

**The level of mycotic and mycotoxigenic Fusaria in
traditional *morogo* and the agro-environment of Dikgale
Demographic Surveillance Site (DDSS)**

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Dissertation submitted in partial fulfilment of the requirements for the degree of

**MAGISTER OF ENVIRONMENTAL SCIENCE
(M.Env.Sci)**

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2006

“All praise be to Allah and peace and blessings be upon His messengers”

This work is dedicated to my wife, Lebogang, and my son, Thato. Your patience, understanding and encouragement were the main driving force throughout my entire study period. Thank you for your support. I love you.

DECLARATION

The experimental work conducted and discussed in this dissertation was performed in the School for Environmental Science and Development at the North-West University, Potchefstroom Campus.

I declare that, the study hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

Elijah Mogakabe

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to the following people and institutions for their contributions in enabling me to complete this study:

The following persons of the School for Environmental Sciences and Development, Microbiology, North-West University (Potchefstroom Campus)

Mrs Retha van der Walt, for her invaluable assistance and patient guidance in all aspects of this study;

Prof. Carlos Bezuidenhout, for his solid approach and assistance in ensuring that I complete this work;

Dr. Mahomed Ibrahim, for his assistance and invaluable advice;

Miss Cecilia Sizana, for her technical assistance;

***Morogo* Research Program (MRP) students, for their assistance and support;**

***Morogo* Research Program (MRP) and the Indigenous Knowledge Systems (IKS) of the National Research Foundation (NRF), South Africa, for financial support;**

Ms Perpetua Modjadji, Human Anatomy Laboratory Technician, School of Health Science Department of Medical Science, University of Limpopo, for her support and facilitating sampling of *lerocho* and *thepe* from villages in DDSS;

South African National Biodiversity Institute (SANBI), Pretoria, for botanical species identification of *lerocho* and *thepe*;

Dr Elna van der Linde and Ms Riana Jacobs, Biosystematics Division, Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI), Pretoria, for training and verification of *Fusarium* species isolate identification;

My wife and son, for their patience, understanding and support;

My parents, for their support and love;

My Creator, Allah Akbar, He is the perfectly wise, in His knowledge and in His deeds. There is no doubt or uncertainty in His knowledge, nor does it have an end.

ABSTRACT

Ubiquitous in agro-environments, *Fusarium* species infect and damage economically important crops and contaminate food commodities with harmful secondary metabolites called mycotoxins. In addition, human infection by pathogenic *Fusarium* strains has now emerged as a major problem particularly among individuals with suppressed immunity. Trichothecenes, deoxynivalenol, nivalenol, moniliforme and fumonisins are potent toxins produced by *Fusarium* species including *F. poae*, *F. nygami*, *F. oxysporum*, *F. proliferatum*, and *F. verticillioides*. The last three, together with *F. solani* and *F. chlamydosporum* are presently recognised as major role players in the occurrence of fusarioses in individuals with compromised immunity.

In subsistence situations in rural areas of South Africa, a variety of traditional leafy vegetables, collectively known as *morogo*, supplement maize-based staple diets with minerals and vitamins. The utilisations of these traditional vegetables are generally based on indigenous knowledge pertaining to production and processing. *Morogo* plants are not natural hosts to mycotoxigenic and mycotic *Fusarium* species that are mainly associated with pathogeneses of grain crops such as maize. However, *morogo* growing in close proximity of maize in typical subsistence agricultural situations might be at risk of *Fusarium* contamination from maize.

The study was conducted in the Dikgale Demographic Surveillance Site (DDSS), a rural area in the Limpopo Province characterised by the production of maize and different types of traditional *morogo* for household subsistence. HIV/AIDS is prevalent in the Limpopo Province. Chronic dietary exposure to *Fusarium* toxins and disseminated fusarioses might enhance disease outcomes associated with AIDS in affected individuals, thus adding to the burden of disease in DDSS communities.

The aim of the study was to investigate the occurrence of mycotic and mycotoxigenic *Fusarium* species in traditional *morogo* and agro-environments in DDSS. Questionnaires were employed to obtain relevant information and indigenous knowledge from communities of Sefateng, Madiga, Mantheding and Moduane related to the utilisation of traditional *morogo*. At each village *thepe* (amaranth) and *lerocho* (African cabbage) were sampled on two occasions, namely before maize planting (M-) and when maize was fully grown (M+). Maize, soil and air were sampled at the same time. Botanical species identification was carried out on specimens of *lerocho* and *thepe*

from each village. *Lerotho*, *thepe*, maize, soil and air samples were subjected to mycological analysis to determine the average fungal levels and *Fusarium* species that occurred. Samples of fresh and traditionally sun-dried samples of *thepe* and *lerotho* were analysed by HPLC for fumonisins.

Average fungal plate counts of *morogo* from all four villages were notably higher in *lerotho* compared to *thepe*. *Lerotho* sampled from M- fields of Madiga, Mantheding and Moduane exhibited higher average fungal levels than those from the M+ fields. However, in *lerotho* sampled from the M+ field of Sefateng average fungal levels were significantly higher than that of the M- field. Fungal levels in maize growing close to *morogo* were lowest in Sefateng and highest in Moduane. The highest fungal counts in soil were reported for Sefateng's M- field and the lowest for Sefateng M+ field. Fungal levels were high in air samples of M+ fields of all four villages and the lowest in M- field of Sefateng.

The majority *Fusarium* isolates retrieved from *morogo* and environmental samples belonged to known mycotoxigenic and/or mycotic species, though predominant species and levels thereof varied in samples from M- and M+ fields of the four villages. *Fusarium* levels in *thepe* from both M- and M+ fields were shown to be lower as in *lerotho*. In samples of the Sefateng M- field, *F. poae* occurred predominantly in *lerotho*, *thepe*, soil as well as air, while *F. subglutinans* was the predominant species in *lerotho* and air samples of Mantheding. In Sefateng samples from M+ field, *F. chlamydosporum* predominated among isolates retrieved from *lerotho*, *F. proliferatum* and *F. graminearum* among those from maize and *F. solani* among those from soil and air. *F. proliferatum* dominated among isolates from *lerotho*, maize, soil and air of M+ sites of Madiga and *F. chlamydosporum* in soil and air samples of Mantheding. HPLC analysis detected fumonisin B1 in traditionally sun-dried as well as fresh samples of *lerotho* as well as *thepe*.

The occurrence of mycotoxigenic and mycotic *Fusarium* species in traditional *morogo* and agro-environments might be an aggravating health risk factor for DDSS communities.

Key terms: rural subsistence households, traditional *morogo*, mycotoxigenic *Fusarium*, mycotic *Fusarium*; fumonisin B1

OPSOMMING

Alomteenwoordig in landbou omgewings, *Fusarium* spesies infekteer en beskadig ekonomies belangrike gewasse en besmet voedsel kommoditeite met skadelike sekondêre metaboliete naamlik mikotoksiene. Menslike infeksies met patogeniese *Fusarium* het nou in die voorgrond getree as 'n groot probleem besonderlik onder individue met onderdrukte immuniteit. Trigotesene, deoksinivalenol, nivalinol, moniliforme and fumonisiene is kragtige toksiene wat deur *Fusarium* spesies insluitend *F. poae*, *F. nygami*, *F. oxysporum*, *F. proliferatum*, and *F. verticillioides* vervaardig word. Die laaste drie, saam met *F. solani* en *F. chlamydosporum* word bejeën as hoof rolspelers in die voorkoms van fusarioses in individue wie se immuniteit gekompromiteer is.

In onderhoudingsituasies in plattelandse gebiede van Suid Afrika, vul 'n verskeidenheid tradisionele blaargroentes, kollektief bekend as *morogo*, mielie-gebaseerde stapel diëte met minerale en vitamienes aan. Die verbruik van hierdie tradisionele groente is gebaseer op inheemse kennis van die produksie en verwerking daarvan. *Morogo* plante is nie natuurlike gashere vir mikotoksigeniese en mikotiese *Fusarium* spesies wat hoofsaaklik met patogenese van graan gewasse soos mielies geassosieer word nie. *Morogo* plante wat egter in die nabyheid van mielies in tipiese bestaanslandbou situasies groei loop die risiko om met *Fusarium* vanaf mielies besmet te word.

Die studie is uitgevoer in Dikgale Demographic Surveillance Site (DDSS), 'n plattelandse area in die Limpopo Provinsie wat gekenmerk word deur die produksie van mielies en verskillende tipes tradisionele *morogo* vir huishoudelike onderhoud. Die voorkoms van MIV/VIGS is hoog in die Limpopo Provinsie. Langdurige blootstelling aan *Fusarium* toksiene in die dieet en verspreide fusariose mag siekte uitkomst wat met VIGS verband hou in geaffekteerde individue verhoog en sodoende toevoeg tot die siekte las van DDSS gemeenskappe.

Die doel van die studie was om die voorkoms van mikotiese en mikotoksigene *Fusarium* spesies in tradisionele *morogo* en landbou omgewings van die DDSS te ondersoek. Vraelyste is gebruik om relevante inligting en inheemse kennis in verband met die verbruik van tradisionele *morogo* van gemeenskappe in Sefateng, Madiga, Mantheding and Moduane, te bekom. Monsters van *thepe* (gewone misbredie) en *lerocho* (oorpeuljie) is in elke dorpie by twee geleenthede onderskeidelik geneem naamlik, voordat mielies gegroei het (M-) en toe mielies volgroeid was

(M+). Monsters is terselfdertyd van mielies, grond en lug geneem. Botaniese spesies identifikasie is op *lerocho* en *thepe* eksimplare van elke dorpie gedoen. *Lerocho*, *thepe*, mielie, grond en lug monsters is aan mikologiese ontleding onderwerp om die swamvlakke en *Fusarium* spesies wat voorgekom het, te bepaal. Vars en tradisioneel son-gedroogde monsters van *thepe* en *lerocho* is deur HPLC vir fumonisiene ontleed.

Gemiddelde swam plaattellings van *morogo* uit al vier dorpies was opmerklik hoër in *lerocho* in vergelyking met *thepe*. *Lerocho* monsters wat by die M- terreine van Madiga, Mantheding en Moduane onderskeidelik geneem is, het gemiddeld hoër swamvlakke vertoon as dié van M+ terreine. In *lerocho* van die M+ terrein van Sefateng was die gemiddelde swamvlakke egter beduidend hoër as dié van die M- terrein. Swamvlakke in mielies wat naby *morogo* gegroei het, was die laagste in Sefateng en hoogste in Moduane. Die hoogste swamtellings in grond is gerapporteer vir Sefateng se M- terrein en die laagste vir Sefateng se M+ terrein. Swamvlakke was hoog in lug monsters van M+ terreine van al vier dorpies en die laagste in die M- terrein van Sefateng.

Die meerderheid *Fusarium* isolate wat in *morogo* en omgewing monsters opgespoor is, behoort tot bekende mikotoksigeniese en mikotiese spesies, hoewel spesies wat oorheers het en vlakke daarvan in monsters van M- en M+ terreine van die vier dorpies gevarieer het. Dit het geblyk dat *Fusarium* vlakke in *thepe* van beide M- en M+ terreine laer was as in *lerocho*. In Sefateng monsters van M- terreine is *F. poae* as die oorwegend spesie uit *lerocho*, *thepe*, grond sowel as lug geïsoleer, terwyl *F. subglutinans* die oorwegende spesie in *lerocho* en lug monsters van Mantheding was. In Sefateng monsters van M+ terreine het *F. chlamydosporum* tussen isolate wat in *lerocho* opgespoor is, oorheers, *F. proliferatum* en *F. graminearum* tussen dié van mielies en *F. solani* tussen dié uit grond en lug. *F. proliferatum* het tussen isolate uit *lerocho*, mielies, rond en lug van die M+ terrein van Madiga oorheers, en *F. chlamydosporum* dié uit grond- en lugmonsters van Mantheding. HPLC analise het fumonisin B1 in tradisioneel son-gedroogde sowel as vars monsters van *lerocho* sowel *thepe* opgespoor.

Die teenwoordigheid van mikotoksigeniese en mikotiese *Fusarium* spesies in tradisionele *morogo* en landbou-omgewings mag 'n verswarende gesondheidsrisiko wees vir DDSS gemeenskappe.

Kernterme: plattelandse onderhoudingshuishoudings; tradisionele *morogo*; mikotoksigene *Fusarium*, mikotiese *Fusarium*; fumonisien B1

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ASSA	Actuarial Society of South Africa
bp(s)	base pair(s)
BSA	Bovine Serum Albumin
CADRE	Centre for AIDS Development, Research and Evaluation
CFU	Colony Forming Units
CLA	Carnation Leaf Agar
CTAB	Cetyl-tri-methyl-ammonium-bromide
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DDSS	Dikgale Demographical Surveillance Site
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleotide tri-phosphate
dTTP	Deoxythymidine triphosphate
EDTA	Ethylene-di-amine-tetra-acetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FB	Fumonisin B-type
FDA	Food and Drug Administration
<i>Fum- F</i>	Fumonisin forward
<i>Fum- R</i>	Fumonisin reverse
g	gram
GC	Gas Chromatography
GPS	Global Positioning System
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HSRC SA	Human Science Research Council of South Africa
IAC	Immuno-affinity column
IARC	International Agency for Research on Cancer

ICP-MS	Inductively Coupled Plasma- Mass Spectrometry
IK	Indigenous Knowledge
kb	kilobase
kV(s)	kilovolt(s)
M	molarity
Mad	Madiga
Man	Mantheding
mg	milligram
MgCl₂	Magnesium chloride
ml	milliliter
mM	millimolar
Mod	Moduane
MRC	Medical Research Council
MS	Mass spectrometer
NaCl	Sodium chloride
ng	nanograms
NTDs	Neural Tube Defects
OPA	<i>o</i>-Phthaldialdehyde
PBS	Phosphate buffered saline
PCNB	Penta-chloro-nitrobenzene
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PVP	Polyvinyl-pyrrolidone
rpm	revolutions per minute
rRNA	ribosomal Ribonucleic acid
SABS	South African Bureau of Standards
SANBI	South African National Botanical Institute
Sef	Sefateng
SNA	Synthetic Nutrient Agar
TAE	Tris-acetate
TE	Tris EDTA
TLC	Thin Layer Chromatography
Tris	Tris(hydroxymethyl) aminomethane

USA	United States America
UV	Ultra-violet
V	volt
v/v	Volume per volume
w/v	Weight per volume
WA	Water Agar
WHO	World Health Organization
YPD	Yeast Peptone Dextrose
°C	degrees Celsius
μl	microlitre
μm	micrometer

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The genus *Fusarium* comprises a number of phytopathogenic filamentous fungi with a widespread cosmopolitan distribution and capable of infecting a wide range of crop plants (Nelson *et al.*, 1983). Over 20 *Fusarium* species have been distinguished based on their morphological characteristics and phylogenetic groupings (Summerell *et al.*, 2003). Some *Fusarium* species are economically important plant pathogens causing root and stem rot, vascular wilt and fruit rot that can dramatically reduce the quality and yield of crops (Nelson *et al.*, 1994; Baxter *et al.*, 1999). Other species cause storage rot and/or are important mycotoxin producers. Exposure to *Fusarium* toxins is associated with serious chronic and acute human diseases (Jurado *et al.*, 2006).

Fusarium species may also cause human infections. Diseases that develop as a result are commonly referred to as mycoses (Bennett and Klich, 2003). Primary pathogens affect otherwise healthy individuals with normal immune systems (immune competent). The majority of human mycoses, however, are caused by opportunistic fungi that produce illness by taking advantage of immune compromised hosts e.g. AIDS/ HIV (Van Burik and Magee, 2001; Bennet and Klich, 2003). *Fusarium* species most frequently implicated in human infections include *F. solani*, *F. oxysporum* and *F. verticilliodes* (Guarro and Gené, 1995; Dignani and Anaissie, 2004). Mycoses caused by fusaria are collectively referred to as fusariosis, which are grouped as primary / invasive and opportunistic / disseminated mycoses (Dignani and Anaissie, 2004). Species such as *F. solani*, *F. oxysporum* and *F. verticilliodes* have been reported as causative agents for mycotic keratitis, onychomycosis and hyalohyphomycosis, particularly in immune competent individuals that have experienced burns and in bone marrow transplant patients (Austen *et al.*, 2001; Dignani and Anaissie, 2004). According to Vismer *et al.* (2002), *Fusarium* infections have increased markedly in communities where the HIV/ AIDS incidence is high.

Some *Fusarium* species also produce low-molecular weight natural products as secondary metabolites, called mycotoxins, which may be detrimental to human and animal health (Sternberg, 1994). Diseases caused by mycotoxins are commonly referred to as mycotoxicoses

(Van Burik and Magee, 2001). The majority of mycotoxicoses results from eating mycotoxin-contaminated foods. Skin contact with mould-infested substrates and inhalation of spore-borne toxins are also important sources of exposure. Poor handling of food, improper storage practices and malnutrition problems are believed to expose an individual to mycotoxins (Nelson *et al.*, 1994; Bennet and Klich, 2003).

Mycotoxins produced by *Fusarium* species include the trichothecenes deoxynivalenol (DON), nivalenol (NIV) and T-2 toxin, fumonisins and zearalenone (Nelson *et al.*, 1994). The fumonisin-group of toxins are produced in varying amounts by *F. verticillioides*, *F. proliferatum*, *F. nygamai* and several other *Fusarium* species that grow on agricultural commodities in the field or during storage (Marasas, 2001; Rheeder *et al.*, 2002). *F. verticillioides* and *F. proliferatum* are reported as the most proliferative fumonisin producers commonly associated with maize and other grain crops (Shephard *et al.*, 1996). More than ten chemically different fumonisin classes have been characterized of which fumonisin B₁, B₂, and B₃ are reported to be produced in the largest quantities (Rheeder *et al.*, 2002; Soriano and Dragacci, 2004). Fumonisin B₁ have been shown to occur in maize and maize based foods (Shephard *et al.*, 1996) The most prevalent of these mycotoxins contaminating corn is fumonisin B₁, which is also believed to be the most toxic (Thiel *et al.*, 1992; Marasas *et al.*, 2001; Rheeder *et al.*, 2002).

Epidemiological studies have linked the consumption of fumonisin-B₁ (FB₁) contaminated food to the occurrence of human oesophageal cancer in, China (Yang 1980), Italy (Franceschi *et al.*, 1990), Transkei region of Eastern Cape Province in South Africa (Rheeder *et al.*, 1992) and Iran (Shephard *et al.*, 2000). Fumonisin B₁ has been implicated as a risk factor for neural tube defects (NTDs) in humans (Voss *et al.*, 2003) and is classified by the W.H.O as a probable human carcinogen (WHO-IARC, 1993). Due to its inhibitory effect on sphingolipid metabolism, fumonisin B₁ has been shown to produce a range of negative impacts on the immune cell functioning. It affect cell proliferation, inter- and intracellular communication, T- and B cell activation, antibody production and mechanism for the destruction of infecting bacteria by phagocytic neutrophils (Berek *et al.*, 2001; Baumrucker and Prieschl, 2002). Van der Walt *et al.* (2005) suggested that dietary fumonisin exposure could significantly add to the burden on compromised immune system of HIV/AIDS individuals.

Morogo is a collective term for a group of indigenous and traditional leafy green vegetable plants, some of which grow naturally, while others are cultivated in traditional subsistence farming (Van der Walt *et al.*, 2006). They form part of the staple diet of many households. In rural settings of South Africa, communities supplement grain-based staple diets consisting of maize porridge, maize rice, mabele/sorghum soft porridge with legumes and *morogo* (Khumbane, 1996). Leaves of some *morogo* plants can be sun-dried and stored for later use. Plants utilised as traditional *morogo* include, amaranth (*Amaranthus spp.*), African cabbage (*Cleome gynandra*), Jew's mallow (*Corchorus olitorius* and *C. tridens*), cowpea leaves (*Vigna unguiculata*) and cucurbits (*Cucumis spp.*, *Citrillus spp.* and *Cucurbita spp.*; Schippers, 2002; Van Wyk and Gericke, 2003).

Leafy vegetables are good sources of calcium (Ca), iron (Fe), pro-vitamin A, vitamin C (<http://www.fao.org/DOCREP/003/X6877E00.htm#TOC>, 20/10/2006) and folate (Tapiero *et al.*, 2001). Folate can be obtained from dietary sources. It is thought to be essential for the biosynthesis of nucleic acids and important in methylation reactions (Das, 2003). Dietary intake of sufficient amounts of folate has been shown to reduce the risk of having a baby with birth defects of the brain and/or spinal cord collectively referred to as neural tube defects (Missmer *et al.*, 2006). These traditional food plants are well adapted to local growing conditions and grow either naturally as weedy plants in cultivated lands or in the field. Those cultivated need low inputs of water and agrochemicals (Schippers, 2002; Aphane *et al.*, 2003; Van der Walt *et al.*, 2005).

The present study was conducted in the Dikgale Demographic Surveillance Site (DDSS) situated $\pm$ 50 km north-east of Polokwane, the capital of the Limpopo Province. Prof. Marianne Alberts, School of Health Sciences, University of Limpopo established DDSS for research purposes. The DDSS is situated in a semi-arid, low rainfall region and is made up of eight informal resident sites (villages) situated close to one another. Infrastructure is poor. The community of DDSS are mainly Pedi speaking and rates of unemployment and illiteracy are high. Four of the eight villages were chosen for the purpose of this study, namely Sefateng, Madiga, Mantheding and Moduane. These villages represent rural settings where subsistence cultivation of maize and utilisation of traditional leaf vegetables (*morogo*) have an important role in providing food for resource-limited households. Open fields surrounding villages are used for cattle grazing and

provide dung for soil improvement of lands. *Morogo* plants generally grow in maize lands and/or are collected from surrounding fields.

Sufficient scientific evidence exists to suggest that mycotic and mycotoxigenic *Fusarium* species of concern primarily occur in association with maize. Growing among or close maize hosting harmful *Fusarium* strains predisposes *morogo* to fusarial contamination. In view of the nutritional advantages of traditional *morogo* for households in rural subsistence settings such as DDSS, their mycological quality was considered an important aspect for investigation. The DDSS is in the Limpopo Province where overall HIV prevalence is estimated at 8.0% and death due to HIV/AIDS related diseases was 38 % (HSRC SA, 2005; ASSA 2006). In addition, the occurrence of neural tube defects (NTDs) in the DDSS was unexpectedly high and reported as 3.55 cases per 1000 live births (Venter, 1995). Exposure to mycotic and mycotoxigenic fusaria implicate health risks that could enhance disease outcomes associated with HIV/AIDS and NTDs.

1.2 RESEARCH AIMS AND OBJECTIVES

The aim of the study was to investigate the levels of mycotoxigenic and mycotic fusaria in two widely consumed *morogo* plants (*lerocho* and *thepe*) and the agro-environment of the Dikgale Demographic Surveillance Site (DDSS) where maize and *morogo* often grow in close proximity of each other. Objectives for the study were the following:

1. To document the botanical species identification of plants used as traditional *morogo* in the DDSS as well as the indigenous knowledge related to their utilisation;
2. To determine fungal levels in *thepe*, *lerocho* and their immediate surroundings;
3. To investigate the occurrence of mycotic and mycotoxigenic *Fusarium* species occurring in association with *thepe*, *lerocho* and their immediate environment;
4. To analyse *lerocho* and *thepe* samples for the presence of fumonisin B group toxins.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Fungi are eukaryotic, spore-producing, achlorophyllous organisms reproducing both sexually and asexually. Vegetatively, true fungi (Eumycota) consist of filamentous-branched structures (hyphae) that are surrounded by chitin-containing cell walls (Alexopoulos *et al.*, 1995; Baxter *et al.*, 1999). Hyphae extend on the tip and enable the fungus to penetrate a host and form a network of filaments (mycelium). Fungi may live either as saprotrophs on dead plants or animals, or as parasites on living plants or animals (Baxter *et al.*, 1999).

Fungal species useful to man include the yeast *Saccharomyces cerevisiae* used in the baking and brewing industries, and mushrooms that are appreciated as food. Some fungi, e.g. *Penicillium* species, are useful in the dairy industry to give distinctive flavours during the ripening of cheeses (Alexopoulos *et al.*, 1995; Baxter *et al.*, 1999). Others find application in the pharmaceutical and medical fields as they are employed in biotechnological processes to produce antibiotics, e.g. penicillin from *Penicillium chrysogenum* (Malloch, 1981; Alexopoulos *et al.*, 1995; Baxter *et al.*, 1999). Mycorrhizal fungi form associations with plant roots enhancing mineral uptake of the plant host. In agriculture, these associations increase crop yield. Fungi that share in endophytic relationships with plants protect their hosts from pathogen invasion, insect attacks or damage by grazing mammals (Alexopoulos *et al.*, 1995; Baxter *et al.*, 1999).

Many fungi are pathogens of agricultural crops and others may spoil goods produced of leather, petroleum products or foodstuffs. Certain fungal species cause diseases in animals and humans by direct infection (mycoses) or by producing toxins while growing on edible plants (mycotoxicoses). Fumonisin toxins produced by *Fusarium* species and ochratoxins from *Aspergillus* species have been implicated in human oesophageal cancer and renal atrophy respectively. Fumonisin B₁, the most abundant toxin produced by a number of *Fusarium* strains was shown to be the cause of a fatal neurological disease in horses and respiratory conditions in pigs (Alexopoulos *et al.*, 1995). Other potent toxins produced by *Fusarium* species include trichothecenes, moniliformin, fusaric acid, beauvericin and hyperestrogenic zearalenone (Placinta *et al.*, 1999).

2.2 THE GENUS *FUSARIUM*

Fungi are divided into two main groups based on the nature of their vegetative structure during the non-reproductive phase. Fungi that are unicellular or have a mycelia vegetative structures falls in the group Eumycota that branches into five divisions. The genus *Fusarium* belongs to the Deuteromycota branch of the Eumycota having septate mycelia and forming asexual spores called conidia (Baxter *et al.*, 1999). The genus *Fusarium* contains a number of phytopathogenic species occurring widely distributed in soil, plants and air (Nelson *et al.*, 1983). However, fusaria are common in cultivated fertile and rangeland soils in tropical and temperate regions, they are also found in extreme environments such as deserts (Burgess *et al.*, 1988; Jeschke *et al.*, 1990; Nelson *et al.*, 1994). In agricultural ecosystems *Fusarium* species are typically associated with grain crops such as maize (Munkvold and Desjardins, 1997) and were also reported to occur in association with non-agricultural plants such as prairie grasses (Zeller *et al.*, 2003). Phytopathogenic species affecting maize plants include *F. verticillioides*, *F. proliferatum*, *F. graminearum* and *F. anthophilum* (Scott, 1993; Munkvold and Desjardins, 1997). Presently over twenty *Fusarium* species are distinguished based on their morphology and phylogenetic grouping (Nelson *et al.*, 1983). In the agro-environment, *Fusarium* spores survive in plant residue and can be spread by irrigation water, wind, rain, and contaminated farm equipment (http://www.extento.hawaii.edu/kbase/crop/Type/fus_prim.htm, 13/07/2006). *F. verticillioides* was shown to produce abundant airborne spores that can spread some distances away from the main source of contamination (Munkvold and Desjardins, 1997).

2.3 *FUSARIUM* SPECIES CONCEPTS

Morphological, biological and phylogenetic species concepts are presently used in *Fusarium* taxonomy (Summerell *et al.*, 2003). The morphological species concept developed by Nelson *et al.* (1981) and Gerlach *et al.* (1982) is based on visible characteristics including colony morphology, spore size and shape. The genus *Fusarium* produces two types of spores called microconidia and macroconidia (banana shaped). Chlamydospores are thick-walled structures produced to survive extreme environmental conditions. The primary structure used for accurate microscopic identification is the macroconidia (Nelson *et al.*, 1983). Macroconidia are larger than microconidia and distinguishing properties used for identification would be observed more accurately provided cultures were initiated from single spores under favourable sporulation conditions. Morphological species were in some cases found to be too broad. Based on

morphological characteristics, the *Gibberella fujikuroi* complex was shown to comprise of between one and four species, but molecular analysis conducted by O'Donnell *et al.* (1998) demonstrated that it included more phylogenetic species (Summerell *et al.*, 2003). Nirenberg *et al.* (1998) played an important role in the development of the phylogenetic and biological species concepts. The phylogenetic species concept is based on DNA sequencing to generate a unique profile of molecular characteristics common to a specific group of organisms (Nelson *et al.*, 1994; Summerell *et al.*, 2003). Errors in phylogenetic description, however, can occur by splitting isolates into groups that are not biologically meaningful. The requirement for the biological species concept is that the progeny resulting after sexual-crossing of members of the same species must be fertile and viable (Summerell *et al.*, 2003). A problem with applying the biological species description is the possibility that an appropriate tester strain would be unavailable. According to Steenkamp *et al.* (2002), the taxonomy of *Fusarium* isolates of the *Gibberella fujikuroi* complex can only be consistent when morphological, biological, as well as phylogenetic species concepts are applied.

2.3.1 Isolation and identification of *Fusarium* species

Fusarium can be isolated from environmental samples using an appropriate medium such as penta-chloronitrobenzene (PCNB) agar, a semi-selective medium for *Fusarium* containing bacteria inhibiting substances (Nelson *et al.*, 1983). Once isolated different media formulations are employed to examine specific macroscopic and microscopic features based on which isolates are assigned to a morphological species. Molecular methods are subsequently applied to verify morphological species identification.

Culture media generally used for the isolation and purification of *Fusarium* isolates according to Fisher *et al.* (1982), Nelson *et al.* (1983) and Klotz *et al.* (1988) includes Penta-chloronitrobenzene (PCNB), Potato Dextrose Agar (PDA), Water Agar (WA), Carnation Leaf-piece Agar (CLA) and Synthetic Nutrient Agar (SNA; Nelson *et al.*, 1983).

PDA is a carbohydrate rich medium used for examining macroscopic colony characteristics including texture and pigmentation, thickness and height of the hyphal growth and position of the sporodochia (Nelson *et al.*, 1983; Seifert, 1996; Summerell *et al.*, 2003). While colonies on this medium might look similar, they are not necessarily belonging to the same species, while those that are different in colour may be of the same species. Sporulation is very poor on PDA and

macroconidia produced are not uniform for proper identification, hence it is not used for this purpose (Nelson *et al.*, 1983; Summerell *et al.*, 2003).

CLA medium promotes sporulation and reduces phenotypic variation. Conidia and conidiophores are produced in abundance on CLA, and the morphology of the conidia and conidiophores closely resembles what are seen under natural conditions. Macroconidia produced from a single-spore culture on CLA medium are uniform in shape and ideal for accurate identification (Nelson *et al.*, 1983). Important macroconidial characteristics include shape and size, the number of septa and the shape of the apical (top) as well as basal (bottom) cells. Apical cells can be hooked, nipple-like, blunt or conical in shape, whereas basal cells may be papillate, notched or foot-shaped, have an extended foot or be blunt or not notched. Microconidia and chlamydospores are also observed on CLA plates. Microconidia are examined for the following characteristics: shape and size; presence of a septum; whether they are occurring single, in chains or on false heads; the nature of their conidiogenous cells on which they are borne. Two types of conidiogenous cells are distinguished, namely monophialides having a single opening in the conidiogenous cell and polyphialides with two or more openings. Chlamydospores may be present or absent and are examined on both CLA and SNA. Distinguishing characteristics in relation to chlamydosporum include the type of wall (e.g. rough or smooth) and pattern of arrangement that can be in single, in pairs, clumps or chains (Nelson *et al.*, 1983; Seifert, 1996; Summerell *et al.*, 2003). According to Summerell *et al.* (2003), PDA and CLA can be used for initial evaluation and depending on information gathered. SNA medium are also used for the production of microconidia and chlamydospores should additional information be required. Cultures on PDA, CLA and SNA are incubated for 7 to 14 days at 20°C - 25°C with day and night cycle light exposure to promote sporulation on CLA and pigmentation on PDA (Nelson *et al.*, 1983; Summerell *et al.*, 2003).

Proper microscopic examination is crucial for accurate *Fusarium* species identification. Since stains and fixatives can change the appearance of spores (Nelson *et al.*, 1983), stains are preferably not employed for slide preparation but colourless lactophenol in stead (Baxter *et al.*, 1999).

2.3.2 Molecular analyses

Molecular methods are applied to confirm morphological species identification. Information is further used to determine phylogenetic relationships of isolates (Geiser *et al.*, 2004). Molecular

markers used in various studies for polymerase chain reaction (PCR)-based DNA sequencing include calmodulin, Histone *H3*, translation elongation factor (EF-1 α), β - tubulin, mitochondrial rRNA and mtSSU rDNA (Steenkamp *et al.*, 2002; Zeller *et al.*, 2003; Schroers *et al.*, 2004). The PCR process involves the separation of individual DNA strands (denaturation) by heat applied for a specific time. A small segment of DNA (probe) is targeted to anneal with the piece of DNA of interest (target strand). The probe is extended to equal the number of kilobase (kb) of the product to be amplified and yield double stranded DNA (Russell and Paterson, 2006). EF-1 α and β -tubulin are useful biomarkers to discriminate between *Fusarium* species (O'Donnell, 2000; Schmidt *et al.*, 2004; Yergeau *et al.*, 2005). A simple BLAST server web-accessible DNA sequence database (*FUSARIUM-ID*) was created based on translation elongation factors sequences representing the phylogenetic diversity of *Fusarium*. The sequence produced from an unknown *Fusarium* species can be used as a query against this database to determine the identity of the unknown species or its closest relative (Geiser *et al.* 2004). A useful molecular marker for fumonisin-producing *Fusarium* strains is the *FUM1* gene encoding the polyketide synthase enzyme involved in the biosynthesis of fumonisin (par. 2.6). The reliability of the *FUM1* gene alone was questioned on the basis that polyketide synthase can be coded for by between 7 and 25 different genes (Kroken *et al.*, 2004; Bezuidenhout *et al.*, 2006). Bezuidenhout *et al.* (2006) showed that using the primers EF-1 α and *FUM1* together in a multiplex PCR can successfully produces the expected PCR fragments of each primer, 700 bps and 800 bps respectively.

2.4 ECOLOGICAL AND ECONOMICAL IMPORTANCE OF THE GENUS *FUSARIUM*

Members of the genus *Fusarium* have been widely studied because of their ability to cause disease in economically important crops, animals and humans (Nelson *et al.*, 1994). As plant pathogens, some *Fusarium* spp. are capable of causing root and stem rot, wilting or head blight reducing the quality, nutritional value and yields of important agricultural crops often rendering them unfit for human consumption (Baxter *et al.*, 1999; Fandohan *et al.*, 2003). Crops affected by fusarial diseases include amongst others nuts and sunflower seeds (Jiménez *et al.*, 1991) sugarbeets (Bosch and Mirocha, 1992), bananas (Jiménez *et al.*, 1993), asparagus (Elmer *et al.*, 1996) and maize (Munkvold and Desjardins, 1997). Enormous financial losses have been estimated in certain areas (Nganje *et al.*, 2002) and the cost of preventative measures can be high (Baxter *et al.*, 1999).

2.5 THE IMPACT OF THE GENUS *FUSARIUM* ON HUMAN AND ANIMAL HEALTH

A number of *Fusarium* species have been reported as being pathogenic to humans and animals either by direct infection (mycosis) or through the production of mycotoxins (mycotoxicoses). *Fusarium* species produce a range of potent mycotoxins that can contaminate human food and animals feed. Dietary exposure to fusarial toxins has been shown to have deleterious effects on human health (Nelson *et al.*, 1994; Jurado *et al.*, 2006).

2.5.1 Fusarioses

Mycoses due to *Fusarium* infection are commonly referred to as fusarioses. Tissue breakdown caused by trauma, severe burns or invasive organisms increase the risk for fusariosis in immunocompetent individuals. Disease conditions resulting from fusarial infections include keratitis, onchomycosis, peritonitis and cellulites (Vismer *et al.*, 2002; Dignani and Anaissie, 2004). *Fusarium* species implicated regularly as causative agents in both invasive and disseminated fusariosis include *F. solani*, *F. oxysporum* and *F. verticillioides* (Dignani and Anaissie, 2004). According to Vismer *et al.* (2002), *Fusarium* infections have increased in communities where the HIV/AIDS incidence is high. Severely suppressed immune systems, tissue damage and organ transplants are some of the risk factors for disseminated fusariosis (Guarro *et al.*, 2000; Bodey *et al.*, 2002; Dornbusch *et al.*, 2004). Diseases associated with disseminated fusarioses include amongst others skin lesions and persistence refractory fever (Dignani and Anaissie, 2004). *Fusarium* causing infection may enter the body through direct skin contact or inhalation of spores from the air (Bennet and Klich, 2003).

2.5.2 *Fusarium* mycotoxins

Under predisposing environmental factors, fusarial plant pathogens produce harmful low-molecular weight secondary metabolites that can accumulate in quantities detrimental to human health when toxin-containing plant materials are consumed (Nelson *et al.*, 1994; Sternberg 1994). Poor food handling and improper storage could enhance the risk for toxin production and dietary exposure to mycotoxins (Nelson *et al.*, 1994; Bennet and Klich, 2003). Mycotoxins typically produced by *Fusarium* strains include trichothecenes deoxynivalenol, nivalenol, fusarenon-X, diacetoxyscirpenol, neosolaniol, T-2 toxin and a range of fumonisin analogs (Nelson *et al.*, 1994; Rheeder *et al.*, 2002).

2.5.2.1 Fumonisin

Fumonisin is a group of fungal toxins produced on agricultural crops in the field or during storage by several *Fusarium* strains including *F. verticillioides*, *F. proliferatum* and *F. nygami* (Marasas, 2001). *F. verticillioides* and *F. proliferatum* are reported to be the most prolific fumonisin producers (Shephard *et al.*, 1996; Rheeder, *et al.*, 2002). According to Blackwell *et al.* (1993) the backbone structure of fumonisin is of a polyketide origin. The methyl groups ($-\text{CH}_3$) are derived from methionine whereas the carboxylic acid side chains ($-\text{COOH}$) and the amino group ($-\text{NH}_2$) were derived from glutamic acid and serine, respectively. The structure of fumonisin B₁ (FB₁) as adapted from Blackwell *et al.* (1993) is shown in figure 1.1.

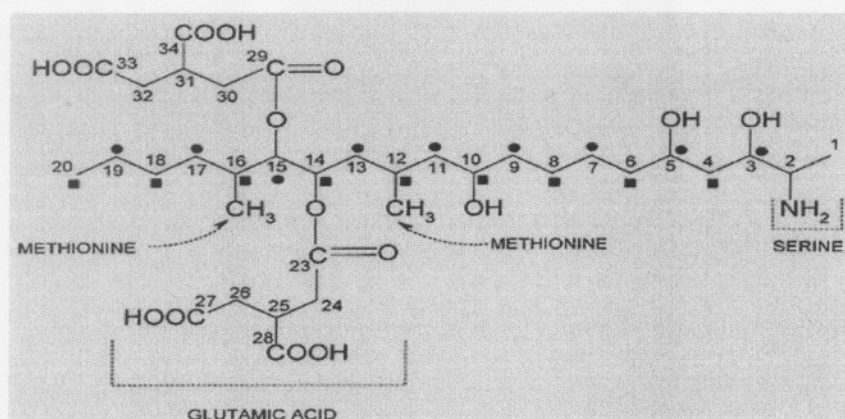


Fig. 2.1- Biosynthetic origin of FB₁ (Blackwell *et al.* 1993)

Fumonisin was found to be common contaminants of maize-based foods and feeds in several countries. A number of 28 fumonisin analogs have been isolated and characterized. The B-group fumonisins, namely fumonisin B₁ (FB₁), fumonisin B₂ (FB₂), and fumonisin B₃ (FB₃) are considered the predominant and most potent of the fusarial toxins (Shephard *et al.*, 1996; Rheeder *et al.* 2002). Fumonisin B₁ accounts for 70 to 80% of the total fumonisins produced, while FB₂ made up to 15 to 25% and FB₃ from 3 to 8% (Marín *et al.*, 1995; Marasas *et al.* 2001; Rheeder *et al.* 2002).

Environmental conditions (temperature, humidity, drought stress, and rainfall), insect infestation and pre- and postharvest handling could influence the level of fumonisins in food commodities (Fandohan *et al.*, 2003). Hot and dry weather, followed by periods of high humidity, or a

moisture content of 18 to 23% during storage of crops may lead to high levels of fumonisin (Bacon and Nelson, 1994; Munkvold and Desjardins, 1997; Miller, 2001). Temperatures of about 20-30 °C and water activity around 0.98 were found to promote production of mycotoxins by *Fusarium* species (Soriano and Dragacci, 2004). Other studies showed that high rainfall areas, prevalence of insect damage and fungal diseases could result into high production of fumonisin (Ono *et al.*, 1999; Soriano and Dragacci, 2004). It is reported that interaction amongst *Fusarium* species and interactions with other fungi can affect fungal infection and mycotoxin production. The population of *F. verticillioides* and *F. proliferatum* were found to be reduced markedly by the presence of *F. graminearum*, and the fumonisin B₁ (FB₁) production by them were significantly inhibited in the presence of *F. graminearum* (Velluti *et al.*, 2000).

2.5.2.2 Disease conditions caused by fumonisin

Epidemiological studies have shown a correlation between the consumption of fumonisin B₁-contaminated food and human oesophageal cancer in China (Yang 1980), Italy (Franceschi *et al.* 1990), Transkei region of Eastern Cape Province in South Africa (Rheeder *et al.* 1992) and Iran (Shephard *et al.* 2000). Other studies related fumonisin B₁ consumption with fatal diseases in animals including leukoencephalomalacia in horses (Kellerman *et al.*, 1990), pulmonary edema in swine (Harrison *et al.*, 1990), and cancer in rats (Gelderblom *et al.*, 1991). Fumonisin B₁, classified by the World Health Organisation (WHO) as a probable human carcinogen (WHO-IARC, 1993), is also implicated as a potential risk factor in the occurrence of neural tube defects (NTDs; Voss *et al.* 2003; Marasas *et al.*, 2004; Missmer *et al.*, 2006). The US Food and Drug Administration (FDA) determined a fumonisin level not greater than 4 µg/g in human foods (<http://vm.cfsan.fda.gov/~dms/fumongui.html>, 20/02/2006), whereas the Joint FAO/WHO Expert Committee on Food Additives recommended maximum tolerable daily intake of 2 µg/kg body weights to fumonisin B₁, B₂, and B₃ either alone or in combination (WHO, 2002).

2.5.2.3 Fumonisin and sphingolipid metabolism

Fumonisin B₁ (FB₁) is a structural analogue of the sphingolipid intermediate sphinganine. It is suggested that the presence of FB₁ could interfere with sphingolipid metabolism by inhibiting the sphinganine *N*-acetyltransferase enzyme (Wang *et al.*, 1991; Spiegel and Merrill, 1996). Sphingolipids are a class of membrane lipids essential for maintaining membrane function and lipoprotein structure in eukarotic cells. Fumonisin B₁ inhibits ceramide synthase and thus blocks the biosynthesis of complex sphingolipids and cause sphinganine/sphingosine to accumulate. The

accumulation of sphingoid bases is thought to be the primary cause of the toxicity of fumonisin B₁ though other biochemical events are also involved (Merrill *et al.*, 2001). Due to its inhibitory effect on sphingolipid metabolism, fumonisin B₁ negatively impacts on the immune cell functioning including cell proliferation, inter- and intracellular communication, T- and B cell activation, antibody production, mechanism for the destruction of infecting bacteria by phagocytic neutrophils (Baumrucker and Prieschl, 2002). Dietary exposure to fumonisins is therefore expected to add to the burden on the compromised immune system of HIV/AIDS individuals (Van der Walt *et al.* 2005).

2.6 FUMONISIN GENETICS

Fumonisin compounds are metabolites of a polyketide pathway. The *FUM1* gene encodes a polyketide synthase involved at an early step in the biosynthesis of fumonisin (Proctor *et al.*, 2003). *FUM1* is thought to be one of 15 *FUM* genes clustered on chromosome 1 that are involved in fumonisin biosynthesis or possible self-protection from fumonisin toxicity. The amino acid sequence of the 15 gene *FUM* cluster suggested that 11 genes encode biosynthetic enzymes, 2 encode transporters, and 2 encode proteins supposed to be involved in self-protection. These 15 *FUM* genes are believed to co-ordinately expressed during fumonisin biosynthesis (Proctor *et al.*, 2003). It was discovered that when the *FUM1*, *FUM6*, or *FUM8* are disrupted, the fumonisin biosynthesis ceases (Seo *et al.*, 2001). Two genes (*ZFR1* and *FCC1*) responsible for regulating fumonisin biosynthesis and thought not to be linked to the *FUM* gene cluster have been characterized (Shim and Woloshuk, 2001; Flaherty and Woloshuk, 2004). Experimental evidence suggest that *FCC1* encodes a cyclin-like protein that is considered to be part of a signal transduction pathway affecting both fumonisin biosynthesis and development (Shim and Woloshuk, 2001) whereas *ZFR1* encoding a zinc finger protein is a positive regulator of *FUM* genes (Flaherty and Woloshuk, 2004).

2.7 FUMONISIN DETERMINATION

Different methods can be applied for the detection and quantification of fumonisins in contaminated food samples namely chromatography, immunoassay and capillary electrophoresis (Dutton, 1996; Shephard, 1998). An immunoassay procedure e.g. enzyme-linked immunosorbent assay (ELISA) provides an appropriate method for the rapid screening of samples and is less costly in terms of equipment. The success of this method depends on having FB specific antibodies. It can be useful as primary screening procedure to monitor the safety of food and

check legislative tolerance imposed on certain foods commodities (Pestka *et al.*, 1994; Dutton, 1996; Shephard, 1998). Chromatographic methods include thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC; Shephard, 1998). GC has the disadvantage of requiring expensive instrumentation and hydrolytic pretreatment, but is useful when used with mass spectrometry (MS) in quantitative determination of fumonisins in heavily contaminated field samples (Shephard *et al.*, 1992; Shephard, 1998). Both the TLC and HPLC require extraction of fumonisins from the food sample and ‘‘clean up’’ procedures. They both lack significant UV chromophores because they are not inherently fluorescent. As a result they need a fluorescent derivatisation reagent for sensitive detection. Since the TLC detection limit is 0.1 mg/g, this method is not used for the determination of fumonisin in contaminated food samples. HPLC allows fumonisin detection levels at as low as 50µg/kg and has thus found useful application for the quantification of fumonisins (Dutton, 1996, Shephard, 1998).

2.8 MOROGO - TRADITIONAL LEAFY VEGETABLES

Morogo is a collective term used in South Africa for a group of indigenous and traditional leafy vegetables some of which grow naturally, while others are cultivated in traditional subsistence farming (Van der Walt *et al.*, 2006). Growing under a wide range of environmental conditions examples of *morogo* include cowpea leaves (*Vigna unguiculata*), African cabbage (*Cleome gynandra* and *C. monophylla*), cucurbits (*Cucurbita* spp.) and vegetable amaranth (*Amaranthus hybridus*, *A. thunbergii*). These plants are easier to grow and resistant to pests and diseases compared to introduced vegetable crops (Schippers, 2002). Leafy vegetables are also important sources of micronutrients including pro-vitamin A, vitamin C, iron, calcium, magnesium and others (Aphane *et al.*, 2003). Rural communities supplement grain-based staple diets consisting mainly of maize or *mabele* (sorghum) soft porridge with legumes, *morogo* and tuber-type of traditional vegetables (Questionnaire information; appendix 1). Grain-based foods are reported to be poor sources of protein, calcium, iron, zinc, riboflavin, nicotinic acid, vitamin C, and carotene (pro-vitamin A), but are good sources of magnesium, potassium, and thiamine. When the *morogo* leaves are cooked and eaten with white maize porridge a more balanced diet results (Aphane *et al.*, 2003). Leafy vegetables are also known to be rich sources of folate (Van der Walt *et al.*, 2005). Some studies have shown that folate supplementation for women of child bearing age can help to reduce the risk of having a baby with birth defects of the brain and spinal cord called neural tube defects (NTDs; Missmer *et al.*, 2006). Consumption of green leafy vegetables like *morogo* should be encouraged in pregnant women.

2.9 AMARANTH AND AFRICAN CABBAGE

Some widely utilised *morogo* plants are species of the plant genera *Amaranthus* and *Cleome* both preferring warm temperate and semi-arid regions. Of the various amaranth species *Amaranthus thunbergii* Moq is believed to have originated in Southern Africa (Schippers, 2002) and grows throughout the summer periods. The species *Cleome gynandra* is scarce in the cooler and high rainfall parts of South Africa (Van Wyk and Gericke, 2003). Leaves of both *Cleome gynandra* (*lerotho* - Pedi) and *Amaranthus thunbergii* (*thepe* - Pedi) are appreciated as vegetables in the Capricorn District of South Africa where this study was conducted. As leafy vegetables of Western and Asian diets, traditional *morogo* is expected to provide high levels of essential micronutrients. Table 2.1 compares the nutritional value of Amaranth and African cabbage with that of cabbage and spinach. This information was obtained from the UN Food and Agriculture Organization (FAO) food composition table. According to data in Table 2.1, Amaranth and African cabbage have almost three times the protein and seven times the iron content found in the same weight of cabbage.

Table 2.1 Nutrient compositions per 100 grams of edible portion of raw amaranth and African cabbage compared to cabbage and spinach
(<http://www.fao.org/DOCREP/003/X6877E00.htm#TOC>, 20/10/2006)

Nutrient	Amaranth	African cabbage	Cabbage	Spinach
Energy (calories)	42	34	26	26
Moisture (%)	84	86.6	91.4	90.6
Proteins (g)	4.6	4.8	1.7	2.1
Carbohydrate (g)	8.2	5.2	6.0	5.3
Fibre (g)	1.8	-	1.2	0.8
Calcium (g)	410	288	47	61
Phosphorus (g)	103	111	40	46
Iron (g)	809	6.0	0.7	1.7
β -carotene (mg)	5716	-	100	-
Thiamine (mg)	0.05	-	0.04	0.03
Vitamin C (mg)	64	13	54	46
Riboflavin (mg)	0.42	-	0.1	0.27

Abbreviation: - no values reported, or data are questionable and omitted

2.10 PROCESSING OF AMARANTH AND AFRICAN CABBAGE IN TRADITIONAL AFRICAN SETTINGS

Local names used for amaranth and African cabbage species in Africa, vary according to region. In the Limpopo Province of South Africa Pedi-speaking people, call amaranth leaves *thepe* and the African cabbage *lerotho*. In most African countries, African cabbage and leaf amaranth are processed similarly for consumption (Schippers, 2002). According to Schippers, (2002) *A. thunbergii* and *Cleome gynandra* leaves both have a bitter taste and to improve the taste salt is added. Amaranth and African cabbage leaves are sun-dried and stored until needed for cooking

and it is then served with starch-based staple food. In Kenya, Botswana and Namibia, fresh leaves are cooked with salt to remove the bitter flavour and the boiled leaves kneaded into small balls, which are then sun-dried. After drying, the processed product can be stored for few months before consumption. To improve taste groundnut paste is added in Zambia whereas in Zimbabwe buds of *Aloe greatheadii* are added (Schipper, 2002). The two traditional African vegetables can also be used for purposes other than food. In some African countries such as Zimbabwe, the amaranth plant flower heads are dried and grinded into powder and mixed with tobacco snuff for elderly people. African cabbage leaves have medicinal applications in some countries (Schipper, 2002).

Mycotic and mycotoxigenic *Fusarium* species are ubiquitous in the agro-environment. They are capable of infecting and damaging economically important agricultural crops, resulting in low yields and huge economical loss. Their ability to be pathogenic to humans and animals is also of great concern. Although, some *Fusarium* species are capable of producing a number of mycotoxins, fumonisin B group toxins have been suggested to be capable of harming the health of humans and animals in a deleterious way. Mycotoxigenic and mycotic *Fusarium* species have been shown to be common contaminant of maize. *Morogo* plants are not natural hosts to mycotoxigenic and mycotic *Fusarium* species. Contamination of *morogo* by *Fusarium* species could have high health risk implications. Consumption of these plants could afford a measure of health protection provided they are microbiologically safe.

CHAPTER 3

STUDY AREA AND INDIGENOUS KNOWLEDGE

3.1 STUDY AREA

The study was conducted at Dikgale Demographic Surveillance Site (DDSS) an area $\pm$ 50 km north-east of Polokwane, the capital city of the Limpopo Province in South Africa. The DDSS is a semi-arid, low rainfall region made up of eight (8) informal residential sites (villages) situated close to one another. The infrastructure in those villages is poor. Villagers are mainly Pedi people and the local language spoken is *Sepedi*. Four villages were chosen for the present study namely Sefateng, Madiga, Mantheding and Moduane. The geographical orientations of the villages, as recorded using the Global Position System (GPS), were as follows: Sefateng-S23°4.092'; E029°46.647', Madiga-S23°48.542'; E029°43.268', Mantheding-S23°46.349'; E029°44.572' and Moduane-S23°48.279'; E029°44.837'.

These villages were representative of sites where subsistence farming was practised. Open fields used for grazing surround villages. Subsistence cultivation of maize, vegetable crops including indigenous leafy vegetables (*morogo*) has an important role in providing food for households in DDSS. *Morogo* plants grow either among maize or in proximity of maize fields.

Rates of unemployment and illiteracy are high. Statistics released for 2005 by the Human Science Research Council of South Africa (HSRC SA) estimated the overall (males, females, adults and children) prevalence of HIV/AIDS in the Limpopo Province at 8.0 % (HSRC SA, 2005). Actuarial Society of South Africa (ASSA) reported that 38 % of deaths during 2005 in the Limpopo Province were due to HIV/AIDS related disease outcomes (ASSA, 2006). In addition to the high incidence of HIV/AIDS, the occurrence of Neural Tube Defects (NTDs) was of great concern in this region. A study by Venter, (1995) reported 3.55 cases per 1000 live births. No latest studies of NTDs have been reported so far.

3.2 INDIGENOUS KNOWLEDGE (IK) IN DDSS

Indigenous knowledge is the local knowledge that is unique to a given society and is the basis for local-level decision-making with regards to hunting and fishing, agriculture, health, food production, education, adaptation to environmental or social change, and other activities concerning rural community life

(<http://www.scidev.net/dossiers/index.cfm?fuseaction=dossierfulltext&Dossier=7>,

22/10/2006). Questionnaires (Appendix 1.1) were used and interviews conducted with female members of selected households in the DDSS villages to obtain information related to indigenous knowledge relevant to this study. Information pertained to: types of *morogo* plants utilized; sources of *morogo* (veldt / fields); methods of cultivation (where applicable); harvesting, processing, storage practices as well as awareness of any health related properties.

3.2.1 Socio-economic aspects

Most of the households composed of not less than four members with at least two children per household. Most teenagers are not staying with their families, as they preferred living with their relatives in townships or attending schools elsewhere. In many instances breadwinners like teachers, policemen and soldiers are staying and working away from the family in townships, towns or elsewhere. Generally, occupations of breadwinners staying and working in the villages included construction builders, gardeners, domestic workers and research assistance workers for the Limpopo University. Salaries per month for these local workers range between R200 and R400. Adults staying behind are mostly women who work on household or field cultivation schemes. There is a high rate of unemployment and some of the reasons supplied for being jobless included lack of suitable jobs while in a number of cases serious illness like ulcers, tuberculosis (TB) or asthma were mentioned. Other source of income includes pension money as well as children grants.

3.2.2 Dietary information

Generally, the staple food of communities consisted of maize porridge and bought wheat bread. The consumption frequency of the maize porridge is mostly once a day and bread twice a day. Other foods that are cooked include meat, beans, rice, fish and vegetables like potatoes, cabbages and tomatoes. Fish and rice, affordable only by households of the high-income group are eaten at least twice a week. Other foods mentioned as a source of protein are eaten once or twice a week. Some people interestingly prefer also *mopani* worms while most dislike it. DDSS has a variety of *morogo* plants that are appreciated as leafy vegetables for consumption. These *morogo* plants grow naturally as weeds in household schemes though few are cultivated and broadcasted along with maize when seeds are available. When available, traditional *morogo* are eaten about four times a week usually as relish to supplement maize porridge. The average weight of *morogo*

eaten by members of the household per meal is usually two full serving spoons for children and 3 to 4 for adults. *Ditoro* (prickly pears) are usually picked from the field and eaten uncooked by both adults and children.

3.2.3 *Morogo* information

Morogo is a group of indigenous and traditional leafy green vegetable plants some of which grow naturally and others cultivated in traditional subsistence farming (Van der Walt *et al.*, 2006). Consumption of *morogo* is more popular with older people than with children and teenagers. The South African National Botanical Institute (SANBI, Appendix 1.2) identified some plant species used, as *morogo* in the DDSS. *Thepe* and *lerocho* were the most abundant and preferred *morogo* type. *Mophotse*, *monawa* and *mochacha* are cultivated but other types of *morogo* grow naturally.

3.2.4 Cultivation of *morogo*

Morogo plants usually grow with or near to maize and most types were available during the 2005 and 2006 seasons. Some women are reported to cultivate *morogo* for profit making by selling, especially when these plants are not available during winter periods. Because of the unavailability of irrigation systems, the community of DDSS relies on rainfall. During rainfall people are heard screaming and singing of joy. Most households have ploughed the previous (2005) as well as the current season (2006).

When little rainfall is experienced, as was the case towards the end of 2005, plant growth is poor and yields lower. Young *morogo* leaves are harvested one month after planting and those growing naturally are collected after full growth, usually one month after the first rain. In rural subsistence settings seed of cultivated *morogo* plants are harvested and used for planting in the following season. Livestock droppings from surrounding grazing fields are collected and used for soil improvement where *morogo* is cultivated for household purposes. As a measure of quality control, older leaves that appear mouldy are thrown away in the field or collected in a container and buried to a pit.

3.2.5 Methods of preparing *morogo*

According to questionnaire information households might prepare *morogo* in different ways. Sometimes leaves of different kinds of *morogo* are mixed for cooking especially *thepe* and *lerocho*. Women mostly prefer to prepare young rather than older leaves which are believed to

have a bitter taste and sometimes have developed “surface hairs” - a sign of mould infection. To improve the taste, leaves are cooked along with salt, tomatoes, potatoes and oil when these commodities are available. Others prefer to add onions to *mokhuša* (*morogo* dried after cooking) leaves. Those who can afford them add sometimes spices. Cooking time for *leroto* usually does not exceed 20 minutes depending on leaf quality.

Morogo leaves that are stored for future use are processed mostly similarly. Hand picked fresh leaves are cooked for 20 to 30 minutes. Following cooking, *morogo* leaves are kneaded into small balls for sun-drying. The duration for sun drying depends on the weather and how quick the processed product dries. There is no specific container where drying can be made. Kneaded balls are allowed to dry on top of any bare zinc or tin container. After drying, the processed product can be stored for few months in closed containers to be consumed during winter months when *morogo* would be unavailable. Traditionally dried *morogo* can be cooked for less time compared to freshly picked leaves.

3.2.6 Health and medicinal aspects of *morogo*

Most people prefer *morogo* plants growing amongst maize crops because they believe it taste better and grow best. Others stated that *morogo* cultivated separately from maize dry quicker before being processed, while some reported no difference between those growing with or without maize. Some believe *mophutse* causes diarrhea. It is also believed that *mokgadi wa sega-sega* cure high blood pressure. *Thepe* and *thelele* are used against constipation and roots of *monawa* are thought to heal severe pains experienced by some women after childbirth (*chilwane*). *Monawa* is used to clean wounds.

Morogo can grow easily under a wide range of environmental conditions, these plants are reported to be more resistant to pests, and diseases compared to introduce vegetable crops (Aphane *et al.*, 2003). In addition, some *morogo* plants were also found to be important sources of micronutrients that include vitamins A (carotene) and C (Ascorbic acid), iron, calcium, magnesium and rich in folic acids (Schippers, 2002; Aphane *et al.*, 2003). Folic acid is believed to be essential in the reduction of the risk of having a baby with certain birth defects of the brain and spinal cord e.g. neural tube defects (NTDs; Missmer *et al.*, 2006). *Morogo* plants can add nutritional value and balance grain-based staple diets with health supporting elements, especially in rural settings depending on subsistence farming.

According to Khumbane (1996), food security involves production, preservation and storage of food. Areas such as Sekhukhune, Tzaneen, Venda, Bushbuckridge and Athol in the Limpopo Province and Mpumalanga are reported to have maintained the usefulness of indigenous knowledge related to production, preservation and storage of food for future use (Khumbane, 1996). Practices that are carried out in preparation for ploughing prior to the first rain in August involves: scattering of kraal manures from cattle, sheep, donkeys and goats in the field, blessing of the seeds by the chief, environmental cleansing ceremony by the tribal traditional doctor, collection of water from the river to the chief's kraal by traditionally dressed women and young girls and performance of a ritual by the traditional doctor. The traditional doctor then led the community to the veldt to collect rubbish to be burnt in the chief's kraal. Afterwards, people celebrate by dancing and carrying out a feast. As soon as the first rainfall, ploughing then starts and ends up until January. Melons, cowpeas and pumpkins leaves are normally collected and preserved as dried green vegetables (*morogo*). Wood-ash is added to keep pests away and the *morogo* stored for 3 years without pest infestation. Another method of preservation and keeping pests away involves cooking of green maize, cowpeas and jugo beans in big pots with the skin on, dried, and stored in big pots or bags (Khumbane, 1996).

Problems in rural communities include difficulties in obtaining water for irrigation and lack of funds to buy pesticides and chemical fertilizers. Schippers, (2002) reported that traditional plants are resistant to drought and common diseases. Based on indigenous knowledge rural subsistence communities secure food availability by targeting rainy periods for cultivation. Fields are fertilized and insect pests controlled by traditional means.

CHAPTER 4

MATERIALS AND METHODS

4.1 GEOGRAPHICAL POSITIONING OF SAMPLING SITES

The Global Positioning System (GPS; satellite navigation system) was used to determine geographical orientation of the four sampling sites. These sites were Sefateng, Madiga, Mantheding and Moduane in the Dikgale Demographic Surveillance Site (DDSS), a rural area close to Polokwane in the Limpopo Province.

4.2 INDIGENOUS KNOWLEDGE (IK)

Household questionnaires (Appendix 1.1) were used and interviews conducted to extract relevant Indigenous Knowledge (IK) from local villagers in Dikgale Demographic Surveillance Site (DDSS) in relation to dietary patterns, *morogo* types consumed, methods of cultivation, storage practices and awareness of any health related issues from *morogo* plants.

4.3 BOTANICAL IDENTIFICATION

Two types of *morogo* widely consumed by rural households in the study area were chosen for the present investigation, namely *thepe* and *leroto*, growing naturally in household schemes. Herbarium specimens of the collected *morogo* plants were submitted to the South African National Botanical Institute (SANBI) in Pretoria for scientific species identification. Specimen collection and preparation were conducted in accordance with methods provided by SANBI for collecting and processing of plants from the field for identification (Appendix 2).

4.4 MYCOLOGICAL ANALYSIS

Morogo plants were subjected to standard procedures for mycological analysis to determine the presence and levels of *Fusarium* strains reported to be mycotoxigenic and /or mycotic. Environmental samples including maize components, air and soil were collected at the same time to be included for mycological analysis.

4.4.1 Sampling

Representative samples of the two *morogo* types and the surrounding environment were collected on two occasions at selected sampling sites at each of the four villages.

4.4.1.1 *Morogo* samples

Morogo samples were obtained from four villages, Sefateng, Madiga, Mantheding and Moduane, all in Dikgale Demographic Surveillance Site (DDSS) in Limpopo Province, before and after planting of maize during February 2006 and May 2006 respectively. Two different types of *morogo* namely *thepe* and *leroto* were sampled from each of the four villages. One household scheme in each village where subsistence farming is practiced was selected for sampling. Ten leaves from each *morogo* plant were collected. In total 80 *thepe* leaves and 80 *leroto* leaves were collected. The leaves were put in marked Ziploc bags and transported to the North-West University's Microbiology laboratory in a cooler box and stored at 4 °C until subsequent analysis. These samples were used for isolation, purification and identification of *Fusarium* species. Additional *morogo* from the two *morogo* types were collected for fumonisin and nutritional analyses.

4.4.1.2 Environmental samples

Other components in the environment where *morogo* plants grew namely, maize, soil and air, were also sampled for isolation of fusarial species. Three maize cobs together with its leaves growing in vicinity of the *morogo* at each of the four sites, were also sampled during the May survey. All samples were transferred to Ziploc bags, transported in a cooler box to the laboratory, and stored at 4 °C until analysis. These samples were all subjected to mycological analysis for the isolation and identification of *Fusarium* strains. Three Penta-chloro-nitrobenzene (PCNB) agar plates were put apart at the growing site of each *morogo* plant to trap fungal spores from the air where plants were growing. The plates were sealed with parafilm and taken to the laboratory where they were incubated at 25 °C. PCNB agar is a selective medium for *Fusarium* species and contains bacterial inhibiting substances (Appendix 3). Three soil samples were also collected at three different positions on the site of each *morogo* plant. The three soil samples were mixed together and transferred to a Ziploc bag, and transported to the laboratory in a cooler box where it was stored at 4 °C until analysis. A total of 24 air plates and 8 combined soil samples were collected on each sampling occasion in February and May 2006. Both samples were also subjected to mycological analysis for the isolation and identification of *Fusarium* strains.

4.4.2 Isolation of *Fusarium*

All samples (*morogo* leaves, soil and, both internal and external surfaces of maize) were subjected to mycological analyses to determine the levels of potential mycotoxigenic and mycotic *Fusarium* spp in traditional vegetables and the agro-environment of the DDSS. Recipes for growth media according to Nelson *et al.* (1983) are provided in Appendix 3.

4.4.2.1 Isolation of *Fusarium* from *morogo*

To isolate fungi from external surfaces, *morogo* leaves were washed by a standard mycological washing method (Medina-Martinez and Martinez, 2000). Washing removed colonizers from the host substrate. A total of 80 *leroto* and 80 *thepe* leaves from the respective collection sites were treated separately. Each leaf was added to 99 ml sterile 1% peptone water (Biolab, Merck, South Africa) containing 0.01% Tween 80 which served to break the surface tension between colonizers and dislodge spores that might be present and the leaf surface to which they were attached. The mixture was shaken for 10 minutes on a rotary shaker at room temperature to wash off surface fungal colonisers into the diluents. From the washing (10^{-2} dilution) a dilution series of 10^{-3} to 10^{-5} for each leaf was prepared and subsequently used for surface plating by transferring 0.1 ml aliquots from each dilution to PCNB medium. Plates were incubated at 25 °C for a minimum of 20 days.

4.4.2.2 Isolation of *Fusarium* from soil

At the laboratory, soil collected at three positions around the sampled *morogo* plant, were thoroughly mixed and used as a combined sample for subsequent isolation of fusaria. From the mixed soil sample of each sampling site, 1g was accurately measured and transferred to 9ml sterile distilled water (10^{-1} dilution of the sample). A vortex was used to suspend and evenly distribute soil particles before preparing dilution series of 10^{-2} to 10^{-5} for subsequent surface plating on PNCB agar. From each dilution 0.1 ml aliquot was transferred and plated out onto PNCB agar. In total 5 plates per site were incubated at 25 °C for a minimum of 20 days.

4.4.2.3 Isolation of *Fusarium* from maize samples

Maize leaves, kernels and hairs were used for the recovery of *Fusarium* using PNCB agar plates. For isolation of surface colonizers, maize leaves were subjected to the same washing procedure as *morogo* leaves. To isolate internal *Fusarium* species, the maize leaf was first surface sterilized

in 1% hydrogen peroxide solution for 1 min and rinsed 3 times in sterile distilled water to remove fungal colonizers from the surface. The leaf was then cut aseptically into squares of 1cm² and 5 squares per leaf plated out on PCNB agar plates (Baxter *et al.*, 1999). Maize kernels were also used for the isolation of both endophytic and epiphytic isolates. For external isolation 10 kernels were subjected to the same procedure for washing and dilution preparation as described for *morogo* and maize leaves. For the isolation of *Fusarium* from internal surfaces the kernels were surface sterilized according to the method described by Baxter *et al.* (1999). Kernels were aseptically crushed and 5 kernels per sampled cob were plated out on PCNB agar plates. Maize hairs from each maize cob sample were directly plated out on PCNB agar to isolate *Fusarium* colonizers. All plates were incubated at 25 °C for a minimum of 20 days.

4.4.2.4 Isolation of *Fusarium* from the air

The three PCNB agar plates used to trap fungal spores from the air for each sampling site during field sampling were incubated at 25 °C for a minimum of 20 days. A total of 24 air plates represented both sampling occasions.

4.4.3 Enumeration of fungal species

Though PCNB is selective for *Fusarium*, colonies of other fungi were also present following incubation of plates. Developed fungal colonies were counted according to dilution, the number of colonies per sample calculated and expressed as colony forming units per milliliter (CFU/ml) of the washing liquid of each leaf or CFU/g of soil.

4.4.4 Purification and identification of *Fusarium* isolates

Following isolation, purification and identification of isolates were conducted according to the methods of Nelson *et al.* (1983).

4.4.4.1 Purification of colonies on CLA and preparation of single-spore cultures

Colonies for subsequent purification were selected on the basis of characteristics described in Nelson *et al.* (1983). For subsequent identification of *Fusarium* species, selected isolates were purified by transferring them aseptically from PNCB plates to Carnation Leaf Agar (CLA; Appendix 3). Plates were incubated at 25 °C for a minimum of 7 to 10 days during which time they were exposed to a 12:12- hour light/dark cycle. Following incubation, isolates were used for

the preparation of single-spore cultures according to Nelson *et al.* (1983). CLA plates were flooded with 9 ml of sterilized distilled water and the mixture transferred to Water Agar (WA; Agar Bacteriological, Biolab, Merck, South Africa; Appendix 3). The water was drained off the WA plates which were subsequently incubated at 25 °C in an inclined position to allow the spores to separate in a gradient pattern. After 16-24 hours plates were examined under a stereomicroscope and germinated single spores aseptically transferred to Potato Dextrose Agar (PDA; Biolab, Merck, South Africa), Synthetic Nutrient Agar (SNA; Appendix 3), and CLA. All the plates were incubated at 25 °C for 7-14 days (Nelson *et al.* 1983).

4.4.4.2 Microscopic examination of Fungal isolates for morphological identification

Following incubation, PDA, SNA and CLA single spore cultures of *Fusarium* isolates were subjected to microscopic investigation for morphological identification according to procedures described in the illustrated manual for identification of *Fusarium* species (Nelson *et al.* 1983). Recorded macroscopic observations on single-spore colonies growing on PDA plate included colony morphology, colony colour, the presence and position of the sporodochia. Microscopic characteristics of cultures on CLA plates recorded included microconidia, macroconidia and chlamydospores. The microconidia were noted for their shape, size, position of the septum, pattern of formation (e.g. single, in chains or in false heads), and for the nature of their conidiogenous cells (e.g. monophialides or polyphialides). Macroconidia were observed for their shape, size, number of septa and the shape of the apical (top) and basal (bottom) cells. Apical cells can be hooked, nipple-like, blunt or conical in shape, whereas basal cells may be papillate, notched or foot-shaped, have an extended foot or be blunt or not notched. If present, chlamydospores can be observed on both the CLA and SNA and noted characteristics include the type of wall (rough or smooth) and formation whether in singles, pairs, clumps or in chains (Nelson *et al.* 1983).

4.5 MOLECULAR VERIFICATION OF *FUSARIUM* SPECIES IDENTIFICATION

Molecular analyses of *Fusarium* specific gene sequences were employed to verify microscopically based species identification.

4.5.1 Sample preparation for DNA extraction

Single spore cultures of morphologically identified isolates on PDA plates were used to extract DNA from *morogo* isolates representative of fumonogenic and mycotic fusarial strains. Approximately 2 cm² of each culture was transferred from PDA plate to 9 ml Yeast Peptone Dextrose (YPD) broth (Appendix 3). Inoculated YPD broth cultures were incubated at room temperature (25 °C) on a rotary shaker (200 rpm) for 7-10 days. Mycelial biomass was separated from the YPD broth by centrifugation at 4000 rpm for 5 min. The supernatant was discarded and the remaining mycelia stored at -65 °C before freeze-dried. The freeze-dried mycelial biomass was grounded to a fine powder in liquid nitrogen using a mortar and a pestle. The finely grounded mycelia were then transferred to sterile 2 ml microfuge tubes for the DNA extraction.

4.5.2 DNA isolation

DNA isolation was carried out in accordance with the procedure described by Saghai-Marooof *et al.* (1984). Double strength CTAB isolation buffer was prepared by mixing 100 mM Tris pH 8.0, 20 mM EDTA, 2% w/v CTAB and 1.5 M NaCl. β -mercaptoetanol (1 μ l v/v; Appendix 4) was added just prior to it being used. A volume of 500 μ l hot (65°C) CTAB isolation buffer, 100 μ l 5 % PVP solutions (Appendix 4) and 30 μ l of proteinase K (20 mg/ml) were added to a 2 ml microfuge tube containing the freeze-dried sample. The microfuge tubes were subsequently incubated in a water bath at 65°C for 90 minutes and gently inverted every 30 minutes. An equal volume of TE Buffered Phenol: Chloroform: Isoamyl alcohol (25:24:1; Appendix 4) was added to each microfuge tube at room temperature and the tubes were gently inverted 3 or 4 times (every 5 min) for 20 min. The tubes were centrifuged at 13 400 rpm for 10 minutes in a bench-top centrifuge (Eppendorf, Germany) and the aqueous phase containing the extracted DNA transferred to a new sterile microfuge tube. After measuring the volume of the aqueous phase, it was re-extracted with equal volume of TE Buffered Chloroform: Isoamyl alcohol (24:1; Appendix 4) for 20 min at room temperature while gently inverting the tubes 3 or 4 times (every 5 min). The tubes were again centrifuged at 13 400 rpm for 10 min and the aqueous phase transferred to a new sterile microfuge tubes. A volume of 100 μ l of 5M NaCl (Appendix 4) was added to the liquid in the microfuge tube followed by the addition of 1 ml of ice-cold 95% ethanol. The DNA was precipitated overnight at -80°C. Centrifugation at 13 400 rpm (4°C) for 10 minutes was subsequently applied to separate the supernatant which was gently poured off and discarded. Care was taken not to dislodge the pellet. The pellet was dried at 65°C with the

microfuge tubes opened to remove ethanol. Pellets were re-suspended in 50 µl DNase/RNase free water to inhibit DNase and RNase and incubated at 65 °C for an hour to reconstitute the DNA. The DNA samples were stored at 4 °C until required for use.

4.5.3 DNA Amplification (Polymerase Chain Reaction)

For the Polymerase Chain Reaction (PCR), an ICycle (BioRad, UK) thermal cycler was used. PCR mix contained 12.5 µl of double strength PCR master mix, 4 mM MgCl₂; primer (50 pmole); DNase/RNase-free distilled water; 1 U of Supertherm *Taq* polymerase (JM Holdings, UK); 100 ng of DNA and 50 ng of bovine serum albumin (BSA). The final volume of the PCR mix was 25 µl. The double strength PCR master mix contained 2.5 U *Taq* polymerase in 20 mM Tris-HCl, 100 mM KCl, 3.0 mM MgCl₂, Brij 35, 0.01% (v/v), dNTP mix (dATP, dCTP, dGTP, dTTP each 0.4 mM) with pH of 8.3 at 20°C (PCR Master; Roche, Germany). The primers used are indicated in the Table 4.1.

Table 4.1 Primer type and composition

Primer	Sequence	Gene Target	Product Size (bp)	Reference
<i>FUM-1</i>	5'GTTCGAGTTGTTGACCACTGCG3'	Polyketide Synthase (PKS)	800	Bluhm <i>et al.</i> 2002
<i>FUM-2</i>	5'CGTATCGTCAGCATGATAGC3'			
α - <i>EF</i> 1	5'ATGGGTAAGGAGGACAAGAC3'	Translation elongation factor 1- α	700	Geiser <i>et al.</i> 2004
α - <i>EF</i> 2	5'GGAAGTACCAGTGATCATGTT3'			
β <i>tub</i> -1	5'AACATGCGTGAGATTGTAAGT3'	Beta-Tubulin	650	Leslie <i>et al.</i> 2006
β <i>tub</i> -2	5'TAGTGACCCTTGGCCCAGTTG3'			

The thermocycler (ICycler Thermal Cycler, BioRad, UK) was programmed as follows:

- 5 minutes at 95°C, followed by 30 cycles of
- 30 seconds at 95°C for denaturing,
- 60 seconds at 65°C for annealing
- 30 seconds at 72°C for extension

After 30 cycles, an additional cycle were completed to ensure final extension, this cycle was run for 5 minutes at 72°C. Tubes containing the PCR products were stored at 4°C prior to gel electrophoresis.

4.5.4 Agarose gel electrophoresis

Amplified products were visualized by 1.5% w/v agarose gel (Roche, Germany) electrophoresis in 1x TAE buffer after staining with 10% w/v ethidium bromide (BioRad, UK). A mixture of loading buffer (5 µl) and the PCR product mix (5 µl) were loaded onto the agarose gel. A DNA molecular weight marker (100 bp Molecular Weight Marker; BioRad, UK) was used to compare the size of the resultant DNA bands. The electrophoresis process was performed for 45 minutes at 80V. Gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software.

4.5.5 Sequence analysis

The amplified PCR fragments were submitted for sequencing to Inqaba Biotec, South Africa. BlastN in GeneBank and the *Fusarium* database (Geiser *et al.*, 2004) was used for the confirmation of the identity of the amplified sequences.

4.6 FUMONISIN ANALYSIS

The Enzyme-Linked Immunosorbent Assay (ELISA) technique was employed to screen in *morogo* plants for the presence of fumonisins. High Performance Liquid Chromatography (HPLC) was subsequently used to characterise (e.g. FB₁, FB₂ or FB₃) and determine the amount of each fumonisin B type in *morogo* sample for which ELISA results were positive (Shepard *et al.*, 1990)

4.6.1 ELISA method

The commercially available quantitative ELISA assay kits (Neogen Corporation, Lansing, MI, USA/Canada) was used according to the manufactures instructions to detect the presence of total fumonisins.

4.6.1.1 Plant extracts

Five samples of traditionally dried *morogo* plants were used for this analysis. Each sample was grounded into fine powder using a mortar and a pestle and used for the analysis. Five grams (5g) of each sample was homogeneous in a 25 ml mixture of methanol/water (3:1) for 5 min. The extract was then filtered through Whatman no. 4 filter paper and the filtrate collected in a clean vessel.

4.6.1.2 The kit assay method

A volume of 100 μ l of the filtrate from each sample was diluted with fumonisin diluents provided in the kit. An accurately measured volume of 100 μ l of fumonisin conjugate solution was added to each red marked mixing well. Five standards (100 μ l each) and diluted samples were added to their respective wells marked on the well tray. A multi-channel pipettor was used to mix the mixture from the red wells thoroughly and 100 μ l transferred to antibody wells, and incubated for 10 min at room temperature. The liquid from antibody wells were dumped and the wells washed 5 times thoroughly with distilled water. The water was tapped out on an absorbent paper towel. The substrate volume of 100 μ l was transferred from reagent boat to antibody wells using the multi-channel pipettor and the contents were allowed to react for 10 minutes. A volume of 100 μ l sulphuric acid (red stop solution) was added to antibody wells to stop the reaction. The results were read using a micro-well reader with a 650 nm filter (ELISA assay kits, Neogen Corporation, Lansing, MI, USA/Canada)

4.6.2 HPLC method

The HPLC method was used for the determination of fumonisin B₁, B₂ and B₃ levels in samples according to Shephard *et al.* (1990). Five samples of traditionally dried *morogo* plants used during the ELISA method were used for this analysis.

4.6.2.1 Fumonisin extraction and IAC (Immunoaffinity column) clean up

Each sample was grounded into a fine powder using a mortar and pestle. Two grams (2 g) of each sample and sodium chloride (0.4 g) were homogenized in a 30 ml mixture of methanol/water (8:2) for 5 minutes. The extract was filtered through Whatman no. 4 filter paper and the filtrate collected in a clean vessel. The filtered extract (10 ml) was diluted with 40 ml of a solution of phosphate-buffered saline (PBS; pH 7.0) containing 0.5% Tween 20. The extract was subsequently filtered through a micro-fiber filter (Schleicher & Schuell) and the filtrate transferred into a polypropylene syringe barrel that was attached to the FumoniTest immunoaffinity (IA) column (Vicam). The extract was passed through the IA column at a rate of about 1-2 drops per second until air passed through the column. Thereafter, a volume of 15 ml PBS was passed through the column at a rate of 1-2 drops/second. Fumonisin were eluted from the IA column under gravity by passing 3 ml of HPLC grade methanol through the column at a rate of 1 drop/second. The eluate was collected into a glass vial, and dried under a stream of nitrogen at 60 °C to concentrate it at the base of the vial with a capacity of 4 ml.

4.6.2.2 Derivatization and HPLC analysis

FB₁, FB₂ and FB₃ standard solutions were prepared by dissolving 1mg/ml in acetonitrile/ water (1:1). The working standard solutions were prepared from stock standard solutions by transferring 100 µl aliquots of each solution to clean glass vials and adding 200 µl acetonitrile-H₂O (1:1) to yield a working standard solution containing the three (3) fumonisin analogues. Forty milligrams (40 mg) of *o*-phthaldialdehyde (OPA) were dissolved in 1 ml methanol and diluted with 4 ml disodiumtetraborate (0.1 M). Afterwards, 50 µl of 2-mercaptoethanol was added. The reagent solution was stored in capped amber or aluminium foil covered vials and stored for no more than a week at room temperature in dark. Fifty microliters (50 µl) of the fumonisin mixtures (FB₁, FB₂ and FB₃) working standard solutions (10 – 100 µg/ml) were transferred to the base of small vials and mixed with 225 µl with *o*-phthaldialdehyde (OPA) reagent. Ten microliters (10 µl) of the reaction mixture was injected to HPLC within 2 min. It is critical to adhere to reproducible time between addition of OPA reagent and injection into HPLC system. Fluorescence of OPA-fumonisin began to decrease after 2 min. The purified dry film residues of sample extract were dissolved in 200 µl methanol. Fifty microliters (50 µl) of this extract were transferred to the base of the small vial. Afterwards, 225 µl of OPA reagent was added, mixed, and 10 µl of the derivative was injected into the HPLC system. The HPLC system

consisted of Waters (Milford, MA) Millennium software system coupled to a Waters 474 fluorescence detector set at excitation and emission wavelengths of 335 and 440 nm, respectively. The fumonisins were separated on a 250 mm internal diameter (i.d.), 4 μ m, Nova pack column (Waters, MA) using a mobile phase of methanol/0.1 M sodium phosphate buffer (75:25, pH 3.35) pumped at a flow rate of 1 ml/min. Quantification was by peak area comparison with a similarly derivatized standard in acetonitrile/water (50:50).

4.7 STATISTICAL ANALYSIS

Statistical processing of some data was done by one-way analysis of variance for comparison of data (Microsoft Excel 2000).

CHAPTER 5 RESULTS

5.1 BOTANICAL SPECIES IDENTIFICATION

Botanical species identification of *lerocho* and *thepe* utilised by rural communities in the villages of Sefateng, Madiga, Mantheding and Moduane in the Dikgale Demographic Surveillance Site (DDSS), Limpopo Province by the South African National Biodiversity Institute (SANBI) are reported in Table 5.1.

Table 5.1 Botanical species identification of *thepe* and *lerocho*

Sampling Site	Genspec no.	<i>Morogo</i> type	Botanical species name
Sefateng	308200017	<i>Lerocho</i>	<i>Cleome gynandra</i> L.
Sefateng	229900033	<i>Thepe</i>	<i>Amaranthus thunbergii</i> sensu Suess. & Podlech
Madiga	308200017	<i>Lerocho</i>	<i>Cleome gynandra</i> L.
Madiga	229900032	<i>Thepe</i>	<i>Amaranthus thunbergii</i> Moq.
Mantheding	308200017	<i>Lerocho</i>	<i>Cleome gynandra</i> L.
Moduane	308200017	<i>Lerocho</i>	<i>Cleome gynandra</i> L.
Moduane	229900032	<i>Thepe</i>	<i>Amaranthus thunbergii</i> Moq.

Note: *Thepe* was not sampled at Mantheding for the purpose of plant species identification

Lerocho specimens from Sefateng, Madiga, Mantheding and Moduane were all identified as *Cleome gynandra* L.. *Thepe* specimens were identified as *Amaranthus thunbergii* Moq. except *thepe* from Sefateng which was a member of the subspecies *Amaranthus thunbergii* sensu Suess. & Podlech.

5.2 MYCOLOGICAL ANALYSES

Average fungal plate counts of (i) *lerocho* and *thepe*; (ii) leaves, hair and kernels of maize growing close to the *morogo*; (iii) air and soil sampled at sites where *morogo* were growing

before maize planting (M-) and with grown (M+) maize are reported in this section. *Fusarium* species recovered from *lerocho*, *thepe*, air and soil samples and their relative occurrences are reported. Raw data are attached as appendices (Appendix 4). HPLC analysis of fumonisins in *lerocho* and *thepe* are presented in tables. Gel images of molecular analysis for the verification of microscopically-based species identification are included with results of gene sequencing showed in table.

5.2.1 Average fungal plate counts

Figures 5.1 to 5.4 compare fungal levels in *lerocho* and *thepe* leaves sampled from the villages of Sefateng (Sef), Madiga (Mad), Mantheding (Man) and Moduane (Mod). Standard errors and significance of data are indicated in Tables 5.A to 5.D (Appendix 5). Average fungal plate counts in Sefateng *lerocho* and *thepe* growing before maize planting (M-) and with grown (M+) maize is shown in Figure 5.1.

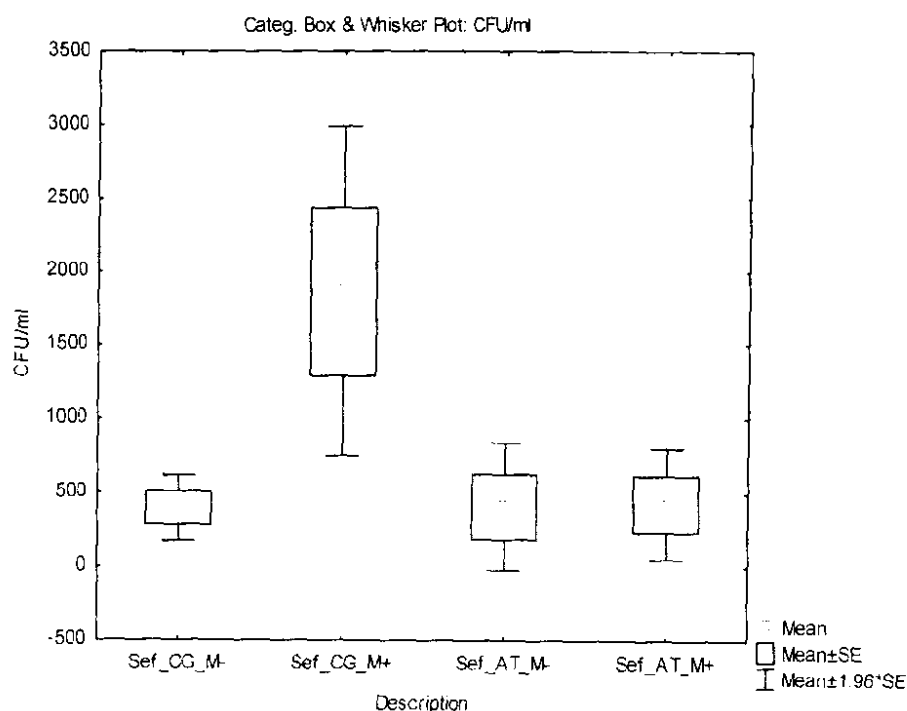


Fig. 5.1- The average number of fungi isolated from *lerocho* and *thepe* sampled from M- and M+ sites in the village of Sefateng (Sef)

Results indicated that fungal counts in *leroitho* sampled from a site close to maize in the village of Sefateng (Sef_CG_M+) were significantly higher than those in *leroitho* from a site away from maize (Sef_CG_M-). Compared to *leroitho* from the M+ site, fungal levels in *thepe* were relatively low in samples from both the M+ and M- sites and were in the same numerical order as that of *leroitho* from the M- site (Sef_CG_M-; Table 5.A, Appendix 5).

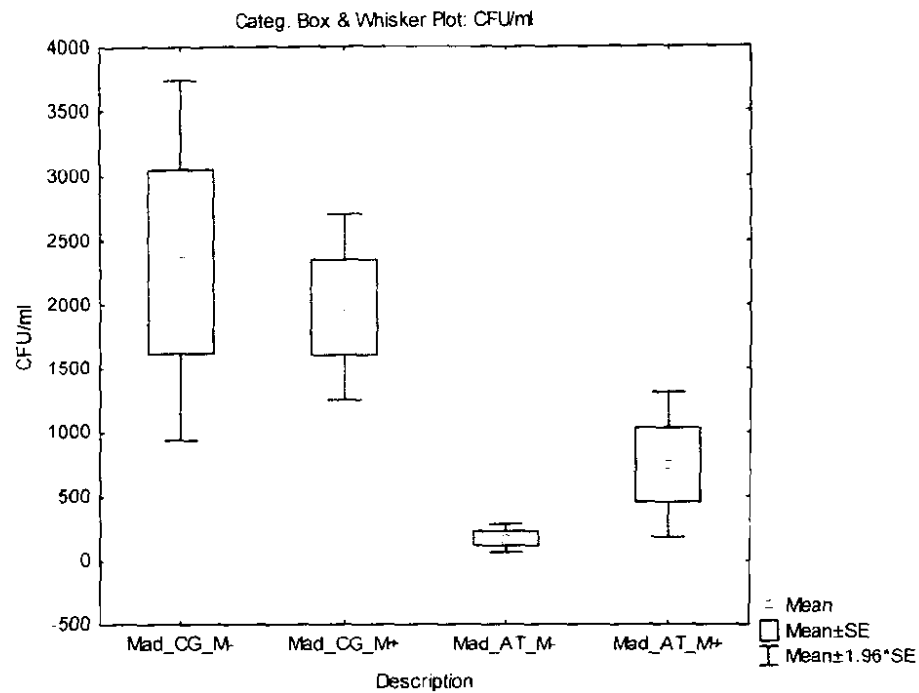


Fig. 5.2- The average number of fungi isolated from *leroitho* and *thepe* sampled from M- and M+ sites in the village of Madiga (Mad)

Figure 5.2 illustrates that mean values of average fungal plate counts in *leroitho* from the village of Madiga showed high fungal levels in *leroitho* from both M- (Mad_CG_M-) and M+ (Mad_CG_M+) sites compared to *thepe* from the same sites (Mad_AT_M- and Mad_AT_M+). The difference in mean values indicated a notable higher fungal level in *thepe* from the M+ site (Mad_AT_M+) compared to *thepe* from the M- site (Mad_AT_M-), fungal levels of which were significantly lower compared to that of *leroitho* from the M- site (Mad_CG_M-; Table 5.B, Appendix 5).

Figure 5.3 compares fungal levels in *lerocho* and *thepe* sampled from the village of Mantheding.

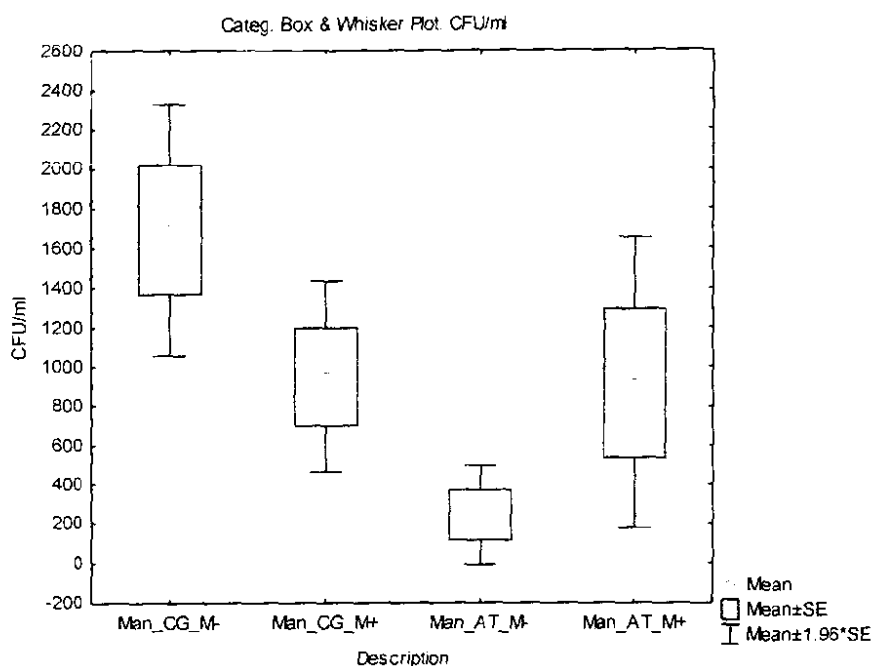


Fig. 5.3- The average number of fungi isolated from *lerocho* and *thepe* sampled from M- and M+ sites in the village of Mantheding (Man)

Mean values of fungal counts indicated that fungal levels were notably lower in *lerocho* from the M+ site (Man_CG_M+) compared to that of the M- site (Man_CG_M-) which were significantly higher than fungal levels in *thepe* from the M- site (Man_AT_M-; Table 5.C, Appendix 5). Mean values showed that fungal levels were of the same numerical order in *lerocho* and *thepe* from the M+ site (Man_CG_M+ and Man_AT_M+).

Figure 5.4 depicts fungal levels associated with *lerocho* and *thepe* sampled from the village of Moduane.

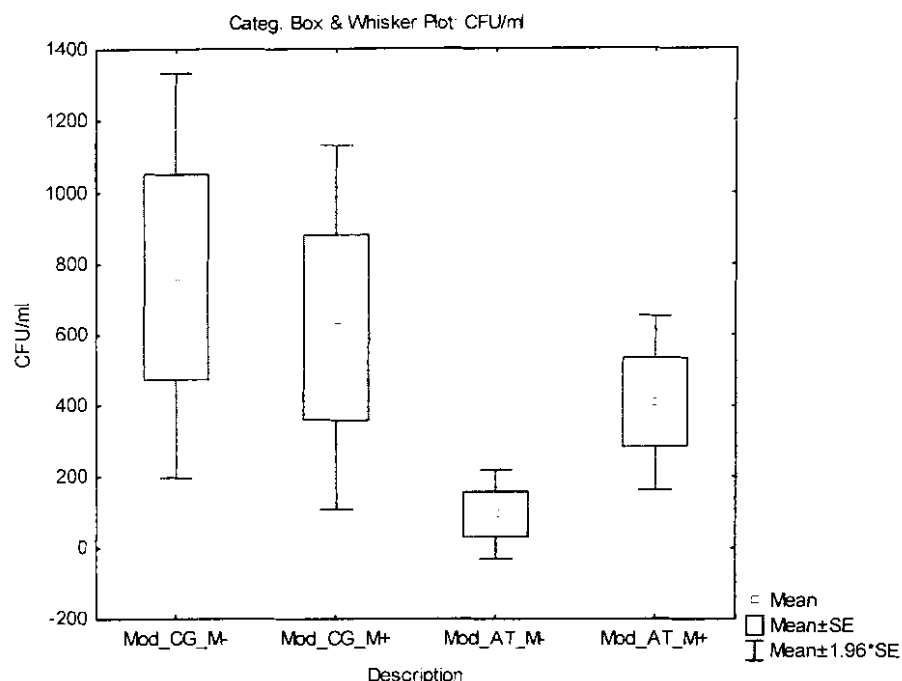


Fig. 5.4- The average number of fungi isolated from *lerocho* and *thepe* sampled from M- and M+ sites in the village of Moduane (Mod)

Mean values indicate slightly higher fungal levels in *lerocho* sampled from the M- site (Mod_CG_M-) compared to *lerocho* of the M+ site (Mod_CG_M+), both values being notably higher than that of *thepe* from the M- site (Mod_AT_M-) and slightly higher than *thepe* from the M+ site (Mod_AT_M+). Fungal levels were considerable but not significantly lower in *thepe* from the M- site (Mod_AT_M-) compared to fungal levels indicated for *thepe* from the M+ site or fungal levels in *lerocho* from either the M- or M+ sites (Table 5.D, Appendix 5).

Figures 5.5 to 5.7 illustrates fungal levels in components (maize, soil and air) of the immediate environment where *lerocho* and *thepe* were sampled from each of the four villages at sites before maize planting (M-) or with grown (M+) maize. Numbers of fungi isolated from external and internal maize leaf surfaces, as well as those from maize kernels and hair were combined and averaged. Three soil samples taken at each site of *morogo* sampling were combined and analysed as a composite soil sample. Counts of the three air plates taken at each sampling point were

likewise treated as one sample. Figure 5.5 show fungal levels in maize, while Figures 5.6 and 5.7 compares fungal counts in soil and air samples respectively.

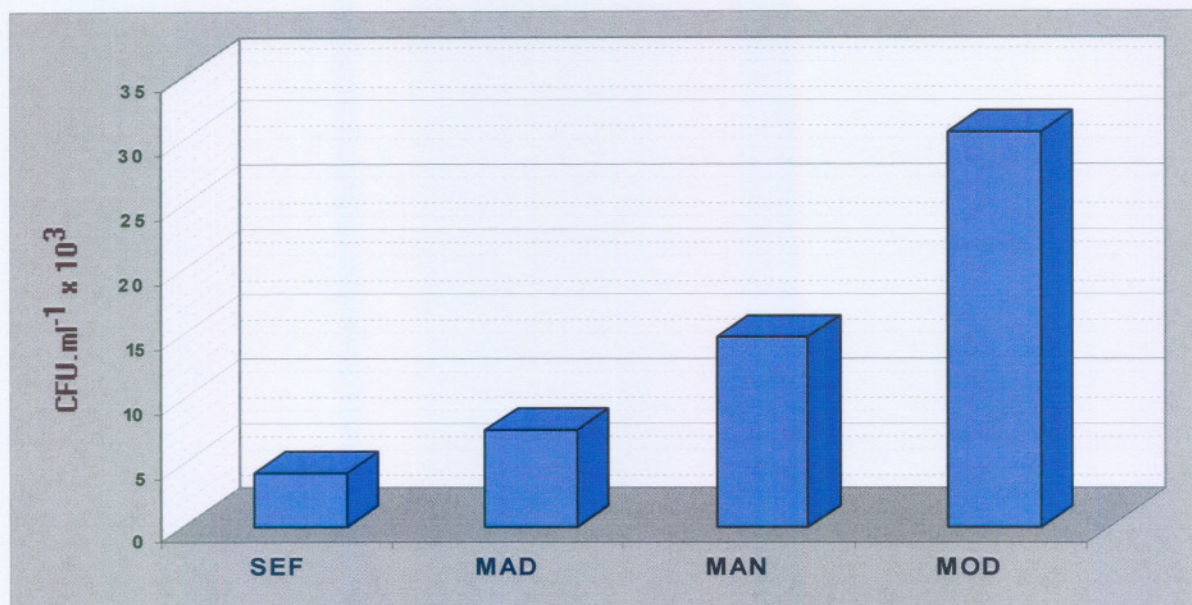


Fig 5.5- Average fungal counts in maize growing close to *morogo*

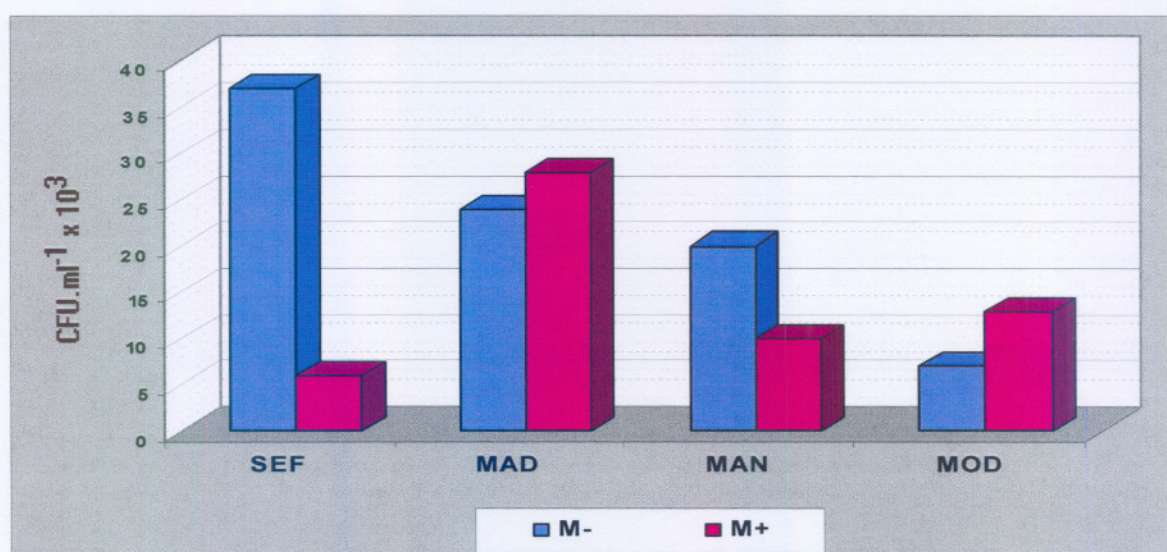


Fig. 5.6- Average fungal counts in soil where *morogo* plants were growing before maize planting (M-) and with grown maize (M+)

Results in Figure 5.5 show that maize fungal counts varied considerably between the four villages. The lowest counts were observed in maize of Sefateng and the highest in maize of Moduane. Figure 5.6 illustrates the respective average fungal counts in soil where *morogo* plants were growing before maize planting (M-) and with grown (M+) maize. In Sefateng and Mantheding, fungal counts were higher in soil of the M- than in soil of M+ sampling period, while in Madiga and Moduane fungi were retrieved in higher numbers from soil of the M+ sampling sites. The highest soil counts were observed in soil from the M- site of Sefateng and the lowest in soil of the M- site of Moduane. Figure 5.7 depicts fungal counts in air at the time of *morogo* sampling.

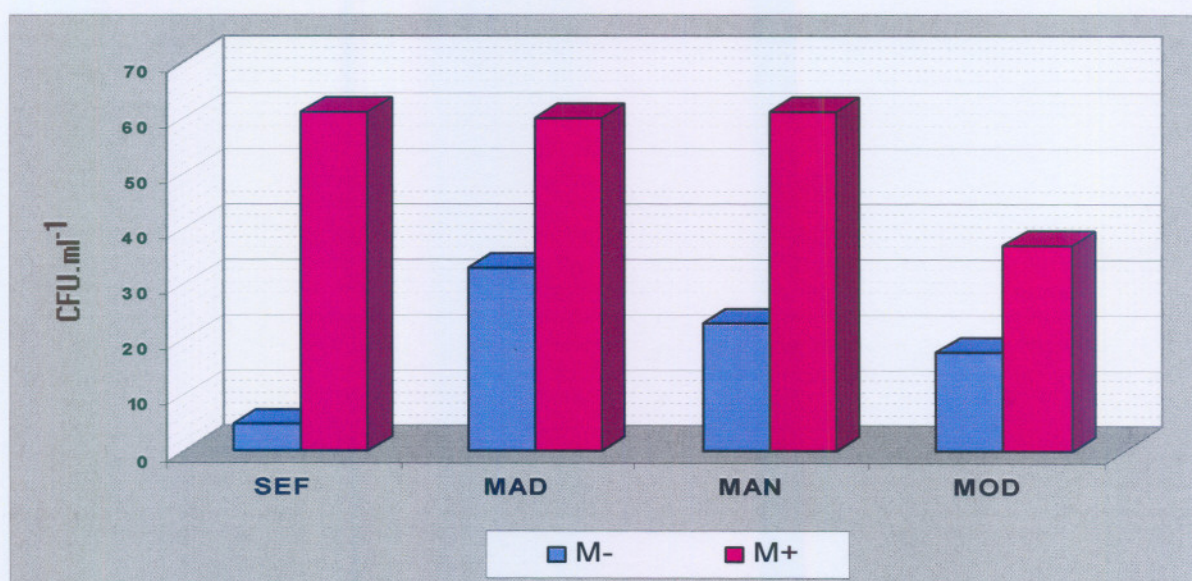


Fig. 5.7- Average fungal counts in air where *morogo* plants were growing before maize planting (M-) and with grown maize (M+)

In all four villages fungal counts were higher in the air of M+ sampling period (M+). Compared to the high fungal levels in air of the Sefateng, Madiga and Mantheding M+ sampling period, fungal counts of the Moduane air samples during the M- period were obviously lower. The lowest fungal counts in air at sampling sites before maize planting (M-) were demonstrated for Sefateng and the highest from Madiga.

5.2.2 *Fusarium* species and levels in *lerotho*, *thepe* and the agro-environment

Results of species identification of *Fusarium* isolates retrieved from *lerotho* and *thepe* growing before maize planting (M-) and with grown (M+) maize, as well as from maize structures (leaves, kernels and hair), soil and air from each sampling site, is illustrated in Figures 5.8 to 5.10.

The relative occurrence of the different *Fusarium* species isolated from *lerotho*, *thepe*, soil and air samples from sites before maize planting (M-) are illustrated in Figure 5.8.

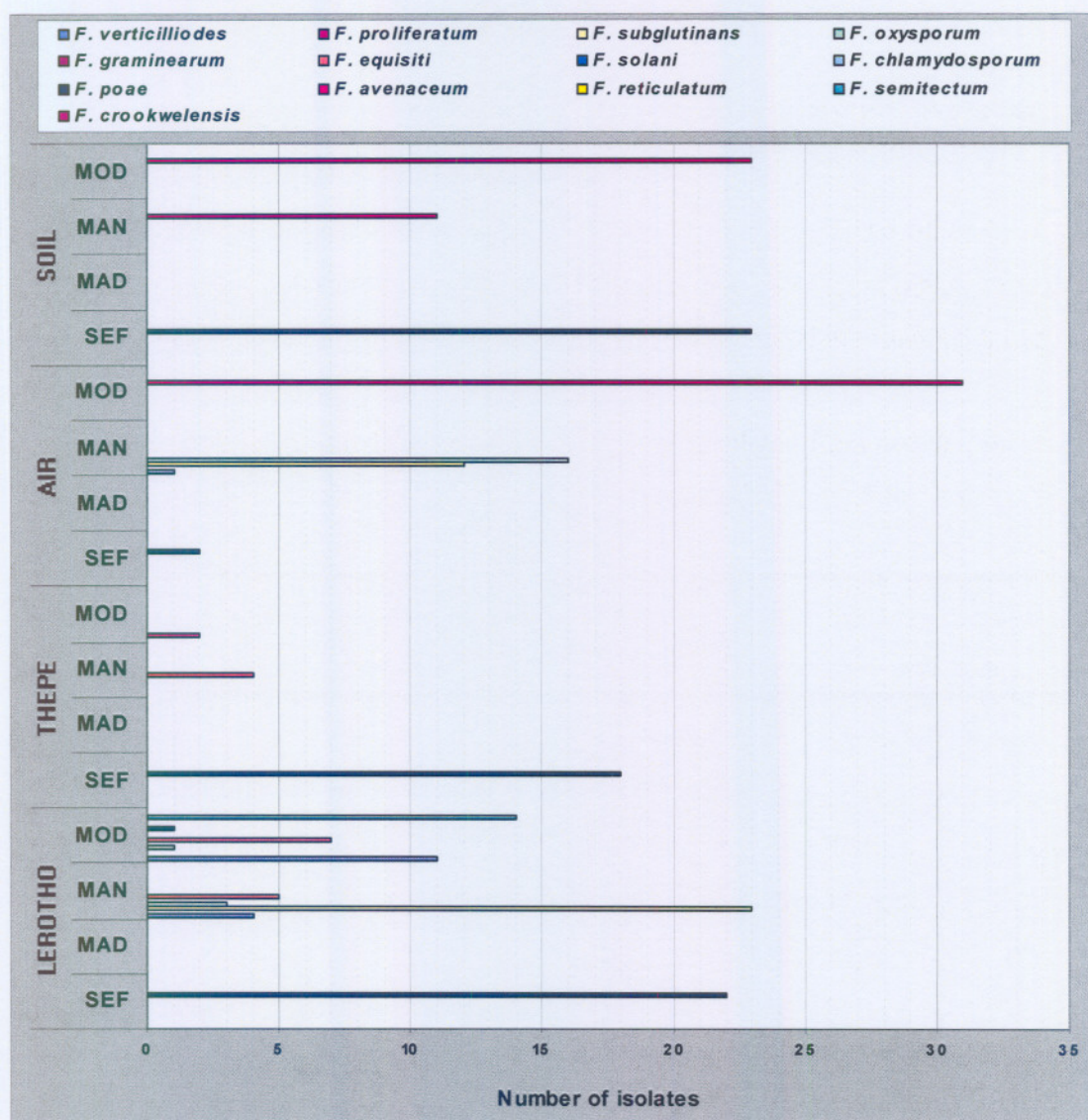


Fig. 5.8- Relative numbers of different *Fusarium* species isolated from *lerotho*, *thepe*, soil and air before maize was planted

Three *Fusarium* species were isolated from *thepe* growing before maize planting (M-): *F. poae* – 18 (Sefateng); *F. equisiti* – 4 (Mantheding) and *F. proliferatum* – 2 (Moduane). *Fusarium* species retrieved from *lerotho* included: *F. poae* – 22 (Sefateng); *F. verticillioides* – 4, *F. subglutinans* – 22, *F. oxysporum* – 3 and *F. equisiti* – 5 (Mantheding); *F. verticillioides* – 11, *F. oxysporum* – 1 and *F. equisiti* – 7 and *F. poae* – 1 (Moduane). Two *Fusarium* species were isolated from soil: *F. poae* – 23 (Sefateng); *F. avenaceum* – 11 and 23 (Mantheding and Moduane respectively). From air samples the following species were identified: *F. poae* – 2 (Sefateng); *F. verticillioides* – 1; *F. subglutinans* – 12 and *F. oxysporum* – 16 (Mantheding); *F. avenaceum* – 31 (Moduane).

Figure 5.9 show the relative occurrence of different *Fusarium* species in *lerotho* and *thepe* sampled from M+ sites of Sefateng, Madiga, Mantheding and Moduane.

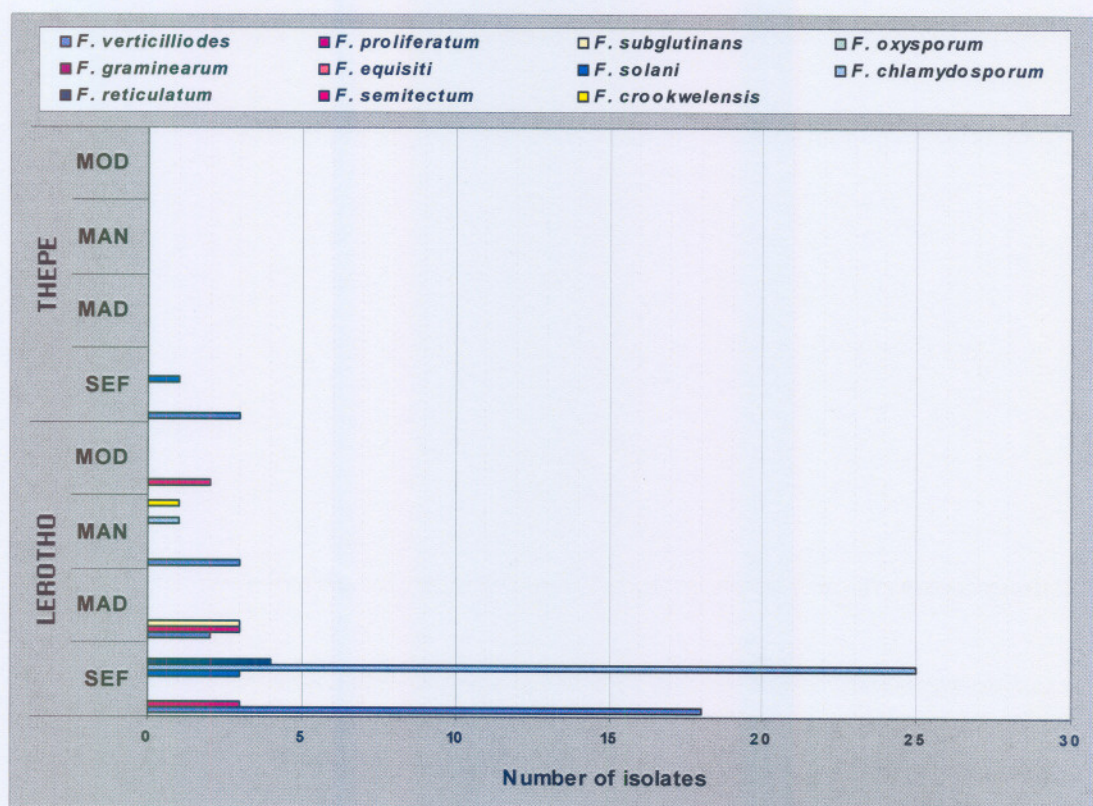


Fig. 5.9- Occurrence of different *Fusarium* species in *lerotho* and *thepe* sampled from sites close to maize

Two *Fusarium* species were identified among the three isolates from Sefateng *thepe*: *F. verticillioides* (3) and one *F. solani* (1). *Fusarium* was not isolated from *thepe* sampled from Madiga, Mantheding or Moduane. *F. chlamydosporum* (25) and *F. verticillioides* (18) were isolated from Sefateng *lerotheo*. *F. verticillioides* were retrieved from *lerotheo* of Madigo (2) and Mantheding (3). *F. proliferatum* was isolated from *lerotheo* of Sefateng (3), Madiga (3) and Moduane (2). *F. subglutinans* (3) was obtained from Madiga and *F. chlamydosporum* (1) from Mantheding *lerotheo*. Relative numbers of different *Fusarium* species in maize, soil and air at sites where *lerotheo* and *thepe* were growing, are illustrated in Figure 5.10.

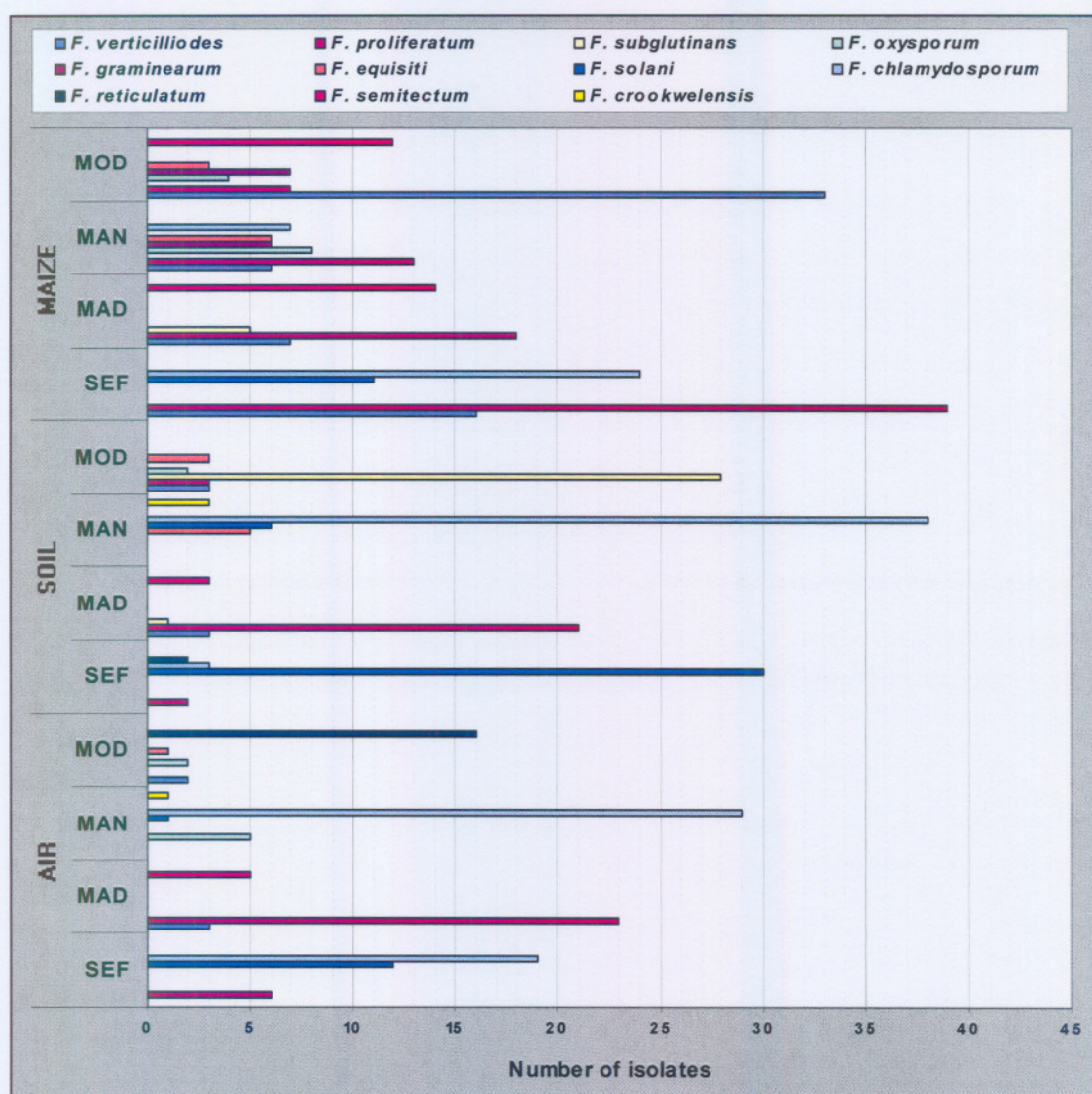


Fig. 5.10- Relative numbers of different *Fusarium* species isolated from maize, soil and air where *lerotheo* and *thepe* were growing

Of the eleven *Fusarium* species seven occurred in maize of Mantheding and Moduane the respective numbers of which were the following: *F. verticillioides* - 6 and 33; *F. proliferatum* - 13 and 7; *F. oxysporum* - 6 and 4; *F. graminearum* - 7 and 7; *F. equisiti* - 6 and 3; *F. solani* - 8 and 4; *F. semitectum* - 12 (Moduane). *F. chlamydosporum* was not retrieved. Four of the eleven *Fusarium* species were isolated from maize of Sefateng and Madiga respectively in the following numbers: *F. verticillioides* - 16 and 7 and *F. proliferatum* - 37 and 18; *F. subglutinans* - 5 (Madiga); *F. graminearum* - 24 (Sefateng); *F. chlamydosporum* - 11 (Sefateng) and *F. semitectum* - 14 (Madiga). Most *Fusarium* species were retrieved in relatively low numbers (between 1 and 3) from soil samples with the exception of the following: *F. solani* - 6 (Sefateng); *F. proliferatum* - 31 (Madiga); *F. chlamydosporum* - 38 (Mantheding) and *F. subglutinans* - 28 (Moduane). *Fusarium* species were isolated in relatively low numbers from air samples the highest numbers being: *F. proliferatum* - 6 (Sefateng) and 23 (Madiga); *F. chlamydosporum* - 25 (Mantheding) and *F. reticulatum* - 16 (Moduane).

Results illustrated in Figures 5.1 to 5.10 indicate the following important trends in fungal load and the occurrence of the different *Fusarium* species in *lerocho*, *thepe* and the agro-environments of Sefateng, Madiga, Mantheding and Moduane: (i) the fungal load in *lerocho* was higher than that of *thepe* in all four villages; (ii) at sites before maize was planted no *Fusarium* isolates were recovered from Madiga *morogo*, soil or air; (iii) in Sefateng *F. poae* occurred as the predominant *Fusarium* species in *lerocho*, *thepe*, soil as well as air samples; (iv) in Mantheding the predominant species were *F. subglutinans* retrieved from *lerocho* as well as in air samples together with *F. oxysporum* and *F. equisiti* from *thepe*; (v) In Moduane samples the predominant species were *F. verticillioides*, *F. equisiti* and *F. semitectum* in *lerocho*, *F. proliferatum* in *thepe* and *F. avenaceum* in soil and air samples; (vi) predominant species from *morogo* of M+ periods were *F. verticillioides* and *F. chlamydosporum* in Sefateng *lerocho*; (vii) *F. verticillioides*, *F. proliferatum* and *F. chlamydosporum* were the predominant species retrieved from maize samples; (viii) from soil samples *F. proliferatum*, *F. subglutinans*, *F. chlamydosporum* and *F. solani* were the species predominantly recovered and (ix) from air samples *F. proliferatum*, *F. chlamydosporum* and *F. poae*.

5.3 MOLECULAR ANALYSIS

Selected isolates of potential fumonisin producing fusarial species were subjected to molecular analysis. The selected species were previously morphologically identified as *F. verticilliodes*, *F. oxysporum*, *F. subglutinans* and *F. proliferatum* (Nelson *et al.*, 1983). All the isolates were recovered from *morogo* samples. Polymerase chain reaction (PCR) was used to amplify β -tubulin, translation elongation factor (EF-1 α) and *FUM1* genes using primers in Table 4.1.

5.3.1 PCR detection of *Fusarium* species in pure mycelial cultures

The freeze-dried and frozen mycelial samples from these four *Fusarium* species produced DNA suitable for PCR amplification. Successful PCRs using primers EF-1 α , β -tubulin and *FUM1* are indicated in Figure 5.11 to Figure 5.13. The amplified DNA fragments were of expected sizes.

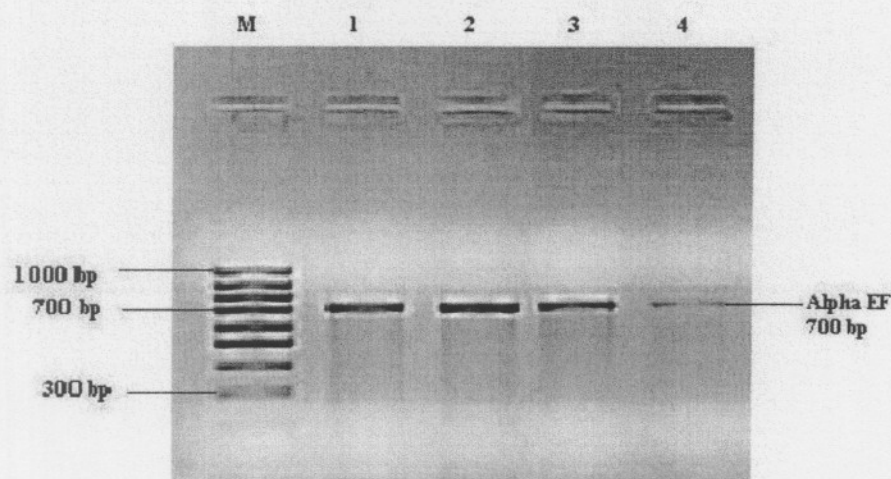


Fig. 5.11- Negative image of an ethidium bromide stained gel representing the elongation factor 1- α amplicon. M-Molecular marker; Lane 1-4 represent: *F. oxysporum*; *F. verticilliodes*; *F. proliferatum*; *F. subglutinans*

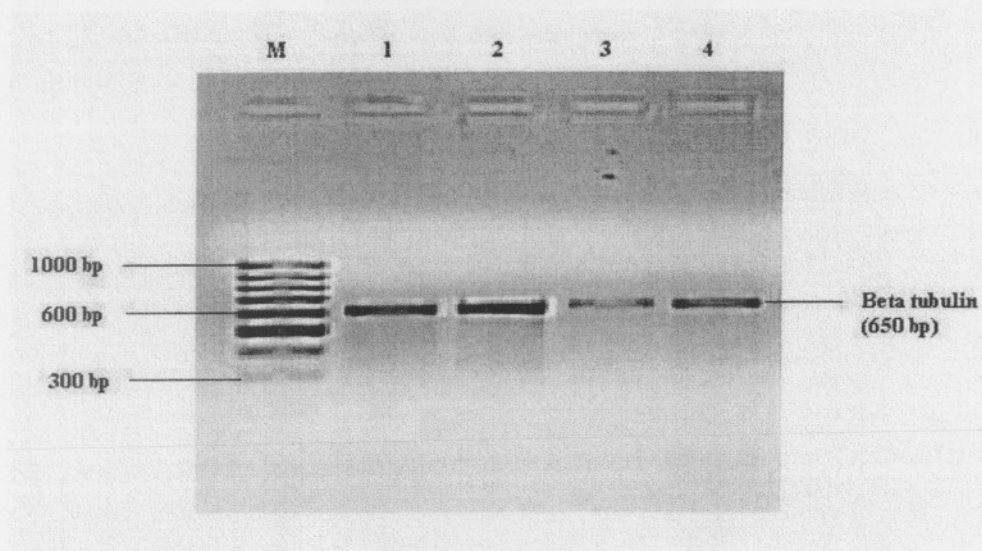


Fig. 5.12- Negative image of an ethidium bromide stained gel representing the β -tubulin amplicon. M-Molecular marker; Lane 1-4 represent: *F. oxysporum*; *F. verticillioides*; *F. proliferatum*; *F. subglutinans*

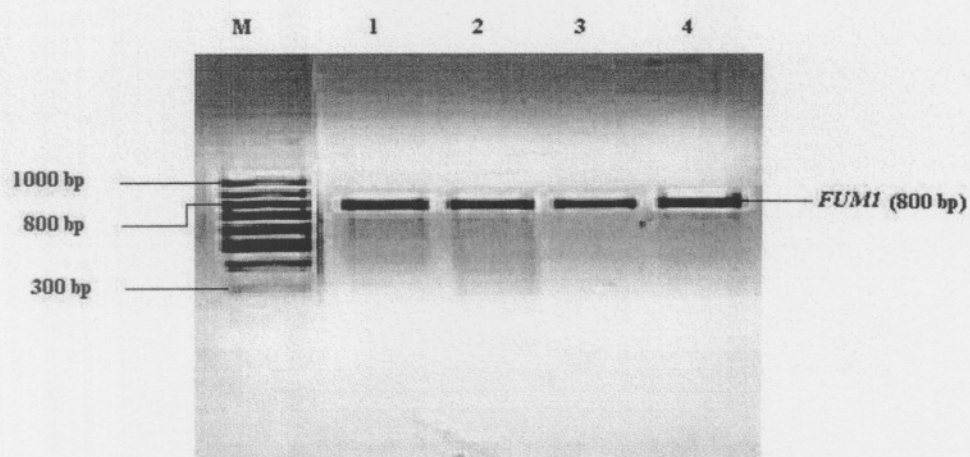


Fig. 5.13- Negative image of an ethidium bromide stained gel representing the *FUM 1* amplicon. M-Molecular marker; Lane 1-4 represent: *F. oxysporum*; *F. verticillioides*; *F. proliferatum*; *F. subglutinans*

In Fig. 5.11 to Fig. 5.13, DNA fragments sizes of 650, 700 and 800 bps were produced using the β - tubulin, EF-1 α and *FUMI* primer sets, respectively.

5.3.2 Blast search result from the EF -1 α and β - tubulin sequences

The β -tubulin and EF-1 α PCR products from these isolates were sequenced by Inqaba Biotech (SA) and analysed by BLASTN search using ChromasPro version 1.33. The results are indicated in Table 5.2 and 5.3. Varying degrees of backgrounds (noise) are shown in Appendix 6 and in two cases (Table 5.3) there were too much background in the sequences rendering them unusable. Some of the sequences had very little background. Generally 500 bp of the sequences were sufficient for identification.

Table 5.2 B-tubulin sequence identification

Code used and Morphological identification	Nucleotides Used	Noise		GeneBank ID	% Similar	Seq. ID	Nearest relative (s) ID	% Similar	Seq. ID
		No	Yes						
1b <i>Fusarium oxysporum</i>	50 - 550		✓	<i>Fusarium oxysporum</i> gi 82791276 gb DQ289145.1	94	<u>430</u> 456	—	—	—
10b <i>Fusarium subglutinans</i>	50 - 380		✓	<i>Fusarium nygamai</i> gi 1808710 gb U34426.1 U34426	97	<u>319</u> 327	<i>Fusarium</i> sp. NRRL26793 gi 9230304 gb AF160324.1 AF160324	97	<u>318</u> 327
2PB <i>Fusarium proliferatum</i>	20 - 530	✓		<i>Fusarium proliferatum</i> NRRL 31071gi 15637118 gb AF291055.1 AF291055	99	<u>510</u> 511	—	—	—
2SB <i>Fusarium subglutinans</i>	20 - 540	✓		<i>Fusarium nygamai</i> gi 1808710 gb U34426.1 U34426	100	<u>503</u> 503	<i>Fusarium</i> sp. NRRL26793 gi 9230304 gb AF160324.1 AF160324	99	<u>501</u> 503
2VB <i>Fusarium verticillioides</i>	20 - 540	✓		<i>Fusarium nygamai</i> gi 1808710 gb U34426.1 U34426	100	<u>501</u> 501	<i>Fusarium</i> sp. NRRL26793 gi 9230304 gb AF160324.1 AF160324	99	<u>499</u> 501
							<i>Fusarium verticillioides</i> gi 1808748 gb U34413.1 U34413	97	<u>491</u> 502

Table 5.3 Translation elongation factor 1- α sequence identification.

Code used and Morphological identification	Nucleotides Used	Noise		GeneBank ID	% Similar	Seq. ID	Nearest relative(s) ID	% Similar	Seq. ID
		No	Yes						
1E2 <i>Fusarium proliferatum</i>	50 - 500		✓	<i>Gibberella fujikuroi</i> var. <i>intermedia</i> gi 121491304 emb AM404120.1	92	$\frac{369}{398}$	<i>Fusarium proliferatum</i> strain NRRL22944 gi 9230402 gb AF160280.1	92	$\frac{369}{398}$
3EE <i>Fusarium verticillioides</i>	50 – 500		✓	<i>Fusarium lactis</i> strain NRRL25200 gi 9230386 gb AF160272.1	87	$\frac{62}{71}$	<i>Fusarium</i> sp. NRRL 26793 gi 9230460 gb AF160309.1	87	$\frac{61}{70}$
							<i>Gibberella fujikuroi</i> var. <i>moniliformis</i> gi 121491376 emb AM404156.1	84	$\frac{60}{71}$
10E <i>Fusarium subglutinans</i>	Sequence unusable. See Appendix 6								
15 E <i>Fusarium oxysporum</i>	Sequence unusable. See Appendix 6								

From Table 5.2 it is evident that the molecular identification for *F. oxysporum* and *F. proliferatum* confirmed morphological identification of these isolates. The sequences of isolate *F. subglutinans* had background from 190 bps onwards and its immediate identification (*F. nygamai*) and nearest relatives were contrary to morphological identification. Repeated PCR and subsequent sequencing (Inqaba Biotech, SA) of the same isolate (2SB) showed cleaner background. However, its molecular identification was still in contrast with morphological identification and the immediate and nearest relatives were also the same as before.

All of the recognised sequences (501) of isolate 2VB were used by database to identify (100 %) the isolate as *Fusarium nygamai*. This result was contrary to morphological identification that indicated 2VB as *F. verticilliodes*. However, according to Table 5.2 it is evident that the closest *F. verticilliodes* sequences was 97% similar to the query sequence submitted to GeneBank.

The EF-1 α sequences results are shown in Table 5.3. According to the table, the identity of isolates 1E2 and 3EE and were positively confirmed by their nearest relatives as *F. proliferatum* and *F. verticilliodes*, respectively. *Gibberella fujikuroi* var. *moniliformis* is a teleomorph of *Fusarium verticilliodes*. The 10E and 15E sequences were unusable due to extensive backgrounds (Appendix 6)

5.4 FUMONISIN ANALYSES

Household sun-dried samples of *lerocho* and *thepe* harvested before maize grew (M-) and freshly picked oven-dried samples of *lerocho* and *thepe* growing close to maize (M+) were analysed for fumonisins. Enzyme-linked immunosorbant assay (ELISA) was employed to screen *lerocho* and *thepe* samples for the presence of fumonisin B-group toxins. High performance liquid chromatography (HPLC) was subsequently applied to fumonisin-positive samples to determine levels of FB1, FB2 or FB3. Results for ELISA detection of fumonisin B-group toxins and HPLC determination of their respective levels are reported in Table 5.4.

Table 5.4 Levels of fumonisin B-group toxins in sun-dried and freshly sampled oven-dried household *lerocho* and *thepe* growing close to maize (M+).

Village	<i>Morogo</i> type	ELISA	Fumonisin concentration (ng/g)		
			FB1	FB2	FB3
SEF	<i>Lerocho</i> + <i>Thepe</i> ¹	135.5	25.0	nd	nd
MAD	<i>Lerocho</i> ¹	376.0	46.2	nd	nd
MAN	<i>Thepe</i> ¹	90.8	53.0	nd	nd
	<i>Thepe</i> ²	nt	nd	nd	nd
MOD	<i>Lerocho</i> + <i>Thepe</i> ¹	93.3	26.4	nd	nd
	<i>Lerocho</i> ¹	93.3	14.5	nd	nd
	<i>Lerocho</i> ²	nt	48.8	nd	nd

Abbreviations: SEF – Sefateng; MAD – Madiga; MAN – Mantheding; MOD – Moduane; nd - not detected; nt - not tested

¹ Sun-dried household sample

² Freshly picked leaves oven-dried upon arrival at laboratory

ELISA results depicted in Table 5.4 indicated fumonisin levels in *lerocho* and *thepe* ranging from 90.8 to 376 ng/g. HPLC analysis detected FB1 in all samples except fresh *thepe* from Mantheding. FB1 levels ranged between 14.5 ng/g in sun-dried household *lerocho* of Moduane and 53.0 ng/g in sun-dried household *thepe* of Mantheding. FB2 and FB3 were not detected by HPLC in any of the samples.

CHAPTER 6

DISCUSSIONS AND CONCLUSIONS

6.1. DISCUSSIONS

Figures 5.1 to 5.4 illustrate average fungal plate counts in *lerotho* and *thepe* sampled respectively before maize planting (M-) and when maize was fully grown (M+). Average plate counts of surface-colonizing fungi were notably higher in *thepe* sampled from M+ fields of Madiga, Mantheding and Moduane compared to that of *thepe* from M- fields. Fungi occurred in almost equal levels in *thepe* from M- and M+ fields of Sefateng. These fungal levels seem to correspond with that of maize and soil of M+ fields that were higher in Madiga, Mantheding and Moduane and lower in Sefateng (Figures 5.5 and 5.6). Fungal spores are ubiquitous in agro-environments (Nelson *et al.*, 1983; Alexopoulos *et al.*, 1995).

Cotton and Munkvold (1998) found that maize-associated fungi can survive in maize stalk residue for as long as 630 days. Fungal levels in dead maize plants remaining on the land for animal grazing and maize debris on the ground are thus expected to reflect, at least to some extent, levels in growing maize plants though, according to Nesci *et al.* (2006), their abundance would be influenced by seasonal variation. Fungal spores might become suspended in the air when grazing cattle or human agricultural activities disturb the soil surface. According to Nesci *et al.* (2006), tillage would change the physical soil environment related to soil water, porosity, aeration and temperature – factors that would in turn influence organic matter decomposition rate and affect soil microbial soil communities. The aerospora would be deposited on plant leaves surfaces. *Morogo* and maize would have been equally exposed to fungal levels in the air. Fungal levels in the air of M+ fields of all four villages were shown to be higher than in M- fields (Figure 5.7). Where soil is not disturbed, fungal levels would probably be high in soil and low in air, a trend that was demonstrated by Nesci *et al.* (2006) in a study comparing the effect of tillage practices on fungal levels in soil of maize ecosystems in Argentina. Low soil and maize counts but high air counts in M+ fields of Sefateng seem to reflect this trend (Figures 5.5 to 5.7).

The opposite trend was observed in *lerotho* which appears to have carried a higher fungal load in M- fields than those in M+ fields of Madiga, Mantheding and Moduane (Figures 5.2 to 5.4). Air fungal counts in M- fields of these villages corresponded with that of soil (Figures 5.6 and 5.7).

The difference in soil and air fungal levels in M- fields of the respective villages possibly reflects the influence of these variables on survival of fungi in soil where maize are grown annually. After harvest, soil is likely to be disturbed by cattle grazing the dead maize or humans harvesting *morogo*, activities which would have suspended fungi from the soil into the air from where they were likely to be trapped onto *morogo* plants growing there. Though *thepe* and *lerocho* were exposed to the same fungal levels in air, leaf surface qualities could have contributed in keeping fungal spores attached to *lerocho* leaves. Other crops or agricultural activities at M- fields were not recorded but could have influenced fungal species composition in soil and dispersal of fungal spores differently in M+ and M- fields of the respective settings. However, high fungal levels in air samples of Sefateng probably explain why fungal plate counts in Sefateng *lerocho* from the M+ site were significantly higher in *lerocho* from the M- site (Table 5.A, Appendix 5).

Pathogenesis of *Fusarium* in humans result either from infection or dietary exposure to their mycotoxins (Bennett and Klich, 2003; Jurado *et al.*, 2006). The following *Fusarium* species, indicated in literature as mycotic strains, were identified among those recovered from *lerocho*, *thepe*, maize and air samples: *F. chlamydosporum*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, *F. solani* and *F. verticillioides*. In literature these strains have been reported as causative organisms in the following case studies: *F. chlamydosporum* - nasal septum abcess and lesions; *F. oxysporum* - opportunistic infection in renal transplant patient, keratitis and endophthalmitis, cutaneous infections in immunocompetent patients and catheter-related fungemias; *F. proliferatum* and *F. semitectum* - disseminated skin lesions; *F. solani* – invasive or disseminated skin lesions, deep cutaneous infection in HIV positive individual; *F. verticillioides* - skin lesions in immune competent as well as immune suppressed individuals, nasal septum abcess in immune suppressed child and mucositis in HIV positive individual (Young and Meyers, 1979; Jones, 1975; Raad and Hachem, 1995; Romano *et al.*, 1998; Segal *et al.*, 1998; Guarro *et al.*, 2000; Bodey *et al.*, 2002; Dornbusch *et al.*, 2004; Dignani and Anaissie, 2004; Nakamura *et al.*, 2006).

From these case studies health risks for DDSS communities implied by the presence of these *Fusarium* species are evident, particularly in view of the high level of HIV/AIDS reported in the Limpopo Province.

The following *Fusarium* species, predominantly identified among those isolated from the DDSS agro-environment, have been reported in literature as producers of a range of potent mycotoxins:

F. poae - trichothecenes nivalenol and T-2 toxin; *F. subglutinans* - fumonisin B₁; *F. verticillioides* and *F. proliferatum* - fumonisin B-group, fusaroproliferin, beauvericin and deoxynivalenol; *F. oxysporum* - fumonisin B₁, moniliformin and fusaric acid; *F. equisiti* – fusarochromanones and zearalenone; *F. graminearum* - deoxynivalenol and nivalenol (Placinta *et al.*, 1999; Rheeder *et al.*, 2002; Bennett and Klich, 2003; Sudakin 2003; Edwards, 2004).

One study related fumonisin B₁, deoxynivalenol and zearalenone with cytotoxicity and lipid peroxidation (Kouadio *et al.*, 2005). Zearalenone was shown in animal studies to be hyperestrogenic (Bennet and Klich, 2003; Placinta *et al.*, 1999). Sudakin (2003), reported on the protein-inhibitory and immune modulatory effects of trichothecenes deoxynivalenol and T-2 toxin, the last also being responsible for life-threatening alimentary toxic aleukia (ATA), symptoms of which include inflammation of the skin and damage to the haematopoietic tissue (Bennett and Klich, 2003).

Epidemiological studies have linked the occurrence of human oesophageal cancer with the consumption of fumonisin B₁-contaminated food worldwide (Yang 1980; Franceschi *et al.*, 1990; Rheeder *et al.* 1992, Shephard *et al.* 2000). FB₁ is classified by the W.H.O as a probable human carcinogen (WHO-IARC, 1993) and the US Food and Drug Administration (FDA) recommended a fumonisin level not greater than 4 µg/g in human foods (FDA, 2000). Animal and cell culture studies have related the toxicological effect of fumonisin B₁ (FB₁) to the ability of this compound to disrupt the sphingolipid metabolism. Sphingolipids are a class of membrane lipids that play essential function in membrane and lipoprotein structure. FB₁ structurally resembles the sphingolipid intermediate sphinganine and is thought to disrupt sphingolipid metabolism by inhibiting the enzyme ceramide synthetase (Wang *et al.*, 1991; Spiegel and Merrill, 1996) In the presence of FB₁ ceramide synthase activity is inhibited. Ceramide depletion affects biosynthesis of complex sphingolipids and leads to the accumulation of sphinganine/sphingosine. Accumulation of sphingoid bases is believed to be the primary cause of the toxicity of fumonisin B₁ and possible carcinogenicity in cell or tissues (Meivar-Levy *et al.*, 1997; Mobio *et al.*, 2000; Merrill *et al.*, 2001). The inhibitory effect of FB₁ on sphingolipid metabolism affects many biochemical reactions among which the disruption of inter- and intracellular cell signalling systems reportedly have important consequences for coordinated immune cell functioning (Baumrucker and Prieschl, 2002). Van der Walt *et al.* (2006) proposed that dietary exposure to FB₁ could add to the burden on the compromised immune system of HIV/AIDS individuals. FB₁

was reported by Carratù *et al.* (2003) to have antinutritional effects similar to those induced by nutritional deficiencies.

F. verticillioides and *F. proliferatum* both are recognised as prolific producers of fumonisin B-group toxins (Rheeder *et al.*, 2002). Velluti *et al.* (2000) found that *F. verticillioides* and *F. proliferatum* levels were markedly reduced by the presence of *F. graminearum* and that fumonisin B1 production by the former species was also inhibited. In this study *F. proliferatum* was, however, retrieved in larger numbers from both subsistence maize and air samples of DDSS agro-environments compared to *F. graminearum*. According to Fandohan *et al.* (2003), environmental conditions such as temperature, humidity, drought stress, and rainfall, insect infestation and pre- and post-harvest handling would influence fumonisin levels in crops. Previous studies showed that hot and dry weather, followed by periods of high humidity or when moisture content of crops during storage is 18 to 23%, lead to high levels of fumonisin (Bacon, 1994; Munkvold and Desjardins, 1997; Miller, 2001). A temperature of about 20-30 °C and water activity of around 0.98 were shown to promote production of mycotoxins by *Fusarium* species (Ono *et al.*, 1999; Soriano and Dragacci, 2004)

These reports strongly support the view that mycotoxigenic fungi in the subsistence agro-environment imply health risks that could further burden the compromised immunity of HIV positive individuals in DDSS communities.

In the present study HPLC analysis detected varying levels of FB1 in fresh as well as traditionally sun-dried *lerocho* and *thepe* samples (Table 5.6). FB1 levels in *lerocho* and *thepe* were notably lower than amounts reported for *lerocho* and *thepe* of the DDSS in a previous study (Van der Walt *et al.*, 2006). Data of the previous study by Van der Walt *et al.* (2006) showed that in one *lerocho* sample FB1 was detected in an extremely high concentration (38860 ng.g⁻¹) as well as FB2 (6511 ng.g⁻¹) and FB3 (1565 ng.g⁻¹). In another *lerocho* sample the combined concentration of FB1 and FB2 were 2500 ng.g⁻¹. FB1 was detected in a small amount in a DDSS sample of *thepe* with tomato (143 ng.g⁻¹), but in a higher amount in a sample of *lerocho* and *thepe* (661 ng.g⁻¹). These results strongly support findings of the present study that *morogo* growing in subsistence maize ecosystems may be at risk of fumonisin contamination.

Morphologically-based identification of these species was subjected to molecular procedure to verify their identification. *Fusarium* species specific PCR using translation elongation factor 1-

alpha (EF-1 α) and beta tubulin (β -tub) primer set, amplified the expected DNA fragments in all tested isolates (Fig. 5.11 – Fig 5.13).

The presence of *FUM1* gene indicated the presence of the polyketide synthase gene that is believed to be responsible for fumonisin biosynthesis (Proctor *et al.*, 2003). The results indicate that the isolates recovered from *morogo* in DDSS are capable of producing fumonisin under certain environmental conditions cited by Fandohan *et al.* (2003). The *FUM1* gene was successfully sequenced by Bezuidenhout *et al.* (2006).

With the exception of isolates from *F. subglutinans*, there were generally good agreement between molecular and the morphologically identifications (Table 5.2 and 5.3). Isolate 1b and its repeated isolate (2SB) were identified as *F. nygamai* contrary to their morphological identifications (*F. subglutinans*). The illustrated manual for *Fusarium* identification by Nelson *et al.* (1983) does not have *F. nygamai* as one of the species described for morphological identification. Some of the species based on morphological characters as documented by Nelson *et al.*, 1983 are now presented by a number of independent species due to sequences data obtained for a number of genes. According to the manual for *Fusarium* identification compiled by Leslie and Summerell (2006), morphological characters common to these two species include oval shaped microconidia, arrangement of microconidia in false heads, slender and thin walled macroconidia with three (3) septates. The presence of chlamydospores and the arrangement of microconidia in chains distinguish *F. nygamai* from *F. subglutinans* (Leslie and Summerell, 2006). However, some strains of *F. nygamai* might not have chlamydospores and a mutant of *F. subglutinans* that produce short chains of microconidia has been reported (Tiedt and Jooste, 1988; Leslie and Summerell, 2006). The result in Figure 5.13 confirmed the presence of the *FUM1* gene which correlated with the reports confirming their potential in fumonisins production (Thiel *et al.*, 1991; Nelson *et al.*, 1992; Reynoso *et al.*, 2004). With the exception of *F. subglutinans*, all the morphologically-based identifications of the *Fusarium* isolates were confirmed by molecular sequencing.

6.2. SUMMARY AND CONCLUSION

Results reported in this study show that mycotoxigenic and mycotic *Fusarium* species such as *F. chlamydosporum*, *F. equisiti*, *F. graminearum*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, *F. solani*, *F. subglutinans* and *F. verticillioides* occurred in varying levels on subsistence maize and

traditional household *morogo*, as well as in soil and air of Sefateng, Madiga, Mantheding and Moduane. Based on results presented here, *lerotheo* plants appear to be more strongly predisposed for contamination with *Fusarium* which is suspected of being dispersed in the agro-environment through spore dissemination. In Madiga, such harmful *Fusarium* species were largely absent from both *morogo* types as well as soil and air before maize was planted (M- field), but occurred in *lerotheo*, soil and air after maize was planted (M+ field). Sefateng *lerotheo* from the M+ field contained five harmful fusarial species in relatively high numbers in comparison with *lerotheo* that was growing before maize planting (M- field).

These trends seem to indicate that maize could have been a major factor in the occurrence of *Fusarium* species in the DDSS agro-environment. Fusarial spores surviving in dead maize plants, maize plant debris and soil might become suspended and dispersed into the air as a result of agricultural activities (e.g. tilling, ploughing) and environmental factors (e.g. rain, wind) from where they land on *morogo* leaves. *Morogo* plants appeared healthy and no scientific reports could be found referring to *lerotheo* or *thepe* being pathogenised by any of these *Fusarium* species.

The conclusion would be that *morogo* plant surfaces become contaminated with spores of harmful *Fusarium* species when they grow in maize ecosystems. Health risks implied by the presence of mycotic *Fusarium* species are evident from literature reports. Infections caused by mycotic fusaria are more often reported to occur in individuals with suppressed or compromised immunity.

Human portals of entry include skin damage, alimentary system as a result of ingestion of contaminated food of the respiratory system through inhalation (Dignani and Anaissie, 2004). All these portals of entry are possible in the DDSS considering the occurrence of *Fusarium* species in food (maize as well as *morogo* vegetables) upon which households rely. Contaminated air inhaled by field workers gathering *morogo* from the agro-environment may also put the community at risk to mycotic *Fusarium* infection. The presence of mycotic and fumonogenic *Fusarium* species in subsistence maize, traditional *morogo* and the agro-environment of the DDSS could have important health consequences for communities dependent on subsistence food production. Those who will feel the effect of that would be particularly those affected by the AIDS pandemic.

6.3. RESEARCH RECOMMENDATIONS

Although the current study revealed the level of *Fusarium* species in *morogo* and the risk of subsistence cultivation of maize close to these traditional vegetables in the community of DDSS, the following recommendations that would add value to the current findings are suggested:

DDSS is made up of eight (8) rural settlements in proximity of each other. Increasing the sampling sites from the current four to eight will provide useful information on the distribution of mycotic and mycotoxigenic *Fusarium* species in the subsistence agro-environment of the entire DDSS. It would be important to confirm sources of *Fusarium* contamination in subsistence agro-environments.

Isolation and identification of *Fusarium* species from traditionally dried *morogo* plants would be of value to verify the effect of traditional preservation by sun-drying on the levels of harmful *Fusarium*.

Molecular confirmation of morphological identification should be done concurrently on all *Fusarium* isolates to avoid confusing isolate identity. According to the results of this study, β -tubulin was the better primer set to use for molecular identification.

The effect of seasonal variation and environmental factors on fumonisin production should be investigated from specific sites over a certain period from traditionally dried or fresh dried *morogo* samples.

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APPENDICES

APPENDIX 1

1.1 Dikgale Demographic Surveillance Site Questionnaire Form

1.1.1 Household Information

Sampling point number: _____

Household number : _____

Name : _____

Occupation of Breadwinner (s): _____

Number of household members of specific age group:

0- 10 ☐ 11- 20 ☐ 21- 30 ☐ 31- 40 ☐ 50 above ☐

1.1.2 Dietary Information

1.1.2.1 What is the staple diet of the household?

Porridge & *morogo* ☐ sorghum ☐ wheat ☐ legumes ☐

1.1.2.2 What is the consumption frequency of the staple diet per day?

Once ☐ twice ☐ thrice ☐ others ☐ specify _____

1.1.2.3 What other foods do you cook and eat?

Meat ☐ Vegetables ☐ Beans ☐ other ☐ specify _____

1.1.2.4 What is the consumption frequency of these foods per day?

Once ☐ twice ☐ thrice ☐ others ☐ specify _____

1.1.2.5 What is the consumption frequency of *morogo* per day?

Once ☐ twice ☐ thrice ☐ others ☐ specify _____

1.1.2.6 What is the average weight of *morogo* eaten by members of the household per meal? Children _____ Teenagers _____ Adults _____

1.1.2.7 Do some members of the household eat foods from the field that are not cooked?

Yes ☐ No ☐

1.1.2.8 If yes, who are they? Children ☐ Adults ☐

1.1.3 Morogo Information

1.1.3.1 Give the names of *morogo* that you use for consumption.

1.1.3.2 Why do eat *morogo*? You like it ☐ you have no choice ☐

1.1.3.3 Do children like to eat *morogo*? Yes ☐ No ☐

1.1.3.4 Do teenagers like *morogo*? Yes ☐ No ☐

1.1.4 Cultivation of Morogo plants

1.1.4.1 Where do you usually get your *morogo*?

At home ☐ Schemes ☐ Relatives ☐ others ☐ specify _____

1.1.4.2 Do you plough? Yes ☐ No ☐

1.1.4.3 If yes, where do you plough? At home ☐ Fields ☐

1.1.4.4 Do you rely on rain for-water? Yes ☐ No ☐

1.1.4.4 Did you plough this season (2004 - 2005)? Yes ☐ No ☐

1.1.4.5 Does the lack of rain affect ploughing and plant growth? Yes ☐ No ☐

1.1.4.6 When do you get the best yield of *morogo*?

Rainy seasons ☐ dry seasons ☐ others ☐ specify _____

1.1.4.7 How did you cultivate your *morogo*?

From seedlings ☐ from seeds ☐ grow on its own ☐ others ☐ specify ____

1.1.4.8 How long after planting is *morogo* collected?

Less than a month ☐ 1 month ☐ 2 months ☐ 3 months ☐ others ☐ specify ____

1.1.4.9 Which parts of the *morogo* plants do you collect for consumption?

Young leaves ☐ Older leaves ☐ Flowers ☐ Seeds ☐

1.1.4.10 Where do you get the seeds from?

Store ☐ previous years crop ☐ Relatives ☐

1.1.4.11 Do you use anything for soil improvement? Yes ☐ No ☐

1.1.4.12 If yes, what do you use?

Compost ☐ Cow droppings ☐ Chicken droppings ☐ Chemical fertilizers ☐

1.1.4.13 Do you sometimes find any mouldy leaves? Yes ☐ No ☐

1.1.4.14 On which leaves do you recognize moulds?

Young leaves ☐ older leaves ☐ almost all the leaves ☐ Flowers ☐ Seeds ☐

1.1.4.15 Do you apply pesticides on the infected leaves? Yes ☐ No ☐

1.1.4.16 If yes, which type of pesticide? Chemical ☐ Organic ☐ others ☐ specify ____

1.1.4.17 What do you do with those mouldy leaves?

Throw them away ☐ use them with other leaves as food ☐ others ☐ specify ____

1.1.4.18 Where do you throw the mouldy leaves?

In the compost ☐ in the field ☐ others ☐ specify _____

1.1.5 Methods of Preparations

1.1.5.1 Do you store these *morogo* for the winter season? Yes ☐ No ☐

1.1.5.2 How do you preserve these *morogo* during storage?

Dry before cooking ☐ dry after cooking ☐ none of the two ☐

1.1.5.3 Where do you dry these *morogo*? In the sun ☐ in the shadow ☐

1.1.5.4 Where do you store these *morogo*?

In closed containers ☐ open containers ☐ others ☐ specify _____

1.1.5.5 How long do you cook the fresh *morogo*?

Less than 10 min ☐ more than 10 but less than 20 min ☐ more than 20 min ☐

1.1.5.6 How long do you cook the dried *morogo*?

Less than 10 min ☐ more than 10 but less than 20 min ☐ more than 20 min ☐

1.1.5.8 What other ingredients do you add in the *morogo*?

Tomatoes ☐ Salt ☐ Onions ☐ others ☐ specify _____

1.1.5.9 Do you get *morogo* in the form of weeds amongst the maize? Yes ☐ No ☐

1.1.5.10 Do you also collect that *morogo* (*morogo* amongst maize) for consumption? Yes ☐

No ☐

1.1.6 Health related information of *morogo* plants

1.1.6.1 Have you noticed any difference between the *morogo* amongst maize and the one cultivated separately? Explain

1.1.6.2 Can you associate any symptoms like diarrhea, sore throat or other with the consumption of the *morogo*?

1.1.6.3 Are you aware of any known myths of the healing effect caused by these *morogo*?

1.2 Local, English and scientific species names of *morogo* plants utilised in the DDSS

Local (DDSS) name	English name ^{b, c}	Scientific name ^a
<i>Lerotho</i>	African cabbage	<i>Cleome gynanadra</i> L.
<i>Lehlanye</i>	-	<i>Vernonia fastigiata</i>
<i>Thepe</i>	Amaranth	<i>Amaranthus thunbergii</i>
<i>Mochacha</i>	Watermelon	<i>Citrullus lanatus</i>
<i>Thelele/ motelele</i>	Wild jute	<i>Corchorus tridens</i> L
<i>Monawa</i>	Cowpea	<i>Vigna unguiculata</i>
<i>Mophotse</i>	Pumpkin leaves	<i>Cucurbita spp</i>
<i>Monyaku</i>	-	-
<i>Moraka</i>	Calabash	<i>Lagenaria siceraria</i>
<i>Mokgadi wa sega-sega</i>	-	-
<i>Tshehlo</i>	Devil's thorn	<i>Tribulus terrestris</i>

^a SANBI; ^b van Wyk and Gericke, 2003; ^c Bromilow, 2001

APPENDIX 2

PLANTE VIR IDENTIFIKASIE

Direkteur: Naworsing
Nasionale Botaniese Instituut
Privateak 100
0001 PRETORIA

'N HANTERINGSFOOI SAL VIR ELKE EKSEMPLAAR WAT INGEHANDIG WORD, GEHEF WORD.

Wanneer u plante vir identifikasie stuur, moet die volgende aangekom word:

1. VERSAMELING VAN PLANTEKSEMPLARE

Volledigheid en grootte van eksimplare

- Waar moontlik moet die hele plant, volledig met wortels, stengels, blare, blomme en vrugte en voldoende om op 'n herbariumvel (380 x 250 mm) te pas, versamel word. Steriele materiaal kan selds akkuraat bemaat word en is van weinig of geen wesenkaplike waarde nie.
- In die geval van houtagtige plante, soos bome, waar dit onmoontlik is om die hele plant te versamel, moet 'n verteenwoordigende deel gekies word om 'n herbariumvel (380 x 250 mm) te kan volmaak.
- Groot kruidagtige plante en grasse (wat wortels en wortelstokke moet insluit) kan gebuig word of verdeel word om op meer as een vel te pas.
- In die geval van klein plantjies, moet genoeg individuele plante versamel word om min of meer 'n herbariumvel te vul.
- Moenie slegs sade en vrugte vir identifikasie instuur nie, maar stuur altyd 'n volledige eksimplaar soos beskryf in a-d hierbo, saam.
- Foto's, skyfies of tekeninge van plante moet, waar moontlik, vergesel wees van 'n eksimplaar van die plant om te help met identifikasie.

Duplikaadmateriaal

- Wanneer plantmateriaal vir identifikasie gestuur word, hou altyd 'n duplikaatversameling teruggoed planteksemplare wat vir benaming ontvang word, slegs in uitsonderlike omstandighede na die versamelaar teruggestuur sal word.
- Indien moontlik, stuur asseblief een of meer duplikaat-eksemplare saam met die oorspronklike eksimplaar aangesien dit na ander herbariums in Suid-Afrika en oorsse versprei word.

Pers en draging van eksimplare

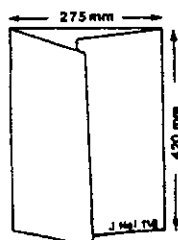
- Plante moet so gou moontlik nadat hulle versamel is in 'n plantpers geplaas word om versuifing te voorkom. Druk moet toegepas word om die eksimplare plat te stryk.
- Indien 'n permaante pers nie beskikbaar is nie, kan koerspapier, blaas van tydkrifte of vloeipapier, plek-plek versier deur stywe kartonblase of soortgelyke materiaal, gebruik word. Matige druk moet toegepas word om die eksimplare plat te druk.
- Plaas pers in die son of naby 'n verwarmde om draging te bespoedig.
- By plante met 'n hoë waterinhoud, bv. sukkulente, moet die droogpapier daagliks verander word tot taamlik droog. Dit is nodig om samentreue te voorkom.

2. VOORBEREIDING VAN CEDROOGDE MATERIAAL

Omset

Plaas elke eksimplaar in 'n skoon en stywe beskermende omslag. Indien moontlik gebruik onbedrukte of bedrukte koerspapier. Moenie die eksimplare op 'n karton moner nie. Elke omslag moet soos volg voorberei word:

- Vou 'n vel soos op skets.
- Skrif versamelaar se naam en nommer regs onder.



- Moenie teekrante of bo- en onderkant neevu nie—plante en etikette val nie maklik uit nie.
- Indien u nie duplikaadmateriaal kan versamel nie en die eksimplaar graag wil terughe, merk die omslag met 'n duidelike stertjie (*) na die versamelaar se nommer.

Etikette

- 'n Etikette waarop die volgende inligting in drukskrif aangegee word, moet elke gesamorde eksimplaar vergesel:
- (1) Naam van versamelaar.
- (2) Versamelnommer (soosmaar eksimplare opeenvolgend beginnende by 1—'n nommer moet nooit meer as een keer gebruik word nie en moenie letters of simbole as deel van 'n nommer gebruik nie).
- (3) Datum versamel.
- (4) Ruikverwyting (bv. 2527 AC).
- (5) Presiese veldplek (lokaliteit).
- (6) Habitat (omgewing)—bv. rivieroeuw, hang van koppie).
- (7) Grondtipe.
- (8) Groeiwurm en grootte van plant.
- (9) Bloemkleur.
- (10) Volksnaam, indien bekend.
- (11) Gebruike, ongewenste eienskappe ens.

Verpakking

- Plante moet nooit in plastieksakke of ander lugdigte houers gestuur word nie.
- Om te verhoed dat plante muf, moet hulle nie verpak word teeny hulle heeltemal droog is nie.
- Droë eksimplare word maklik in die pos beskodig. Daar word aanbeveel dat u ongeveer twintig eksimplare (elk in sy eie omslag) in 'n dubbelvel koerspapier toevou en vasplek; die aantal pakkies moet dan in 'n stewige kartondoos verpak word.
- Plantidentifikasie-versoekpyns (verkrygbaar van die NBI) moet elke besending wat gestuur word, vergesel.
- Elke doos moet die versamelaar se naam en adres in drukskrif daarop he, saam met die verklaring 'Droë eksimplare vir identifikasie'.
- 'n Brief om die versoeding van 'n versameling aan te kondig moet afsonderlik gepos word en nie in 'n pakkie ingesluit word nie. Dit moet ook die nodige inligting oor die aantal dose en eksimplare wat afgestuur word, bevat.
- Personne wat groot versameldings van meer as 100 eksimplare wil stuur, moet eers die nodige toestemming van die NBI verkry alvorens die eksimplare afgestuur word.

3. VOLKSNAME

- Moenie inligting omtrent plante vra deur slegs na die volksnaam te verwys nie, aangesien dieselfde volksnaam dikwels vir verskillende planteties gebruik word.
- Stuur altyd 'n eksimplaar van die plant waarop die volksnaam betrekking het.

4. ERKENNINGSVORM

Nadat u planteksemplare gekompiliseer is, ontvang u saam met 'n finale lys van name 'n erkenningsvorm. Vul asseblief hierdie vorm in en stuur dit aan die NBI terug.

5. ADRES

Pakkies en navre moet aan bogenoemde adres gestuur word.

APPENDIX 3

MEDIA USED FOR FUSARIUM CULTIVATION AND IDENTIFICATION

3.1 Pentachloronitrobenzene (PNCB) agar

15.0g Difco peptone

1.0g KH₂PO₄

0.5g MgSO₄·7H₂O

20.0g Agar

1.0g Pentachloronitrobenzene (Terraclor)

1.0 liter distilled water.

The pH was adjusted to between 5.5- 6.5 and the mixture autoclaved. After autoclaving and allowing the mixture to cool ($\pm 55^{\circ}\text{C}$), the following antibiotics were added:

- Benzyl penicillin Fresenius 1mu=0.2ml/L (prepared by adding 4.6ml benzyl powder to sterile distilled water)
- Pendistrep 20/20=0.6ml/L,
- Chloramphenicol pure 0.02g/l=0.28ml/L (prepared by adding 14.0ml sterile to distilled water).

Sterile syringe for each antibiotic was used when preparing and adding the antibiotics to the PNCB agar medium

3.2 Potato Dextrose Agar (PDA)

39g PDA (Biolab, Merck, South Africa)

1 litre distilled water

The mixture was shaken and autoclaved. After autoclaving, it was dispensed into sterile small Petri dishes

3.2 Water Agar (WA)

20g Bacteriological Agar (Agar Bacteriological, Biolab, Merck, South Africa)

1 litre distilled water

The mixture was shaken and autoclaved. It was subsequently dispensed into 9 mm diameter sterile Petri dishes.

3.3 Carnation Leaf Agar (CLA)

20g Bacteriological Agar (Agar Bacteriological, Biolab, Merck, South Africa)

1 liter distilled water

Sterilized carnation leaf pieces

20g of the Bacteriological agar was added to 1 liter of distilled water and the mixture autoclaved. Following autoclaving, the agar was dispensed into 9 mm sterile Petri dishes. One piece of the carnation leaf was aseptically put in the centre of each agar plate. The contents were allowed to solidify.

3.4 Yeast Peptone Dextrose (YPD) agar

Yeast extracts 10 g

Peptone 20g

Distilled water 900 ml

20% dextrose 100 ml

Autoclave 29% dextrose separately before adding to the other mixture, then mix to other ingredients and autoclave again.

APPENDIX 4

COMPOSITE TABLES OF AVERAGE FUNGAL COUNTS				
Table 1 - Total fungal plate counts				
	Cleome/M+	Cleome/M-	Amaranth/M+	Amaranth/M-
SEF	17	3	2	3
MAD	17	20	6	1
MAN	8	15	7	2
MOD	3	6	3	1
Table 2 - Average fungal plate counts				
	Cleome/M+	Cleome/M-	Amaranth/M+	Amaranth/M-
SEF	1695	310	420	335
MAD	1815	2090	620	115
MAN	925	1101	875	215
MOD	610	595	365	80
Table 3 - Total fungal counts/soil (CFU X10 ⁴)				
	M+	M-		
SEF	1.8	11.2		
MAD	8.4	7.1		
MAN	3.1	6.1		
MOD	3.8	1.4		
Table 4 - Average fungal counts of soil and air (CFU x10 ³)				
	Soil/M+	Soil/M-	Air/M+	Air/M-
SEF	6	37	0.06	0.01
MAD	28	24	0.06	0.03
MAN	10	20	0.06	0.2
MOD	13	5	0.04	0.02
Table 5 - Average fungal counts of maize components				
	Cleome/M+	Amaranth/M+	MLI	MLE
SEF	85	420	28	4100
MAD	59	21	18	7467
MAN	26	23	21	14600
MOD	11	14	17	30433

Before maize was growing - M-; With maize growing - M+

SEF - Sefateng; MAD - Madiga; MAN - Mantheding; MOD - Moduane

MLI - maize leaves internal; MLE - maize leaves external; MK - maize kernels

Relative numbers of different *Fusarium* species isolated from lerotho and thepe growing with maize

LEROTHO				THEPE			
SEF	MAD	MAN	MOD	SEF	MAD	MAN	MOD
18	2	3	0	3	0	0	0
<i>F. proliferatum</i>	3	3	0	2	0	0	0
<i>F. subglutinans</i>	0	3	0	0	0	0	0
<i>F. oxysporum</i>	0	0	0	0	0	0	0
<i>F. graminearum</i>	0	0	0	0	0	0	0
<i>F. equiseti</i>	0	0	0	0	0	0	0
<i>F. solani</i>	3	0	0	0	0	1	0
<i>F. chlamydosporum</i>	25	0	1	0	0	0	0
<i>F. reticulatum</i>	4	0	0	0	0	0	0
<i>F. semitectum</i>	0	0	0	0	0	0	0
<i>F. crookwellensis</i>	0	0	1	0	0	0	0

	MAIZE LEAVES				MAIZE HAIR				MAIZE KERNELS			
	SEP	MAD	MAN	MOD	SEP	MAD	MAN	MOD	SEP	MAD	MAN	MOD
<i>F. verticillodes</i>	13	7	6	33	0	0	0	0	3	0	0	0
<i>F. proliferatum</i>	37	17	13	7	0	0	0	0	2	1	0	0
<i>F. subglutinans</i>	0	5	0	0	0	0	0	0	0	0	0	0
<i>F. oxysporum</i>	0	0	7	4	0	0	0	0	0	0	1	0
<i>F. graminearum</i>	0	0	6	7	0	0	0	0	0	0	0	0
<i>F. equiseti</i>	0	0	3	3	0	0	3	0	0	0	0	0
<i>F. solani</i>	11	0	0	0	0	0	0	0	0	0	0	0
<i>F.</i>												
<i>chlamydosporum</i>	24	0	7	0	0	0	0	0	0	0	0	0
<i>F. reticulatum</i>	0	0	0	0	0	0	0	0	0	0	0	0
<i>F. semitectum</i>	0	9	0	6	0	5	0	6	0	0	0	0

Relative numbers of different *Fusarium* species isolated from lerotho and thepe before maize was growing

LEROTHO				THEPE			
SEP	MAD	MAN	MOD	SEP	MAD	MAN	MOD
F. verticillodes	0	0	4	11	0	0	0
F. proliferatum	0	0	0	0	0	0	2
F. subglutinans	0	0	23	0	0	0	0
F. oxysporum	0	0	3	1	0	0	0
F. graminearum	0	0	0	0	0	0	0
F. equiseti	0	0	5	7	0	4	0
F. solani	0	0	0	0	0	0	0
F.							
chlamydosporum	0	0	0	0	0	0	0
F. poae	22	0	0	1	18	0	0
F. avenaceum	0	0	0	0	0	0	0
F. reticulatum	0	0	0	0	0	0	0
F. semitectum	0	0	0	14	0	0	0
F. crookwellensis	0	0	0	0	0	0	0

Fusarium species	AIR				SOIL			
	SEF	MAD	MAN	MOD	SEF	MAD	MAN	MOD
<i>F. verticillioides</i>	0	0	1	0	0	0	0	0
<i>F. proliferatum</i>	0	0	0	0	0	0	0	0
<i>F. subglutinans</i>	0	0	12	0	0	0	0	0
<i>F. oxysporum</i>	0	0	16	0	0	0	0	0
<i>F. graminearum</i>	0	0	0	0	0	0	0	0
<i>F. equiseti</i>	0	0	0	0	0	0	0	0
<i>F. solani</i>	0	0	0	0	0	0	0	0
<i>F. chlamydosporum</i>	0	0	0	0	0	0	0	0
<i>F. poae</i>	2	0	0	0	23	0	0	0
<i>F. avenaceum</i>	0	0	0	31	0	0	11	23
<i>F. reticulatum</i>	0	0	0	0	0	0	0	0
<i>F. semitectum</i>	0	0	0	0	0	0	0	0
<i>F. crookwellensis</i>	0	0	0	0	0	0	0	0

APPENDIX 5

Table 5.1A - Average fungal counts in *morogo* from Sefateng (Sef)

Sites	Average Fungal counts and Standard Error (CFU/ml)
Sef_CG_M-	395 ± 112.66 ^a
Sef_CG_M+	1865 ± 572.18 ^b
Sef_AT_M-	425 ± 190.36 ^a
Sef_AT_M+	405 ± 218.90 ^a

Table 5.1B - Average fungal counts in *morogo* from Madiga (Mad)

Sites	Average Fungal counts and Standard Error (CFU/ml)
Mad_CG_M-	2340 ± 716.31 ^a
Mad_CG_M+	1980 ± 370.82 ^a
Mad_AT_M-	175 ± 55.40 ^b
Mad_AT_M+	745 ± 286.79 ^{a,b}

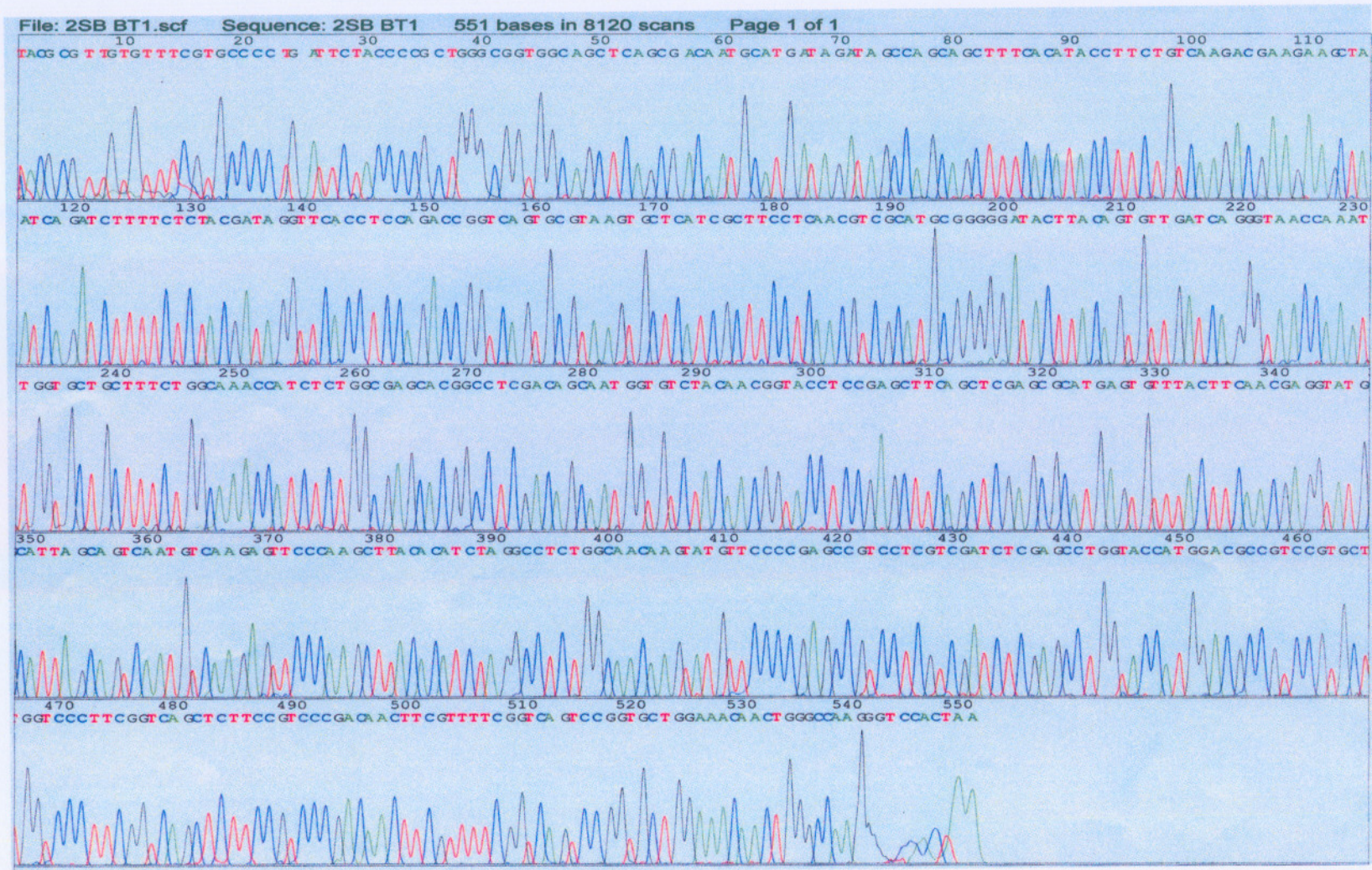
Table 5.1C - Average fungal counts in *morogo* from Mantheding (Man)

Sites	Average Fungal counts and Standard Error (CFU/ml)
Man_CG_M-	1695 ± 324.93 ^b
Man_CG_M+	950 ± 248.10 ^{a,b}
Man_AT_M-	245 ± 127.46 ^a
Man_AT_M+	915 ± 376.17 ^{a,b}

Table 5.1D - Average fungal counts in *morogo* from Moduane (Mod)

Sites	Average Fungal counts and Standard Error (CFU/ml)
Mod_CG_M-	765 ± 289.64 ^a
Mod_CG_M+	620 ± 261.21 ^a
Mod_AT_M-	95 ± 63.44 ^a
Mod_AT_M+	410 ± 124.90 ^a

APPENDIX 6



File: 2PB BT1.scf Sequence: 2PB BT1 543 bases in 8120 scans Page 1 of 1

