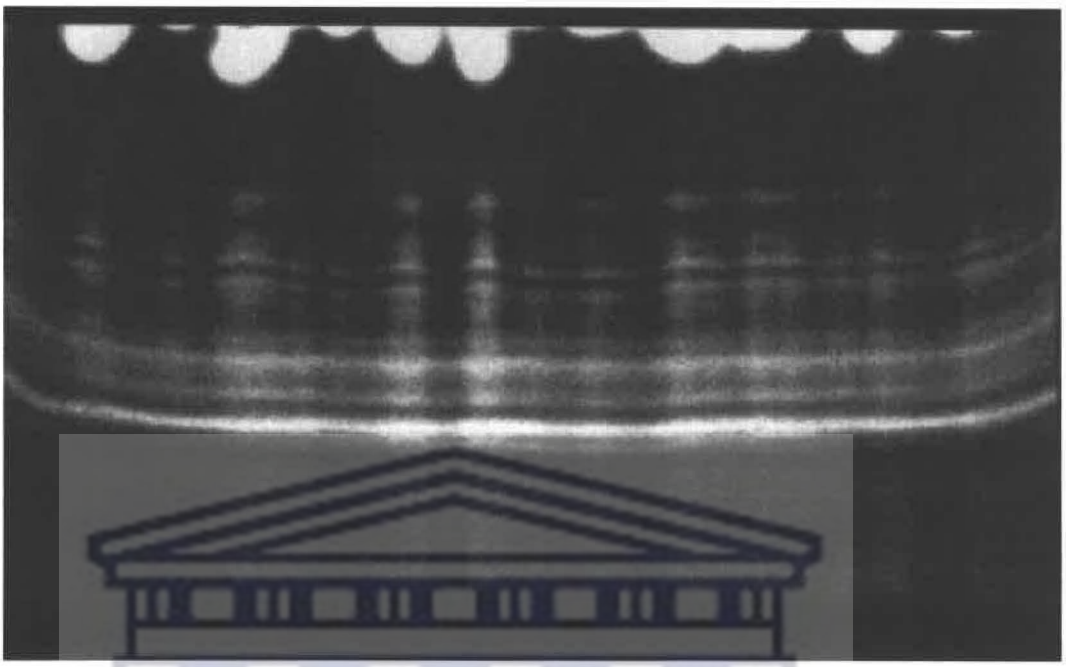


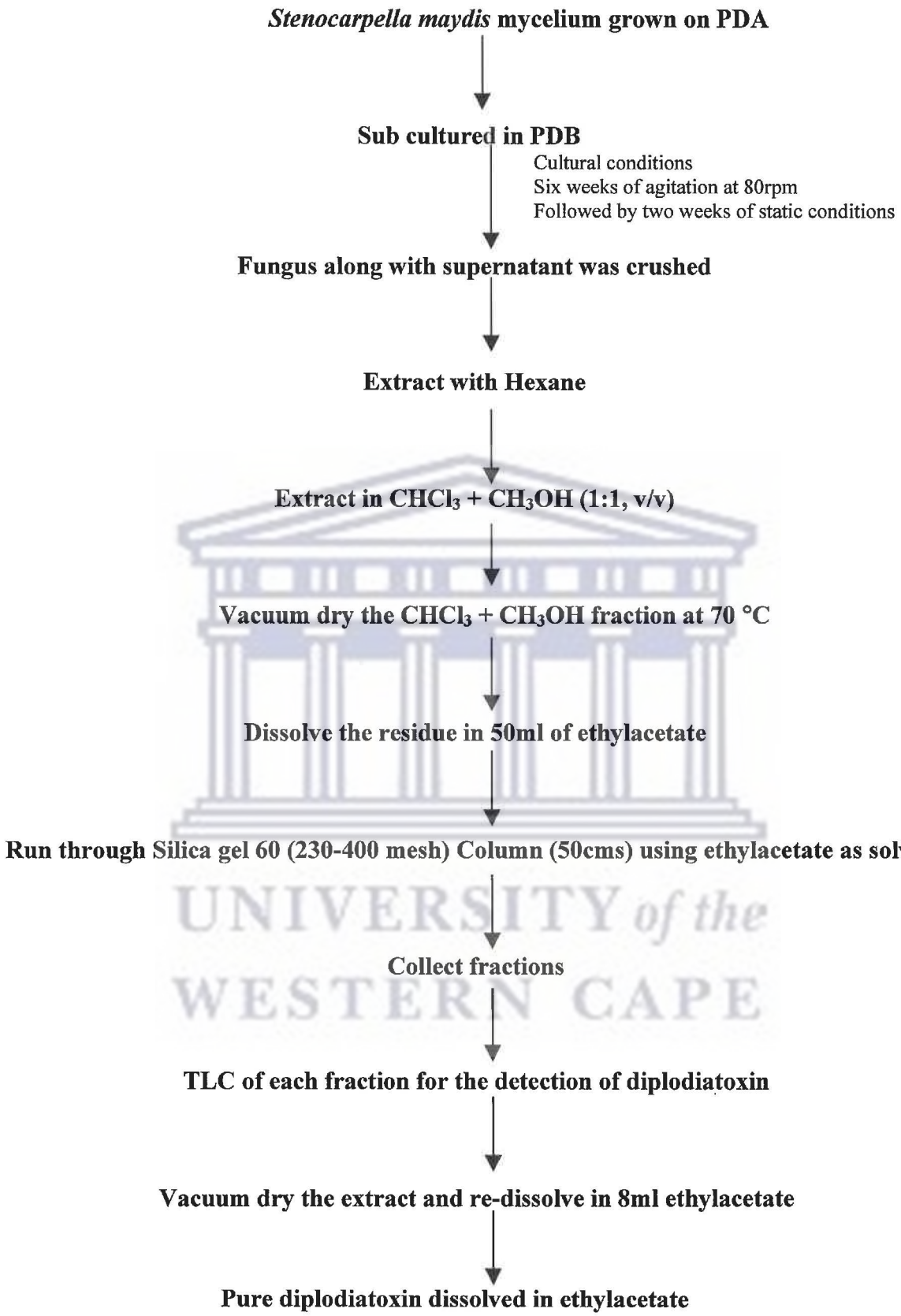
Fig 3.3 Separation of crude extract of MC43 isolate on the TLC plate viewed under the UV showed the presence of several compounds

1. The crude extract of MC43 isolate is developed on Thin Layered Chromatographic plate
2. The plate is viewed under U.V light
3. The plate shows other compounds present in crude extract

Fig 3.4 Schematic presentation of the method used for extraction, isolation and purification of diplodiatoxin

1. The following figure shows the schematic representation of protocol developed for the production of diplodiatoxin, which include

    - Extraction
    - Isolation
    - Purification



3.4.3 Structural confirmation

Diplodiatoxin appeared as minute white needle-like crystals and with the melting point of 187 °C. Mass spectrum analysis confirmed its molecular weight of 308 Daltons and molecular composition of $C_{18}H_{28}0_4$. Proton NMR spectra of the standard i.e., synthetic diplodiatoxin was comparable with the diplodiatoxin isolated by the protocol developed (Fig 3.5) despite a few small additional peaks which represents background impurity no additional dominant peak were observed. Thus, NMR confirmed the structural composition of diplodiatoxin.

3.4.4 Quantification

A standard curve was drawn using a synthetic diplodiatoxin (Fig 3.6) and the sample concentration was calculated to be 2.4mg diplodiatoxin/ml. Further, it was concluded that 60g (fresh weight) mycelia from 8-weeks-old cultures yielded 19.5mg of diplodiatoxin after running through the silica gel column, giving a resultant yield of 0.32mg per gram fresh weight of the fungus. This compound gave an orange spot on TLC when sprayed with 1% vanillin and thus was confirmed as diplodiatoxin.

Fig 3.5 The following figure shows the NMR of diplodiatoxin

1. The NMR data of extracted diplodiatoxin compared with the standard diplodiatoxin
2. The NMR is (Varian Gemini 300 MHz) (University of Natal, S.A) with the Standard
3. The solvent used is CD_3OD
4. The standard diplodiatoxin is from CSIR, South Africa
5. The compound extracted from MC43 isolate is confirmed as diplodiatoxin by comparing data
6. The figure A is standard, where as the figure B is extracted diplodiatoxin

Figure A

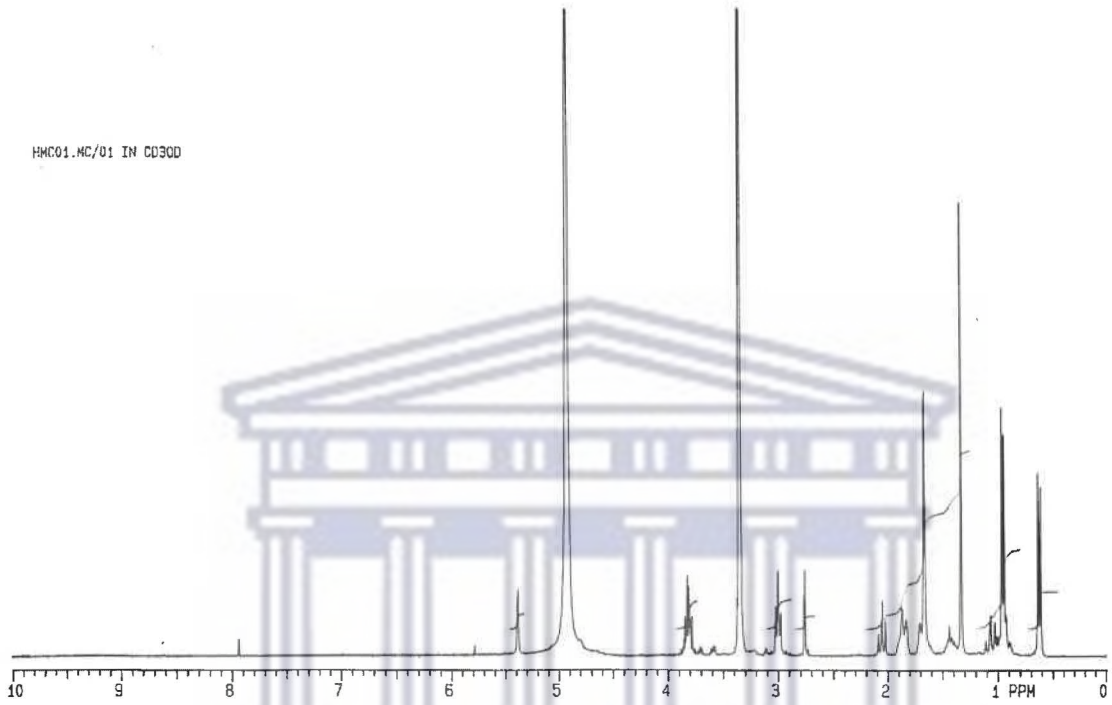
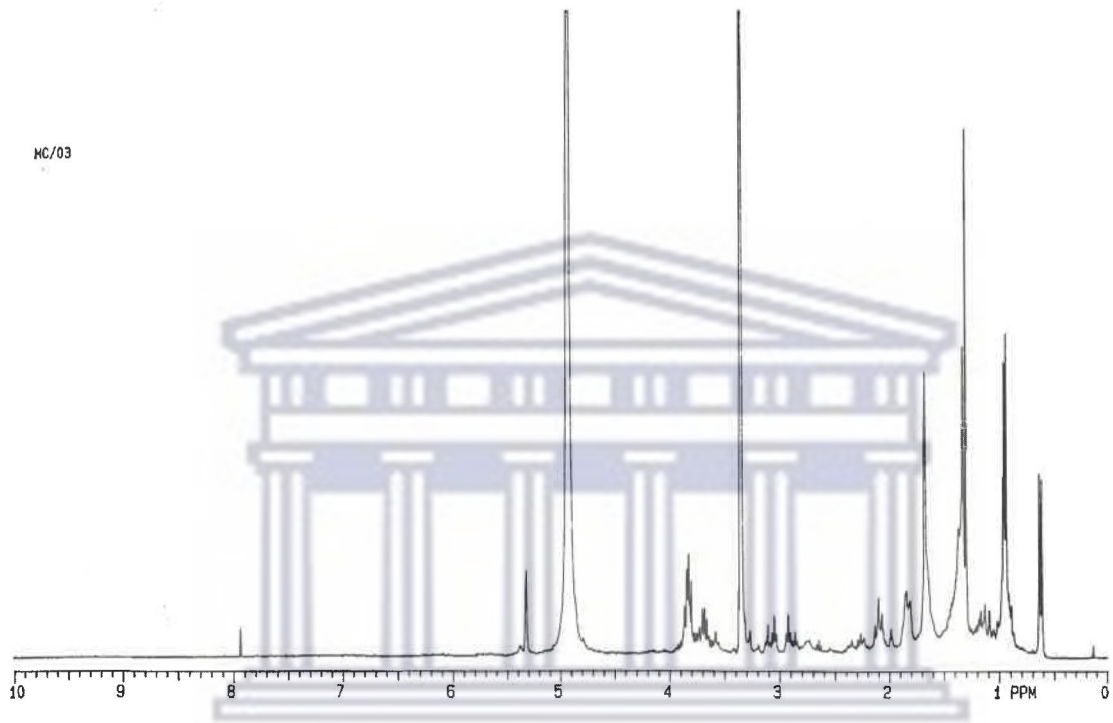


Figure B



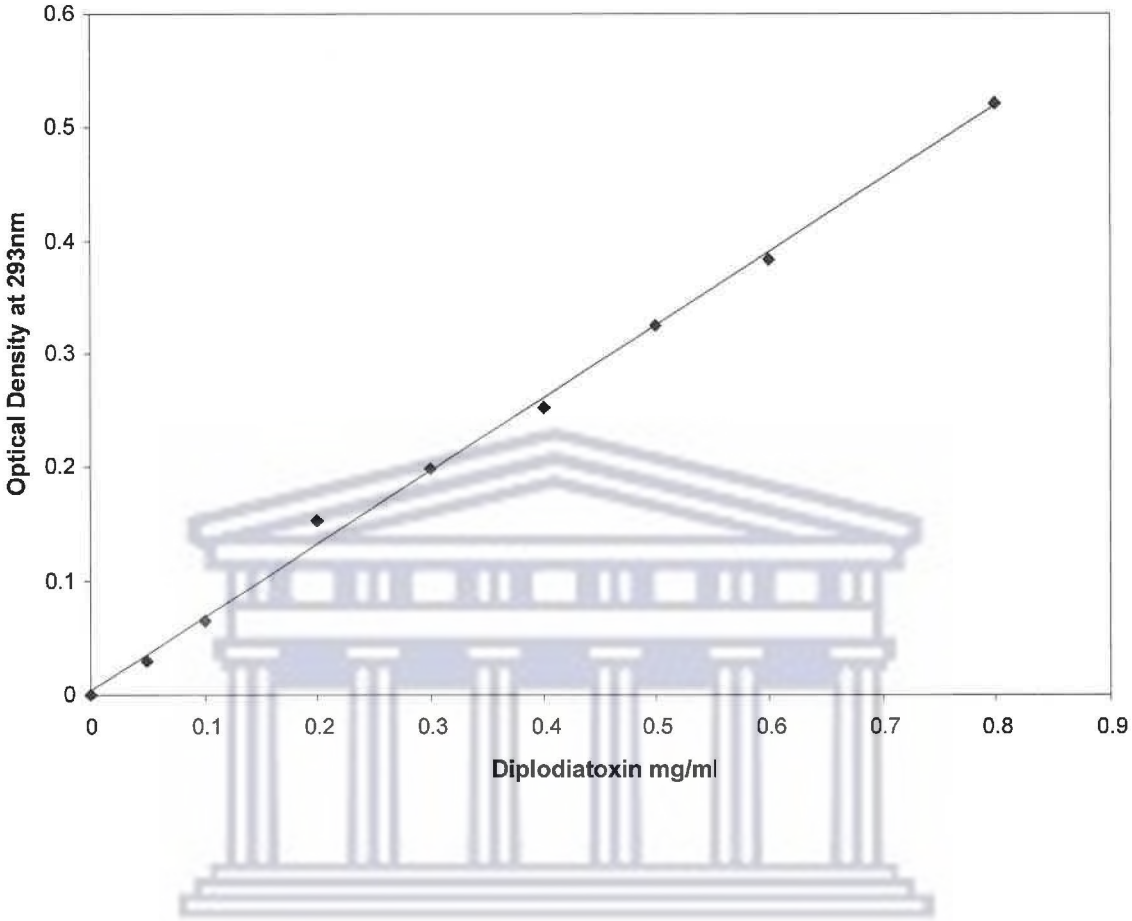


Fig 3.6 Standard curve for the quantification of diplodiatoxin

1. The stock concentration of diplodiatoxin was 1mg/ml
2. Various concentrations of diplodiatoxin toxin were ranging from 0.056 to 0.80mg/ml
3. The estimated concentration of diplodiatoxin was 2.4mg/ml
4. The absorbance was at 293nm (Steyn *et al.*, 1972)

3.5 Discussion

Effect of diplodiatoxin on animals has not yet been reported and the devastating affects on animal husbandry were due to the crude cultures of *S. maydis*. To study the toxicity of pure diplodiatoxin, production of the toxin in bulk amount becomes necessary.

In the previous chapter *in vitro* production of diplodiatoxin from the pure culture of the *S. maydis* isolate MC43 was optimised and detected on the basis of TLC and was confirmed by ApcI-MS. In the present chapter a protocol was developed for bulk isolation of diplodiatoxin in its natural form from the pure culture of *S. maydis*.

The protocol developed for the isolation of diplodiatoxin involves the initial extraction with hexane to remove all the fatty acids present in the crude extract, which may otherwise interfere with the solubility of the compound and evaluation of its toxicity. This was followed by fractional extraction with equal volume of chloroform and methanol. This step was repeated three to four times, which allowed complete extraction of diplodiatoxin from the fungus. Purification of diplodiatoxin was done by Column Chromatography on silica gel column with ethylacetate as a solvent for elution. Proton-NMR-based purity of the isolated compound was comparable with the standard and deuterated methanol was used as a solvent for analysis. The protocol developed was efficient to yield diplodiatoxin with excellent purity.

The protocol developed yielded 19.5mg of diplodiatoxin from 60g (fresh weight) mycelia from 8 weeks old cultures. After passing through the silica gel column the yield was about 0.32mg of diplodiatoxin per gram fresh weight of the fungus. The diplodiatoxin appeared as minute white needle-like crystals with a melting point of 187 °C which was supported by the literature (Steyn *et al.,* 1972). Diplodiatoxin showed low solubility when dissolved in water and was completely soluble in methanol and ethylacetate at room temperature.

Diplodiatoxin when isolated in its natural form is advantageous over the synthetic compound as the synthetic compound is a raceme mixture and may not be as active as natural form. The natural compound gives the exact toxicity in animal systems and its property of anti-bacterial activity.

Diplodiatoxin has been artificially synthesised (Steyn *et al.,* 1972, Ichihara *et al.,* 1986). The chemistry of diplodiatoxin was discussed in section (2.1.7) and the assumed stereostructure was confirmed by the synthesis using highly stereocontrolled strategy, in which the intramolecular Diels-Alder reaction of a (E, E, E)-triene is involved. The synthetic compound is a racemic mixture (a mixture of L and D isomers) therefore, is not suitable for knowing the exact toxicity of diplodiatoxin on animals or other experimental models. Also literature on the effect of pure form of diplodiatoxin on animals and its *in vitro* production is not available. Thus, isolation of diplodiatoxin in its natural form from *S. maydis* to known the toxicity becomes necessary.

Finally, the protocol developed for the isolation of diplodiatoxin from the pure culture of *S. maydis* is recommended for the best quality and quantity of diplodiatoxin and with purity comparable to that of the standard synthetic compound (Rao and Achar, 2001). Further, the extraction of diplodiatoxin in the pure form from the pure culture of *S. maydis* will reveal the nature of the toxicity of diplodiatoxin, the level of toxicity and effects of the toxin on the experimental models.

Chapter 4

EFFECT OF DIPLODIATOXIN ON BACTERIA AND MAMMALIAN CELL LINES

4.1 Introduction

Mycotoxins are ubiquitous, potent, biologically active toxins, which even at low concentrations may cause numerous diseases in animals and humans (Mally and Marr, 1997). In general mycotoxins affect the nervous system, induce liver and kidney damage and are carcinogenic. Clinical syndromes in farm animals range from acute to chronic diseases and from reproductive deficiencies to overall debilitations. Several studies have indicated that mycotoxins directly inhibit the growth of micro-organisms and exhibit bacteriostatic properties. Mycotoxins may either slow down the growth of bacteria or act as bactericidal compounds, which depend upon the chemical nature of the mycotoxin, bacterial strain and the mode of action of mycotoxin. Besides the mycotoxins, other fungal metabolites also exhibit anti-microbial activity. Further, to evaluate the effect of unknown mycotoxins bacteria serves as an excellent indicator for predicting the toxicity.

The development of sensitive, rapid, and reproducible *in vitro* assays is an absolute requirement for the screening of mycotoxins. The use of different bioassay systems using micro-organisms in the diagnosis of mycotoxicoses has been reported (Yates, 1988). These assays are less expensive, consume less time and also can be tested on a wide range of bacterial strains. Some mycotoxins are mutagenic and have genotoxic effects, so bacteria serves excellently the purpose of predicting the results in short period of time (Said *et al.*, 1999). Further, the mode of action of each mycotoxin on several bacterial strains can also be evaluated with quick results. Turbidimetric assays are widely used as they are the most reliable methods to test the anti-bacterial activity of mycotoxins. The difference between the growth rate in the presence and absence of mycotoxin is an accurate indicator of bacteriostatic effect of the mycotoxin. In modern technological studies even bacteria, which are antibiotic resistant, are used to predict the level of toxicity of the compound (Conti *et al.*, 2000). Micro-turbidimetric assays also provide a

rapid, conventional and high throughput capacity system to analyse bacteria- mycotoxin interactions.

Several mycotoxins have been tested against bacteria, but extensive efforts are still required for potential applications. Toxicity of different mycotoxins on several species of bacteria and yeast and the inhibitory effect has been observed by (Madhyasta *et al.,* 1994). Ali-Vehmas *et al.*, 1998 demonstrated the antibacterial activity of T-2 toxin (*Trichothecium* sp), deoxynivalenol, ochratoxin A (*Penicillium*), aflatoxin B (*Aspergillus*) and fumonisin B (*Fusarium*) by evaluating growth delays using a fully automated microturbidimetric method. Different bacterial strains used for this study were *Escherechia, Streptococcus, Staphylococcus, Yersinia, Salmonella, Erysipelothrix* and *Lactobacillus*. They reported that *Staphylococcus agalactiae* was most sensitive to all the toxins tested with an exception of ochratoxin A and T-2 toxin. Fumonisin B was the most effective in inhibiting the growth of *Staphylococcus aureus*. Aflatoxin B_1 affected the growth of *Yersinia enterocolitica.* Recently, *in vitro* effect of aflatoxin B_1 on bifidobacteria was studied in detail by (Oatley *et al.,* 2000*).* The growth rate of *E. coli* and *S. infantis* was decreased by fumonisin B_1, while the growth rate of *Erysipelothrix rhusiopathiae* was inhibited by ochratoxin A.

The genus *Penicillium* is counted as one of the most successful group of micro-organisms on the earth and has been both an asset as well as a source of misery. The antibiotic, penicillin was isolated from *P. notatum* and was a remarkable discovery. It is used as a potential antibiotic drug against a wide range of bacterial infections. Since then several antibiotics of fungal origin have been discovered. 1-Hydroxycrisomicin A and 9-hydroxycrisomicin A, new isochromaquinones from *Micromonospora* species was found to exhibit antibiotic properties (Yeo *et al.,* 1997, 1998). The involvement of toxic oxygen intermediates in the bacteriostatic effects of mycotoxin T-2, aflatoxin B_1, fumonisin B_1, ochratoxin A and deoxynivalenol have also been investigated and the data obtained indicated that bacterial growth can be inhibited especially by T-2 toxin, aflatoxin B_1 and ochratoxin A (Atroshi *et al.,* 1998).

Recently, some endophytic fungi were found to have anti-microbial activity (Rodrigues *et al.*, 2000). Some mycotoxins also have antiviral properties like anthraquinones and xanthones and their derivatives have an activity *in vitro* against *Leishmania* species. Due

to a wide range of anti-microbial activities of mycotoxins, they are used as insecticides and viral pesticides (Wright *et al.,* 1982).

Apart from bacteria, cell lines also serve as an excellent model for toxicity tests, Mycotoxins have measurable affect on cell lines. Aflatoxin B_1 was found to be cytotoxic when tested on human epithelioid lung cells (Palanee *et al.*, 2001). Fumonisin B_1 had cytotoxic and genotoxic effects on rabbit kidney cell lines (Rumora *et al.*, 2002). FB_1 is also known to cause apoptosis (Programmed cell death) in the cell lines (Jones *et al.*, 2001). Ochratoxin A had cytotoxic effects on kidney cells (Sauvant *et al.*, 1998). When rat epithelial cells were exposed to ochratoxin A, it led to cytotoxicity (Horvath *et al.,* 2002). Mycotoxins are also tested on human and animal cell lines to determine the cytotoxic and mutagenic effect.

Thus, there is a need to explore the anti-bacterial activity and toxicity on cell lines of mycotoxins. Diplodiatoxin is a mycotoxin produced by *Stenocarpella maydis* and the effect of the crude toxin was only tested on animals, but the *in vitro* effect of pure compound has not yet been reported. So, there arises a need to evaluate the effect of purified diplodiatoxin. For this purpose bacteria and cell lines serve as a useful model to show the toxic effect of diplodiatoxin, the bacteria-diplodiatoxin interactions, and the level of toxicity of diplodiatoxin on eukaryotic cell lines. In the present study the anti-bacterial activity of diplodiatoxin with various bacterial strains has been evaluated and the toxicity of diplodiatoxin on cell lines has been tested.

4.2 Materials

Luria Broth Medium (LB)

Bacto Tryptone	2% (w/v)
Bacto Yeast Extract	1% (w/v)
NaCl	1% (w/v)
Distilled water	1 litre
pH 7	

Luria Broth Agar

Bacto Tryptone	2% (w/v)

Bacto Yeast Extract	1% (w/v)
NaCl	1% (w/v)
Agar	1.5% (w/v)
Distilled water	1 litre
pH 7	

Tissue culture Medium

Medium: 5% FCS

Nutrient mixture: F12 (HAM)

Penicillin-Streptomycin

Supplements: gpt selection- 25mg/ml Mycophenolic acid, 0.143mg/ml sodium xanthine, 1× HAT

Complete F12 (HAM): (F12 (HAM) + L-glutamine + penicillin-streptomycin + 5% FCS)

Trypsin: 2.5% (Diluted with PBS to 0.125%)

APOPercentage™ (Bio Color Ltd)

Cell Lines

Species: Hamster, Cell Lines: CHO 22

Bacterial Strains

Bacillus cereus, *Bacillus subtilis*, *Escherechia coli*, *Pseudomonas fluorescence* and *Staphylococcus aureus*

The materials were from Sigma or Merck laboratories, The tissue culture media and supplements were supplied by Gibco Ltd: Dulbeco's modified medium in South Africa

4.3 Methods

4.3.1 Storage and maintenance

Bacterial strains

The bacterial strains were stored at –70 °C in LB media containing 20% glycerol as a cryoprotective agent and were plated on to LB media when needed. These glycerol stocks were made fresh after every two months. The bacterial strains were maintained on LB agar at 37 °C in an incubator.

Cell lines

Freezing of cell lines were carried out, as required, when cells approached confluency, they were removed using 0.12% trypsin and pelleted by centrifugation. The pellet was resuspended in a solution of 90% FCS and 10% DMSO, dispensed into 1ml aliquots in cryo-vials prior to freezing at –150 °C. The cells were maintained in an incubator at 37 °C in an atmosphere of 5% CO_2. Cells were removed for passage using 0.12% trypsin.

4.3.2 Effect of diplodiatoxin on the growth of bacterial strains

Various bacterial strains viz., *B. cereus, B. subtilis, E. coli, P. fluorescence* and *S. aureus* were plated on LB medium with 1.5 % agar. Loading wells (3x3mm) were made on semi-solid media with a sterile cork borer after spreading the bacterial strains. Different concentrations (0.6-9.7µg/ml) of diplodiatoxin were loaded in these wells (3x3mm) to test the anti-bacterial activity of diplodiatoxin. Plates were incubated at 37 °C in dark for 24hr. The area of inhibition zone was measured around the each well.

4.3.3 Effect of diplodiatoxin on the growth of *S. aureus*

The effect of various concentrations of diplodiatoxin (4.8-49.7µg/ml) on growth of *S. aureus* in the liquid culture was also studied. LB media was inoculated with *S. aureus* cultures and incubated overnight on a rotary incubator shaker at 200rpm, 37 °C and 55-60% relative humidity. The overnight grown culture was diluted with sterile LB liquid media to yield a cell density of 8 x 10^6 cells per 100µl. Further, this dilution stock was used to inoculate fresh LB media with an inoculum of 8 x 10^6 cells per 10ml culture. The

optical density of bacterial cultures grown for 1 to 4 hours at 37 °C at 200rpm was measured at the wavelength of 600nm using a spectrophotometer. LB media was used as blank. The control sample contained LB media devoid of diplodiatoxin inoculated with the same inoculum and grown for 4 hours at similar incubation conditions.

4.3.4 Effect of diplodiatoxin on Chinese hamster ovary cells

CHO 22 cells were cultured in 25cm^2 flasks until confluent. The cells were trypsinised and seeded in a 96-well tissue culture plate at a cell concentration of 0.25×10^4 cells/ml. The plate was incubated for 24 hours at 37 °C. After 24hrs the media was removed and 100µl media containing diplodiatoxin extract was added to the wells. The concentrations of the diplodiatoxin extract ranged from 0.02µg/µl to 0.24µg/µl in methanol, triplicate wells were prepared for each concentration. The same experiment was done with pure diplodiatoxin with the concentration ranging from 0.02µg/µl to 0.24µg/µl with a stock concentration of 2.4µg/µl diplodiatoxin in methanol. As the toxin was dissolved in methanol the cells were tested with similar concentrations of methanol and were used as controls.

4.3.5. APOPercentage™ apoptosis assay

The APOPercentage™ Apoptosis assay is also a colorimetric assay used to detect and measure apoptosis. When cells undergo apoptosis a so-called "flip flop" mechanism results in the translocation of phosphatidylserine from the inner to the outer membrane surface. With the APOPercentage™ Apoptosis assay one makes use of this mechanism to label apoptotic cells (refer to Figure 4.4). The principle of this assay is based on a purple-red dye, which is trapped inside cells during the translocation of phosphatidylserine. Apoptotic cells can be identified due to their purple-red stain. CHO 22 cells were seeded in a 96-well tissue culture plate as described above (Section 4.3.4). The cells were cultured for 24 hours before treatment with diplodiatoxin extract. The concentrations of diplodiatoxin extract varied between 0.02µg/µl and 0.24µg/µl, triplicate wells were set up for each concentration. The cells were treated for 30min after which the cells were gently washed with PBS. The PBS was replaced with 100µl APOPercentage dye (refer to manufacturers manual) and the cells were incubated for a further 60min at 37 °C. After

60min staining the cells were washed twice with 100µl PBS to remove residual dye. The cells were visualised under a light microscope and photographs were taken.

4.4. Results

4.4.1 Effect of diplodiatoxin on the growth of bacterial strains

Effect of different concentration of diplodiatoxin (0.6 to 9.7µg/well) on the growth of various Gram positive and negative bacterial strains viz., *Bacillus cereus, B. subtilis, Escherechia coli, Pseudomonas fluorescence and Staphylococcus aureus* was studied (Table 4.1). Different bacterial strains showed a varied range of tolerance to the diplodiatoxin. An overnight incubation of these bacteria in the presence of diplodiatoxin showed a distinct zone of inhibition and area of this inhibition zone varies with each treatment and type of bacterial strain. *S. aureus* was found to be the most susceptible to diplodiatoxin among the bacterial strains tested in the present study. *B. subtilis* and *S. aureus* showed zone of inhibition at the concentration as low as 1.2µg/µl but, the area of inhibition was greater in *S. aureus.* On the other hand, *E. coli* was found to be the most tolerant to the concentration of diplodiatoxin tested. Lower concentrations of diplodiatoxin (less than 1.2µg/µl) were not inhibitory for *E. coli* and *P. fluorescence.* The increasing order of tolerance of bacterial strains to diplodiatoxin was *S. aureus* < *B. cereus* < *B. subtilis* < *P. fluorescense* < *E. coli.*

4.4.2 Effect of diplodiatoxin on the growth of *S. aureus*

The effect of different concentrations (4.8 – 49.7µg/ml) of diplodiatoxin was also studied on the growth of *S. aureus* in LB liquid medium using turbidimetry. Among all the bacterial strains tested, *S. aureus* was found to be most susceptible to diplodiatoxin, Reduction in the turbidity indicated the inhibition of growth by diplodiatoxin. The presence of diplodiatoxin in the medium reduced cell growth as compared to the control (Table 4.2) and it was observed that diplodiatoxin was not bactericidal but was acting as

bacteriostatic. All the concentrations of diplodiatoxin tested had an inhibitory effect on the growth of *S. aureus* and all the concentrations (less than 19.5µg/ml) of diplodiatoxin

tested showed that the growth of bacteria drastically reduced after 4 hours and became almost stationary. At much higher concentration (above 29.2µg/ml) the growth of bacterial cultures drastically reduced soon after 2 hours. Thus, anti-bacterial activity of diplodiatoxin was confirmed.

4.4.3 Detection of toxicity of diplodiatoxin on Chinese hamster cells

Chinese hamster ovary cell lines under control conditions had a confluent growth (Fig 4.1). Exposure of these cells to crude extract and pure diplodiatoxin showed a remarkable difference in the growth and survivality of the cells. The concentrations of diplodiatoxin (0.1µg/µl) tested *in vitro* on the Chinese hamster cell lines induced activity like cell elongation and cell death. The activity in the cells was observed within 2 hours of incubation. This indicates that diplodiatoxin is toxic to Chinese hamster cell lines. Further it was evaluated by APOPercentage test whether it was a necrosis or apoptosis (Programmed cell death). The APOPercentage test revealed that the extract containing diplodiatoxin was apoptotic as the cells were stained purple in colour (Fig 4.3). The controls were normal (Fig 4.1, 4.2).

Bacterial strains	Area of Inhibition zone (mm^2) Diplodiatoxin (µg/well)					
	Control	0.6	1.2	2.4	4.8	9.7
Bacillus cereus	0	0	3.5	5.5	6.5	7.5
Bacillus subtilis	0	0	0	3.5	5.0	7.0
Escherechia coli	0	0	0	0	3.5	5.5
Pseudomonas fluorescence	0	0	0	0	4.5	6.0
Staphylococcus aureus	0	0	4.0	5.5	7.0	8.5

Table 4.1 Toxicity of diplodiatoxin on various bacterial strains on semi-solid media

1. The bacteriostatic effect of diplodiatoxin on various bacterial strains increases with the increase in the concentration of the toxin
2. The medium used is LB medium
3. Initial cell density was 8 x 10^6 cells per 100µl
4. The bacteriostatic effect is determined by measuring the inhibition zone in mm^2

Time (h)	Optical density at 600nm					
	Diplodiatoxin (µg/ml)					
	Control	4.8	9.7	19.5	29.2	49.7
1	0.2	0.2	0.1	0.1	0.1	0.1
2	0.4	0.3	0.3	0.3	0.3	0.3
3	0.7	0.5	0.3	0.3	0.3	0.3
4	0.9	0.6	0.5	0.4	0.3	0.3

Table 4.2 Effect of diplodiatoxin on the growth of *S. aureus* in liquid LB media

1. The bacteriostatic effect of diplodiatoxin on *S. aureus* increases with increase in the concentration of the toxin
2. The medium used is LB liquid medium
3. Innoculum size = approx. 8 x 10^6 cells
4. The bacteriostatic effect is measured on a spectrophotometer

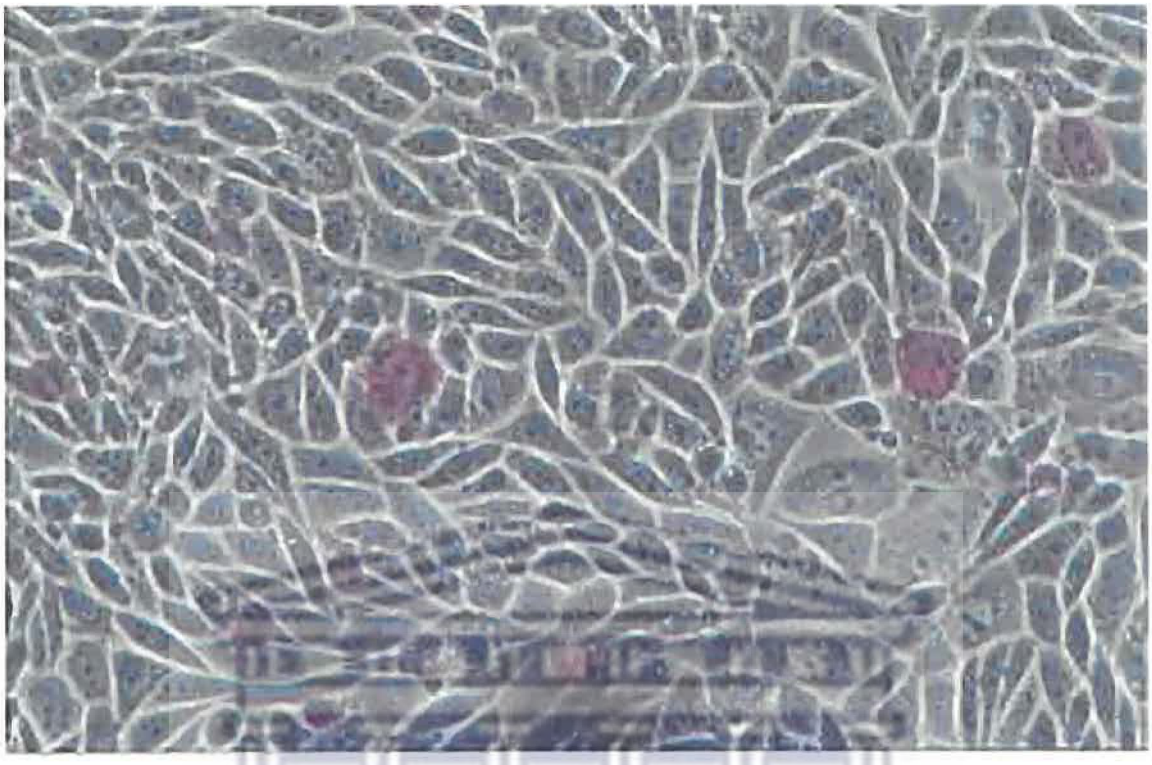


Fig 4.1 The effect of diplodiatoxin and APOPercentage test showing the control Chinese hamster cells

1. The CHO 22 cells were cultered in 25cm^2 flasks until confluent
2. The medium used is HAM (F12)
3. Tissue culture plate was with a cell concentration of 0.25×10^4 cells/ml
4. Apoptosis is not induced in the cell lines as the cells are not stained purple

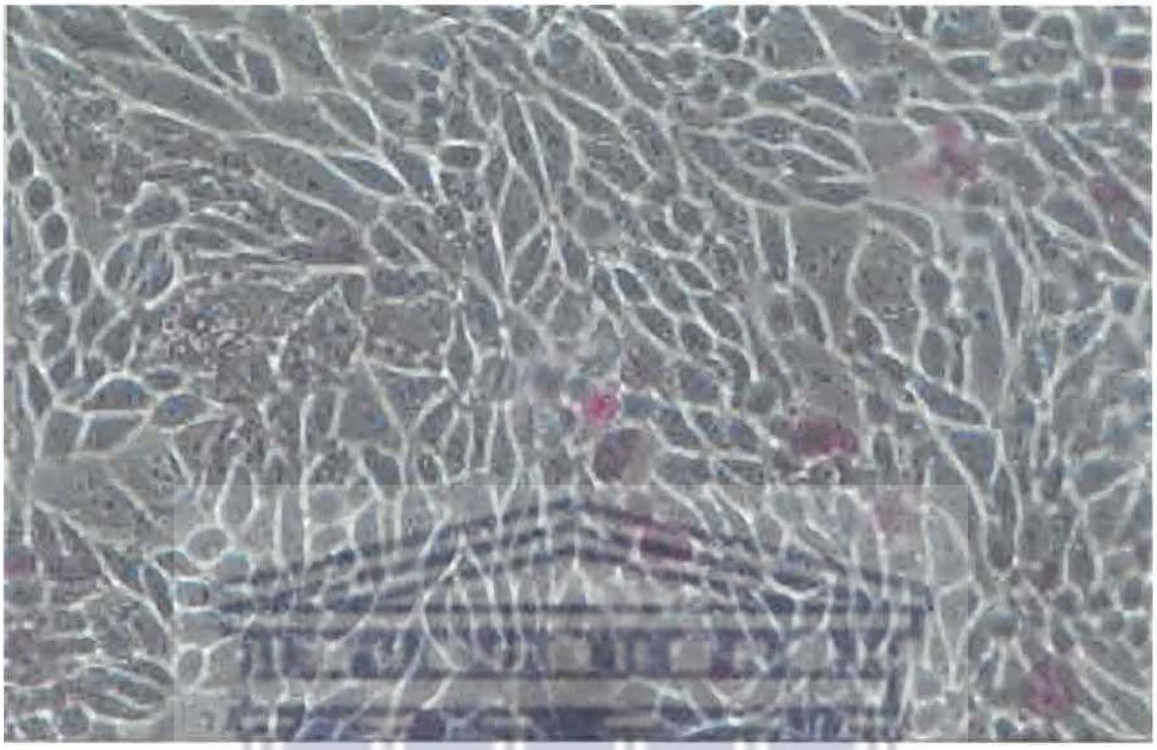


Fig 4.2 The effect of diplodiatoxin and APOPercentage test showing the methanol control Chinese hamster cells

1. The CHO 22 cells were cultered in 25cm^2 flasks until confluent
2. The medium used is HAM (F12)
3. Tissue culture plate was with a cell concentration of 0.25×10^4 cells/ml
4. The concentration of methanol is 0.12μg/μl
5. Apoptosis is not induced in the cell lines as the cells are not stained purple

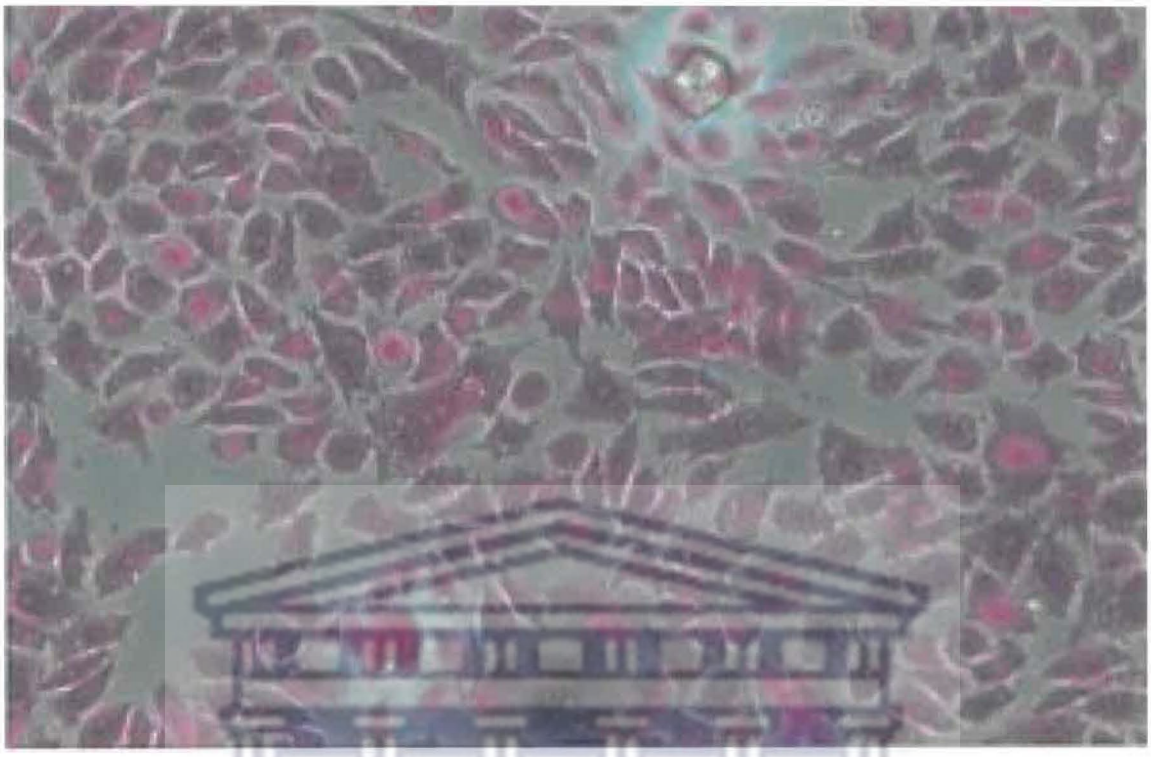


Fig 4.3 The effect of diplodiatoxin and APOPercentage test showing the apoptosis in Chinese hamster cells

1. The CHO 22 cells were cultered in 25cm^2 flasks until confluent
2. The medium used is HAM (F12)
3. Tissue culture plate was with a cell concentration of 0.25×10^4 cells/ml
4. The concentration of *S. maydis* extract is 0.12μg/μl
5. The cells are coloured purple indicating apoptosis

4.5 Discussion

Effect of diplodiatoxin on bacterial strains

Mycotoxins are usually bacteriostatic and bactericidal. So far the anti-bacterial activity of diplodiatoxin produced by *S. maydis* has not been reported although the hepatotoxic and neurotoxic nature of crude cultures of *S. maydis* in sheep, cattle, horses, monkeys and poultry has been well established (Blaney *et al.,* 1981; Rabie *et al.,* 1986; Prozesky *et al.,* 1994). In the present study, the anti-bacterial activity of diplodiatoxin on *B. cereus, B. subtilis, E. coli, P. fluorescence* and *S. aureus* using diffusion/well-diffusion and turbidimetry methods has been undertaken and a strong anti-bacterial property of diplodiatoxin was established. Anti-bacterial activity depends upon the concentrations used and the type of bacterial strain. *S. aureus* was found to be the most susceptible to diplodiatoxin and *E. coli* was the most tolerant among the bacterial strains tested.

The disc-diffusion assay, the technique which is similar to the well diffusion method is used in the present study (Boutibonnes *et al.,* 1983; Madhyastha *et al.,* 1994). Further, the turbidimetry assays used for testing the anti-bacterial action of mycotoxins are more sensitive. All the concentrations of diplodiatoxin tested had an inhibitory effect on the growth of *S. aureus* in turbidimetry assay.

Thus, the anti-bacterial activity of diplodiatoxin was well established and is supported by the literature available on the anti-bacterial activity of mycotoxins. The growth of bacteria was inhibited in the initial stages of growth (lag or log phases). Diplodiatoxin was found to be bacteriostatic rather than bactericidal. Other mycotoxins like T-2 toxin, aflatoxin B_1, fumonisin B_1, ochratoxin A and deoxynivalenol are known to be bacteriostatic (Ali-Vehmas *et al.,* 1998). This property of diplodiatoxin can be explored for its potential use as a bacteriostatic compound but a detailed investigation of the mode of action of diplodiatoxin would be required.

Effect of diplodiatoxin on Chinese hamster cells

The effect of diplodiatoxin on Chinese hamster cell revealed the toxic nature of diplodiatoxin on mammalian cells. The toxin showed activity at 0.1µg/µl after 2 hours of incubation. The activity increased as the incubation period increased and it was observed that the morphology of the cells changed completely and cells clearly showed a difference when compared with that of the control cells. Further the experiments were conducted to know the apoptotic nature of pure diplodiatoxin and the crude extract of *S. maydis*. The APOPercentage test with the crude extract (0.1µg/µl) showed the apoptotic activity. The pure diplodiatoxin showed activity at the concentration ranging from 0.02µg/µl to 0.2µg/µl so it can be predicted that diplodiatoxin may also be apoptotic in nature.

Thus the diplodiatoxin has a significant effect on bacteria and eukaryotic cell lines, further a detailed study of acute and sub acute toxicosis of diplodiatoxin is necessary to confirm the nature of toxicity of compound.

Chapter 5

ACUTE TOXICITY OF DIPLODIATOXIN ON RATS

5.1 Introduction

Mycotoxins are a particular problem in developing countries, causing acute toxicity in animals and human beings. Acute toxicity refers to the immediate effect of exposure to a toxin. Different routes of exposure lead to various toxicity effects, for example acute oral, dermal or aerosol exposure to mycotoxins produces intestinal lesions. Acute toxicity in the central nervous system causes anorexia, lassitude, suppression of reproductive organ functions and acute vascular effects leading to hypo-tension and shock. Specific effects have been observed in different animal models. Early symptoms of acute toxicity of mycotoxins include severe nausea, vomiting, discomfort, weakness, and loss of co-ordination within minutes to hours.

Aflatoxins are the most potent acutely toxic compounds. The acute toxicity of aflatoxin B_1 caused severe liver damage in Fischer rats (Kumagai *et al.,* 1998). Aflatoxin B_1 exposure in the rats lead to severe histological changes, and death of about half the animals after 3-4 weeks of exposure (Mandel *et al.,* 1992). When the toxicity level was estimated in fish as a model system, reduction in the levels of all anti-oxidant enzymes was observed (Madhusudhan, 2000). Acute affects of mycotoxin causes a measurable change in the hormonal activity for example aflatoxin inhibits prolactin secretion by rat pituitary cells in the culture (Abdel-Haq *et al.,* 2000). In the acute toxicity tests the levels of hepatic enzymes play a vital role in predicting the toxicity levels (Mandel *et al.,* 1992).

Fumonisins are also potent mycotoxins which have measurable effects on animals and humans. Fumonisin B_1 when given to pregnant rats causes maternal toxicity like lethargy,

death and foetal toxicity (Collins *et al.,* 1998). Ingestion of fumonisin decreases cardiac contractility in swine (Constable *et al.,* 2000). Clinical abnormalities in guinea pigs which were exposed to T-2 toxin were observed by Masood and Ranjan, 1994. Thus, acute doses of mycotoxins have adverse effects on both human and animal health (Hussein *et al.,* 2001).

Trichothecenes are mycotoxins produced by fungus *Stachybotrys chartarum,* when the spores of this fungus were tested in rats, severe lung haemorrhage was observed. A single acute pulmonary exposure to a large quantity of *S. chartarum* spores by intratracheal instillation causes severe injury detectable by bronchoalveolar lavage. The primary effect appeared to be cytotoxic and inflammation with haemorrhage. There were measurable effects of trichothecene on rats (Rao *et al.,* 2000).

Acute toxicity of mycotoxins leads to changes in the activity of biochemical target enzymes. Thus, biochemical parameters are a sensitive index of the changes caused by any toxicant and can constitute an important diagnostic tool in toxicological studies. Acute toxicity studies also give information regarding the toxicity ranking. Most mycotoxins are neurotoxins. Acetylcholinestrase (AChE), being a target enzyme for toxic metabolites acts as an excellent indicator of acute toxicity. It plays a key role in many organisms, including humans. Inhibition of AChE by toxins like chlorpyrifos and methyl parathion in pregnant rats was observed by (Abu-Quare *et al.,* 2001).

Enzymatic activity estimations have powerful predicting roles in toxicity assessments. ASAT, ALAT, AkP, AcP are the enzymes which are affected due to acute toxicity and the toxicity level of a mycotoxin can be easily predicted by monitoring these enzyme levels. Exposure to certain toxins with reference to these enzymes in the kidney was observed by Verplanke *et al.,* 2000 and in liver it was observed by Gole and Dasgupta, 2002. Generally AChE predicts the neurotoxic nature of the compound, and ASAT, ALAT, AcP and AkP predict the hepatotoxic nature of the compound.

Thus, the present study is targeted to test the acute toxicity of diplodiatoxin in male and female rats with special reference to biochemical enzymes.

5.2 Materials

I. Protein estimation

1. Copper Reagent: 2% (w/v) Sodium carbonate in the 0.1M NaOH, 1% (w/v) copper sulphate and 2% (w/v) sodium potassium tartarate were mixed in the ratio of 100:1:1
2. 0.5M Sodium hydroxide
3. 1% (w/v) Bovine serum albumin (BSA)
4. 1N Folin-phenol reagent

II. Estimation of Alanine aminotransferase (ALAT) and Aspartate aminotransferase (ASAT)

1. Aspartate aminotransferase substrate (pH 7.4)

∝ keto glutaric acid (0.002M) and 2grams of dipotassium hydrogen phosphate (0.115M) were dissolved in about 80ml of distilled water. The pH was adjusted to 7.4 with sodium hydroxide and the final volume was made up to 100ml with distilled water. Few drops of 1% chloroform was added as a preservative

2. Alanine aminotransferase substrate (pH 7.4)

1.78gram of dL-alanine (0.2M), 30mg of ∝ ketoglutaric acid (0.002M) and 2gram of dipotassium hydrogen phosphate (0.115M) were dissolved in about 80ml of distilled water. The pH was adjusted to 7.4 with sodium hydroxide and the final volume was made up to 100ml with distilled water. Few drops of 1% chloroform was added as a preservative

3. Aniline citrate

To 5gram of citrate, 5ml distilled water and 5ml of reduced alanine were added. The reagent thus prepared was diluted 1:5 with distilled water before use

4. Dinitro phenyl hydrazine (0.002M)

To 40mg of 2, 4-dinitro phenyl hydrazine, 5ml concentrated HCl was added and the solution was made upto 100ml with distilled water. This reagent was stored in a brown bottle

5. Sodium Hydroxide (0.75M)

6. 0.1% (w/v) pyruvic acid stock solution

III. Estimation of Acetlycholinesterase (AChE)

1. Tris-HCL Buffer (pH 7.4)

7.90gms of Tris-HCl (0.1M) and 0.145gms of Acetlythiocholine Iodide (1.0) dissolved in about 480ml of distilled water and pH adjusted 7.4. Then the volume made up to 500ml with further distilled water

2. Tris HCL diluting buffer (pH.7.4)

7.9gms of Tris HCL (0.1M) dissolved in about 480ml of distilled water and pH adjusted to 7.4 and then the volume was made up to 500ml with more distilled water

3. 0.1 % (w/v) Quinidine Sulphate (0.1 percent)

4. 4. 5, 5-dithiobis (2-benzoi acid) (DTNB) and Sodium dodecyl sulphate (SDS) solutions: 0.1gm of DTNB and 1.10gms of SDS dissolved in 250ml of Tris HCL diluting buffer (which yields final concentration of 0.04 and 0.44 percent respectively in the reaction mixture)

5. Sucrose 0.8M, Sucrose 0.25M

IV. Estimation of Alkaline Phosphatase

1. All the reagents for the determination of this parameter were used from the AkP kit from Sigma Diagnostics Inc (St. Louis, MO)

This includes alkaline phosphatase substrate and citrate buffer

2. 0.02N Sodium hydroxide

V. Estimation of Acid Phosphatase

1. All the reagents for the determination of this parameter were used from the AcP kit from Sigma Diagnostics Inc (St. Louis, MO)

This includes acid phosphatase substrate and citrate buffer

2. 0.02N Sodium hydroxide

5.3 Methods

5.3.1 Maintenance of rats in animal house

Male and female Sprague-Dawley rats weighing 100 – 120gms were obtained from the Biomedical Resource Centre, University of Durban-Westville and acclimatised for one week prior to the experiment. They were kept in individual polypropylene cages (25x40cms) (Fig. 5.1) and maintained under standard housing conditions (12hr light/dark cycle, temperature 25 ± 2 °C and relative humidity (20% ± 5%). The rats were provided with standard feed and water *ad libitum*.

5.3.2 Treatment of rats

For the acute toxicity study rats were divided into four groups (1, 2, 3 & 4) containing 6 rats in each group. The first group consisted of male rats chosen for treatment. The second group consisted of female rats for treatment. Third and fourth group consisted of control male and female rats, respectively. The rats were given a single dose of 5.7mg of diplodiatoxin/kg body weight. As diplodiatoxin was insoluble in water, it was mixed with coconut oil and the administration of diplodiatoxin was through oral intubation (Fig 5.2 and Fig 5.3). Simultaneously, the control group of male and female rats were kept separately and received coconut oil only.

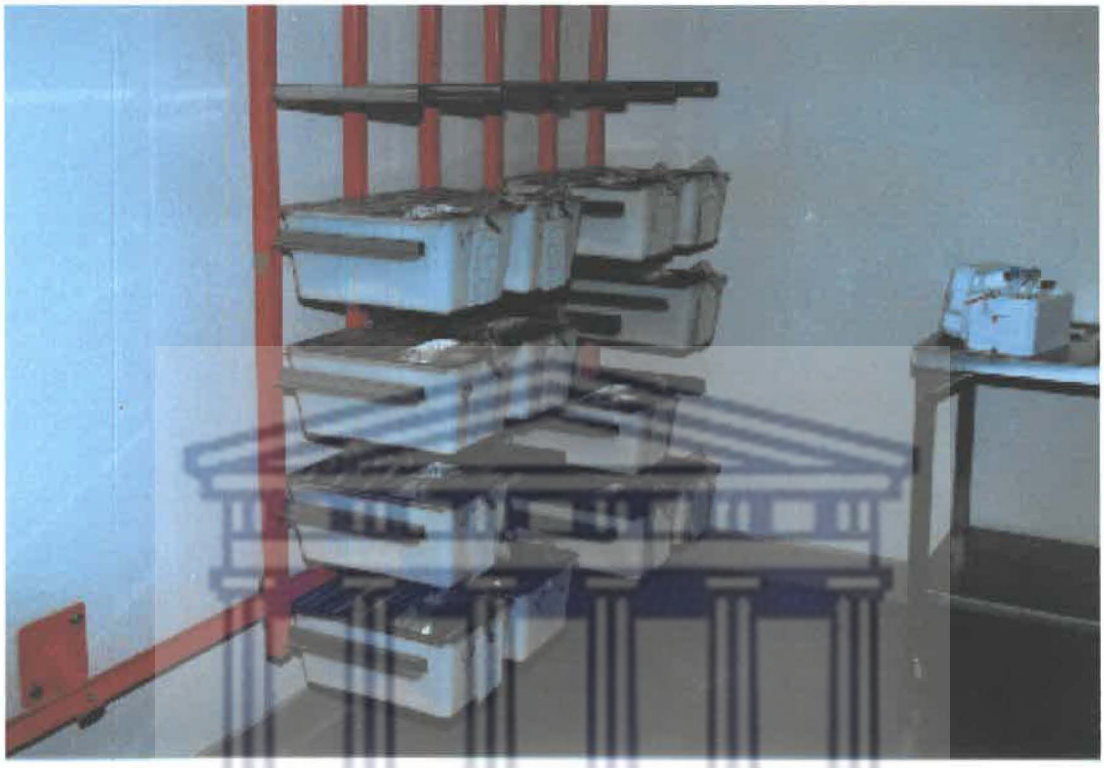


Fig 5.1 Maintenance of rats in animal house with automated control of temperature, humidity and light/dark period

1. The experimental rats were kept in the animal house in Biomedical Resource Centre (BRC)
2. The animal house was facilitated with automated control of temperature (25 ± 2 °C) and humidity (20% ± 5%)
3. The animals were kept at 16 hours light and 8 hours dark periods
4. The rats were kept as groups in individual polypropylene cages (25x40cms)

Fig 5.2 Handling the rats prior to dose administration

1. The rats were monitored before and after their dosage administration
2. The rats were observed closely for behavioural change and compared with the control rats

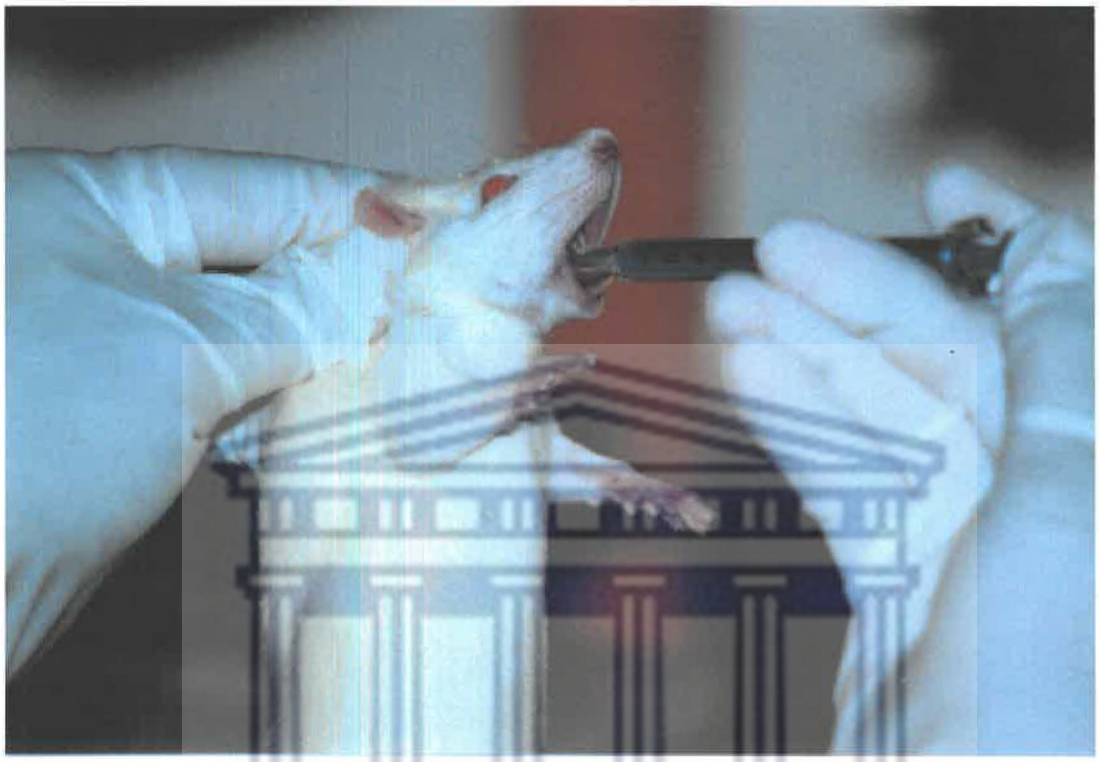


Fig 5.3 Oral administration of the acute dose of diplodiatoxin

1. The figure illustrates the oral administration of the acute dosage
2. The compound is diplodiatoxin

5.3.3 Biochemical study

For the acute study the rats from every group were killed by decapitation after 24 hours of dose administration. Blood was collected in preheparinised vials for RBC AChE determination. Serum was obtained by centrifuging blood without any anticoagulant at 150rpm for 10min at 4 °C. The liver and brain of both control and treated rats were dissected out and quickly homogenised, separately, in ice-cold sucrose (0.25M, 0.8M) respectively and a 10% homogenate (w/v) was made. The homogenate was centrifuged at 10,000g for 10min at 4 °C. The supernatant was used as an enzyme source and the pellet was discarded. Serum and supernatant of the homogenate of the liver were used to determine ASAT and ALAT following the method of Yatzidis (1960). The level of AcP and AkP were determined by Jacobson (1960) and (Bessey *et al.*, 1946) methods. RBC AChE was determined according to the method of Ellman as modified by Chambers *et al.*, (1989). Protein was determined as described by (Lowry *et al.*, 1951). Details of these methods are given in the following sections. Feed intake and body weight were measured every day. Withdrawal study was done after seven days of the treatment.

5.3.3.1 Estimation of protein in different tissues of rats

The protein was estimated in homogenates of liver and brain in control and treated rats with folin-phenol reagent using the standard procedure described by Lowry *et al.*, 1951.

Principle

A deep blue colour is formed when protein is treated with the phenol reagent of Folin ciocalter. Two colour reactions take place simultaneously. Firstly, the peptide bonds of protein react with copper in alkaline solution and secondly a reduction of phosphomolybdic acid and phosphotungstic acid take place by the aromatic amino acids tyrosine and tryptone present in the protein.

Calibration curve for protein

The calibration curve for protein was drawn with different aliquots of BSA solutions at the room temperature, the optical density of these samples was recorded at 750nm using a Shimdsu double beam Spectrophotometer. The concentration of the protein in (mg) was plotted on X-axis against their optical densities on Y-axis (Fig 5.6).

Procedure for estimation of protein in tissues of rat

Tissue samples were diluted with 0.5M NaOH and made up to a final volume of 1ml. Then 5ml of Copper reagent was added to all the samples including the blank and mixed thoroughly and kept for 10 minutes at room temperature. After incubating for 10 minutes, 0.5ml of 1N Folin reagent was added to all the test tubes and the test tubes were kept at the room temperature for 30 minutes. The absorbance of the sample was read at 750nm against the blank. The protein present was calculated by taking the values of optical density obtained with the help of standard calibrations.

5.3.3.2 Determination of acetlycholinesterase in RBC and brain of rats

Acetlycholinesterase (AChE) activity in RBC and brain of rats was estimated by the method of Ellman (1961) as modified by Chambers and Chambers (1989).

Principle

The principle of this method is based on the measurement of the rate of production of thiocholine. As acetlythiocholine is hydrolysed it is accomplished by the continuous reaction of the thiol with 5, 5 dithio-bis-z-nitrobenzoate at 412nm.

H_2O + (CH3) N + CH2CH2S + RSSR

H_2O + (CH3)3 N + CH2CH2S.COCH3 ⟶ (CH3)3 N + CH2CH2S + CH3COO + 2H (CH3)3 NCH2CH2S + (CH3)3 NCH2CH2SSR + RS

Preparation of the enzyme

For the assessment of enzyme in RBC the blood samples were diluted 25 times with Tris–HCl diluting buffer and for the brain AChE determination, rats were killed by decapitation and the whole brain dissected out and homogenised in ice cold 0.8M sucrose using Potter-Elvehjem homogeniser. The homogenate was centrifuged at 10,000g for 10 minutes at 40 °C and the supernatant was used as the enzyme source.

Procedure for the enzyme assay

The test tubes contained 4ml total volume of incubating mixture comprising 0.1M Tris.HCl buffer, pH 7.4, 1.0mM acetyl thiocholine iodide, 25µl (25 times) diluted blood and 15µl of 0.1% quinidine sulphate. In case of brain AChE determination instead of blood, 10µl of 10% (w/v) homogenate was taken and the addition of quinidine sulphate was omitted (to get total AChE activity). The tubes were incubated for 15min with shaking at 37 °C. The reaction was then stopped by adding a mixture of DTNB and SDS (0.5ml) yielding final concentrations of 0.04% and 0.44 %, respectively. Assays were run in triplicate. The absorbance was read at 412nm on a Shimadzu double beam spectrophotometer. Protein content in the brain homogenate was estimated by the method of (Lowry *et al.*, 1951).

Calculations

$$\text{RBC} = \frac{\text{OD of test} \times 4 \times 1000 \times 1000 \times 25}{15 \times 13600 \times 1000 \times 0.025}$$

(µM hydrolysed/min/ml RBC)

$$\text{Brain} = \frac{\text{OD of test} \times 4 \times 1000 \times 1000}{\text{mg protein} \times 15 \times 13600 \times 1000 \times 0.01}$$

(µM hydrolysed/min/mg protein)

Where 4 is the total volume of the solution used in the reaction
15 is the incubation time and 1000 is for converting M into mM
13600 is extn coefficient in Moles
1000 is for converting mM into µM and another 1000 is for converting per litre to per ml
25 is the times the blood is diluted
0.025 is for the total volume of blood taken in ml and
0.01 is for total volume of brain homogenate taken in ml
The amount of AChE per sample was calculated by using the above formula

5.3.3.3 Estimation of Aspartate aminotransferase and Alanine aminotransferase in the serum and different tissues of rats

The activity of ASAT and ALAT in serum and liver was estimated by the method of Yatzidis (1960).

Principle

In the transamination reaction an amino group is transferred from an alpha amino acid to an alpha keto group to form a different alpha amino acid and keto acid.

$$R.CH.NH_2.COOH + R^{|}C(COOH) \rightleftharpoons R.CO.COOH + R^{|}CH.NH_2.COOH$$

ASAT and ALAT catalyse the reaction as follows

$$\text{∝-oxoglutarate + L-aspartate} \xrightleftharpoons{\text{ASAT}} \text{L-glutamate + oxaloacetate}$$

$$\text{∝-oxoglutarate + L-alanine} \xrightleftharpoons{\text{ALAT}} \text{L-glutarate + Pyruvate}$$

The oxaloacetate in the presence of aniline citrate is converted into pyruvate. In both the cases pyruvate reacts with 2, 4-DNPH and forms a red colour complex, dinitrophenyl hydrozone, which can be measured on a spectrophotometer.

Calibration curve for aminotransferase

To draw a calibration curve for aminotransferase, different concentrations of pyruvic was measured at 500nm against the blank set at zero optical density using a spectrometer (Shimadzu). The blank was prepared by replacing pyruvic acid with saline. A graph was plotted with the concentration of pyruvic acid in µg/ml on the X-axis and optical density on Y-axis (Fig. 5.7).

Procedure for estimation of ASAT and ALAT in tissues of rat

Required amount of serum (25µl) was taken in test tubes and diluted to 1ml with saline. To the test tubes 0.5ml of the substrate for ASAT or ALAT was added, respectively and the test tubes were incubated at 37 °C for 60 minutes. Then the tubes were kept at room temperature

and about 0.5ml of diluted citrated aniline was added to stop the enzymatic reaction. Then 0.5ml of dinitro phenyl hydrazine was added immediately and the contents were vigorously mixed. 3ml of NaOH was added and the tubes were incubated at room temperature for 30 minutes. The colour change was measured in a Shimadzu Double Beam Spectrophotometer at 500nm against the blank. Pyruvic acid formed by the enzyme was determined using a standard curve and protein content in the tissue was determined by folin phenol method described by Lowry *et al* (1951) as described earlier. For the estimation of ASAT and ALAT in the liver, 20 times diluted liver homogenate was taken instead of serum and the experiment was repeated in the same way as discussed above.

Calculations

Serum (x moles/hr /ml) = x / 0.025 X 1 / 88
Tissue (x moles/hr /mg) =x / mg protein X 1 / 88

Where x is the pyruvic acid formed in micro grams
0.025 is the volume of serum in ml
88 is the molecular weight of pyruvic acid for converting micrograms into x moles

5.3.3.4 Estimation of Alkaline phosphatase in the serum and different tissues of rats

Alkaline phosphatase (AcP) activity in the serum and liver of rats was determined by the modified method of Bessey (1946) using kits obtained from Sigma Diagnostics Inc (St. Louis, MO).

Principle

Alkaline phosphatase hydrolyses p-nitrophenyl phosphate to p-nitrophenol and disodium phosphate at 5.0 pH. The p-nitrophenol so formed reacts with 4-amino antipurine in alkaline

medium in the presence of an oxidising agent, potassium ferricyanide to form a yellow coloured complex, which is measured at 400-420nm in a spectrophotometer.

AkP (pH 5.0)

Disodium phenyl phosphatase + H_2O ⟶ Phenol + Disodium hydrogen phosphate

Alkaline medium

Phenol + 4 Amino antipurine ⟶ Yellow coloured complex

Potassium ferricyanide

Calibration curve for Alkaline phosphatase

To the different concentrations of alkaline phosphatase substrate, different proportions of p-nitrophenol (1-10ml) and 0.02N NaOH (1-10ml) was added. Optical density was recorded at 420nm. A standard graph was plotted with Pi concentration on the X-axis and their optical densities on the Y-axis (Fig 5.8).

Procedure of estimation of Alkaline phosphatase in the serum or liver

Alkaline buffer (0.5ml) was placed in a test tube and 0.5ml of stock substrate was added. The tubes were then incubated at 37 °C in a water bath and 0.1ml serum/100ml whole homogenate of liver was added and incubated at 37 °C for 15 minutes. To stop the enzymatic reaction, 10ml of NaOH (0.05N) was added and optical density was measured at 420nm. To the same test tubes, 0.2ml of HCl (Concentrated) was added and the colour difference was observed at 420nm. The blank contained 0.1ml of distilled water instead of serum/homogenate of liver.

Calculations

$$\text{Serum alkaline phosphatase} = \frac{X \times 10 \times 100}{\text{OD of Std.} \times 1000 \times \text{serum in ml}}$$

(K-A units/100ml)

$$\text{Tissue alkaline phosphatase} = \frac{X \times 10 \times 60}{\text{mg protein} \times \text{OD of Std.} \times 15 \times 94}$$

(µMoles phenol released/hr/mg protein)

Where X stands for absorbance of test –absorbance of control

10 is phenol concentration in standard in µg

60 is a conversion factor for per hour

15 is incubation time in minutes

94 is molecular weight of phenol for converting into µmoles

5.3.3.5 Estimation of Acid phosphatase in the serum and different tissues of rats

Acid phosphatase (AcP) activity in the serum and liver of rats was determined by the modified method of Jacobson (1960) using kits obtained from Sigma Diagnostics Inc (St. Louis, MO).

Principle

Acid phosphatase hydrolyses p-nitrophenyl phosphate to p-nitrrophenol and disodium phosphate at 5.0 pH. The p-nitrophenol so formed reacts with 4-aminoamino antipurine in

alkaline medium in the presence of an oxidising agent, potassium ferricyanide to form a yellow coloured complex, which is measured at 400-420nm in a spectrophotometer.

AcP (pH 5.0)

Disodium phenyl phosphate + H_2O ⟶ Phenol + Disodium hydrogen phosphate

Alkaline medium

Phenol + 4 Amino antipyrine ⟶ Yellow coloured complex

Potassium ferricyanide

Calibration curve for Acid phosphatase

To the different concentrations of Acid phosphatase substrate, different proportions of p-nitrophenol (1-10ml) and 0.02N NaOH (1-10ml) were added. Optical density of the solution was recorded at 420nm. A standard graph was plotted with Pi concentration on the X-axis and their optical densities on the Y-axis (Fig 5.9).

Procedure of estimation of Acid phosphatase in the serum or liver

Acid phosphatase substrate (0.5ml) was placed in a test tube and incubated at 37 °C in a water bath. To that 0.5ml citrate buffer was added and the mixture was kept at 37 °C. To these test tubes, 50µl serum or/10µl of the liver homogenate was added and incubated at 37 °C for 30 minutes. The tubes were then held at room temperature and 5ml of 0.1N NaOH was added to stop the enzymatic reaction. The colour intensity so developed was measured against the blank (distilled water) at 420nm using a Shimadzu Double beam Spectrometer. For the reagent blank the same procedure was repeated, but instead of serum/liver homogenate, 50µl /10µl of distilled water was used. Optical density was measured at 420nm. And for the serum blank, 6ml of 0.1N NaOH was taken in a test tube, 50µl serum was added, and the optical density was measured at 420nm.

Calculations

$$\text{Serum} = \frac{X \times 60}{0.5 \times 15}$$

(µ Moles Pi released/hr/ml enzyme)

$$\text{Tissue} = \frac{X \times 60}{\text{mg protein} \times 15}$$

(µ Moles Pi released/hr/mg protein)

Where X stands for phosphatase concentration read from the standard graph

The value 0.5 for volume of the enzyme

60 is a conversion factor for conversion into (per) hour

The value 15 represents incubation time

In case of tissues instead of volume of enzyme it is divided by the mg protein content

5.3.4 Statistical analysis

The experimental data were analysed by Student's 't' test ($p<0.05$) to determine the significant changes in the treated rats from the control. Further the significance of the activity of male rats was compared with female rats by analysing Student's 't' test to observe sexual dimorphism. The correlation coefficient was also determined to observe the relative changes for serum versus liver.

5.4 Results

5.4.1 Behavioural changes in rats

The acute treatment of diplodiatoxin caused 50% mortality after the dose administration but no death was observed in the control rats. Sudden loss of body weight was observed up to 48 hours and which was regained during the following days (withdrawal period) but remained less than that of the control rats (Fig 5.4). Female rats showed drastic loss in body weight compare to male rats.

Further, feed intake was also drastically reduced in both male and female rats after 48 hours of the dose administration but female rats were found to be more susceptible (Fig 5.5). Female rats consumed almost negligible feed on the second day. On the first day, feed intake by male and female rats reduced to 1.5-2 times respectively over the control. After 48 hours, feed intake by male and female rats was reduced to 2.7 times and 3.5 times, respectively over the control on second day. This showed that diplodiatoxin severely reduced the growth of the animals. During the subsequent days of growth (withdrawal study), feed intake was improved but always remained lower than the control rats.

Other than the loss in body weight and feed intake, dullness, irritation, bulky stomach, tremors and convulsions were observed in the treated rats. Rats were scared while handling and their movements were very slow. These symptoms indicate growth retardation and the stressed condition of the treated animals.

5.4.2 The Standard curves for the biochemical parameters

The standard curve for protein with concentration of the protein in mg was plotted on X-axis against their optical densities on Y-axis is shown in the Fig (5.6).
The standard curve for ASAT, ALAT was plotted taking the concentration of pyruvic acid in µg/ml on the X-axis and optical density on Y-axis (Fig 5.7).
The standard curve for AkP was plotted taking Pi concentration on the X-axis and the optical densities on the Y-axis (Fig 5.8).
The standard curve for AcP was plotted taking Pi concentration on the X-axis and the optical densities on the Y-axis (Fig 5.9).

5.4.3 Changes in the level of AChE in the brain of male and female rats

Activity of AChE is an excellent indicator of the early toxicity. The level of AChE in the brain was reduced by 14% and 20% in male and female rats, respectively (Table 5.1). Thus, the level of AChE reduced significantly in female rats. After seven days of post-treatment (withdrawal study), the level was still lower but was almost close to that of control rats.

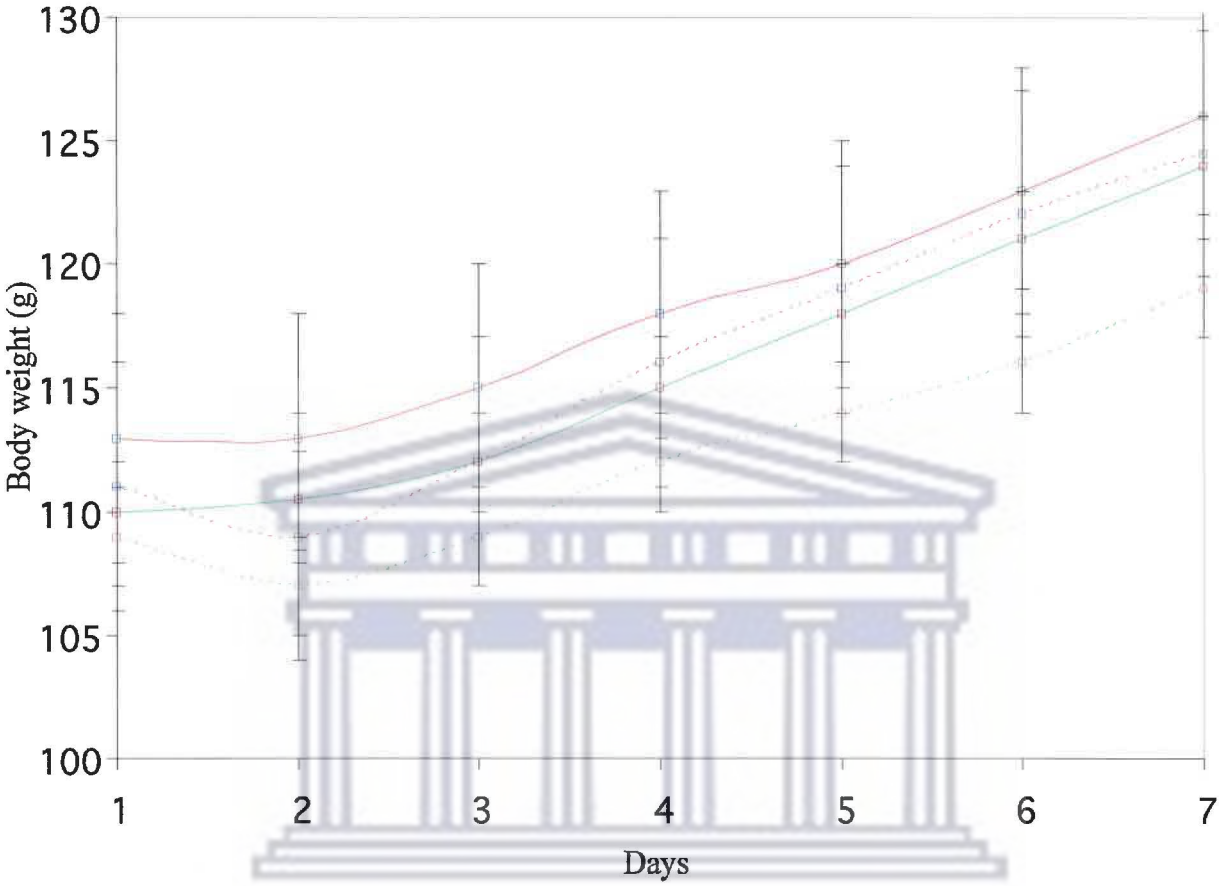


Fig 5.4 Graph showing the deviation in the body weight (g/day) in the treated rats when compared with control rats

1. The behavioural response with reference to body weight after acute dosage
2. The line (---------) represents the male rats in response to acute dose of diplodiatoxin
3. The line (-------) represents the female rats in response to acute dose of diplodiatoxin

3.3 The dotted line represents the treated rats and the solid line represents the control rats

4. The error bar represents the mean of 6 rats from each group

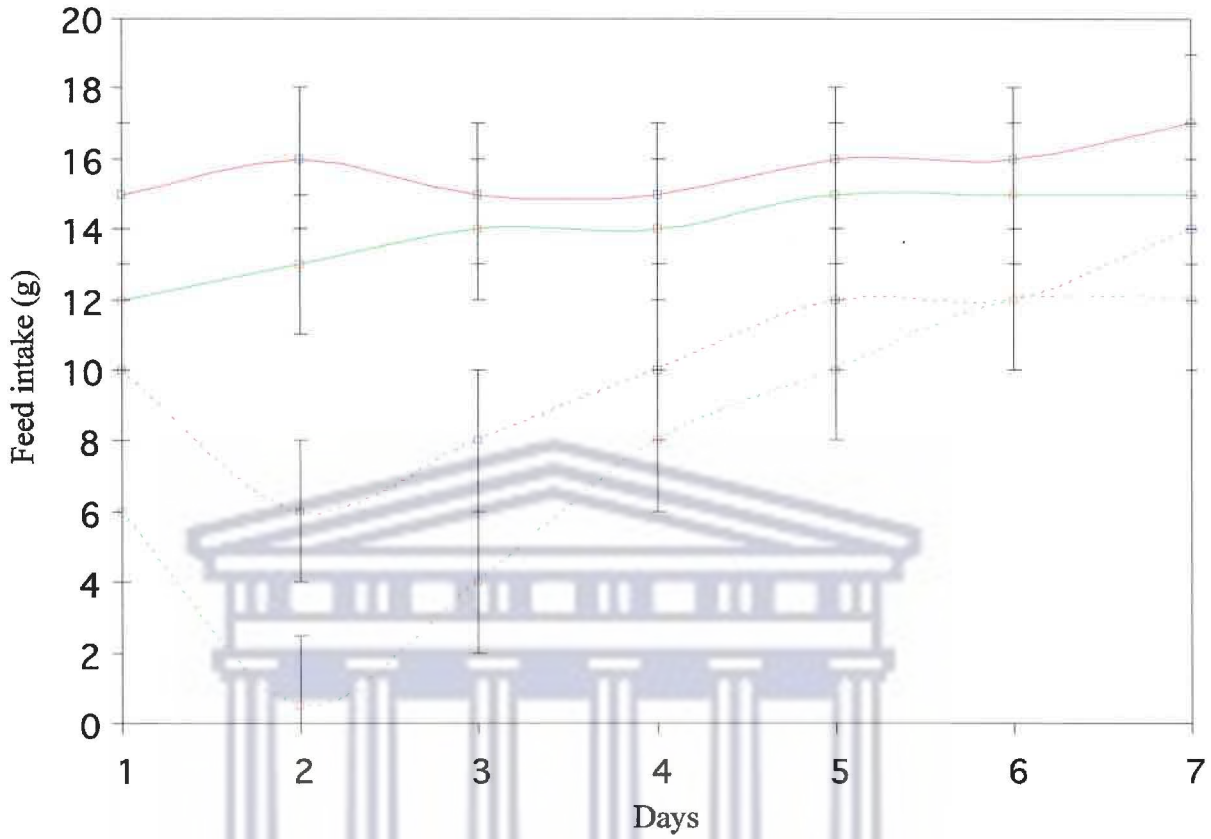


Fig 5.5 Graph showing the deviation in feed intake (g/day) in treated rats when compared with control rats

1. The behavioural response with reference to feed intake after acute dosage
2. The line (-------) represents the male rats in response to acute dose of diplodiatoxin
3. The line (-------) represents the female rats in response to acute dose of diplodiatoxin
4. The dotted line represents the treated rats and the solid line represents the control rats
5. The error bar represents the mean of 6 rats from each group

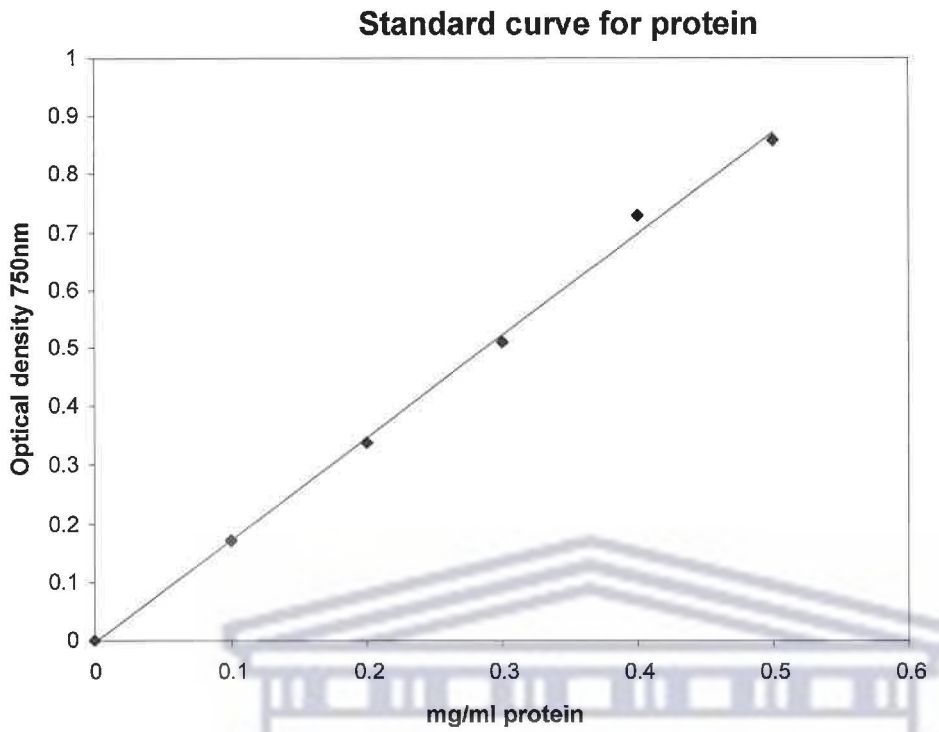


Fig 5.6 Standard curve for protein

1. The calibration curve for protein was done with different aliquots of BSA
2. The concentration of protein is in mg/ml

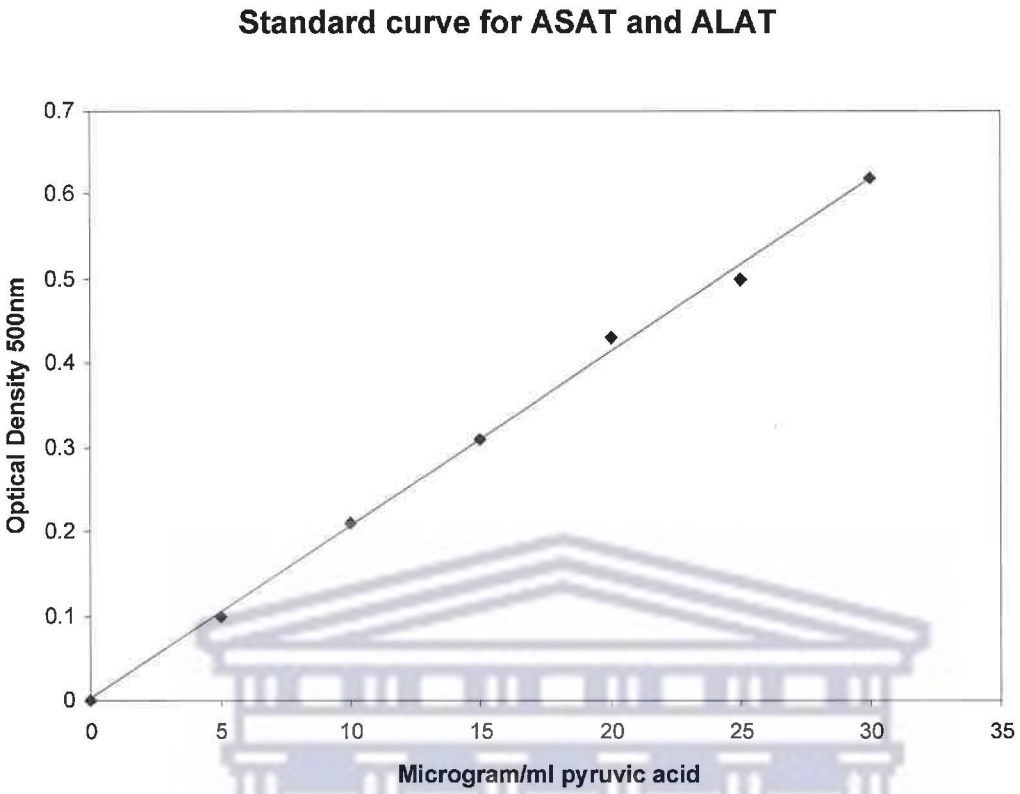


Fig 5.7 Standard curve for ASAT and ALAT

1. The calibration curve for aminotransferase was done with different aliquots of pyruvic acid
2. The concentration of pyruvic acid is in µg/ml

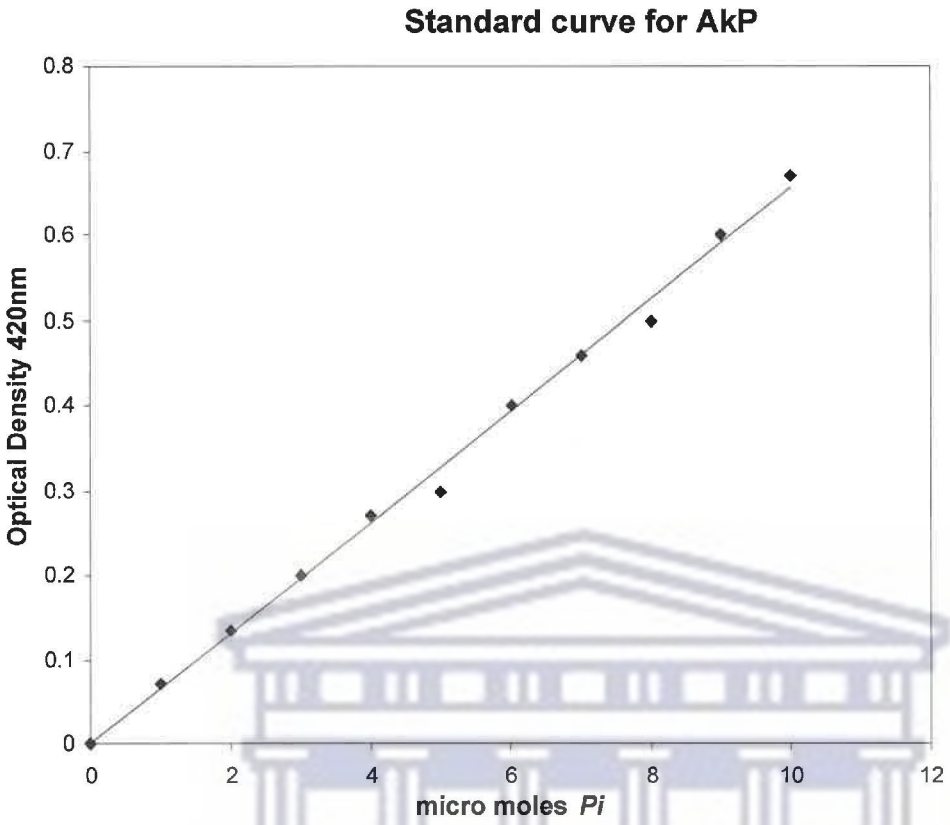


Fig 5.8 Standard curve for AkP

1. The calibration curve for alkaline phosphatase is drawn with different concentrations of alkaline phosphatase substrate added with different proportions of p-nitrophenol 10 and 0.02N sodium hydroxide

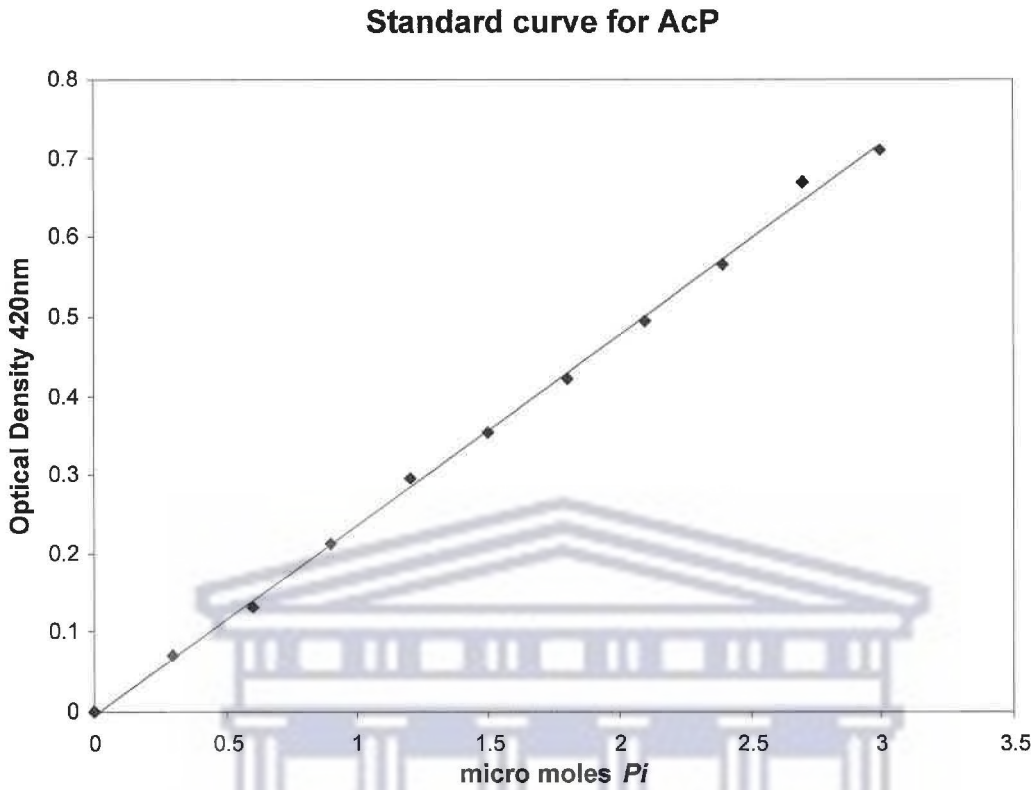


Fig 5.9 Standard curve for AcP

1 The calibration curve for acid phosphatase is drawn with different concentrations of acid phosphatase substrate added with different proportions of p-nitrophenol 10 and 0.02N sodium hydroxide

Sex	Control	Treated
Acute Toxicity Study		
Brain AChE [a] **After 24 hours of treatment**		
Male	15.9 ± 0.8	13.6 ± 0.2
Female	14.7 ± 0.4	11.7 ± 0.5
After 7 days of post treatment (withdrawal study)		
Male	16.6 ± 0.2	15.7 ± 1.1
Female	13.9 ± 0.5	13.3 ± 0.4

Table 5.1 Acute effect of diplodiatoxin on brain acetylcholinestrase levels in rats

1. The table shows the data of Acute effect of diplodiatoxin on rats
2. The parameter is acetlycholinesterase in brain
3. [a] is in μ moles hydrolysed/min/mg Protein
4. The table shows the deviation between control and treated rats
5. Data represent mean ± S.E (Standard Error) of three replicate
6. The *p<0.05 significantly different from control

5.4.4 Changes in the enzymatic levels in the serum of male rats

Acute doses of diplodiatoxin in male rats caused 7.69% and 10.87% increase in the levels of ASAT and ALAT, respectively over the control in the serum. Due to the acute exposure of diplodiatoxin in male treated rats the level of AcP, AkP and RBC AChE was inhibited significantly after 24 hours of treatment (Table 5.2). The level of AcP was reduced to (40%) and AkP was reduced to (15.15%). The decrease in case of RBC AChE was (12 %) when compared with the control rats.

After seven days of the treatment (withdrawal effect), the level of ASAT, ALAT, AkP, AcP and blood AChE reached near to the normal

5.4.5 Changes in the enzymatic levels in the serum of female rats

In the female rats the enzymatic changes were drastic as compared to male rats. All the parameters showed considerable change when compared with that of the control rats. After 24 hours of treatment, the level of serum ASAT and ALAT increased to 26.5% and 13.7%, respectively over the control rats (Table 5.3). Whereas, the level of AcP, AkP and RBC AChE was reduced by 29.5%, 33% and 14.6 % respectively over that of the control rats. The reduction in the level of AkP and RBC AChE was much higher in the female rats compare to male rats. Inhibition of the level of AkP in female rats was almost double that in the male rats.

Like male rats, female rats also became normal after seven days of the treatment (withdrawal effect). The level of ASAT, ALAT, AkP, AcP and blood AChE reached near to the normal. But still there was considerable difference between the treated and normal rat.

Sl.No.	Parameter	Control	Treated
After 24 hours of treatment			
1.	ASAT [b]	6.8 ± 0.1	7.4 ± 0.1
2.	ALAT [b]	6.9 ± 0.1	7.7 ± 0.1
3.	AcP [c]	7.7 ± 0.1	4.6 ± 0.1*
4.	AkP [c]	29.8 ± 1.7	25.3 ± 0.5
5.	RBC AChE [a]	11.6 ± 0.03	10.2 ± 0.03*
After 7 days of treatment (withdrawal study)			
1.	ASAT [b]	6.5 ± 0.1	6.5 ± 0.1
2.	ALAT [b]	6.4 ± 0.0	6.8 ± 0.0
3.	AcP [c]	7.2 ± 0.2	7.0 ± 0.1
4.	AkP [c]	29.6 ± 0.4	29.3 ± 0.2
5.	RBC AChE [a]	11.0 ± 0.1	10.9 ± 0.2

Table 5.2 Acute effects of diplodiatoxin on biochemical parameters in serum of male rat

1. The table shows the data of acute effect of diplodiatoxin on serum of male rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [a] μ moles hydrolysed/hr/ml
4. [b] μ moles/hr/ml
5. [c] μ moles/hr/ml
6. The table shows the deviation between control and treated rats
7. Data represent mean ± S.E (Standard Error) of three replicate
8. The *$p<0.05$ significantly different from control

Sl.No.	Parameter	Control	Treated
After 24 hours of treatment			
1.	ASAT [b]	6.2 ± 0.2	7.95± 0.2*
2.	ALAT [b]	6.5 ± 0.1	7.5 ± 0.1*
3.	AcP [c]	8.9 ± 0.1	6.2 ± 0.3*
4.	AkP [c]	28.2 ± 0.2	18.9 ± 0.2*
5.	RBC AChE [a]	11.1 ± 0.0	9.4 ± 0.1*
After 7 days of treatment (withdrawal study)			
1.	ASAT [b]	6.1 ± 0.1	6.5 ± 0.1
2.	ALAT [b]	6.7 ± 0.03	6.8 ± 0.1
3.	AcP [c]	6.6 ± 0.1	5.4 ± 0.6
4.	AkP [c]	28.0 ± 0.4	27.4 ± 0.7
5.	RBC AChE [a]	10.9 ± 0.1	10.6 ± 0.1

Table 5.3 Acute effects of diplodiatoxin on biochemical parameters in serum of female rat

1. The table shows the data of acute effect of diplodiatoxin on serum of female rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [a] µ moles hydrolysed/hr ml
4. [b] µ moles/hr/ml
5. [c] µ moles/hr/ml
6. The table shows the deviation between control and treated rats
7. Data represent mean ± S.E (Standard Error) of three replicate
8. The *$p<0.05$ significantly different from control

5.4.6 Changes in the enzymatic levels in the liver of male rats

Acute doses of diplodiatoxin resulted in a significant decrease in the level of ASAT and ALAT in the liver of male rats, whereas, the levels of AcP and AkP increased (Table 5.4). Reduction in the level of ASAT and ALAT in liver was 17.5% and 19%, respectively over the control after 24 hours of the dose administration. Further, the level of AcP was found to be 37.5% higher than the control in response to the acute dose of diplodiatoxin. The liver AkP level drastically increased after 24 hours of the treatment.

After seven days of the treatment (withdrawal study), levels of these enzymes were found to be the same as that in the control rats, but the level of ASAT was still 6.8% lower than that of the control rats although the enzymes showed a trend of returning to the normal.

5.4.7 Changes in the enzymatic levels in the liver of female rats

Female rats were found to be more susceptible to diplodiatoxin than the male rats. Levels of ASAT and ALAT in the liver increased to 19.43% and 41.67%, respectively over the control rats after 24 hours of the dose administration (Table 5.5). There was a decrease in the level of AkP (about 59%) after 24 hours. The level of AcP in the liver was increased to 45% over the control rats.

The levels of ASAT, AcP and AkP returned to nearly normal after seven days of treatment (withdrawal effect) but the level of ASAT was 6.8% lower than that in the control rats. Withdrawal study showed the recovery of rats.

Sl.No.	Parameter	Control	Treated
After 24 hours of treatment			
1.	ASAT [d]	63.1 ± 0.9	52.0 ± 0.4*
2.	ALAT [d]	77.9 ± 2.9	63.1 ± 0.9*
3.	AcP [e]	66.6 ± 1.6	91.6 ± 4.2*
4.	AkP [e]	8.1 ± 1.4	4.4 ± 1.2
After 7 days of treatment (withdrawal study)			
1.	ASAT [d]	61.1 ± 2.2	60.6 ± 1.5
2.	ALAT [d]	75.7 ± 3.7	69.9 ± 2.5
3.	AcP [e]	76.1 ± 2.9	78.0 ± 0.7
4.	AkP [e]	8.4 ± 0.4	7.8 ± 0.6

Table 5.4 Acute effects of diplodiatoxin on biochemical parameters in liver of male rat

1. The table shows the data of acute effect of diplodiatoxin on liver of male rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [d] μ moles/hr/mg protein
4. [e] μ moles/hr/mg protein
5. The table shows the deviation between control and treated rats
6. Data represent mean ± S.E (Standard Error) of three replicate
7. The *p<0.05 significantly different from control

Sl.No.	Parameter	Control	Treated
After 24 hours of treatment			
1.	ASAT [d]	57.3 ± 2.0	46.2 ± 0.3*
2.	ALAT [d]	74.3 ± 1.2	43.3 ± 0.3*
3.	AcP [e]	66.0 ± 0.6	95.8 ± 5.2*
4.	AkP [e]	7.5 ± 0.4	3.0 ± 0.8*
After 7 days of treatment (withdrawal study)			
1.	ASAT [d]	55.8 ± 0.9	54.1 ± 1.5
2.	ALAT [d]	72.6 ± 1.5	67.6 ± 1.3
3.	AcP [e]	64.2 ± 1.3	66.1 ± 1.2
4.	AkP [e]	6.2 ± 1.7	5.3 ± 1.3

Table 5.5 Acute effects of diplodiatoxin on biochemical parameters in liver of female rat

1. The table shows the data of acute effect of diplodiatoxin on liver of female rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [d] μ moles/hr/mg protein
4. [e] μ moles/hr/ mg protein
5. The table shows the deviation between control and treated rats
6. Data represent mean ± S.E (Standard Error) of three replicate
7. The *$p<0.05$ significantly different from control

5.4.8 Statistical analysis

The changes caused by diplodiatoxin in serum and liver were statistically significant when compared to the changes in male and female rats. The results suggested sexual dimorphism.

The correlation coefficient studies between serum and liver showed a negative correlation in ASAT, ALAT, AcP and a positive correlation in AkP. In acutely treated rats a high degree of correlation was observed in male ASAT (-0.56), ALAT (-0.44), AcP (–0.83) and AkP (+0.76) and female ASAT (-0.66), ALAT (-0.83), AkP (+0.65) and AcP were insignificant.

5.5 Discussion

Acute toxicity test of diplodiatoxin in male and female rats with reference to changes in the levels of specific target enzymes viz., ASAT, ALAT, AkP, AcP and AChE in blood, liver, serum and brain is conducted in the present study. Withdrawal studies were also undertaken to check the recovery levels in the rats.

Acute dose of diplodiatoxin caused sudden loss in the body weight and feed intake in both male and female rats. Other than loss in body weight and feed intake, dullness, irritation, bulky stomach, tremors and convulsion symptoms were also observed which indicated growth retardness and stressed conditions. Female rats were more susceptible to the diplodiatoxin than the male rats. Similar loss in body weight and feed intake was noted in the aflatoxin treated mice (Nair and Verma, 2000).

The level of the enzyme AChE in the brain drastically reduced in both male and female rats. When AChE is reduced or inhibited, the ACh is not hydrolysed and thus it accumulates at cholinergic sites causing over stimulation of nerve impulse (Moser, 2000). Diplodiatoxin also inhibited RBC AChE showing its interference with the normal synaptic transmission of nerve impulse. Inhibition of RBC AChE as in the present study has been reported in rats treated with diethyl phosphoric ester of dicyclopropylokeloxime (Machera and Kostakis, 1992). It has been reported that *Diplodia maydis* caused demyelination of nerves, atrophy,

degeneration and necrosis of muscle and hepatitis in vervet monkeys (Fincham *et al.*, 1991). Similarly, perinatal mortality in lambs was observed when they were exposed to the cultures of *D. maydis* (Prozesky *et al.*, 1994). The inhibition of AChE by diplodiatoxin clearly indicates the neurotoxic nature of the compound. When the recovery studies were conducted the male rats showed positive response than females, but still the level was much less than that of control rats. Diplodiatoxin also caused alterations in some of the target biochemical enzymes like ASAT, ALAT, AcP and AkP.

ASAT and ALAT are membrane enzymes and are simple, reliable to test for the detection of hepatic necrosis (Kaur *et al.,* 2000). The present study demonstrated an increase in ASAT and ALAT in serum, whereas in the case of liver these enzymes decreased in both female and male rats. Increased levels of these enzymes in the serum as reported might be due to the leakage of soluble tissue enzymes into the blood as a result of necrosis of the tissue (Rahman *et al.,* 1999). This was observed in goats treated with chloropyriphos (Kaur *et al.*, 2000) and in rabbits treated with zearalenone indicating liver damage (Conkova *et al.,* 2001) and the decrease in liver ASAT and ALAT was also reported in rats treated with polychlorinated biphenyl organ chlorine compound (Rao and Banerji, 1990). Also increased levels of these enzymes in the serum and a decrease in the liver might be due to liver necrosis caused by diplodiatoxin treatment.

Drastic changes were also observed in the phosphatases when the rats were treated with diplodiatoxin. AcP and AkP levels decreased in the serum and increased in the liver, these results clearly indicate the cell damage caused by diplodiatoxin. As in the present study, increase levels of liver AcP were reported in dichlorvos-treated rats (Srivastava *et al.*, 1989), while decreased levels of liver AkP was observed when rats were treated with poly-chlorinated biphenyl (Rao and Banerji, 1990).

These enzymes are particularly used as diagnostic markers for tissue damage, particularly in liver. Hepato-cellular damage such as necrosis is generally associated with alterations in

tissue and serum enzymes (Kackar *et al.,* 1999). The pathomorphologic changes in the liver in the present study may be correlated with the disturbances in biochemical enzymes in the serum. These are membrane bound biomarkers and the changes in these enzymes might be due to interactions of diplodiatoxin with these enzymes causing alterations in cell membrane permeability. The decreased activity of serum AcP and AkP observed might be due to slow enzyme synthesis or liver damage, whereas the increased AcP activity in liver might be due to increased synthesis of this enzyme as an adaptive mechanism to chemical stress. These results clearly indicate the hepatotoxic nature of diplodiatoxin.

Withdrawal studies showed a recovery pattern in rats as the levels of these enzymes returned to the normal after seven days of withdrawal. This clearly revealed the reversal of the toxicity once the mycotoxin is removed. Likewise reversal of the toxicity has been reported in rats treated with RPR-V by Khan *et al.*, 2001.

The changes observed in the activities of the enzymes in female rats were significant when compared to male rats at 24 hours in acute studies. These results clearly indicate sexual dimorphism. Similar sexual dimorphism was observed in Vepacide treated rats (Rahman *et al.*, 1999).

The correlation studies indicate a negative correlation between serum and liver, as the enzymes increased in serum with parallel decrease in liver. The present study suggest that the oral administration of diplodiatoxin resulted in severe alterations in serum and also in the cellular activities of vital organs such as the liver, causing hepatic and neurological damages, thus can upset the physiology of the individual.

Finally, it is concluded that diplodiatoxin produced by *S. maydis* is a toxic compound as its hepatotoxic and neurotoxic nature are confirmed. In the acute toxicity experiments the symptoms of paralysis were not observed where as it was observed in the animals fed with crude cultures of *S. maydis*. So the study of sub-chronic affect of diplodiatoxin becomes necessary.

Chapter 6

SUB-ACUTE TOXICITY OF DIPLODIATOXIN ON RATS

6.1 Introduction

Sub-acute toxicity of mycotoxins causes carcinogenesis, mutagenesis and immunosuppression (Hussein *et al.,* 2002). Sub-acute or chronic mycotoxicoses causes fatty changes in the liver, enlargement of the gall bladder, peripheral fibrosis with proliferative changes in bile duct epithelium icterus and impaired growth rate or production. In addition to the liver, the thymus is also a primary target organ of damage. The signs of sub-acute or chronic mycotoxin poisoning are less definitive and so may go unrecognised because an animal may show only a reduced growth rate or increased susceptibility to infectious diseases to which the mycotoxicoses has predisposed it. Major economic losses from mycotoxicoses in animals are associated with sub-acute or chronic forms. Than acute toxicity sub-acute toxicity has more adverse effects on animals and human beings. Sub-acute toxicity of certain mycotoxins like aflatoxin, ochratoxin and fumonisin are known to cause cancer in humans (Fink-Gremmels, 1999).

Sub-acute toxicity of aflatoxin has been reported to have a deleterious effect on the reproductive capacity of laboratory and domestic animals (Casado *et al.,* 2001). When fertile female rats were exposed to aflatoxin B_1 significant reduction in the number of ova fertilisation even at the lowest concentration was observed. The results also demonstrated the adverse effects of aflatoxin B_1 on oocytes, spermatozoa and *in vitro* fertilisation (Ibeh *et al.,* 2000). Short-term exposure to sub-acute doses of aflatoxin-induced depressed nitrogen responses in young mallard ducks (Hurley *et al.,* 1999). Sub-acute toxicity of T-2 toxin in rats was characterised by a primary defect in the liver, thymus, spleen and intra-organ arteries. In 75% of animals increase of the size and adipose infiltration of liver was observed and the size of thymus and spleen in animals were reduced (Pozdniakov, 2000).

Chronic exposure to fumonisin B1 resulted in a harmful effect to foetuses of pregnant cows (Zomborszky *et al.,* 2000). There was significant damage of liver and kidney when rodents were exposed to *Fusarium moniliforme* (Voss *et al.,* 2001). Liver tumours were observed when the mice were exposed to sub-acute doses of fumonisin B_1 (Kodell *et al.,* 2001). Unlike other mycotoxins, fumonisin causes, liver cancer and kidney damage. Sub-acute doses of fumonisin resulted in severe liver damage in rats (Theumer *et al.,* 2002). Fumonisin are also noted to be embryo toxic at high maternally toxic doses (Voss *et al.*, 2001).

Sub-acute toxic doses of ochratoxin A in female rats resulted in age-related difference in toxicity levels. It was observed from clinical and pathological data that old rats were more susceptible to this toxin than young ones. The target organs identified in this study were liver and kidney (Dortant *et al.,* 2001).

In the sub-acute toxicity studies, rats are preferred as experimental models as to determine the exact nature of the toxic compounds in mammalian systems. The reproductive cycle for rats is fast and they can be managed easily. They also acclimatise themselves easily to the laboratory conditions. The present study become necessary to know the sub-acute effect of diplodiatoxin as in the acute study there were remarkable changes observed in biochemical parameters. Target enzymes play a vital role in predicting the sub-chronic effects of the toxin. These enzymes are very sensitive and can be used to indicate the early symptoms of toxicity. The aim of the present study is to determine the chronic effects of diplodiatoxin on rats with reference to biochemical enzymes.

6.2 Materials

All the materials used in the present chapter are described in Chapter 5 in the section 5.2

6.3 Methods

6.3.1 Animal house conditions

Male and female Sprague-Dawley rats were obtained from Biomedical Resource Centre, University of Durban West-Ville and kept separately in polypropylene cages (25 x 40cms) and maintained at 25 ± 2 °C temperature, 20 ± 5% relative humidity and 12 hour light/dark period and were fed with standard feed and water *ad libitum*.

6.3.2 Treatment of rats

For the sub-acute study rats were divided into six groups (I, II, III, IV, V and VI) containing five rats of each sex. The rats were treated with 0.27mg/kg body weight/day of diplodiatoxin mixed in coconut oil through oral intubation. Rats in group I and II received the diplodiatoxin for 10 days, whereas those in groups of III to IV received the compound for 21 days. Simultaneously control group (V and VI) of male and female rats were kept separately and received coconut oil only.

6.3.3 Biochemical study

For the sub-acute study, rats were sacrificed on day 10 (group I and II), 21 (group III and IV) and 28 (group V and VI) corresponding to, during medication, after medication and withdrawal study, respectively. Blood (5ml) was collected from the eye in preheparinised vials for RBC AChE determination. Serum was obtained by centrifuging the blood without any anticoagulant at 1500rpm for 10min at 4 °C.

Immediately after killing the animal, liver and brain of both control and treated rats were dissected out quickly and homogenised separately in ice-cold sucrose (0.25M, 0.8M) and 10% homogenate (w/v) was made. The homogenate was centrifuged at 10,000g for 10min at 4 °C. The supernatant was used as an enzyme source and the pellet was discarded. Serum and supernatant of the homogenate of liver were used to

determine ASAT and ALAT following the method of Yatzidis (1960). RBC AChE was determined according to the method of Ellman *et al.,* 1961 as modified by Chambers *et al.*, 1989. AcP and AkP were determined by method of Jacobson (1960) and Bessey *et al.*, 1946. Protein was determined as described by Lowry *et al.,* 1960. The detailed procedures for the estimations of biochemical enzymes are mentioned in detail in chapter 5 in section 5.3.

6.3.4 Statistical analysis

Data obtained were analysed by Student's 't' test ($p<0.05$) to determine the significance of the changes from the control. Further the significance of the activity of male rats was compared with female rats by analysing Student's 't' test to observe sexual dimorphism. The correlation coefficient was also determined to observe the relative changes for serum versus liver.

6.4 Results

6.4.1 Behavioural changes in rats

In the sub-acute treatment mortality was not observed but all the animals were weak and scared while handling (Fig 6.1). Moreover, gradual loss in the body weight was observed in both male and female rats (Fig 6.2) and females were found to be more susceptible to diplodiatoxin than male rats. In the beginning, weight was not affected in both male and female rats. After 8 days of the treatment, male rats started loosing weight gradually. The

weight loss was severe after 11 days of post-treatment and always remained lower than that of control male rats, up to 21 days of dose administration. Withdrawal of dose resulted in an increase in the body weight but still body weight remained lower than the control rats but was increasing to become normal. Female rats showed drastic loss in the body weight after 4 days of post-treatment and remained less than the control during the 21 days period of dose

administration. Withdrawal of diplodiatoxin resulted in an increase in the body weight but was found to be less than the control rats.

Other than the loss in body weight, feed intake drastically reduced in both male and female rats as a result of sub-acute toxicity of diplodiatoxin (Fig 6.3). Feed intake in male rats reduced to 50% during the first week, 76.9% during the second week and 86.6% during the third week of treatment over the control rats. Withdrawal of the diplodiatoxin resulted in a slight increase in the body weight, which was still 33.3% less than the control male rats on the fourth week of the study (i.e., after one week of the withdrawal). Similar pattern of feed intake was also observed in female rats but the loss in feed intake drastically reduced when compared to male rats. After first week, loss in feed intake was 63.66% over the control and then started declining more rapidly. During the second and third weeks of sub-acute treatment of diplodiatoxin, loss in feed intake by female rats was 83.33% and 92.3%, respectively and withdrawal dose resulted in an increase in feed consumption but was still 78.5% less than that in the control rats.

Other than the loss in body weight and feed intake, symptoms like dullness, irritation, tremors and convulsion were also observed in the treated rats. Collectively these changes clearly indicate growth retardness and stressed conditions in the treated rats.

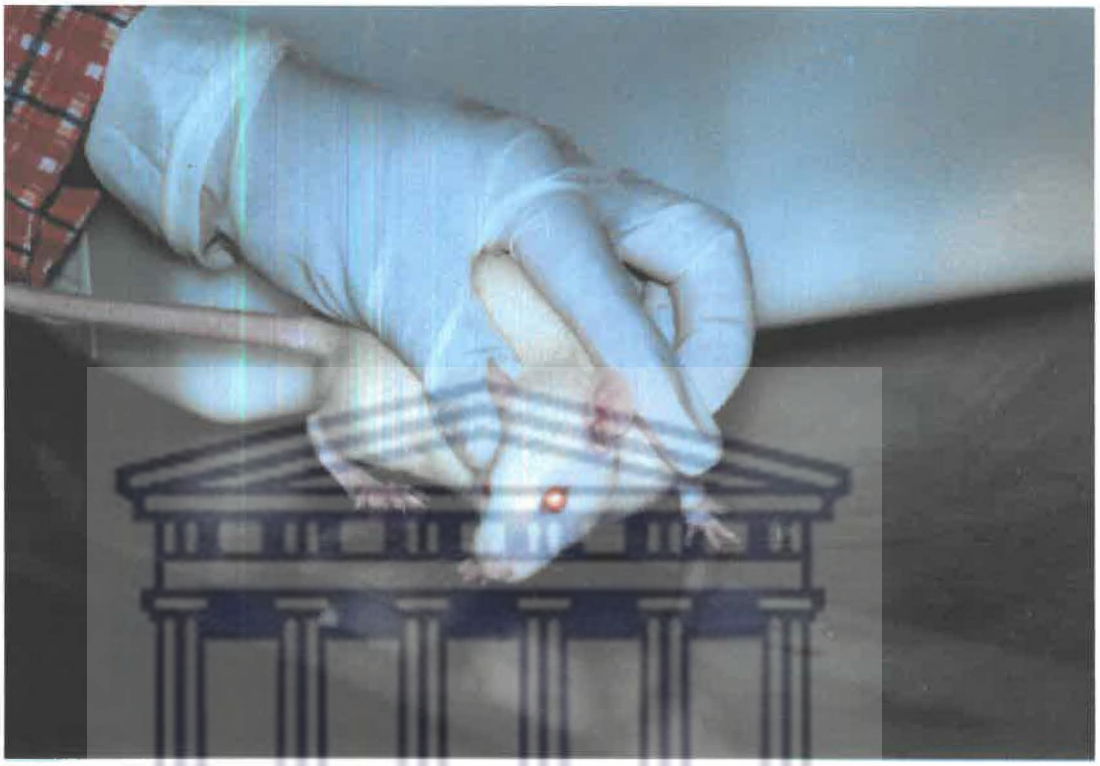


Fig 6.1 Observing the behavioural response of rats after dose administration

1. The treated rats were observed after the administration of dose
2. The treated rats were monitored for the behavioural responses during the treatment
3. The behaviour of treated rats was completely different when compared with the control rats

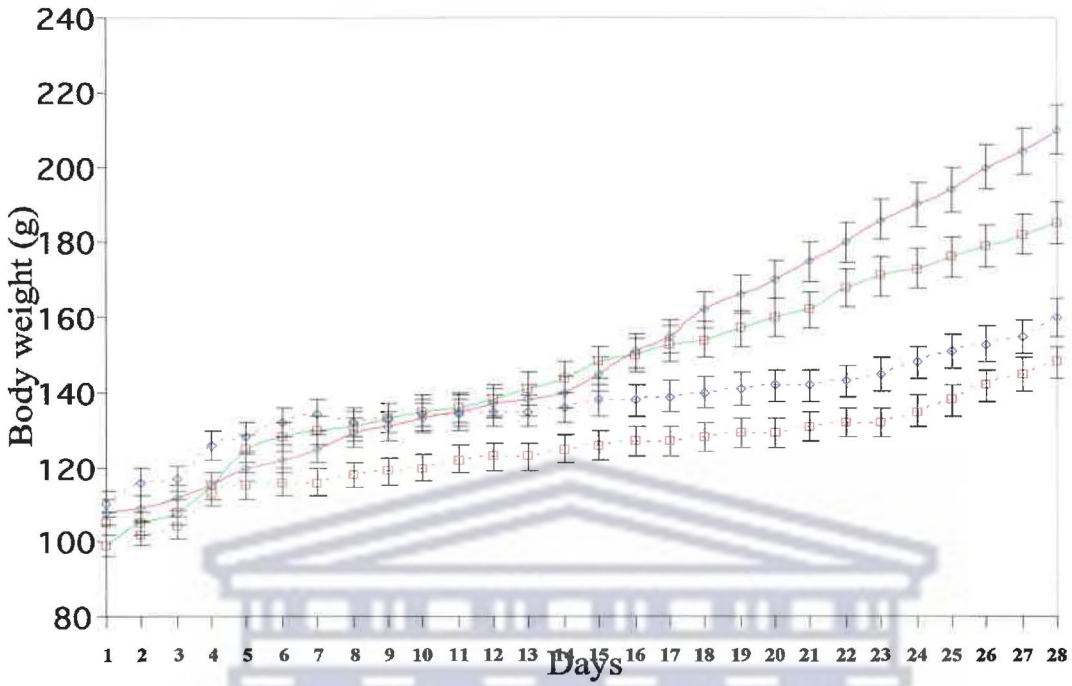


Fig 6.2 Graph showing the deviation in body weight (g/day) in treated rats when compared with control rats

1. The behavioural response with reference to body weight after sub-acute dosage
2. The line (———) represents the male rats in response to sub-acute dose of diplodiatoxin (1-21 days) and its withdrawal (22 to 28 days) effect
3. The line (- - - - - -) represents the female rats in response to sub-acute dose of diplodiatoxin (1-21 days) and its withdrawal (22 to 28 days) effect
4. The dotted line represents the treated rats and the solid line represents the control rats
5. The error bar represents the mean of 6 rats from each group

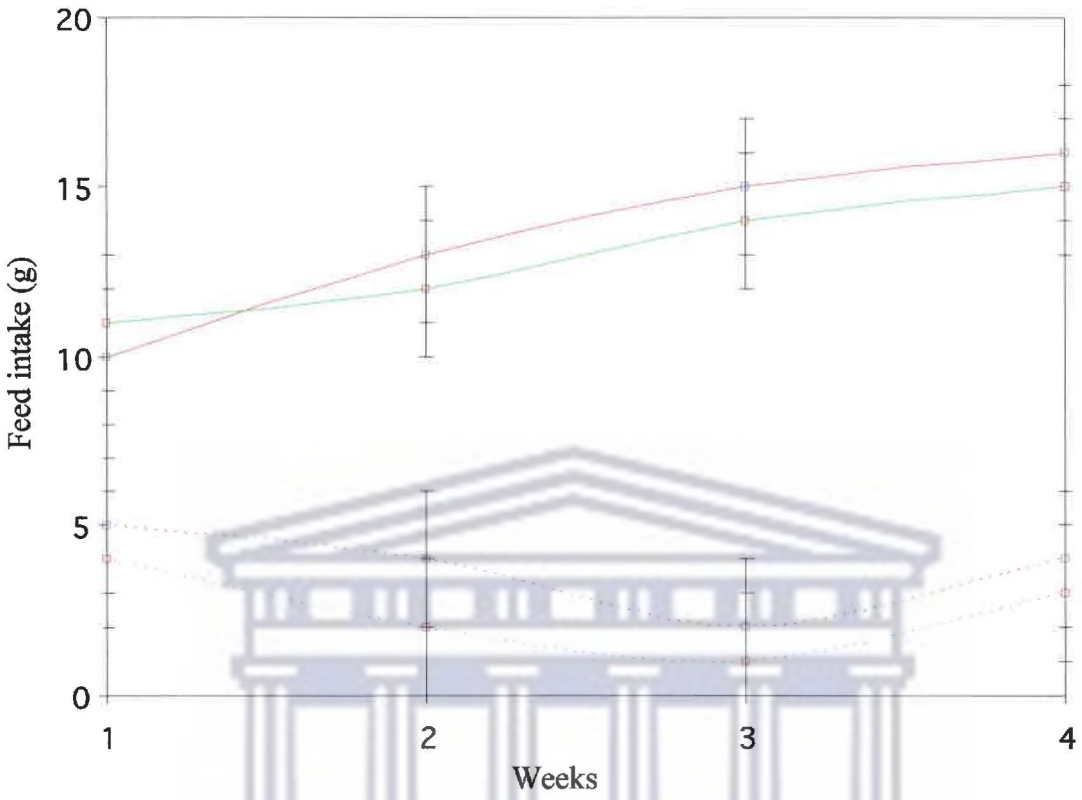


Fig 6.3 Graph showing the deviation in the feed intake (g/day) in the treated rats when compared with control rats

1. The behavioural response with reference to feed intake after sub-acute dosage
2. The line (--------) represents the male rats in response to sub-acute dose of diplodiatoxin (1-3 weeks) and its withdrawal (3-4 weeks) effect
3. The line (--------) represents the female rats in response to sub-acute dose of diplodiatoxin (1-3 weeks) and its withdrawal (3-4 weeks) effect
4. The dotted line represents the treated rats and the solid line represents the control rats
5. The error bar represents the mean of 6 rats from each group

6.4.2 Changes in the level of AChE in brain of male rats

The level of AChE in the brain is an excellent indicator of an early toxicity of a toxic compound. The biochemical experiments which were conducted after 10 days of sub-acute administration of diplodiatoxin resulted in a significant reduction in the level of AChE enzyme as compare to the control rats. The estimation of decrease in the level of AChE enzyme in the brain of male rats was 18.79% over the control. After 21 days of treatment, there was a further reduction in AChE enzyme levels. The percentage of reduction was 41% over the control. Thus there was a measurable change in the level of the enzyme in the treated rats as compared to the control rats. The withdrawal studies showed rapid recovery of the level of AChE but was still less (3.2%) than the control (Table 6.1). Thus we can predict that diplodiatoxin is a toxic compound.

6.4.3 Changes in the level of AChE in brain of female rats

In the sub-acute toxicity the female rats were more susceptible than the male rats. The biochemical studies after 10 days of sub-acute dosage administration revealed that in the female rats the AChE enzyme level reduced and the reduction was drastic as compared to the male rats, and the percentage of reduction was estimated to be 48% After 21 days of sub-acute administration of diplodiatoxin maximum reduction in the level of AChE enzyme was observed. In the withdrawal studies i.e. after 28 days, the level of enzyme AChE was increased but was still 4.3% lower than the control rats (Table 6.1). This shows that the diplodiatoxin is a toxic compound and female rats being severely affected.

Sex	Control (μM /hr /mg protein)	Treated (μM /hr / mg protein)
Brain AChE [c] **After 10 days of treatment**		
Male	16.3 ± 0.6	10.2 ± 0.7
Female	15.8 ± 0.6	10.8 ± 0.5
After 21 days of treatment		
Male	13.2 ± 0.7	7.7 ± 0.9*
Female	16.6 ± 1.0	8.6 ± 0.2*
After 7 days of post treatment		
Male	15.2 ± 0.3	14.7 ± 1.2
Female	13.5 ± 0.3	12.9 ± 0.5

Table 6.1 Sub-acute effect of diplodiatoxin on brain acetylcholinesterase in rats

1. The table shows the data of sub-acute effect of diplodiatoxin on rats
2. The parameter is acetylcholinesterase in brain
3. [c] is μ moles hydrolysed/min/mg protein
4. The table shows the deviation between control and treated rats
5. Data represent mean ± S.E (Standard Error) of three replicate
6. The *p<0.05 significantly different from control

6.4.4 Changes in the enzymatic levels in the serum of male rats

The level of ASAT, ALAT, AcP, AkP and RBC-AChE was determined after 10 and 21 days of the regular sub-acute administration of diplodiatoxin and also after seven days of the withdrawal. Sub-acute doses of diplodiatoxin caused an increase in the level of ASAT and ALAT in the serum of male rats (Table 6.2). Increase in the levels of ASAT and ALAT after 10 days were 5.32% and 5.90%, respectively over the control and was found to increase drastically after 21 days. ASAT and ALAT levels after 21 days of dose administration increased significantly to 22.12% and 11.95%, respectively over the control. Significant reduction in the level of AcP, AkP and RBC AChE was observed after 21 days of the treatment. The level of AcP in the serum of male rats was reduced to 18.4% over the control after 10 days. After 21 days it was further increased to 22.12%. Similar reduction was observed in the level of AkP, and the reduction was 32% after 21 days. Whereas, the level of AChE reduced gradually and was 6.87% less than the control rats after 10 days and after 21 days it was found to be 53.52% over the control. Maximum and rapid reduction was found in the level of AChE in the serum.

Withdrawal study showed a recovery trend in terms of the level of these enzymes in the serum but was deviating from the control rats. The levels of ASAT and ALAT were still 3.7% and 3.53% higher over the control rats, respectively. AcP, AkP and RBC AChE were 16.22%, 4.26% and 5.45%, lower than that in the control rats even after seven days of the withdrawal. In general the level of these enzymes showed a tendency to return to the normal levels. The values showed a narrow range of standard deviation from the mean ranging but were not significant.

Sl.No.	Parameter	Control	Treated
After 10 days of treatment			
1.	ASAT [a]	7.1 ± 0.08	7.5 ± 0.1
2.	ALAT [a]	6.4 ± 0.08	6.8 ± 0.1
3.	AcP [b]	6.5 ± 0.1	5.3 ± 0.3
4.	AkP [b]	32.5 ± 0.2	30.4 ± 0.4
5.	RBC AChE [c]	11.4 ± 0.05	10.7 ± 0.07
After 21 days of treatment (withdrawal study)			
1.	ASAT [a]	6.5 ± 0.2	7.9 ± 0.1
2.	ALAT [a]	6.3 ± 0.1	7.1 ± 0.08*
3.	AcP [b]	7.2 ± 0.2	4.9 ± 0.1
4.	AkP [b]	30.1 ± 0.5	28.5 ± 0.2
5.	RBC AChE [c]	11.2 ± 0.07	5.2 ± 0.4*
After 7 days of post treatment (withdrawal study)			
1.	ASAT [a]	6.2 ± 0.08	6.4 ± 0.2
2.	ALAT [a]	6.5 ± 0.1	6.7 ± 0.08
3.	AcP [b]	8.2 ± 0.3	6.9 ± 0.2
4.	AkP [b]	30.9 ± 0.2	29.6 ± 0.4
5.	RBC AChE [c]	11.1 ± 0.1	10.7 ± 0.2

Table 6.2 Sub-acute effects of diplodiatoxin on biochemical parameters in serum of male rat

1. The table shows the data of sub-acute effect of diplodiatoxin on serum of male rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [a] µ moles/hr/ml.
4. [b] µ moles/hr/ml
5. [c] µ moles hydrolysed/hr/ml
6. The table shows the deviation between control and treated rats
7. Data represent mean ± S.E (Standard Error) of three replicate
8. The *p<0.05 significantly different from control

6.4.5 Changes in the enzymatic levels in the serum of female rats

In response to the sub-acute dose of diplodiatoxin, the level of ASAT, ALAT, AcP, AkP and RBC-AChE were determined after 10 and 21 days and also after seven days of the withdrawal. A significant increase in the level of ASAT and ALAT in the serum of female rats was observed (Table 6.3). After 10 days of the treatment, the level of ASAT and ALAT increased to 22.91% and 8.53%, respectively over the control. The enzyme levels further increase drastically after 21 days. ASAT and ALAT levels after 21 days of dose administration increased significantly to 33.33% and 15.48%, respectively over the control. Unlike ASAT and ALAT, a significant reduction in the level of AcP, AkP and RBC AChE were observed after 10 and 21 days of the treatment. The level of AcP in the serum of male rats reduced to 8.43% over the control, after 10 days and after 21 days it further increased to 33%. Similar reduction was observed in the level of AkP, the reduction was 35% after 21 days. Level of AChE drastically reduced. It was 37.6% less than the control after 10 days and after 21 days it was found to be 53.19% over the control. Maximum and rapid reduction was found in the level of AChE in the serum.

When the dose was withdrawn, levels of these enzymes in the serum returned to normal but were not exactly the same as that of the control rats. Level of ASAT and ALAT were still 3% and 2.5% higher over the control, respectively. Levels of AcP, AkP and RBC AChE were 11.4%, 2.8% and 2.7% lower than that of the control rats after seven days of the withdrawal. Thus, it may be concluded from the withdrawal study that these enzymes showed a tendency to become normal and the female rats were more susceptible to diplodiatoxin than male rats. Results were significant at 5% level.

Sl.No.	Parameter	Control	Treated
After 10 days of Treatment			
1.	ASAT [a]	5.9 ± 0.08	7.3 ± 0.1*
2.	ALAT [a]	6.2 ± 0.08	6.7 ± 0.08*
3.	AcP [b]	6.2 ± 0.1	5.7 ± 0.5
4.	AkP [b]	32.0 ± 0.4	21.8 ± 0.2*
5.	RBC AChE [c]	11.7 ± 0.11	7.30 ± 0.3*
After 21 days of Treatment (withdrawal study)			
1.	ASAT [a]	6.3 ± 0.13	8.4 ± 0.08*
2.	ALAT [a]	6.3 ± 0.23	7.3 ± 0.2
3.	AcP [b]	6.9 ± 0.13	4.6 ± 0.1*
4.	AkP [b]	29 ± 0.27	18.9 ± 0.7*
5.	RBC AChE [c]	11.67 ± 0.12	5.5 ± 0.07*
After 7 days of post Treatment (withdrawal study)			
1.	ASAT [a]	6.5 ± 0.2	6.8 ± 0.1
2.	ALAT [a]	6.4 ± 0.08	6.5 ± 0.1
3.	AcP [b]	7.0 ± 0.1	6.2 ± 0.2
4.	AkP [b]	29.0 ± 0.2	28.2 ± 0.7
5.	RBC AChE [c]	11.3 ± 0.06	11.0 ± 0.05

Table 6.3 Sub-acute effects of diplodiatoxin on biochemical parameters in serum of female rat

1. The table shows the data of sub-acute effect of diplodiatoxin on serum of female rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [a] µ moles/hr/ml
4. [b] µ moles/hr/ml
5. [c] µ moles hydrolysed/hr/ml
6. The table shows the deviation between control and treated rats
7. Data represent mean ± S.E (Standard Error) of three replicate
8. The *p<0.05 significantly different from control

6.4.6 Changes in the enzymatic levels in the liver of male rats

The liver is also a target organ of toxins and the hepatic enzymes are excellent indicator of early toxicity. Levels of ASAT, ALAT and AkP drastically reduced during the entire period of sub-acute administration of diplodiatoxin, the level of AcP in treated male rats increased and was found to be higher than that of the control rats (Table 6.4). The level of ASAT reduced to 27.41% over the control in the liver of male rats after 10 days of treatment and continued to remain low throughout. ALAT also showed a similar reduction trend. The level of AkP was 11.76% lower than that of the control, after 10 days of the treatment and further reduced to 37.9% over the control after 21 days, thus there was continuously declining. Unlike these enzymes, the level of AcP increased drastically to 82% on day 10 and it increased further after day 21 in response to the diplodiatoxin. Results obtained were significant at 0.5% level.

Withdrawal study showed a recovery pattern, levels of these enzymes were near to the control after seven days of withdrawal but were still found to be lower than that of the control rats. The level of ASAT, ALAT and AkP were still 1.4%, 4.7% and 5.9% less than that of the control, respectively.

Results indicated that diplodiatoxin is toxic to rats and severely affected the levels of various enzymes in the liver during the period of sub-acute dosage. Except the level of AcP, all the enzymes tested drastically reduced. These enzymes tend to return to their normal level upon withdrawal of the dose within seven days.

Sl.No.	Parameter	Control	Treated
After 10 days of treatment			
1.	ASAT [d]	66.2 ± 1.8	48.1 ± 1.0*
2.	ALAT [d]	77.6 ± 0.9	63.1 ± 2.1*
3.	AcP [e]	83.3 ± 1.2	151.8 ± 2.1*
4.	AkP [e]	7.1 ± 0.4	6.2 ± 0.8
After 21 days of treatment (withdrawal study)			
1.	ASAT [d]	65.3 ± 4.3	52.0 ± 3.4
2.	ALAT [d]	71.9 ± 0.7	56.8 ± 0.9*
3.	AcP [e]	73.3 ± 1.9	108.8 ± 4.4*
4.	AkP [e]	5.8 ± 0.3	3.6 ± 0.2*
After 7 days of post treatment (withdrawal study)			
1.	ASAT [d]	62.0 ± 1.0	61.1 ± 2.2
2.	ALAT [d]	69.6 ± 5.4	66.2 ± 0.7
3.	AcP [e]	81.0 ± 3.6	82.1 ± 3.1
4.	AkP [e]	6.8 ± 0.2	6.3 ± 0.3

Table 6.4 Sub-acute effects of diplodiatoxin on biochemical parameters in liver of male rat

1. The table shows the data of sub-acute effect of diplodiatoxin on liver of male rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [d] μ moles/hr/mg protein
4. [e] μ moles/hr/mg protein
5. The table shows the deviation between control and treated rats
6. Data represent mean ± S.E (Standard Error) of three replicate
7. The *p<0.05 significantly different from control

6.4.7 Changes in the enzymatic levels in the liver of female rats

The female rats were found to be more susceptible to diplodiatoxin. Levels of ASAT, ALAT, AcP and AkP showed a similar pattern of change in response to sub-acute dose of diplodiatoxin as in the male rats (Table 6.5). The levels of ASAT, ALAT and AkP reduced during the entire period of dose administration and their levels were 25.7%, 7.7% and 54.3% over the control after 10 days of treatment, respectively. Thus, indicating that AkP level in the liver severely reduced. Continuation of dose up to 21 days showed a similar trend, but after 10 days the reduction was much more drastic. After 21 days, the levels of ASAT, ALAT and AkP were 33.18%, 21.82% and 69% lower than that of the control rats. Indicating that the females had a drastic decrease in the levels of these enzymes compared to the male rats.

Like male rats, female rats also showed an increase in the level of AcP in response to diplodiatoxin and the increase was 71.12% over the control after 10 days of the treatment. After 21 days of the administration of continuous sub-acute dose the level was 55% higher than the control rats.

Level of these enzymes after seven days of the withdrawal of diplodiatoxin were almost same as that of the control, but the recovery was not complete within seven days. Thus, it is concluded that female rats were more susceptible to diplodiatoxin and the changes in the level of ASAT, ALAT, AcP and AkP in liver were same as observed in male rats and withdrawal of diplodiatoxin dosage resulted in the recovery.

Sl.No.	Parameter	Control	Treated
After 10 days of treatment			
1.	ASAT [d]	63.7 ± 0.8	47.3 ± 1.8
2.	ALAT [d]	72.6 ± 1.5	67.0 ± 0.7
3.	AcP [e]	85.1 ± 2.1	145.8 ± 4.1*
4.	AkP [e]	7.0 ± 0.7	3.2 ± 0.3*
After 21 days of treatment (withdrawal study)			
1.	ASAT [d]	65.15± 0.7	43.5 ± 0.4*
2.	ALAT [d]	66.9 ± 2.0	52.3 ± 0.5*
3.	AcP [e]	80.0 ± 8.8	125.0 ± 4.8*
4.	AkP [e]	6.6 ± 0.6	2.0 ± 0.2*
After 7 days of post treatment (withdrawal study)			
1.	ASAT [d]	64.3 ± 0.9	60.4 ± 0.7
2.	ALAT [d]	67.0 ± 0.7	66.2 ± 1.3
3.	AcP [e]	78.8 ± 13.9	81.8 ± 6.9
4.	AkP [e]	6.0 ± 0.3	5.7 ± 1.0

Table 6.5 Sub-acute effects of diplodiatoxin on biochemical parameters in liver of female rat

1. The table shows the data of sub-acute effect of diplodiatoxin on liver of female rats
2. The parameters are ASAT, ALAT, AcP, AkP and RBC AChE
3. [d] μ moles/hr/mg protein
4. [e] μ moles/hr/mg protein
5. The table shows the deviation between control and treated rats
6. Data represent mean ± S.E (Standard Error) of three replicate
7. The *p<0.05 significantly different from control

6.4.8 Statistical analysis

The changes in the enzyme levels caused by diplodiatoxin in serum and liver were statistically significant when compared to the changes in male and female rats. The results suggested sexual dimorphism.

The correlation coefficient studies between serum and liver showed a negative correlation in ASAT, ALAT, AcP and a positive correlation in AkP. In sub-acutely treated rats a high degree of correlation was observed in male ASAT (-0.49), ALAT (-0.83), AcP (–0.69) and AkP (+0.64) and female ASAT (-0.82), ALAT (-0.71), AkP (+0.90), Acp (-0.64).

6.5 Discussion

Sub-acute toxicity is also important for further confirmation of the toxicity of diplodiatoxin. It was observed that sub-acute dosage of diplodiatoxin did not cause mortality but, mortality was observed in acute study. Similar results were obtained when fumonisin was fed to growing barrows (Harvey *et al.*, 2002). In the behavioural responses gradual loss in body weight and feed intake was observed. Rats also showed dullness, irritation, tremors and convulsions indicating growth retardness and stressed conditions. Similar results were obtained in the acute study.

A significant reduction in the level of AChE in the brain was observed after 10 days of the administration of sub-acute dose of diplodiatoxin and further reduced after 21 days, The AChE level also reduced drastically in RBC of male and female rats after 10 days and 21 days, similar changes in the levels of these enzymes were observed in the acute study.

ASAT and ALAT are membrane enzymes, they are simple and reliable for the detection of hepatic necrosis (Kaur *et al.*, 2000). The present study demonstrated an increase in ASAT and ALAT in serum, whereas in the case of liver these enzymes decreased in sub-acutely treated rats. The increased levels of these enzymes in the serum and the parallel decrease in the liver

might indicate liver necrosis caused by diplodiatoxin treatment. Similar results were observed when rats were treated with aflatoxin B_1 (3mg/kg body weight) for 28 days (Yin *et al.*, 1980). Thus the results confirm neurotoxicity of diplodiatoxin. The increase in the level of these enzymes in liver is similar to the activity of cypermethrin on albino rats when treated for 6 months (sub-acute study) (Shakoori *et al.*, 1988). The changes observed in the activities of liver ASAT and ALAT of female rats were significant when compared to male rats in sub-acute treatment and also the RBC AChE drastically reduced in the sub-acutely treated rats on day 10 and it further reduced after day 21. With the reference to the enzymes ASAT and ALAT sexual dimorphism was observed in the treated rats. Similar sexual dimorphism was observed in MCP treated rats (Janardhan and Sisodia, 1990). The correlation studies indicate a negative correlation between serum and liver in both males and females. These alterations demonstrated that these enzymes (ASAT, ALAT) increased in serum with parallel decrease in liver. Therefore, results of the present study suggest that oral administration of diplodiatoxin resulted in severe alterations in serum and also in the cellular activities of vital organs such as the liver, causing hepatic and neurological damages.

The sub-acute dosage of diplodiatoxin caused serum AcP, AkP and liver AkP to decrease and liver AcP to increase in both male and female rats. Similar significant inhibition of AcP was reported in methyl mercury treated rats (Vinay *et al.*, 1992). As in the present study a decrease in serum AkP was observed in rats treated with D-tagatose (Kruger *et al.*, 1999). A decrease in the serum AkP activity has been reported in case of severe anaemia and arrested growth in the children (Varley *et al.*, 1980). The decreased activity of serum AcP and AkP observed in the present study may be due to slow enzyme synthesis or due to liver damage, whereas the increased liver AcP activity might be due to increased synthesis of this enzyme in response to stress conditions.

The changes caused by diplodiatoxin in serum and liver were statistically significant when male and female rats were compared. The results suggest sexual dimorphism. Similar sexual dimorphism was observed in quails when treated with fenitrothion (Szubartowska and Gromysz-Kalkowska, 1992). The correlation studies also reveal a high degree of correlation for liver AkP activity in female rats. Results suggest that diplodiatoxin interacted with these

enzymes to cause a simultaneous decrease in serum and liver AkP. However, the decrease in serum AcP and a parallel increase in liver AcP provide the evidence that the diplodiatoxin had measurable harmful effects on serum and vital organs such as liver and could impair the cellular, physiologic and metabolic activities of the individual. Thus, the hepatotoxic nature of diplodiatoxin is also confirmed.

Activity of all these enzymes in brain, liver, serum and RBC recovered to near normal after 28 days of post-treatment in both male and female rats. This clearly revealed the reversal of the toxicity once the diplodiatoxin was removed. Likewise a similar reversal was also observed in the acute toxicity and also when rats were sub-acutely treated with RPR-V (Khan *et al.*, 2001)

Results obtained from the present study confirm the hepatotoxic and neurotoxin nature of diplodiatoxin. Moreover, diplodiatoxin can be considered as a toxic mycotoxin and it should not be ignored due to its severe effects. In the sub-acute studies conducted so far the paralytic syndrome was not observed so further investigations should be conducted by future workers to determine if diplodiatoxin combines with other toxic metabolites to cause these symptoms or whether *S. maydis* produces certain toxins other than diplodiatoxin which are capable of producing paralytic syndrome in the animals. So there arises a need to explore these aspects.

Chapter 7

GENETIC RELATIONSHIP OF TOXIN PRODUCING AND NON- PRODUCING ISOLATES OF *STENOCARPELLA MAYDIS* ASSESSED BY ITS SEQUENCING

7.1 Introduction

Molecular techniques represent a powerful tool for the identification of the genetic variation among various organisms, to confirm the taxonomic placement and to clarify the taxonomic controversies. The technique of random amplified polymorphic DNA (RAPD) has become increasingly popular due to its speed, simplicity and low cost, although there are criticisms (Staub *et al.*, 1996). An alternative approach for genetic diversity and phylogenetic analysis has been the use of ribosomal DNA (rDNA) genes. In fungi, these genes are usually found in multiple copies with regions that are very highly conserved (White *et al.*, 1991) joined together by spacers that can be highly variable. The internal transcribed spacers (ITS) and intergenic spacer (IGS) evolve at a more rapid rate. The ITS1 and ITS2 are found between the small (18S) and the large (28S) ribosomal subunit genes and are separated by 5.8S ribosomal subunit genes (Fig 7.1). The ITS1 and ITS2 regions show variability at intraspecific levels but, the 18S, 5.8S and 28S genes do evolve at a slow rate and are used for sorting distantly related organisms. The sequence variations in the rDNA genes are used for classifying organisms at the level of classes and/or families (Burns *et al.*, 1991). Variations within ITS sequences can be most informative for identifying closely related organisms (Burns *et al.*, 1991; Hyun and Clark, 1998).

The PCR amplification of the ITS region followed by restriction fragment length polymorphism (RFLP), has been used to explore variation in fungi (Redecker *et al.*, 1997; Aradhya *et al.*, 2001, Heusser *et al.*, 2002). Sequencing of this region has also been used to detect and quantify variability in several species or a group of fungi (Lloyd-MacGilp *et al.*, 1996). The ITS region has also been used to study the variation at intrageneric level (Arenal *et al.*, 2000) and intraspecific level (Angeles Vinuesa *et al.*, 2001), among the various

isolates of similar species (Chambers *et al.*, 2000, Lyons *et al.*, 2000, Gomes *et al.*, 2000), and sub groups and ecotypes of several fungal species (Salazar *et al.*, 2000).

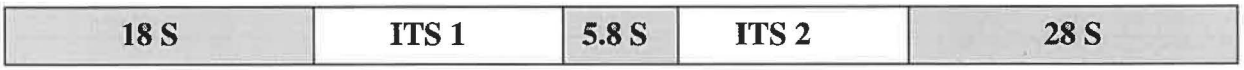


Fig 7.1 The internal transcribed spacer (ITS) region showing that ITS 1 and ITS 2 are separated by 5.8S ribosomal subunit genes and are located between 18S and 28S ribosomal subunit genes

To date molecular detection systems have been successfully established for a number of organisms. They are sensitive tools, which generate enough data for reliable phylogenetic studies. For example, Salazar *et al* (2000) designed primers based on specific rDNA-ITS sequences for PCR detection of the various subgroups and ecotypes of *Rhizoctonia solani.*

Stenocarpella maydis is an economically important plant pathogen and has a worldwide distribution on maize. The fungus produces a mycotoxin called diplodiatoxin. When *S. maydis* is consumed by animals it causes a neurological disorder called diplodiosis. Production of diplodiatoxin is a characteristic feature of the fungus *S. maydis*. No literature is available for the identification of the phylogenetic relationships between different species of the genus *Stenocarpella* on the basis of molecular approaches. The ITS region of *Diplodia quercina (S. quercina)* has been partially sequenced (Jacobs and Rehner, 1998). From the studies conducted earlier it was shown that certain isolates of *S. maydis* collected from different geographical regions in South Africa did not produce diplodiatoxin (Rao and Achar, 2001). Thus, it is important to understand the genetic relationship between these isolates.

In the present chapter sequencing of the ITS region is undertaken to analyse the genetic diversity in the ITS1-5.8S-ITS2 region of the rDNA of the various isolates of *S. maydis* collected from different maize growing areas in South Africa.

7.2 Materials

I Growth of the fungus

Medium used

Potato Dextrose Broth (PDB)

Potato Extract	20% (w/v)
Dextrose	2% (w/v)
Distilled water	1000ml (v/v)

II DNA Extraction

Chemicals

Agarose	1.2% (w/v)
CH_3COONa	3M
$CHCl_3$: Isoamyl alcohol	24:1 (v/v)
Ethanol	70% (v/v)
EDTA	0.5M (pH 8)
Isopropanol	100%
Liquid Nitrogen	
Lysis Buffer:	
(50mM Tris HCl (pH 7.2), 50mM EDTA (pH 8), 3% SDS, β-merceptaethanol 1%)	
RNase	10mg/ml
Sodium Hydroxide	100
Saturated Phenol	pH 8
SDS	20%
Tris HCl	2M (pH 7.2)

III Polymerase Chain Reaction (PCR) and Sequencing

Chemicals

PCR Master Mix (Promega Co. Ltd.)

Primer (0.2 p mol) (ITS1, ITS4)

1x Sequencing Buffer (80mM Tris-HCl and 2mM $MgCl_2$) (Applied Biosystems)

Terminator Ready Reaction Mix

10x loading dye

Ethidium Bromide

TBE, TE Buffer

Kits used

Amersham Pharmacia Biotech GFX™ Gel band purification kit

DNA Sequencing Kit, Big Dye™ Terminator V 3.0 Cycle, Sequencing Ready Reaction (Applied Biosystems)

7.3 Methods

7.3.1 Fungal isolates

Isolates of *S. maydis* viz., MC43, C1A, MC50, C3C, D72, D79, D78, 2Y, 8Y, CH3 and U2 representing a group of toxin producing and non-toxin producing isolates from various regions of South Africa were used for the present study. Collection, maintenance, and source of these isolates are described in chapter 2 in Sec 2.3.1 and Table (2.1). Back-up cultures were also maintained at – 20 °C and were renewed after every two months.

7.3.2 Preparation of DNA

Fungal isolates of *S. maydis* were inoculated in 100ml of Potato Dextrose Broth (PDB) in a 250ml flask and incubated at 26 °C, 100rpm in the dark for 96 hours. Cultures were vacuum filtered through Whatman filter paper (No. 1) to collect the mycelium. Genomic DNA was extracted using the method described by Lee and Taylor (1990) with some modifications. Mycelium (200mg) was grounded to a fine powder in liquid nitrogen using a pestle and mortar and transferred into an eppendorf tube containing 500µl of the lysis buffer. The mixture was vortexed until a homogenous suspension was obtained and incubated at 60 °C for one hour. After incubation, 500µl of saturated phenol (pH 8.0): chloroform: isoamyl alcohol (25:24:1 v/v) was added and the contents were mixed gently. Samples were centrifuged at 10,000rpm for 15 minutes and the aqueous phase containing the DNA was transferred to a new tube. To the aqueous phase 50µl volume of sodium acetate and 300µl of isopropanol was added, mixed gently by inverting the tube, and the DNA was pelleted by

centrifugation at 10,000rpm for 15 minutes. The pellet was air dried and dissolved in 100µl of TE buffer. To the DNA solution 30µg of RNase A was added and incubated at 37 °C for one hour. After the RNase treatment the phenol extraction was performed to eliminate the RNase. The final volume was made up to 200µl by adding 100µl of sterile water and the DNA was precipitated by adding 50µl of 1.5M sodium acetate and 500µl volume of absolute ethanol, followed by centrifugation at 13,000rpm for 15 minute. The supernatant was discarded and pellet was rinsed with 70 % (v/v) ethanol to remove residual salts. The pellet was air dried at room temperature and was re-suspended in 100µl of TE buffer (pH 8.0) and stored at –20 °C. DNA was quantified at A_{260} nm using a UV-spectrometer (Spectronic Genesys 5) and also visually estimated by staring with the ethidium bromide after the electrophoresis on an agarose gel (1.2%).

7.3.3 Designing of Primers for PCR Amplification of the ITS 1-5.8S-ITS2 region

For the PCR amplification of the ITS1-5.8S-ITS2 region of the genomic rDNA from the various isolates of *S. maydis*, universal primer pairs, P_{ITS1} (5'-TCCGTAGGTGAACCTGCGG-3') and P_{ITS4} (5'-TCCTCCGCTTATTGATATGC-3') were used (White *et al.*, 1991). Primers were synthesised using Applied Biosystems 391 DNA Synthesiser. Primers were quantified at A_{260} nm using a UV-spectrophotometer.

7.3.4 PCR Amplification

The ITS1-5.8S-ITS2 region of genomic rDNA of the isolates of *S. maydis* was amplified by PCR. The 25µl reaction mixture for PCR amplification contained the following: 25ng of the genomic DNA, 1 X DNA polymerase buffer, 0.5pM of each P_{ITS1} and P_{ITS4} primers, 1mM $MgCl_2$, 0.2mM of dNTPs mixture and 0.5U Taq DNA polymerase (Promega Co Ltd). The amplifications were performed in a Perkin-Elmer (Gene Amp 9700) PCR System. The thermocycler was programmed for one cycle of initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, 58 °C for 1 minute and 72 °C for 2 minutes, and a final extension at 72 °C for 10 minutes. The annealing temperature was also tested at 55 °C keeping the remaining conditions similar to those kept for 58 °C. Amplification

products were electrophoresed through 1.2% (w/v) agarose gel in 1X TBE buffer, stained with ethidium bromide and photographed using UV transilluminator (UVP image store 5000) and UVP Digital System.

7.3.5 Elution of the PCR product from the gel bands

The PCR product was excised from the agarose gel using a sterile blade and was cut into several smaller pieces. This was transferred to 1.5ml microcentrifuge tube and 300µl of capture buffer was added. Tubes were vortexed vigorously and then incubated at 55 °C until agarose was completely dissolved (5 to 10 minutes). The content of the tube was transferred to the GFX column, incubated for 1 minute at room temperature, and then centrifuged at 13,000rpm for 30 seconds. The flow-through was discarded. The GFX column was placed back inside the collection tube. Then 500µl of Wash Buffer was added to the column and centrifuged at 13,000 rpm for 30 seconds. The flow-through was discarded and GFX column was placed in a new eppendorf tube. To the column, 50µl of TE buffer (pH 8.0) was added, incubated at room temperature for 1 minute and centrifuged at 13,000rpm for 1 minute to collect the sample in the eppendorf tube. Amersham Pharmacia Biotech GFX^{TM} Gel band purification kit was used for this. The concentration of the DNA was quantified at 260nm using an UV-spectrophotometer and The DNA was diluted to 20ng/µl in TE and then was stored at –20 °C.

7.3.6 DNA Sequencing

The 10µl sequencing reaction mixture contained 20ng of the DNA (PCR amplified product), 2µl of Terminator Ready Reaction Mix, 1 X Sequencing buffer and 0.32pM of P_{ITS1} primer or P_{ITS2}. Amplifications were performed in an Applied Biosystems (Gene Amp 9700) PCR System. The thermocycler was programmed for 25 cycles of 94 °C for 15 seconds, 58 °C for 5 seconds and 60 °C for 4 minutes. The content of the tube was transferred to a sterile 1.5ml eppendorf tube. Total volume of the reaction was raised to 36µl with deionized water and 64µl of non-denatured 95% ethanol was added to it and the mixture was incubated at room temperature for 30 minutes. The extension products were pelleted by centrifugation at

13,000rpm for 25 minutes at room temperature and supernatant was aspirated carefully. The pellet was rinsed twice with 70% ethanol to remove unincorporated dye terminators. The pellet was air dried and stored in -20 °C. Before sequencing, DNA pellet was dissolved in 12µl of the template suspension reagent, denatured at 95 °C for 2 minutes, immediately placed on ice for 5 minutes and analysed on Applied Biosystems DNA sequencer (ABI Prism 310).

7.3.7 Sequence analysis

Sequences were aligned with program CLUSTAL-X and the aligned sequences were visually inspected. For a distance-based phylogeny, trees were drawn using the program (Clustal-X, NJ Tree, Bootstrap tree). For the phylogenetic placement of the *S. maydis* isolates the closely related ascomycete members were taken from GenBank (Table 7.1) and the alignment was performed by Clustal-X. The Neighbour Joining (NJ) trees were drawn and the Bootstrap values were analysed for accuracy.

7.4 Results

7.4.1 PCR Amplification of ITS1-5.8S-ITS2 region of rDNA

PCR amplification of the ITS1-5.8S-ITS2 region of all the isolates of *S. maydis* yielded a 560bp fragment. The annealing temperature was optimised and the specificity and yield of the PCR product was optimal at 58 °C (Fig 7.2). The MC43 isolate was used in standardising PCR conditions and the optimal conditions were then applied successfully to all the isolates (Fig 7.3).

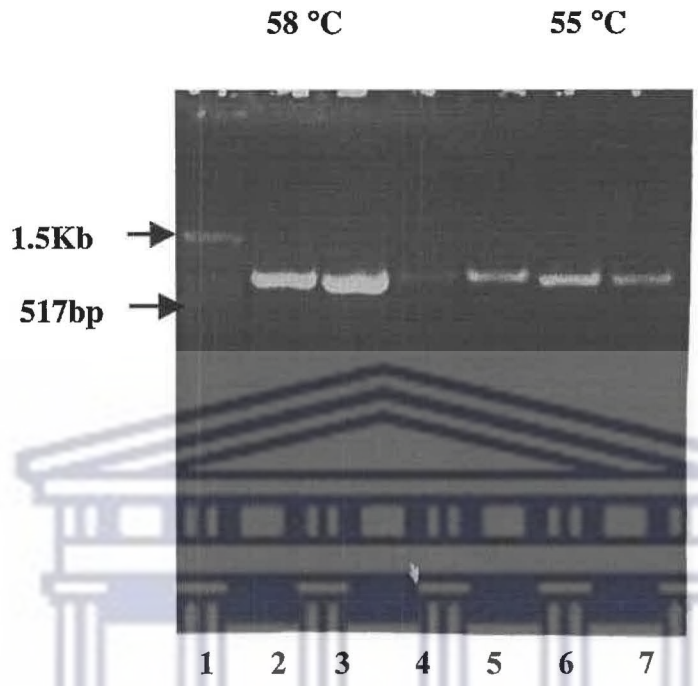


Fig 7.2 Standardisation of the PCR conditions for the amplification of the ITS region

1. The isolate used is MC43
2. The fragment is 560bp in length.
3. The lane 2, 3 represent the temperature at 58 °C
4. The lane 4, 5, 6 represent the temperature at 55 °C
5. The annealing temperature optimised was 58 °C
6. The marker used is the lane no 1 is the PTZ from Dept of Biotechnology, University of Western Cape

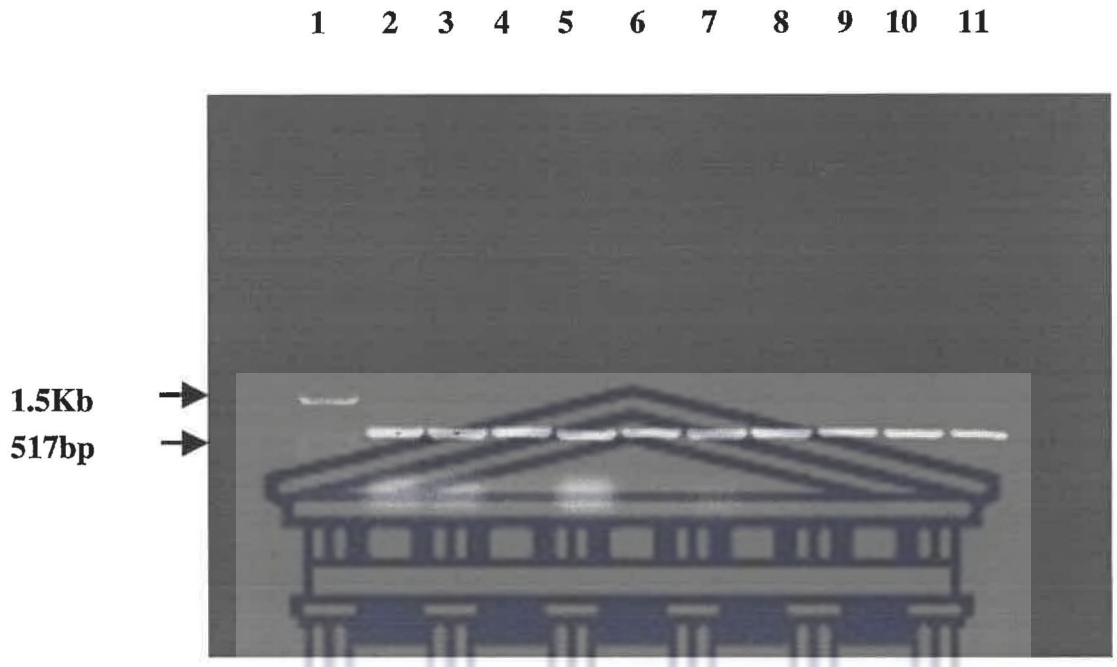


Fig 7.3 PCR amplification of the ITS region of the various isolates of *S. maydis*

1. The PCR amplifications of *S. maydis* isolates from lane one onwards Marker=1, MC50=2, C1A=3, C3C=4, D72=5, D78=6, D79=7, CH3=8, U2=9, 2Y=10, 8Y=11)
2. The PCR amplification is the ITS1-5.8S-ITS2 region
3. The fragment is 560bp in length.
4. The marker used is the lane no 1 is the PTZ from Dept of Biotechnology, University of Western Cape

7.4.2 The ITS1-5.8S-ITS2 region of rDNA of the various isolates of *S. maydis*

The sequence PCR amplifications were performed in an Applied Biosystems (Gene Amp 9700) PCR System and the sequencing was done in Applied Biosystems DNA sequencer (ABI Prism 310). The sequences of the 11 isolates obtained are as follows.

1. MC50

ATTGCTGGAACGCGCCCCAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTG
ACTGTTGCCTCGGCACAGGCCGGCCCCCCATGGGGGGCCCCTCGGAGACGAGGA
GCAGGCCCGCCGGCGGCCAAGTTAACTCTTGTTTTTATACCGAAACTCTGAGCA
GAAAACACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTG
TTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTAC
AGAAGGCAGGCCCTGAAATTCAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGT
AGTTAAACCCTCGCTCCGGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAA
CCTCTGAAAGTT

2. MC43

ACCCTTTGTGAACTTATACCTGACTGTTGCCTCGGCACAGGCCGGCCCCCCATGG
GGGGCCCCTCGGAGACGAGGAGCAGGCCCGCCGGCGGCCAAGTTAACTCTTGTT
TTTATACCGAAACTCTGAGCAGAAAACACAAATGAATCAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTG
GTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTG
GTGTTGGGGCACTGCCTGTACAGAAGGCAGGCCCTGAAATTCAGTGGCGAGCTC
GCCAGGACCCCGAGCGCAGTAGTTAAACCCTCGCTCCGGAAGGCCCTGGCGGTG
CCCTGCCGTTAAACCCCCAACCTCTGAAAGTTTGACCTCGGATCAGGTAGGAAT
ACCCGCTGAACTTAAGCTATCAATAAGCGGGAGG

3. C1A

CAGGCGCACCCAGAAACCCTTTGTGAACTTATACCTGACTGTTGCCTCGGCACA
GGCCGGCCCCCCATGGGGGGCCCCTCGGAGACGAGGAGCAGGCCCGCCGGCGG
CCAAGTTAACTCTTGTTTTTATACCGAAACTCTGAGCAGAAAACACAAATGAAT
CAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAA
CCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTACAGAAGGCAGGCCCTGA
AATTCAGTGGCGAGCTCGCCAGGACCCCGAGCGCAGTAGTTAAACCCTCGCTCC
GGAAGGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACCTCTGAAAGTTTGACC
TCGGATCAGGTAG

4. D79

TCAACCCTGTGAACATACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTG
AAACGGGCCGCCCCCCGCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTCT
GAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGAT
CGGCGGAGCCCCCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGC
CGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAGCGGCGCGGCC
ACGCCGTAAAACACCCAACTCTTCTGAAGTTGACCTCAATCAGGTAGGAA

5. CH3

TACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTGAAACGGGCCGCCCCC
GCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTCTAGAGTAAAACAAGCA
AATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATC
TTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTC
ATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCCCCCCGT
GGGCACACGCCGTCCCCCAAATACTGTGGCGGTCCCG

6. D72

ACTTCAGAAGAGTTGGGTGTTTTACGGCGTGGCCGCGCCGCTCTCCAGTCGCGA
GGTGTTAGCTACTACGCGATGGAAGCTAGCGGCGGGACCGCCACTGTATTTGGG
GGACGGCGTGTGCCCACGGGGGGCTCCGCCGATCCCCAACGCCAGGCCCGGGG
GCCTGAGGGTTGTAATGACGCTCGAACAGGCATGCCCGCCAGAATACTGGCGGG
CGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATTACT
TATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAGCCAAGAGATCCGTTGTTGA
AAGTTTTAATTTATTTGCTTGTTTTACTCA

7. D78

TCAACCCTGTGAACATACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTG
AAACGGGCCGCCCCCCGCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTCT
GAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGAT
CGGCGGAGCCCCCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGC
CGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAGCGGCGCGGCC
ACGCCGTAAAACACCCAACTCTTCTGAAGTTGACCTCAATCAGGTAGGAA

8. 2Y

TCAACCCTGTGAACATACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTG
AAACGGGCCGCCCCCCGCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTCT
GAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGAT
CGGCGGAGCCCCCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGC
CGCA

9. 8Y

TCAACCCTGTGAACATACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGTG
AAACGGGCCGCCCCCGCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTCT
GAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCA
TGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGAT
CGGCGGAGCCCCCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCGC
CGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAGCGGCGCGGCC
ACGCCGTAAAACACCCAACTCTTCTGAAGTT

10. U2

CTGAACCCTGTGAACATACCTAAACGTTGCTTCGGCGGGAATAGACGGCCCCGT
GAAACGGGCCGCCCCCGCCAGAGGACCCTTAACTCTGTTTCTATAATGTTTCTTC
TGAGTAAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTC
AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGC
ATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCCCCGGGCCTGGCGTTGGGGA
TCGGCGGAGCCCCCCGTGGGCACACGCCGTCCCCCAAATACAGTGGCGGTCCCG
CCGCAGCTTCCATCGCGTAGTAGCTAACACCTCGCGACTGGAGAGCGGCGCGGC
CACGCCGTAAAACACCCAACTCTTCTGAAGTTGACCTCGAATCAGGTAGGAATA
CCCGCTGAACTTAAGCATATCAATAAG

11. C3C

CGTTGCTTCGGCGGGAATAGACGGCCCCGTGAAACGGGCCGCCCCCGCCAGAG
GACCCTTAACTCTGTTTCTATAATGTTTCTTCTAGAGTAAAACAAGCAAATAAAT
TAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGA
AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACA
ACCCTCAGGCCCCCGGGCCTGGCGTTGGGGATCGGCGGAGCCCCCT

Table 7.1 The ITS sequences of the ascomycete members and closely related species used in the phylogenetic analysis

1. The ITS sequence of the ascomycete members and their accession numbers were taken from GenBank (http://www.ncbi.nlm.nih.gov/)
2. The ITS region of the closely related species to *S. maydis* isolates done by BLAST search
3. The following strains were used in phylogenetic analysis using a CLUSTAL-X programme

S. No	Ascomycete members From Database	Accession Number
1	*Botryosphaeria vaccinni*	AF243404
2	*Botryosphaeria stevensii*	AF027754
3	*Claviceps viridis*	CVI133404
4	*Claviceps africana*	CAF011784
5	*Cryophonectria cubensis*	AF046895
6	*Cylindrocladium pacificum*	AF493965
7	*Cylindrocladium colhounii*	AF231954.1
8	*Cylindrocladium spathula*	AF307350
9	*Cylindrocladiu perseae*	AF307352
10	*Diaporthe phaseolorum*	AF001025
11	*Diaporthe spp (MS 704)*	AF153737
12	*Diaporthe helianthi*	AJ312365
13	*Diplodia quercina*	AF027753
14	*Diplodia spp*	AJ292761
15	*Fusarium spp*	AF178404
16	*Fusarium oxysporum*	AF440540
17	*Fusarium fujikuroi*	FFU34557
18	*Fusarium tricinctum*	AF111054.1
19	*Fusarium sporotrichioides*	FSU85541
20	*Gibberella avenacea*	AF009186.2
21	*Gibberella tricinctum*	AF111066.1
22	*Leucostoma personni*	AF191178
23	*Nectria haematococca*	AF130142
24	*Oidiodendron maius*	AF307770
25	*Phomopsis spp*	AF317584
26	*Phomopsis spp*	AF317582
27	*Sphearopsis sapinea*	AF243409
28	*Trichoderma herzianum*	AJ507137

7.4 3 Alignment of ITS sequences

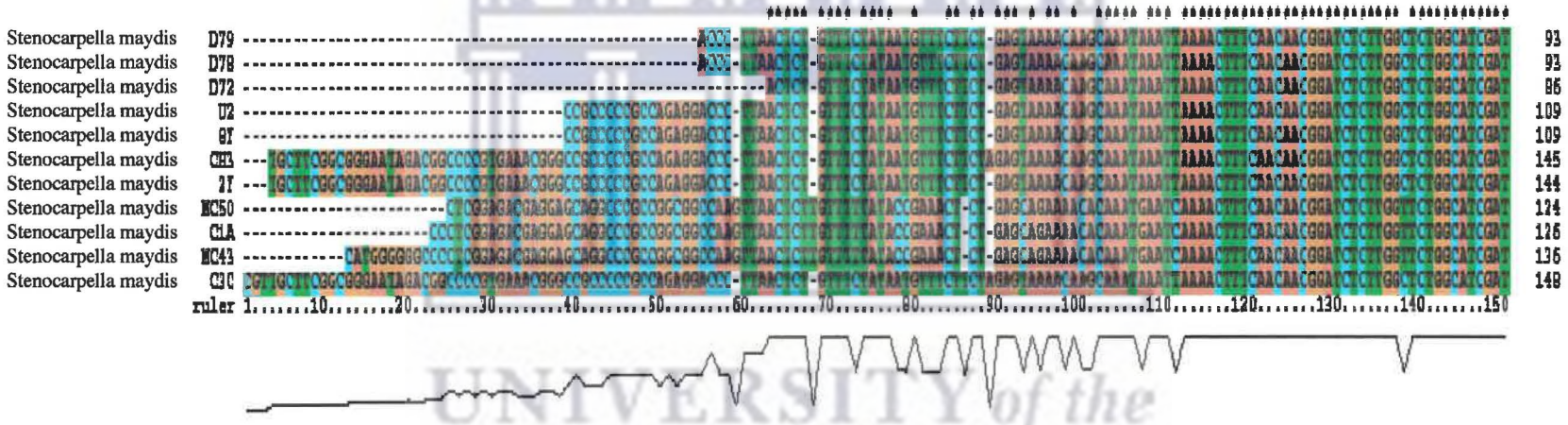
The Alignment of the ITS region of the 11 isolates of the *S. maydis* showed a clear demarcation among the isolates. The isolates C1A, MC43 and MC50 were identical and D79, D72, D78, 2Y, 8Y, CH3, U2 and MC34 isolates showed similarity to each other. The majority of base differences between the isolates were found in the ITS1 and ITS2 regions and were generally base substitutions. In most isolates, there were three small insertions/deletions (1-3 bases) and a large insertion/deletion of 10-11 bases. This large stretch of sequences was present only in C1A, MC43 and MC50 isolates. The 5.8S region was similar and conserved but there was a base deletion and insertion at two places so the isolates were placed into two groups based on these two differences (Fig 7.4). When the NJ tree was drawn it branched into two groups separating the toxin producing isolates and a group with non-toxin, moderate and high toxin producing isolates. Bootstrap values further proved the accuracy of the data (Fig 7.5).

7.4.4 Phylogenetic Relationship

In the phylogenetic analysis the ITS sequences of isolates MC43, MC50 and C1A showed similarity to the sequence data of the ITS1-5.8S-ITS2 region of rDNA of other ascomycete members *viz., Diaporthe spp, Phomopsis spp* while the non-toxin producing isolates D72, D78, D79, the moderate and the high toxin producing isolates showed very close relationship with *Fusarium spp* and *Nectria spp* (Fig 7.6, 7.7). The similarity shown by the toxin producing isolates and *Diaporthe* was around 550bp and the similarity shown by the non-toxin producing isolates with *Fusarium spp* was around 540bp. So the ITS sequence data reveals that the toxin producing isolates were close to *Diaporthe* strains and the isolates which are non-toxin producing may be either *Fusarium* or *Nectria* species. The species *Diaporthe, Fusarium, Nectria* and *Phomopsis* have a wide range of hosts and it is likely that these isolates were misidentified as *S. maydis* species due to their morphological characters. It may be also possible that these isolates belong to *S. maydis* but the factors like recombination and other geographical origin may have reassorted the *S. maydis* into a different genetic background.

Fig 7.4 Alignment of ITS1-5.8S-ITS2 region of rDNA of *S. maydis* isolates

1. The figure shows the alignment of ITS region
2. The isolate used are 11 isolates of *S. maydis*
3. The programme used for alignment is CLUSTAL-X
4. The isolates are aligned into two groups and the bootstrap values are given to show the accuracy of the data



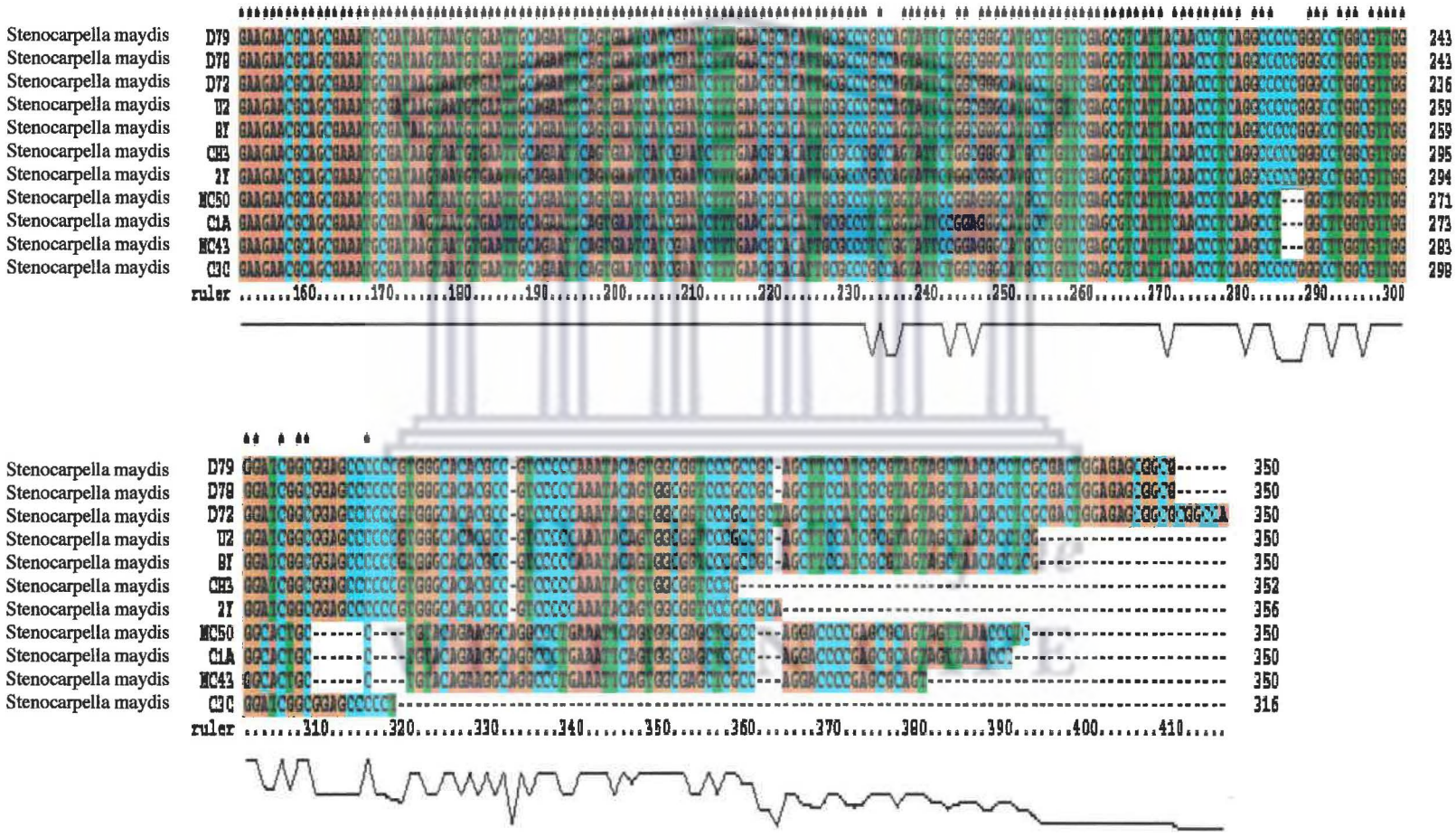
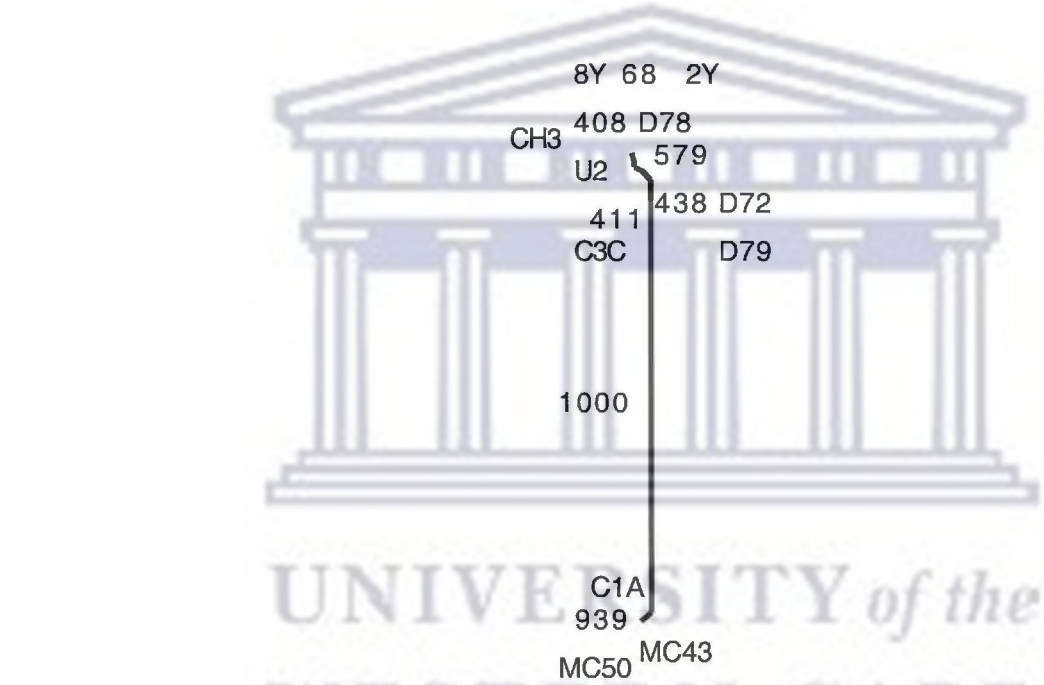


Fig 7.5 Phylogenetic analysis of the *S. maydis* isolates

1. The figure shows the phylogenetic analysis
2. The isolate used are11 isolates of *S. maydis*
3. The tree showing the phylogenetic analysis by NJ (Neighbour joining) tree
4. The bootstrap values are given to prove the accuracy of the tree
5. The programme used for the analysis is CLUSTAL-X



0 1

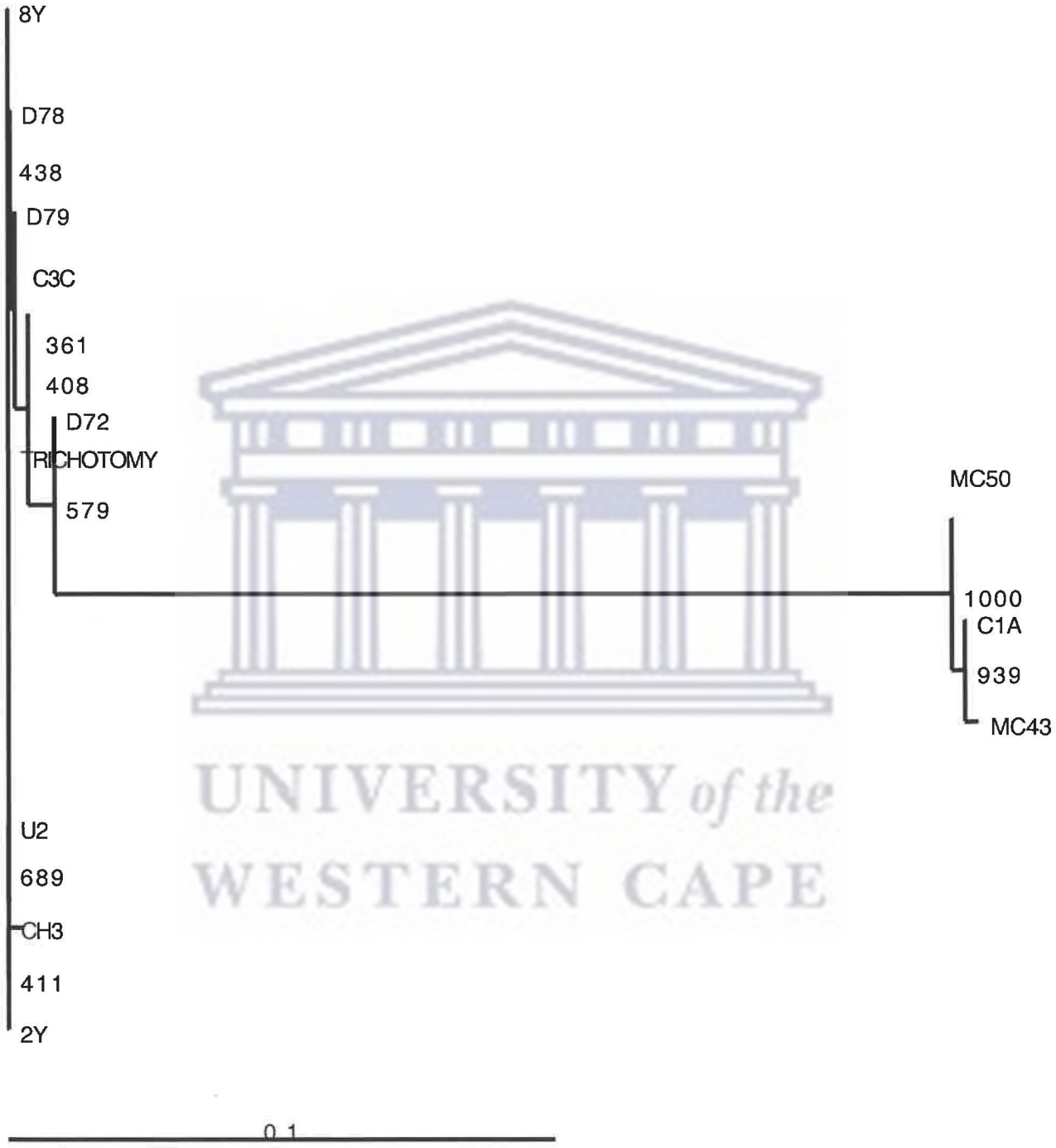
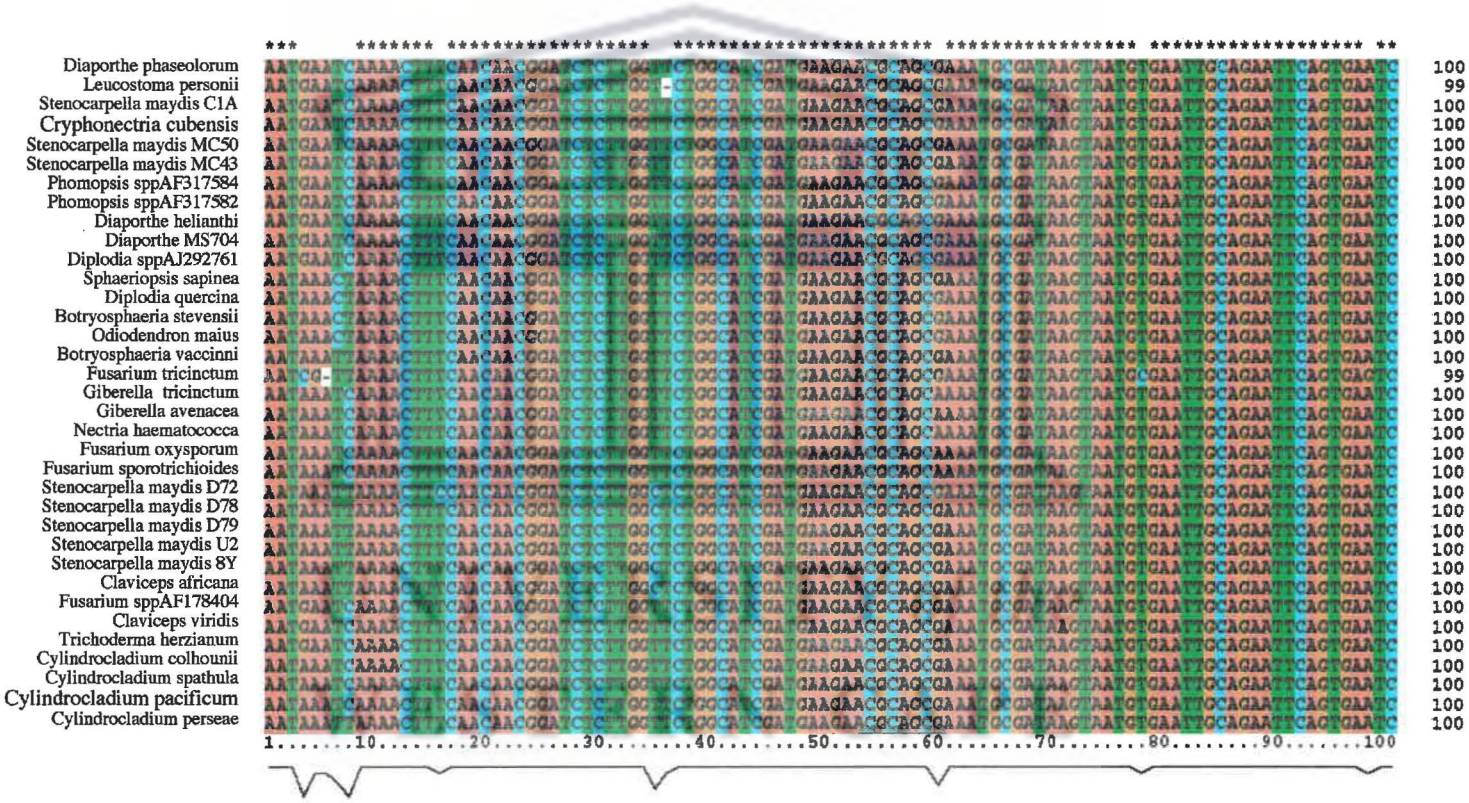
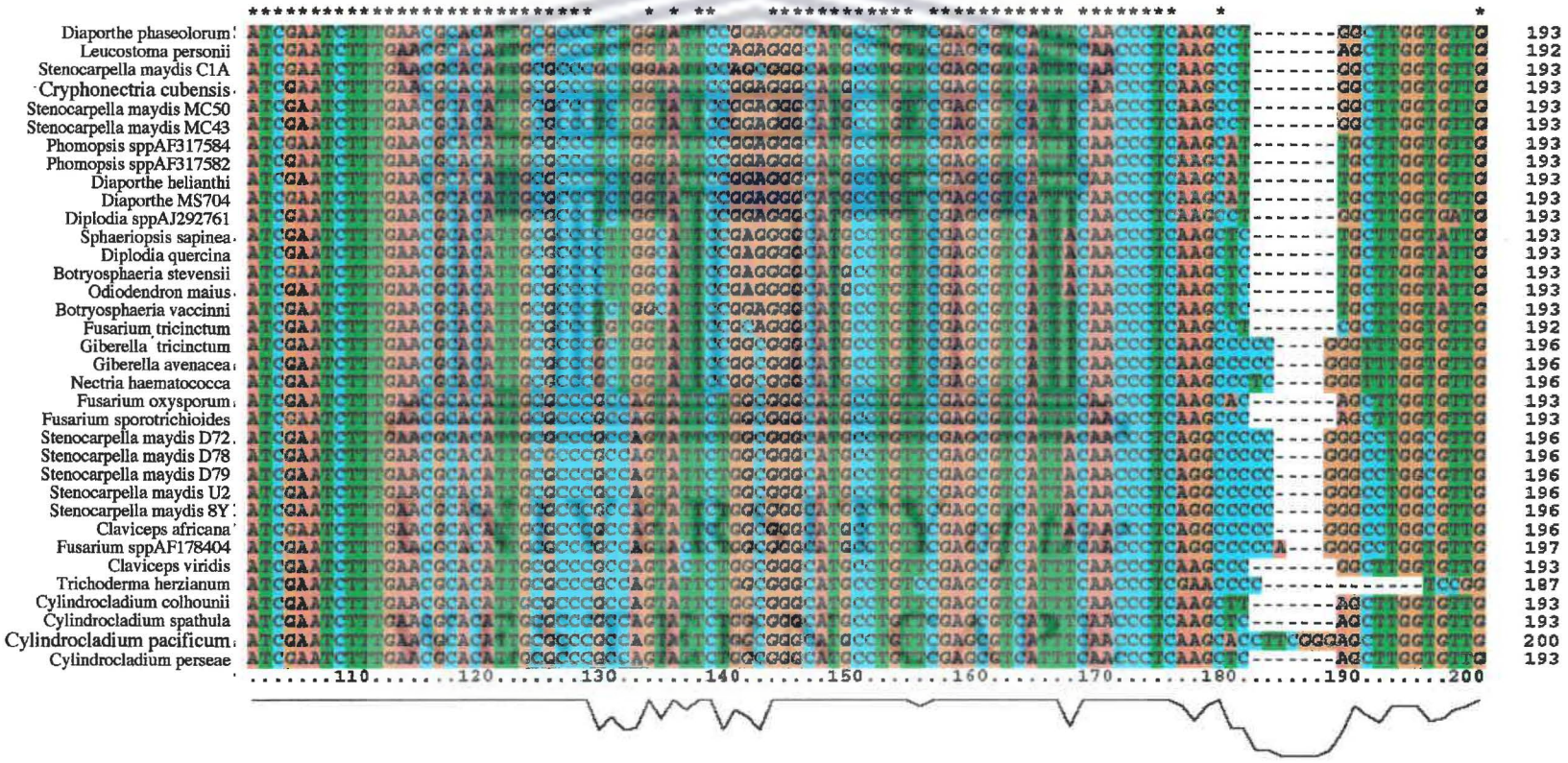


Fig 7.6 Alignment of ITS1-5.8S-ITS2 region of rDNA of *S. maydis* isolates with other ascomycete members

1. The figure shows the alignment of ITS region
2. The isolate used are some of *S. maydis* isolates from different geographical origin
3. The ascomycete members used are from GenBank from NCBI site
4. The programme used for alignment is CLUSTAL-X
5. The isolates are aligned into two groups along with the ascomycete members





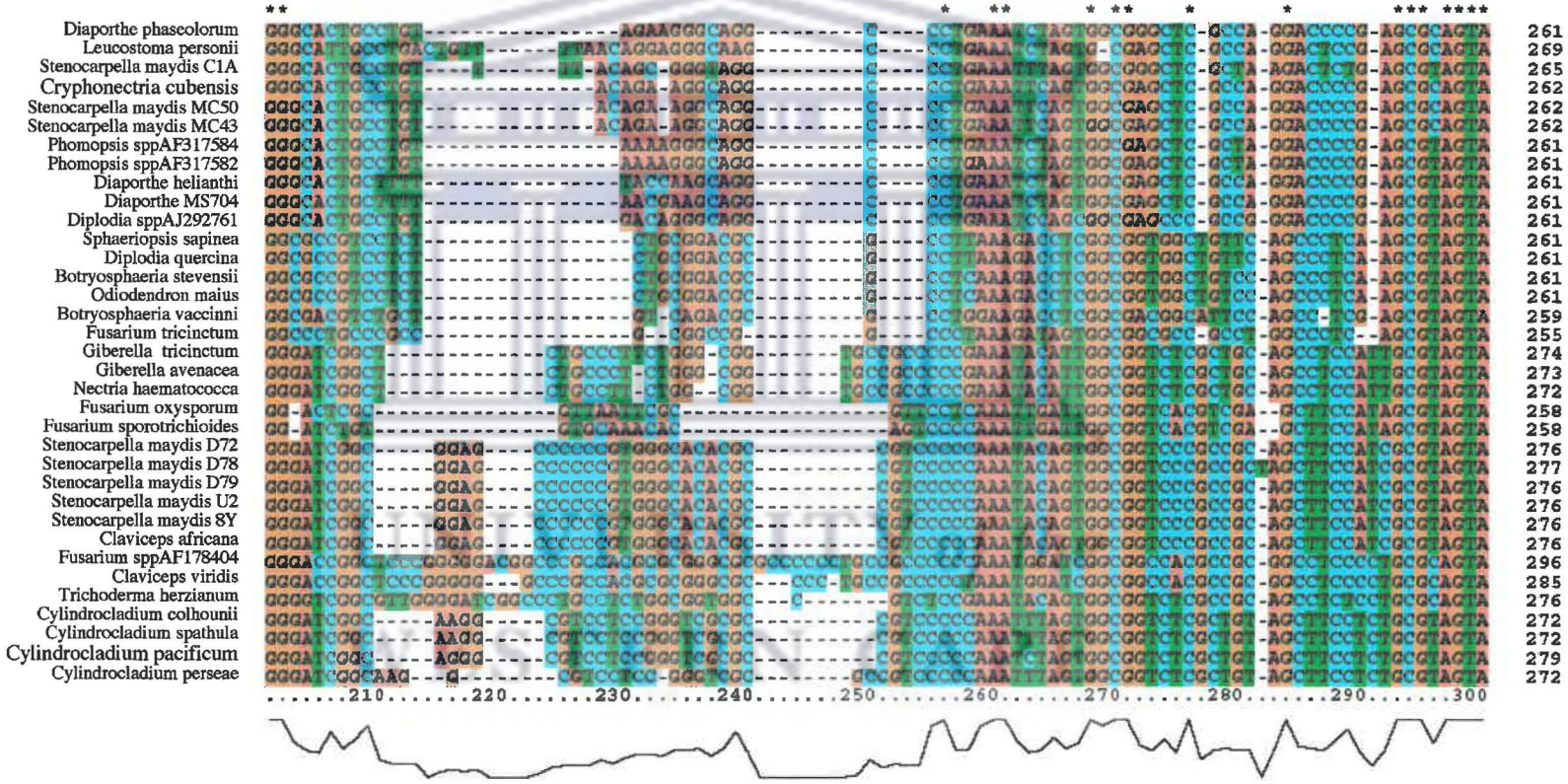
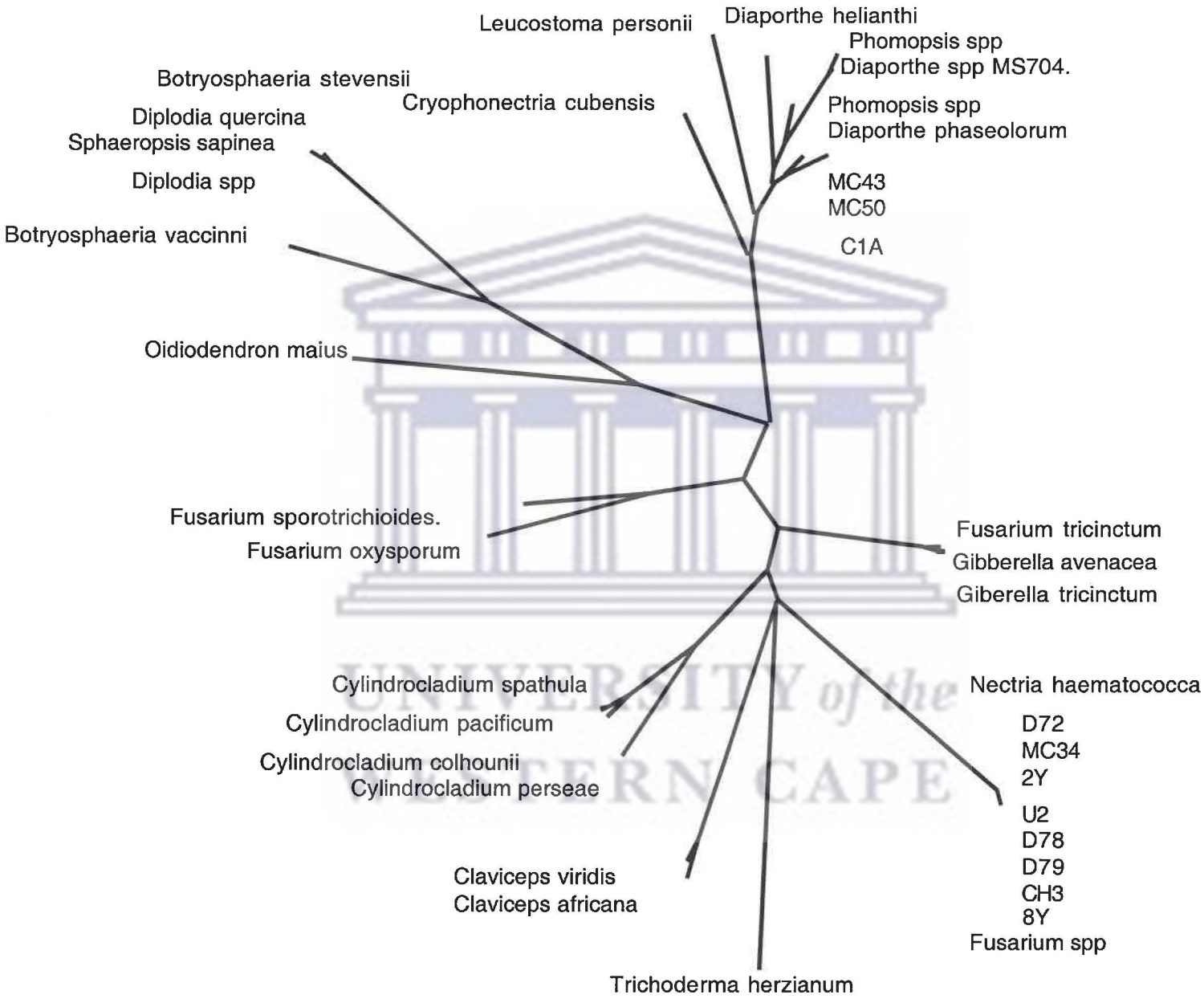
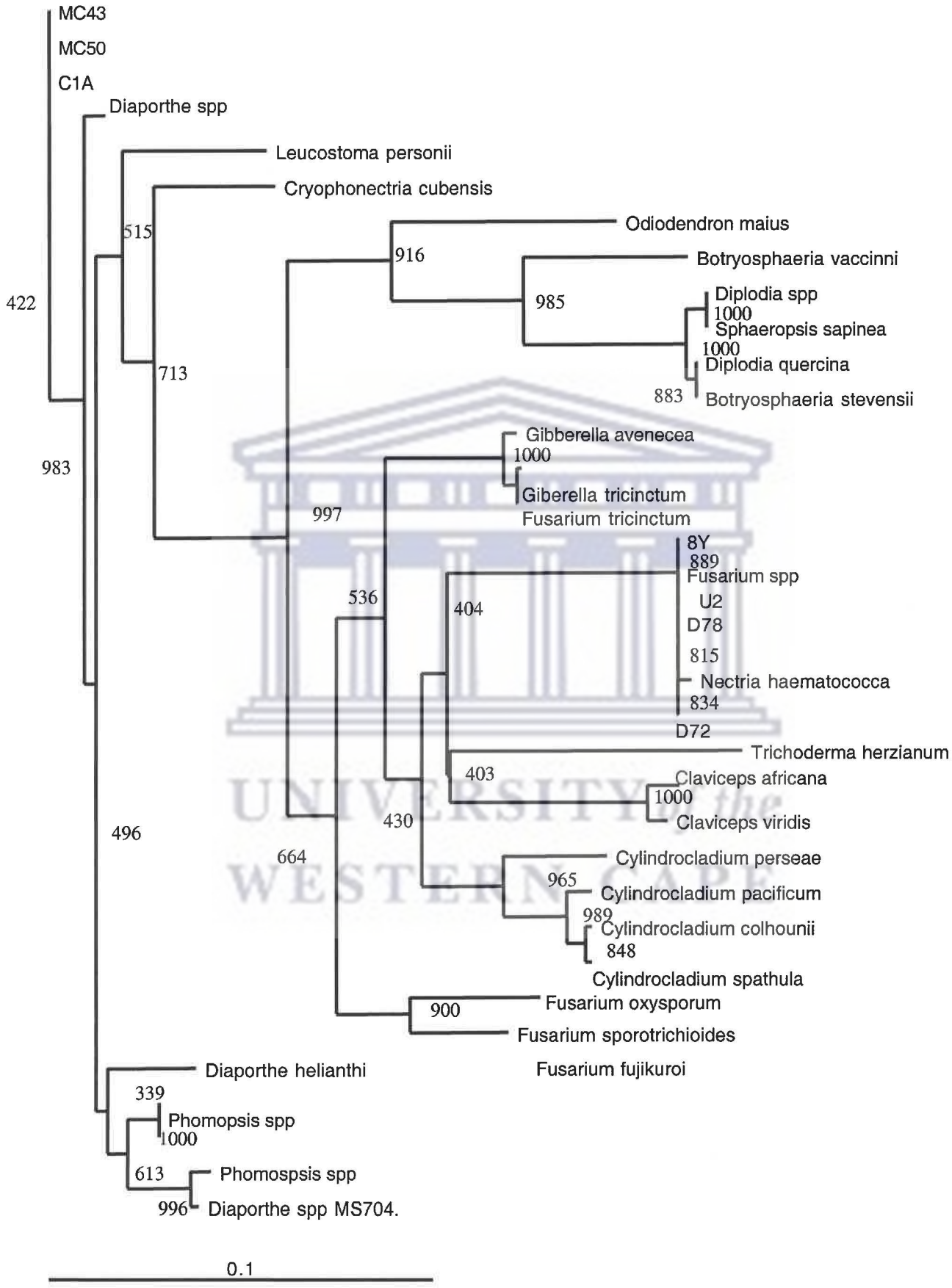
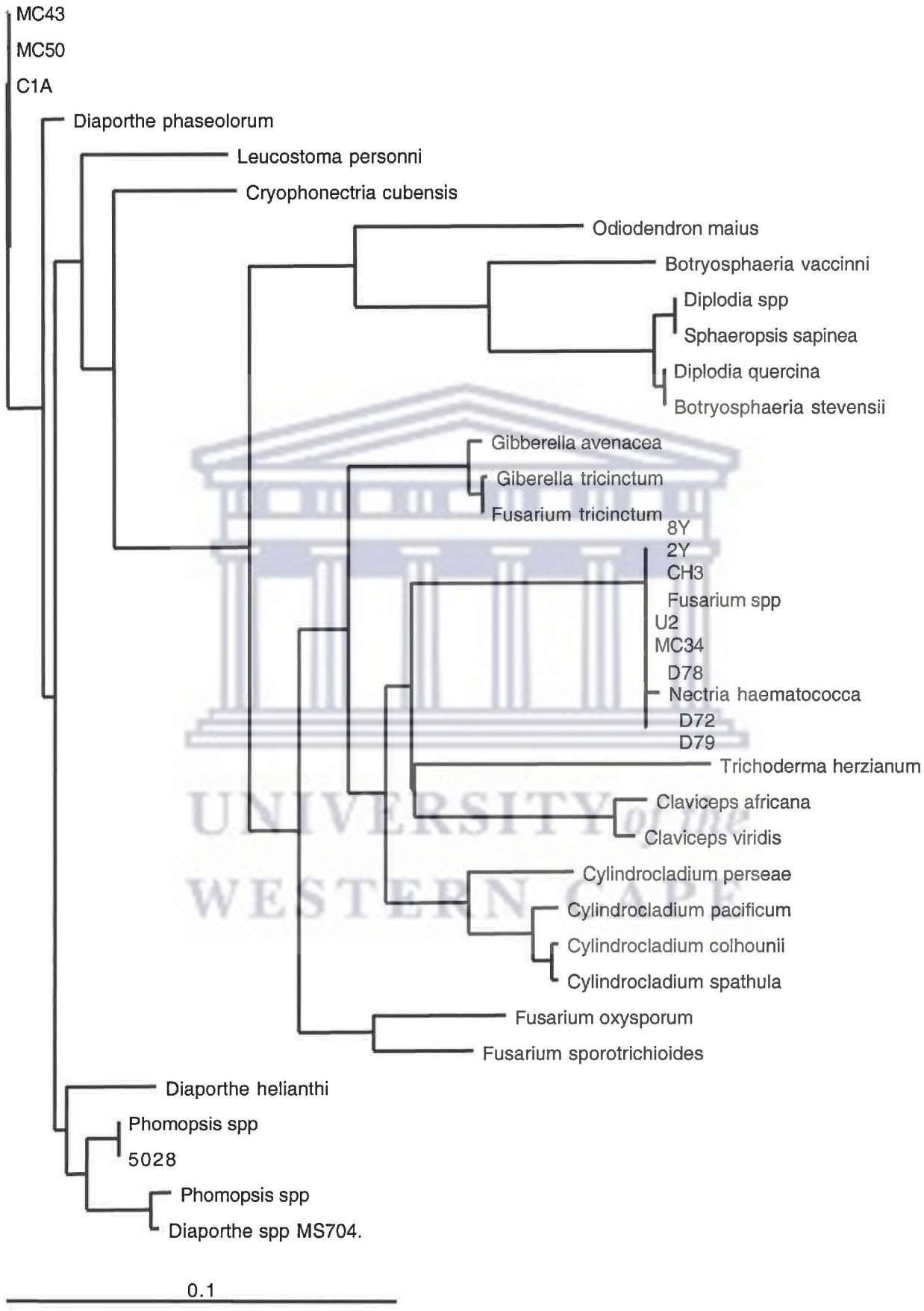


Fig 7.7 Phylogenetic analysis of *S. maydis* isolates with ascomycete members

1. The figure shows the phylogenetic analysis
2. The isolate used are some of *S. maydis* isolates from different geographical origin
3. The ascomycete members are taken from GenBank from NCBI site
4. Two taxonomic entities are shown by *S. maydis* isolates







7.5 Discussion

Stenocarpella maydis is of a great significance due to the severity of its infection on maize and cause loss in the yield and productivity of the crop. It has a severe clinical impact on grazing animals (Rabie *et al.,* 1987). Production of diplodiatoxin is a characteristic feature of *S. maydis* but not all the isolates exhibit the potential for the production of diplodiatoxin even in optimised *in vitro* conditions (Rao and Achar, 2001). Thus, there is a need to study the genetic variation among the isolates of *S. maydis* collected from different geographical regions of South Africa. To study the genetic relationship among the fungi the approach has been the analysis of sequence of the (rDNA) genes. Sequence variation in the rDNA (ITS1 and ITS2) regions are used for the assessment of phylogenetic relationships. The ITS region of *Diplodia quercina* (*S. quercina*) has been partially sequenced (Jacobs and Rehner, 1998), but the sequence data of ITS region of *S. maydis* is not known. In the present study, sequences of 11 isolates of *S. maydis* were compared with each other and with *D. quercina* and outgroups i.e, other members of ascomycetes (Table 7.2)

The *S. maydis* isolates from different geographical regions were taken for the study of genetic relatedness and the ITS1-5.8S-ITS2 region was sequenced. The ITS sequence was about 560bp in length. When the analysis was performed there was a significant difference between the ITS region of 11 isolates of *S. maydis.* When the ITS sequences were aligned by using Clustal-X they grouped into two distinct groups, one with toxin producing isolates C1A, MC43 and MC50, collected from Potchefstroom region in South Africa and the other group with non-toxin producing isolates D78, D72 D79, moderate toxin producing isolates and high toxin producing isolates from other geographical areas in South Africa. The NJ tree clearly represented two divergent branches and the bootstrap values further proved the accuracy of the data. The majority of base differences between the isolates were found in the ITS 1 and ITS 2 region and were generally base deletions/insertions. ITS1 region of C1A, MC43 and MC50 isolates differed from the rest of the isolates in the presence of base insertion/deletion in two places indicating that the two groups are different.

A clear demarcation between toxin producing isolates and the non-toxin producing isolates along with moderate and high toxin producing isolates was observed on the basis of the ITS1-5.8S-ITS2 region of rDNA. Similar results were obtained when toxin and non-toxin isolates of *Aspergillus flavus* and *A. parasiticus* were compared (Tran-Dinh, *et al.*, 1999). When the isolates of *Dothistroma pini* were compared with the non-toxin producing isolates remarkable deviation was observed in the ITS region (Bradshaw *et al.,* 2000). Further, when the isolates of *Fusarium culmorum, Fusarium graminearum, Fusarium avenaceum* were compared they indicated a high degree of deviation (Schilling *et al.*, 1996). These differences observed suggest that either multiple losses of toxigenicity have occurred in the isolates or that the recombination has reassorted the phenotypes into a variety of genetic background. Apart from changes taking place at the molecular level the host range and geographical regions also play an important role in predicting the deviations occurring at the species level, For example, the isolates of *Claviceps purpurea* taken from different geographical origin showed a measurable difference in the ITS region proving that the geographical origin is also important (Jungehulsing and Tudzynski, 1997). The pathogens with a wide range of hosts are also often misidentified as different species as in the case of *Entomophthora musae* which was identified as the *Entomophthora* species after ITS sequencing and also found that it had a wide range of hosts (Annette *et al.,* 2001).

The ITS region of the isolates provided an excellent opportunity to study the phylogenetic relationship between the isolates of a fungal species and to shed light on the taxonomic placement of the isolates. When these *S. maydis* isolates were compared with other ascomycete members taken from GenBank they revealed two taxonomic entities. The toxin producing group C1A, MC43, MC50 was placed near *Diaporthe* and *Phomopsis* species and the group containing D74, D78, D79, CH3, 2Y, 8Y, MC34 and U2 was placed with *Fusarium spp* and *Nectria spp.* The *S. maydis* sequences were similar to the sequences of the species belonging to *Diaporthe, Nectria, Fusarium and Phomopsis.* This data reveals that the isolates were misidentified as different species either based on morphological characters or due to the host range. As the pathogens *Diaporthe, Fusarium* and *Nectria* have, a wide range of hosts like apple, millet, wheat and pinus, which would lead to an inappropriate phylogenetic placement like *Epicoccum nigrum* and *Phoma epicoccina* were recognised as

same biological species with different hosts (Arenal *et al.*, 2000). therefore is difficult to justify the taxonomic placement of the isolates with morphological observations although the pathogens have the same morphological characters.

Thus, it is concluded that the isolates of *S. maydis* collected from different geographical regions in South Africa showed considerable variation and C1A, MC43 and MC50 isolates were almost identical and can be classified as a separate group. These isolates differed considerably from non-toxin producing isolates (D79 and D72) and a clear demarcation between toxin producing and the other isolates is established. The isolates showed a close relationship with other ascomycete members and revealed a different phylogenetic placement when compared to morphological taxonomic placement.

The phylogenetic data suggests that the isolates may have been misidentified as *S. maydis* isolates were as they belong to one of the two species clusters with different hosts. It may also be possible that these isolates belong to *S. maydis* but the factors like recombination and geographical origin may have reassorted the *S. maydis* into a different genetic background. Thus further studies with implications for pathogen reservoirs and disease control strategies have to be undertaken.

Chapter 8

SUMMARY AND CONCLUSIONS

8.1 MYCOTOXINS WITH SPECIAL REFERENCE TO DIPLODIATOXIN

Mycotoxins are considered to be a major problem in developing countries (Coker, 1979). Diplodiatoxin is a mycotoxin produced by *Stenocarpella maydis,* which causes stalk rot and ear rot in maize. *S. maydis* infection is a serious problem all over the world including South Africa (Rheeder *et al.*, 1990). Recently, a *S. maydis* epidemic has also been reported in China (Hamilton *et al.*, 2000). When the infected crop is consumed by grazing animals it causes field outbreaks of mycotoxicosis known as diplodiosis. Diplodiosis is characterised by ataxia, paralysis, lacrimation, neurological disturbances and serious liver damage. The toxic effect of crude extract of *S. maydis* on the laboratory animals like broilers (Rabie *et al.,* 1987), ducklings (Rabie *et al.,* 1987) and horses (Kellerman *et al.,* 1985) has been reported and could be of serious concern to human health. The toxic effect of the crude extract may either be due to the synergistic effect of other metabolites present in the extract or due to unknown mycotoxin present in the extract. Thus, the study with crude extract does not explain the exact principle compound causing toxicity. Therefore research has been undertaken to study the toxigenicity of diplodiatoxin in a pure form.

To study the toxigenicity of diplodiatoxin it is a pre-requisite to produce diplodiatoxin in pure form, therefore screening of various isolates of *S. maydis* for the production of diplodiatoxin, *in vitro* optimisation of the conditions for the production of diplodiatoxin, isolation and purification of diplodiatoxin were necessary. An efficient protocol was developed for bulk production of diplodiatoxin. Before testing on the experimental models it is always recommended to test the nature of toxicity on bacterial strains and cell lines which serve as models for predicting the nature of toxicity. Thus bioassays for anti-bacterial activity on the various bacterial strains and activity on mammalian cell lines (Chinese hamster ovary cells) were undertaken. In the toxicological studies it is necessary to know the immediate and

prolonged effect of the toxic compound so therefore acute and sub-acute toxicity of diplodiatoxin on rats was evaluated. As not all *S. maydis* isolates were producing diplodiatoxin, sequencing of the ITS1-5.8S-ITS2 region of the various isolates was undertaken to characterise their genetic relatedness, diversity and their phylogenetic relationship with other ascomycetes.

8.2 SCREENING OF THE ISOLATES OF *S. MAYDIS* FOR THE PRODUCTION OF DIPLODIATOXIN

*Stenocarpella maydi*s isolates were collected from the different geographical regions in South Africa and were screened for the production of diplodiatoxin using TLC and ApcI-MS. Preliminary screening of isolates of *S. maydis* using TLC showed a considerable variation among these isolates for the production of diplodiatoxin. It was observed that the maximum amount of diplodiatoxin was produced by MC43, C1A and MC50 isolates from the Potchefstroom region. All the isolates collected from Cedara, Shongweni and Transkei showed the presence of lower quantities of diplodiatoxin, except 2Y, CH3, U3H. Some of the isolates from the Potchefstroom region (D79, D72 and D80) did not produce diplodiatoxin. It may be suggested that these isolates differ in their genetic makeup and thus, there is a need to study the molecular aspect of production of diplodiatoxin. The presence or absence of diplodiatoxin was further confirmed by ApcI-MS. The mass spectra were analysed through the range m/z = 100 to 500, no high peaks were observed in the samples. All the samples containing diplodiatoxin showed a strong negative the ion at m/z = 307. The mechanism of ionisation for negative ApcI is by deprotonation, therefore, the ion would be (m-h). The molecular ion would then have mass of 308. The peak at m/z = 233 was a fragment of m/z = 307. For these experiments the ions at m/z = 307 were isolated and fragmented, and the fragments analysed showed that the peak at m/z = 233 derived from 307. Other peaks indicated the presence of other compounds in the fungal extract. The presence of a strong negative ion at m/z = 307 confirmed the molecular weight of 308 which is supported by the available literature (Steyn *et al.,* 1972; Ichihara *et al.,* 1986).

Thus, it may be suggested that these isolates differs in their genetic makeup and therefore genetic analysis by molecular approach is necessary.

8.3 OPTIMISATION OF *IN VITRO* PRODUCTION OF DIPLODIATOXIN

The optimisation of *in vitro* conditions for the production of diplodiatoxin is a pre-requisite for testing the toxicity of diplodiatoxin on various experimental models and also to confirm the toxicity of the crude extract on a wide range of animals.

In the present study, *in vitro* growth of *S. maydis* and production of diplodiatoxin was optimised by changing the media composition, pH of the media and the incubating temperature. The pH 4.5 was found to be optimal for the production of diplodiatoxin and growth of the mycelium. Production of diplodiatoxin in detectable quantity required at least six weeks of incubation. Lower range of pH inhibited the growth of *S. maydis* and increased the production of diplodiatoxin. This was a deviation from the general trend of the increased production of secondary metabolites with the factors limiting the growth of the mycelium.

Among the various media compositions tested, potato dextrose broth was found to be optimal for growth of the mycelium and production of the diplodiatoxin. It was also observed that corn meal extract stimulated the growth of *S. maydis* but the production of diplodiatoxin was not high, it may be due to the reason that maize, being the natural host of *S. maydis,* stimulates its growth rapidly. Similarly, Chen and Strange (1994) reported increased growth of *Ascophyta rabiei* in chickpea cell sap media. This natural media stimulated the growth of fungus drastically, which might be because of the presence of certain stimulatory compounds in the cell sap, as chickpea is the host plant for *A. rabiei* infection.

Production of mycotoxins and other secondary metabolites in response to stress is well known and high incubation temperature is the best way of creating a stressed environment. But, there are examples of deviation from this general rule. The present investigation also showed a deviation from this general trend of high temperature induced production of mycotoxins. Unlike other reports of high temperature stress for the increased production of mycotoxins (Sweeney and Dobson, 1998; Marvin *et al.,* 1995), diplodiatoxin was produced at 28 °C, a temperature found to be optimal for the growth of the mycelium. And a minimum of 5-6 weeks of incubation is required for the production of diplodiatoxin. When the cultures were incubated

with continuous agitation for 6 weeks and a static condition for 2 weeks the growth of mycelia was stimulated and the production of diplodiatoxin was enhanced.

8.4 ISOLATION AND PURIFICATION OF DIPLODIATOXIN

Diplodiatoxin has been artificially synthesised (Ichihara *et al.,* 1986). The synthetic compound is a racemic mixture and therefore, is not suitable for determining the exact toxicity of diplodiatoxin on animals or other experimental models. Thus, isolation of diplodiatoxin in its natural form from *S. maydis* was necessary. There is no literature available on the effect of the pure form of diplodiatoxin on animals and *in vitro* production of diplodiatoxin.

A protocol was designed for bulk isolation and purification of diplodiatoxin in its natural form from the pure culture of *S. maydis.* The protocol involves the initial extraction with hexane to remove all the fatty acids present in the crude extract, which may otherwise interfere with the solubility of the compound and analysis of its toxicity. This was followed by fractional extraction with equal volume of $CHCl_3$ and CH_3OH. Then the extract obtained was concentrated. The concentrated extract was purified by Column Chromatography. Silica gel column was used and the solvent system used was ethyl acetate. The compound isolated was confirmed as diplodiatoxin by comparing it with the standard using NMR. The protocol developed was efficient enough to yield good purity of diplodiatoxin.

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The protocol developed yielded 19.52mg of diplodiatoxin from 60g (fresh weight) mycelia from 8 weeks-old cultures of MC43 isolate after purification through the silica gel column thereby yielding 0.325mg of diplodiatoxin per gram fresh weight of the fungus. Diplodiatoxin appeared as white needle-like crystals and with the melting point of 187 °C as reported in the literature (Steyn *et al.,* 1972). Diplodiatoxin was insoluble in cold water and is completely soluble in methanol and ethyl acetate at room temperature.

The confirmation of the compound was done by nuclear magnetic resonance in comparison with the standard compound obtained from CSIR (Rao and Achar, 2001).

8.5 EFFECT OF DIPLODIATOXIN ON BACTERIA AND MAMMALIAN CELL LINES.

Anti-bacterial activity of diplodiatoxin produced by *S. maydis* has not yet been reported. In the present study, a strong anti-bacterial property of diplodiatoxin was established on *B. cereus, B. subtilis, E. coli, P. fluorescence* and *S. aureus* using well-diffusion and turbitimetry methods. The effect of different amount of diplodiatoxin (0.6 to 0.9µg/well) on the growth of various gram-positive and gram-negative bacterial strains was studied. Different bacterial strains showed varied tolerance to diplodiatoxin. An overnight incubation of these bacteria strains in the presence of diplodiatoxin showed a zone of inhibition. *S. aureus* was found to be the most susceptible to diplodiatoxin and *E. coli* was the most tolerant among the bacterial strains tested.

Order of tolerance of bacterial strains to diplodiatoxin was *S.aureus* < *B. cereus* < *B. subtilis* < *P. fluorescence* < *E.coli.* Further, the effect of different concentrations (4.8-49.7µg/ml) of diplodiatoxin on the growth of *S.aureus* in LB liquid medium was studied and all the concentrations of diplodiatoxin had an inhibitory effect on the growth of the bacterial strains. Thus the anti-bacterial activity of diplodiatoxin was confirmed. Diplodiatoxin was found to be bacteriostatic rather than bactericidal.

Anti-bacterial activity of T-2 toxin, aflatoxin B_1, fumonisin B_1, ochratoxin A and deoxynivalenol on the various species of the genera *E. coli, Streptococcus, Staphylococcus, Yersinia, Erysipelothrix* and *Lactobacillus* has also been established by using a micro-tubidimetry method (Ali-Vehmas *et al.,* 1998). Recently, *in vitro* binding of aflatoxin B_1 to bifidobacteria was studied in detail by (Oatley *et al., 2000*). Thus, the anti-bacterial activity of diplodiatoxin established is supported by the literature available on the anti-bacterial activity of mycotoxins.

The toxicity of diplodiatoxin on eukaryotic cells was analysed by testing the effect of the diplodiatoxin on (Chinese hamster cell lines). The concentrations of diplodiatoxin were from 0.02µg/ml to 0.24µg/ml. The morphology of the cells changed and they became elongated within 2 hours of incubation at a concentration of 0.12µg/ml. Further, to know the nature of

activity of the crude extract, similar concentrations of crude extracts were tested on the cell lines and the same changes were observed in the cell lines at a concentration of 0.12µg/ml. APOPercentage test was undertaken to know whether the activity was due to apoptosis or necrosis. The APOPercentage test revealed that the crude extract showed apoptosis at a concentration of 0.12µg/ml. As the activity shown by diplodiatoxin is also at same concentration, it can be concluded that the morphological changes observed were due to diplodiatoxin or due to the synergistic activity of the other compounds present in the extract, along with diplodiatoxin. The test done with the cell lines gives a clear indication about the activity of the diplodiatoxin and also is helpful in predicting the role of diplodiatoxin in acute and sub-acute toxicosis.

8.6 ACUTE TOXICITY OF DIPLODIATOXIN IN RATS

Acute treatment with diplodiatoxin (5.7mg/kg) caused sudden loss in the body weight and feed intake in both male and female rats. Other than loss in body weight and feed intake, dullness, irritation, bulky stomach, tremors and convulsion symptoms were also observed which indicated growth retardness and stressed conditions.

Mycotoxin – induced inhibition of AChE is an excellent indicator of the level of toxicity (Abu-Quare *et al.*, 2001). The level of AChE in the brain and RBC was drastically reduced in the present study. When AChE is reduced or inhibited the ACh is not hydrolysed and it accumulates at cholinergic sites causing stimulation of nerve impulse (Moser, 2000). The inhibition of the enzyme AChE by diplodiatoxin treatment clearly indicates the neurotoxic nature of the compound. When the recovery studies were conducted the male recovered faster than the female rats but still the levels of enzymes were much less than that of the control rats. Diplodiatoxin also caused alterations in the levels of the target biochemical enzymes like ASAT, ALAT, AcP and AkP.

Levels of ASAT and ALAT increased in serum of male and female rats but, in the liver, the levels were increased in females and reduced in males. These biochemical parameters are a sensitive index to the changes due to any toxicant. The changes in the level of these hepatic

enzymes (ASAT and ALAT) indicate the damage caused to the liver cells. The increased levels of these enzymes in serum and liver indicate the hepatotoxic nature of diplodiatoxin. Increased levels of these enzymes in the serum may be due to the leakage of these enzymes due to necrosis of tissues (Yarbrough *et al.,* 1982). Also increased levels of these enzymes in the serum and the parallel decrease in the liver might be due to the liver necrosis caused by diplodiatoxin treatment.

In rats treated with diplodiatoxin AcP and AkP levels decreased in the serum and increased in the liver, these results clearly indicate the cell damage caused by diplodiatoxin. As in the present study, increased levels of liver AcP were reported in dichlorvos-treated rats (Srivastava *et al.*, 1989), while decreased levels of liver AkP were observed when rats were treated with poly-chlorinated biphenyl (Rao and Banerji, 1990). The enzymes AkP and AcP are membrane bound biomarkers and the changes in these enzymes might be due to interactions of diplodiatoxin with these enzymes causing alterations in cell membrane permeability. The decreased activity of serum AcP and AkP observed might be due to slow enzyme synthesis or liver damage, whereas the increased AcP activity in liver might be due to increased synthesis of this enzyme as an adaptive mechanism to chemical stress. These results indicate the hepatotoxic nature of diplodiatoxin.

The changes observed in the activities of enzymes after 24 hours in acutely treated female rats were significant when compared with male rats. These results clearly indicate sexual dimorphism. The correlation studies indicate a negative correlation in the enzyme activities between serum and liver, as the enzymes increased in serum with parallel decrease in liver. The present study suggests that oral administration of diplodiatoxin resulted in severe alterations in serum and also in the cellular activities of vital organs such as the liver, causing hepatic and neurological damages.

Withdrawal studies clearly revealed the reversal of the toxicity after diplodiatoxin is removed. Finally, it was concluded that diplodiatoxin is a mycotoxin and its hepatotoxic and neurotoxic nature are confirmed.

8.7 SUB-ACUTE TOXICITY OF DIPLODIATOXIN IN RATS

Prolonged exposure to sub-acute doses of mycotoxins results in carcinogenesis and mutagenesis in a wide range of animals and its impact on human health is also well known. The sub-acute toxicity has more adverse effects than the acute toxicity due to prolonged exposure. Sub-acute toxicity of aflatoxins, ochratoxins and fumonisins are known to cause cancer in animals and humans (Fink-Gremmels, 1999).

It was observed that sub-acute dosages of diplodiatoxin (0.27mg/kg/day) caused gradual loss in body weight and feed intake. Rats also showed growth retardation and stressed symptoms as observed in the acute study. Feed intake and body weight recovered after withdrawal of diplodiatoxin.

A significant reduction in the level of AChE in the brain and the erythrocytes was observed after ten days of the administration. The reduction was more severe after 21 days. Females were more susceptible than male rats. Thus these results further confirm the neurotoxic nature of diplodiatoxin.

The present study demonstrated an increase in ASAT and ALAT levels in the serum, whereas in the case of liver these enzymes decreased in sub-acutely treated rats. This might be due to the liver necrosis caused by diplodiatoxin treatment. The changes observed in the activities of liver ASAT and ALAT enzymes of female rats were significant when compared with male rats in sub-acute treatment. The sub-acute dosage of diplodiatoxin caused measurable changes in lysosomal enzymes. The levels of enzymes AcP, AkP in serum and the level of the enzyme AkP in the liver decreased and AcP enzyme in liver increased in both male and female rats. The decreased activity of AcP enzyme and AkP enzyme in serum observed in the study might be due to slow enzyme synthesis or due to liver damage, whereas the increase in the level of enzyme AcP in the liver might be due to increased synthesis of this enzyme in response to stress conditions.

In all the cases females were more susceptible than the male rats and thus the results clearly indicate sexual dimorphism in these enzymes by diplodiatoxin. The correlation studies indicate a negative correlation in the levels of enzymes between serum and liver. These alterations demonstrated that these enzymes increased in serum with a parallel decrease in liver. Therefore, the results of the activity of the enzymes in the present study suggest that oral administration of diplodiatoxin resulted in alterations in the enzyme levels in the serum and also in the cellular activities of vital organs such as the liver, causing hepatic and neurological damages. Thus the hepatotoxic nature of diplodiatoxin is further confirmed.

The activity of all these enzymes in brain, liver, serum and RBC recovered to near normal levels after 28 days in both male and female rats. This clearly revealed the reversal of the toxicity once the diplodiatoxin is removed.

The results obtained from the present study further confirm the hepatotoxic and neurotoxic nature of diplodiatoxin. In the sub-acute studies conducted so far the paralytic syndrome was not observed so further investigations should be conducted by future workers to know whether diplodiatoxin combines with other toxic metabolites to cause these symptoms or *S.maydis* produces toxins other than diplodiatoxin which are capable of producing paralysis in the animals.

8.8 ITS1-5.8S-ITS2 BASED GENETIC RELATEDNESS AMONG THE VARIOUS ISOLATES OF *S. MAYDIS* AND OTHER MEMBERS OF ASCOMYCETES

Production of diplodiatoxin is a characteristic feature of the fungus *Stenocarpella maydis*. The present study revealed that not all isolates of *S. maydis* produce diplodiatoxin and it may be suggested that these isolates differ in their genetic makeup. No literature is available for the characterisation of phylogenetic relationships between different species of the genus *Stenocarpella* on the basis of molecular approaches. ITS region of *Diplodia quercina (S. quercina)* has been partially sequenced (Jacobs and Rehner, 1998). Thus sequencing the ITS region of *S.maydis* is important to shed the light on the genetic relationship among various toxin and non-toxin producing isolates and also their phylogenetic placement.

The sequence of the ribosomal DNA (rDNA) has been used to analyse the interspecific, intraspecific genetic and phylogenetic relationships. In fungi, these genes are usually found in multiple copies with regions that are very highly conserved (White *et al.*, 1990) joined together by spacers that can be highly variable. The ITS 1 and 2 which are found between the small (18S) and the large (28S) ribosomal subunit genes are separated by 5.8S ribosomal subunit genes. ITS1 and ITS 2 regions can show variability at intraspecific levels. Despite this, the 18S, 5.8S and 28S genes do evolve at a slow rate and are used for sorting distantly related organisms. Variations within ITS sequences can be most informative for closely related organisms (Burns *et al.,* 1991; Hyun and Clark, 1998).

Sequencing of the ITS region has also been used to detect and quantify variability in several species or groups of fungi (Lloyd-MacGilp *et al.*, 1996). Sequencing the ITS region has also been used to study the variation at intrageneric level (Arenal *et al.,* 2000), intraspecific levels (Angeles Vinuesa *et al,* 2001), among the various isolates of a species (Chambers *et al.,* 2000). Also sub groups and ecotypes of several fungal species (Salazar *et al.,* 2000).

ITS1-5.8S-ITS2 – based genetic relatedness among the isolates of *S. maydis* and other species of ascomycetes was studied. PCR-amplification of this region yielded a 560bp fragment and it was sequenced. Sequence alignment showed that isolates C1A, MC43 and MC50 were identical to each other and D72, D78 and D79 were also identical to each other. In most of the other isolates, a 1–3 small insertion/deletion was found in the ITS1 and ITS4 regions and a large insertion of 9-10 bases was found in MC43, MC50 and C1A isolates. The 5.8 S region of the rDNA was found to be highly conserved and similar in all the isolates with an exception of insertion/deletion in two places. The ITS sequence showed two distinct groups among these isolates, one containing MC43, MC50 and C1A and the other containing rest of the isolates.

When the isolates were compared with other ascomycete members taken from GenBank the isolates showed two taxonomical entries. The toxin producing group was close to *Diaporthe spp* and *Phomopsis spp* and the non-toxin producing group along with less toxin producing isolates and high toxin producing isolates was close to *Fusarium spp* and *Nectria spp*

indicating that the ITS sequence analysis provides a stronger basis for taxonomic placements when compared to the use of the morphological characters. This data suggests that these isolates were misidentified as the *S. maydis* isolates and in fact they belong to either of the two species clusters. Further, *Fusarium*, *Nectria, Diaporthe* and *Phomopsis* species have been identified as the pathogens of a wide variety of different plant hosts suggesting that the ear rot in maize is a part of a set of diseases of plants caused by a group of fungi. This may have implications for pathogen reservoirs and disease control strategies.

8.9 CONCLUSIONS

The following conclusions were made from the present investigation.

1. Isolates of *S. maydis* collected from different geographical regions in South Africa varied in their ability to produce diplodiatoxin. The screening was done by a preliminary test (TLC) and further confirmed by mass spectrometry.

2. Maximum amount of diplodiatoxin was produced by MC43, C1A and MC50 isolates whereas D72, D79 and D80 isolated did not produce diplodiatoxin. These observations were supported by ApcI-MS.

3. The *in vitro* production of diplodiatoxin was standardised by changing the media composition, pH of the media, and incubating temperature. Under all the optimised conditions, detectable amounts of diplodiatoxin were produced after 6 to 8 weeks of growth. Aeration stress increased the production of diplodiatoxin.

4. Factors enhancing *in vitro* growth of the fungus also enhanced production of diplodiatoxin. But aeration stress further increased the production of diplodiatoxin.

5. A protocol was designed for the isolation and purification of diplodiatoxin from the pure cultures of *S. maydis*. The purity of the isolated compound was found to be comparable to the standard by Nuclear Magnetic Resonance.

6. The protocol developed yielded 19.52mg of diplodiatoxin from 60gram (fresh weight) mycelia of MC43 isolate grown for 8 weeks under optimised conditions.

7. A strong anti-bacterial activity of diplodiatoxin on *B. cereus, B. subtilis, E. coli, P. fluorescence* and *S. aureus* was established by using well diffusion and turbidimetry assays. *S. aureus* and *E. coli* were found to be the most susceptible and tolerant to diplodiatoxin, respectively.

8. Diplodiatoxin is bacteriostatic.

9. When diplodiatoxin was tested on Chinese hamster ovary cells it showed activity at 0.12µg/ml. The cells were elongated and were different when compared with the control cells after 24hrs of incubation.

10. The APOPercentage test was done to determine whether diplodiatoxin was causing necrosis or apoptosis, the test clearly indicated that apoptosis (programmed cell death) was occurring.

11. Acute (5.7mg/kg) and sub-acute (0.27mg/kg/day) toxicity of diplodiatoxin in male and female rats was tested by monitoring the levels of various target enzymes *viz.*, ASAT, ALAT, AcP, AkP and AChE in brain, liver, serum and RBC.

12. A significant reduction in the level of AChE in the brain and RBC was observed in acute and sub-acute studies. Thus, diplodiatoxin – induced alteration in the ability of AChE to breakdown the neurotransmitter, acetylcholine, resulted in the accumulation of acetylcholine. This increases nerve impulse transmission and failure of the central nervous system. These results suggest the neurotoxic nature of the diplodiatoxin.

13. The acute and the sub-acute effect of diplodiatoxin resulted in an increase in ASAT and ALAT levels in the serum, whereas in the case of liver these enzymes decreased in both female and male rats. Increased levels of the enzymes in the serum as reported might be due to the leakage of soluble tissue enzymes into the blood as a result of necrosis of the tissue. Also, increased levels of these enzymes in the serum and parallel decrease in the liver might be due to the liver necrosis caused by diplodiatoxin treatment.

14. Significant changes were also observed in the phosphatases in animals treated with diplodiatoxin. AcP and AkP levels decreased in the serum and increased in the liver suggesting that the cell damage is caused by diplodiatoxin. The pathomorphologic changes in the liver of the present study may be correlated with the disturbances in biochemical enzymes in the serum. The decreased activity of serum AcP and AkP enzymes observed might be due to slow enzyme synthesis or liver damage, whereas the increased level of AcP in liver might be due to increased synthesis of this enzyme as an adaptive mechanism to chemical stress. These results indicate the hepatotoxic nature of diplodiatoxin.

15. Withdrawal studies showed a recovery pattern in rats as the level of these enzymes returned to the normal between 7 and 28 days after withdrawal of the diplodiatoxin.

16. The correlation studies indicated a negative correlation between the level of the enzymes in the serum and liver, as the enzyme levels increased in serum with parallel decreases in liver. The present study suggests that the oral administration of diplodiatoxin resulted in severe alterations in serum and also in the cellular activities of vital organs such as the liver, causing hepatic and neurological damages.

17. ITS1-5.8S-ITS2 based genetic relatedness among the isolates of *S. maydis* and other species of ascomycetes was studied. PCR-amplification of this region yielded a 560bp fragment, which was sequenced. Sequence alignment showed that the ITS sequences of the isolates C1A, MC43 and MC50 were identical to each other and D72 and D79 were identical to each other. In most of the other isolates, small insertion/deletion was found in

the ITS1 and ITS4 regions. The 5.8 S region of the rDNA was highly conserved and similar in all the isolates with an exception of a single insertion/deletion.

18. The ITS sequencing showed two distinct groups among these isolates, one containing MC43, MC50 and C1A and the other containing rest of the isolates.

19. When the isolates were compared with other ascomycete members, the toxin producing group (MC43, C1A, MC50) was closest to *Diaporthe spp* and *Phomopsis spp* and the non-toxin producing (D72, D79, D78) group along with less toxin producing (CH3, 2Y, 8Y andU2) and high toxin producing isolates (C3C) were closest to *Fusarium spp* and *Nectria spp*. Thus two taxonomic entities were shown predicting that the isolates belong to two species clusters and in fact were misidentified as *S. maydis*. Further, *Fusarium spp*, *Nectria spp, Diaporthe spp* and *Phomopsis* species have been identified as the pathogens of a wide variety of different plant hosts suggesting that the ear rot in maize is a part of a set of diseases of plants caused by a group of fungi. This may have implications for pathogen reservoirs and disease control strategies.

Chapter 9

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