The incidence of mycotic and mycotoxigenic *Fusarium* in a peri-urban food-garden environment.

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In the Name of Allah, The Beneficient, The Merciful
I thank the Almighty for giving me the strength and knowledge. To complete my dissertation successfully, and Insha-Allah will carry on giving me the courage and strength to complete the tasks that I have undertaken.

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ABSTRACT

The present study was conducted in Phokeng, a peri-urban residential area outside Rustenburg in the North-West Province. To supplement their restricted diet, households of low socio-economic status often maintain food gardens where traditional African green leafy vegetables (*morogo*) are grown with maize as the traditional staple. Some of *morogo* plants are collected from the field where they grow spontaneously as natural flora. Questionnaires were used to obtain information regarding the composition and socio-economic status of households, the utilisation of *morogo* vegetables and methods of *morogo* preservation. Herbarium samples of 10 *morogo* plants were prepared for botanical species identification.

The study investigated the occurrence of *Fusarium* species in the environment of peri-urban home gardens. Environmental components sampled for mycological analysis included maize, soil, air and the two most commonly-consumed *morogo* vegetables, namely thepe (*Amaranthus* sp.) and lerotho (*Cleome gynandra*). Three home gardens in Phokeng where maize and *morogo* vegetables were growing together in a mixed system, were selected for sampling. Samples were also taken from the food garden of a family living on the nearby Zuurplaat farm and four sampling localities where *morogo* was growing some distance from maize. The presence of eleven *Fusarium* species was of interest to this study, not only for the mycotoxigenic risks to the consumer, but also for their potential to cause opportunistic infections in immune-suppressed and immuno-compromised individuals.

Standard methods were employed for *Fusarium* isolation and morphologically-based species identification of isolates. The following *Fusarium* species were isolated from the different environmental components: *F. avenaceum, F. chlamydosporum, F. dimerum, F. equiseti, F. oxysporum, F. poae, F. proliferatum, F. semitectum, F. solani, F. subglutinans* and *F. verticillioides*. Results indicated fumonisin-producing *F. proliferatum* and *F. verticillioides* as well as *F. solani* were predominantly isolated from localities close to, as well as away from maize. Though *F. subglutinans* and *F. oxysporum* are not considered major producers of fumonisins in maize, these two species were also isolated in higher numbers from sites where maize was growing. HPLC analysis detected fumonisin B_1 in some of the household *morogo* samples.

Molecular techniques were applied to indicate the correct species identification of a selected number of isolates. Primers for PCR amplification of ß-tubulin and translation elongation factor 1-α (TEF1-α) gene fragments, and subsequently sequencing of the amplified DNA, verified the correct identification of *F. proliferatum, F. subglutinans, F. solani* and *F. oxysporum* from *morogo* vegetables. The PCR amplified product of a *FUM1* primer confirmed the presence of fumonisin-encoding genes in these isolates, thus demonstrating their potential to produce fumonisins.

Statistical processing of results indicated *Fusarium* was isolated in significantly higher numbers from the air, and in notably higher numbers from soil, of localities near maize than away from maize. The same species were predominantly recovered from air, and isolated in higher numbers from both lerotho and thepe. Species isolated in highest numbers from lerotho included *F. oxysporum, F. proliferatum, F. solani* and *F. verticillioides.* However, the number of *Fusarium* isolates recovered from thepe was significantly lower than from lerotho. These results suggest that maize play an important role in maintaining fumonigenic *Fusarium* in the environment of peri-urban food gardens. Leaf surface characteristics of lerotho probably was a factor in trapping air-borne *Fusarium* spores more effectively compared to thepe.

Apart from toxigenic risks to consumers, the eleven *Fusarium* species represented by isolates in the present study, have all been reported as etiological agents in cases of human fusariosis. In most cases, opportunistic fusarial infections in immuno-compromised patients were fatal. The presence in a peri-urban environment of *Fusarium* human pathogens, particularly in the air, enhances the risk of life-threatening opportunistic infections in HIV-positive individuals. Inhalation and skin contact are reportedly the two most common ways by which *Fusarium* pathogens gain entry to human hosts. High levels of *Fusarium* in the air thus increases the possibility of infection, particularly in an environment where humans have a larger population density.

The present study demonstrated the presence of eleven mycotoxigenic and mycotic *Fusarium* species in household food gardens and indicated a major role for home-grown maize in maintaining these harmful fungi in the peri-urban environment.

OPSOMMING

Die huidige studie is in Phokeng uitgevoer, 'n naby-stedelike woonbuurt buite Rustenburg in die Noordwes Provinsie. Om hul beperkte dieet aan te vul, bedryf huishoudings van lae sosio-ekonomiese status dikwels voedseltuine waar tradisionele Afrika groen blaargroentes (*morogo*) saam met mielies as die tradisionele stapelvoedsel groei. Sommige *morogo* plante word uit die veld versamel waar hulle spontaan as natuurlike flora voorkom. Vraelyste is gebruik om inligting te bekom met betrekking tot die samestelling en sosio-ekonomiese status van huishoudings, die gebruik van *morogo* groentes en metodes om dit te preserveer. Herbarium monsters is van 10 *morogo* plante voorberei vir die identifisering van botaniese spesies.

Die studie het ondersoek ingestel na die voorkoms van *Fusarium* spesies in die omgewing van naby-stedelike voedseltuine. Omgewingskomponente wat vir mikologiese ontleding gemonster is, sluit in mielies, grond, lug en twee van die mees algemeen verbruikte *morogo* groentes, naamlik (*Amaranthus* sp.) tepe en (*Cleome gynandra*) leroto. Drie huistuine waar mielies en *morogo* groentes saam in 'n gemengde sisteem gegroei het, is vir monsterneming geselekteer. Monsters is ook in 'n voedseltuin geneem van 'n familie wat op die naby-geleë Zuurplaat plaas woonagtig was asook van vier lokaliteite weg vanwaar mielies gegroei het. Die teenwoordigheid van elf *Fusarium* spesies was van belang in hierdie studie, nie alleen vanweë die mikotoksikologiese risiko's vir die verbuiker nie, maar ook vir hul potensiaal om opportunistiese infeksies in immuun-onderdrukte of immuno-gekompromiteerde individue te veroorsaak.

Standaard metodes is gebruik vir die isolasie en morfologies-gebaseerde identifikasie van *Fusarium*. Die volgende *Fusarium* spesies is uit die verskillende omgewingskomponente geïsoleer: *F. avenaceum, F. chlamydosporum, F. dimerum, F. equiseti, F. oxysporum, F. poae, F. proliferatum, F. semitectum, F. solani, F. subglutinans* en *F. verticillioides*. Resultate dui aan dat fumonisin-produserende *F. proliferatum* en *F. verticillioides,* asook *F. solani,* oorwegend vanaf lokaliteite naby, sowel as ver van mielies af, geïsoleer is. Hoewel *F. subglutinans* en *F. oxysporum* nie beskou word as groot produseerders van fumonisiene in mielies nie, is hierdie twee spesies ook in hoër getalle geïsoleer van plekke waar mielies gegroei het. HPLC ontleding het fumonisien B_1 in sommige van die huishoudelike *morogo* monsters opgespoor.

Molekulêre tegnieke is aangewend om die korrekte spesie identifikasie van 'n geselekteerde aantal isolate aan te dui. Peilstukke vir die PKR-amplifisering van ß-tubulin and translasie verlengingsfaktor 1-α (TEF1-α) geen-segmente, en die daaropvolgende volgorde-bepaling van die geamplifiseerde DNS, het die korrekte identifikasie van *F. proliferatum, F. subglutinans, F. solani* en *F. oxysporum* uit *morogo* bevestig. Die geamplifiseerde PCR produk van *FUM1* peilstuk het die teenwoordigheid van die fumonisien-koderende gene in hierdie isolate bevestig en sodoende hul potensiaal of fumonisiene te produseer, gedemonstreer.

Die statistiese verwerking van die resultate het aangetoon dat *Fusarium* in betekenisvolle hoër getalle uit die lug, en merklik hoër getalle uit die grond, vanaf lokaliteite naby mielies geïsoleer is. Spesies wat oorheersend uit die lug verkry is, was dáárdie wat ook in hoër getalle van leroto geïsoleer is, vergelykend met tepe. Spesies wat in die grootste getalle van leroto geïsoleer is sluit in *F. oxysporum, F. proliferatum, F. solani* en *F. verticillioides.* Die getal isolate van tepe was betekenisvol kleiner as van leroto. Uit hierdie resultate wil dit voorkom of mielies 'n belangrike rol daarin speel om fumonigeniese *Fusarium* in die omgewing van naby-stedelike voedseltuine te onderhou. Blaaroppervlak eienskappe van leroto was waarskynlik 'n faktor in die meer effektiewe vasvang van lugggedraagde *Fusarium* spore vergelykend met tepe.

Afgesien van die toksigeniese risiko vir verbruikers, is al elf die *Fusarium* spesies wat deur die isolate in die huidige studie verteenwoordig word, as etiologiese agente in gevalle van menslike fusariose gerapporteer. In meeste gevalle was opportunistiese fusariële infeksies in immun-gekompromiteerde pasiënte fataal. Die teenwoordigheid in die naby-stedelike omgewing van *Fusarium* patogenies vir mense, verhoog die risiko van lewensgevaarlike opportunistiese infeksies in MIV-positiewe individue. Inaseming en velkontak word gerapporteer as die mees algemene wyses vir *Fusarium* patogene om toegang tot menslike gashere te verkry. Hoë vlakke van *Fusarium* spore in die lug verhoog dus die moontlikheid van infeksie, veral in omgewings waar mense 'n groter populasie digtheid het.

Die huidige studie het die teenwoordigheid van elf mikotoksigene en mikotiese *Fusarium* spesies in huishoudelike voedseltuine gedemonstreer en aangetoon dat tuisverboude mielies 'n groot rol daarin speel om hierdie gevaarlike swamme in die naby-stedelike omgewing te onderhou.

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Chapter 1

INTRODUCTION

Members of the fungal genus *Fusarium* are found widely distributed in soil and organic substrates (Alexopoulos *et al.,* 1996). *Fusarium* species have been isolated from adverse environments such as, permafrost in the arctic and sand of the Sahara dessert. They abound in cultivated soil both in temperate and tropical regions (Booth, 1971). Majority of the species is fast growing on culture media, but there is a number of important pathogens, which are relatively slow growing and can be difficult to isolate (Nelson *et al.*, 1983).

Species of the *Liseola* and *Elegans* sections of the genus *Fusarium* have a worldwide distribution and cause diseases in a wide variety of agricultural and horticultural plants (Leslie., 1995; 1999). *Fusarium oxysporum and Fusarium proliferatum* (Matsushima) Nirenberg are the most severe pathogens of asparagus causing *Fusarium* crown and root rot (Elmer *et al.*, 1995). These pathogens can be transmitted by the plant's seed and colonize both vascular and epidermal tissues at the same time (LaMondia & Elmer, 1989). The major species of economic importance is *Fusarium verticillioides* which grows as a corn as an endophyte in both vegetative and reproductive tissues, often without causing disease symptoms in the plant. However, when weather conditions are conducive, insect damage has occurred in the plant host and the fungal pathogen is in contact with an appropriate plant genotype, disease will develop (Nelson et *al.*, 1993).

Maize ecosystems, however, are natural environments for a number of toxigenic *Fusarium* species, notably those producing fumonisin toxins (Munkvold and Desjardins, 1997). *F. verticillioides, F. proliferatum* and *F. subglutinans* are maize plant pathogens causing seedling disease, root and crown rot, stalk rot and ear rot (Cotten and Munkvold, 1998). According to Cardwell *et al.* (2000), *Fusarium* establishes infection when spores in the environment land on the silk, germinate and enter the ear after pollination. Nesci *et al.* (2006) attributed the occurrence of *F. verticillioides* and *F. proliferatum* in soil of pre-harvest maize ecosystems to the survival of these species in soil maize debris from the previous year. Under humid field conditions fusarial spores in crop debris germinate and continue to produce macroconidia (Ross *et al.,* 1990). These

spores become airborne by splash dispersal during rain showers or irrigation and are disseminated in the environment over substantial distances by air currents (Hörberg, 2002; Edwards, 2004).

Fusarium mycotoxins produced as secondary metabolites by several species, commonly contaminate corn, wheat and other cereal grains intended for human and animal consumption, is a worldwide problem (Bullerman, 1996; Corrier, 1991; Bennet & Klich, 2003; Rheeder *et al.,* 2002). *Fusarium* toxins have been shown to exhibit nephrotoxic, immunosuppressive, tetratogenic and carcinogenic properties in animals (Corrier, 1991;Bennet & Klich, 2003; Berek *et al.,* 2001). Fumonisins are a family of food-borne carcinogenic mycotoxins first isolated in 1988 from cultures of *Fusarium verticillioides* (Sacc.) Nirenberg (Gelderblom *et al.*, 1988). Fumonisin B_1 was subsequently shown to cause equine leukoencephalomalacia (Bezuidenhout *et al.,* 1988; Marasas *et al.*, 1988). Considered the most prolific fumonisin producers, *F. verticilliodes* and *F. proliferatum* have a wide geographical distribution and occur frequently in association with maize (Ross *et al.*, 1990; Ross *et al.*, 1992). Dietary fumonisin B_1 has been linked with the prevalence of esophageal cancer (Peraica *et al.*, 1999) and is also implicated as a risk factor in the occurrence of neural tube defects (NTDs) in humans (Voss *et al.* 2003). The W.H.O. has since classified fumonisin B_1 as a probable human carcinogen (WHO-IARC, 1993).

Maize, a staple food for rural populations and resource-limited urban families, is supplemented with leafy vegetables (*morogo*). Edible wild plant resources have a significant role in nutrition, food security and income generation (Johnson and Johnson, 1976). The Setswana term '*morogo*' refers to a collection of traditional African dark green leafy vegetables, some of which are collected from the wild while others either grow spontaneously in disturbed soil or are cultivated (Jansen van Rensburg *et al.* 2007). However, *morogo* plants are often found in maize lands where they would be at risk of *Fusarium* and fumonisin contamination. Furthermore, airborne spores of a number of *Fusarium* species also pose a risk of life-threatening opportunistic infections in immune-suppressed and immuno-compromised individuals (Dignani and Anaissie, 2004). In the North-West Province of South Africa, an estimated 10.9 % of the population is HIV-positive. Van der Walt *et al.* (2005) proposed dietary fumonisin exposure could add an additional burden on the compromised immune system of HIV/AIDS individuals.

To evaluate mycotoxigenic and mycotic risks associated with peri-urban food-gardens, the present study was conducted in Phokeng, a residential area near Rustenburg in the North-West

Province of South Africa. Resource-limited households in Phokeng rely on home-grown food to alleviate food shortages. *Morogo* often co-exist with maize in household food-gardens or families collect them from the field. One of the four sampling localities was situated on a nearby farm. Maize, two types of the most commonly consumed m*orogo* (thepe and lerotho), soil and air were sampled at four sampling localities at sites near, as well as away from maize. Standard techniques were applied for the isolation and morphological identification of *Fusarium* retrieved from food and environmental samples. Molecular techniques were applied to verify morphological-based species identification of isolates and evaluate the risk for fumonisin biosynthesis by potential fumonisin-producing species.

Results indicated maize as a likely source of mycotoxigenic and mycotic *Fusarium* species in peri-urban home gardens. In addition to the risk of dietary fumonisin exposure, the presence of these species enhances the risk of secondary infections in HIV-positive individuals living in the environment.

Results from the present study were presented in the following poster presentation:

Title: Mycotic and mycotoxigenic fusaria in the agro-environment of traditional *morogo* in the Rustenburg district

Authors: S. Alli, M. Ibrahim, C.C. Bezuidenhout and A.M. van der Walt

Conference: 45th Congress of the South African Society for Plant Pathology, 22-24 January 2007, Benoni, South Africa.

Chapter 2

LITERATURE REVIEW

2.1 Introduction

Fungi are eukaryotic, spore-producing, achlorophyllous organisms capable of asexual as well as sexual reproduction. Through their activities, fungi probably have been known to man for thousands of years but, according to Alexopoulos *et al.* (1996), systematic study on this diverse group of organisms only developed over the last 250 years. Presently, it is estimated that about 1.5 million fungal species might exist, though only about 70,000 of these are named (Hawksworth, 2004). Fungi constitute a group of heterotrophic organisms living either saprophytic or parasitic (Moore-Landecker, 1982) and their most important ecological role probably relates to their ability to degrade complex organic materials through the actions of unique extracellular enzymes (Alexopoulous *et al.,* 1996). Figure 2.1 is a schematic representation illustrating positive and negative associations of fungi with humans and animals, indicating detrimental effects as well as useful applications. Though their usefulness seems to outweigh the negative effects, the last have important economic and health consequences. Fungi of importance in the context of this study are members of the genus *Fusarium* that falls under the Class *Deuteromycetes*, which includes the asexual ascomycetes and other asexual fungi (Alexopoulous *et al.,* 1996).

2.2 The genus *Fusarium*

The genus *Fusarium* includes harmless saprophyte species as well as strains pathogenic to plants, animals and humans. Worldwide a considerable degree of research in plant pathology, agriculture, veterinary and medical sciences has focused on ecological and systematical aspects of *Fusarium* and their plant, animal and human associations (Alexopoulos *et al.,* 1996). The genus *Fusarium* includes a number of mycotoxigenic species of economic as well as human health significance (Leslie & Summerell, 2006). Fumonisins are a family of mycotoxins and potent inhibitors of sphingolipid biosynthesis produced by *Fusarium* species. A number of fumonigenic strains have furthermore been identified as etiological agents in various human infectious diseases, particularly in immune incompetent individuals (Dignani & Anaissie, 2004)

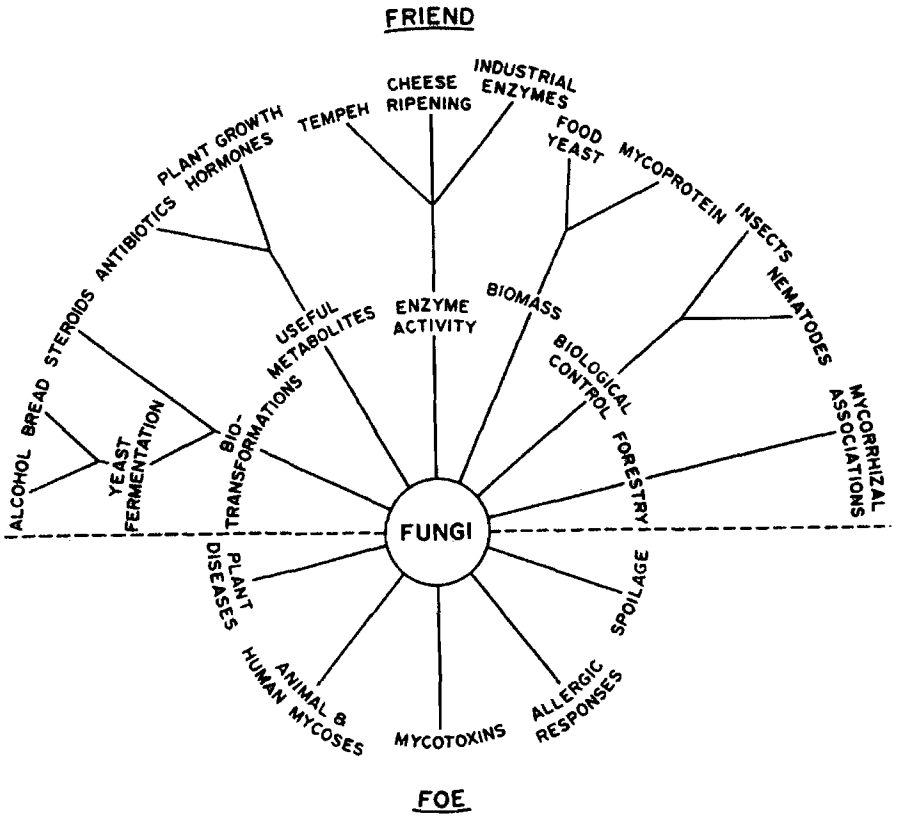


Fig 2.1 - Schematic representation of the various roles and applications of fungi in modern human society (Alexopoulos *et al.,* 1996).

2.2.1 Historical background

Incorporating important toxigenic species, the genus *Fusarium* has had a confusing and unstable taxonomic history (Geiser *et al.,* 2004). This complicated genus was erected by Link (1809) based on *Fusarium roseum,* a species with fusiform, nonsepate spores produced on a stroma. Link's (1809) early research of the *Fusarium* genus was based on diagnosis, identification and enumeration of taxa causing plant diseases. On this basis over a 1000 *Fusarium* species described were associated with various disease outbreaks (Leslie & Summerell, 2006). In 1935 Seifert determined that the production of distinctive macroconidia is not necessarily the key characteristic for *Fusarium* identification, as some coelomycetes are also known to produce spores that could cause them to be erroneously identified as *Fusarium* (Leslie & Summerell, 2006). In the 1940's and 1950's Snyder and Hansen (1940) determined that single spore cultures should solely be used for *Fusarium* identification and based on this approach the number of

Fusarium species were reduced to nine. The nine species that were described by Snyder and Hansen were the following: *F. epishaeria, F. lateritium, F. moniliforme, F. nivale, F. oxysporum, F. rigidiuscul, F. roseum, F. solani* and *F. tricinctum* (Nelson *et al.*, 1983). The presence of fusoid macroconidia with a foot cell bearing some kind of heel was later accepted as a more definite characteristic to distinguish *Fusarium* species (Booth, 1971). Poorly designed taxonomic systems to reflect *Fusarium* species diversity caused many fungal isolates studied in the first three quarters of the 20th century to be incorrectly included in the genus *Fusarium* (Geiser *et al.*, 2004).

2.2.2 Occurrence of *Fusarium* species

Fusarium is found widely distributed on plants, in soil and organic substrates and has been isolated from extreme environments such as permafrost in the arctic and sand of the Sahara Dessert. The genus *Fusarium* is particularly common in sub-tropical and tropical regions, but has also been isolated from overwintered cereals from the former USSR, grains in Norway as well as cereal grains from temperate regions in Europe (Marasas *et al.*, 1984). *Fusarium* strains occur ubiquitously in cultivated soil of agricultural environments and as saprophytes in plant debris (Booth, 1971).

Agricultural ecosystems are most suitable habitats for plant pathogens because it is characterized by plant communities and populations that are genetically and spatially relatively uniform, while regular human activities contribute to effective dissemination of spores (Burdon *et al.*, 1989; Zeller *et al.*, 2004). Various plant pathogenic and / or mycotoxin-producing *Fusarium* species have been isolated in many countries from a range of agricultural and horticultural plants including millet, sorghum and maize (Marasas *et al.*, 1987; Chu & Li, 1994; Fandohan *et al.*, 2003; Shephard *et al.*, 2005), soybean (Xing & Westphal, 2006), cowpea (Kritzinger *et al.*, 2003), rice (Park *et al.*, 2005), oilseeds (Gamanya & Sibanda, 2001), commercialised spices (Zinedine *et al.*, 2005) as well as traditional African vegetables (Van der Walt *et al.*, 2006). Leslie *et al.* (2004), also reported on the occurrence of various toxigenic *Fusarium* species from non-agricultural plants such as native grassland in the vicinity of agricultural lands.

2.2.3 Plant diseases caused by *Fusarium* species

Fusarium species within the sections *Liseola* and *Elegans* have been reported worldwide as being responsible for a wide range of disease conditions in both agricultural and horticultural plants (Leslie *et al.,* 1995; 1999; Jurado *et al.,* 2006). *Fusarium* strains have been implicated in scab or *Fusarium* head blight of cereal grains including maize, wheat, barley and rice (McMullen *et al.,* 1997), wilt of bananas (Ploetz, 1990), root necrosis and sudden death syndrome (SDS) in cowpea (Xing & Westphal, 2006).

2.2.4 Mycotoxins

Mycotoxins are low-molecular weight natural products of secondary metabolism of filamentous fungi. Although toxigenically and chemically heterogeneous, they are grouped together on the basis of diseases caused in plants, humans and other vertebrates (Bennett & Klich, 2003). Mycotoxigenic fungi are well represented in the genera *Acremonium, Alternaria, Aspergillus, Claviceps, Fusarium, Penicillium, Phomopsis* and *Pithomyces,* (Steyn, 1995; Bennett & Klich, 2003). Depending on the duration of exposure, dietary mycotoxins have been demonstrated to have significant and detrimental health effects in humans and animals (Corrier, 1991; Bullerman, 1996; Bennet & Klich, 2003).

Pathologies caused by dietary mycotoxins are collectively referred to as mycotoxicosis (Bennett & Klich, 2003). *Fusarium* mycotoxins commonly linked to human health effects include, amongst many others, deoxynivalenol (DON), fumonisins, giberrellic acid, nivalenol (NIV), ochratoxin T-2 toxin, trichothecenes and zearalenone (ZEN) (Marasas *et al.*, 1986; Cerdà-Olmedo *et al.,* 1994; Bennett & Klich, 2003; Leslie *et al.,* 2004; Serra *et al.,* 2005). Organ toxicity, carcinogenesis, teratogenesis and immune toxicity and / or immune suppression caused by mycotoxins have been demonstrated in laboratory and animal studies (Marasas, 1995; Berek *et al.*, 2001; Bennet & Klich, 2003).

In humans acute forms of mycotoxicosis might produce symptoms such as acute gastrointestinal illness accompanied by nausea, vomiting and diarrhoea or headache. In pigs DON was shown to have growth inhibitory effects and T-2 toxin induced infertility or precipitated abortion. In cows dietary exposure to ZEN caused reduced milk production, infertility and hyperoesrogenism

(Placinta *et al.,* 1999). Toxins produced by *Fusarium* species and their human health effects are well documented in scientific reports.

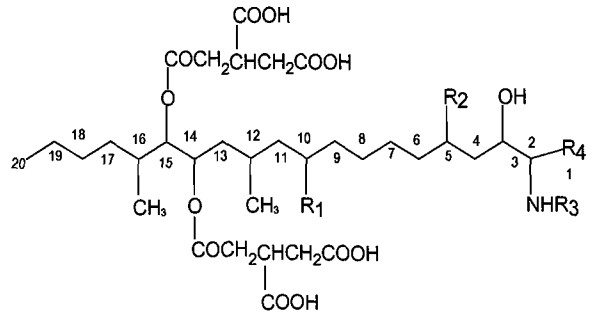
2.2.4.1 Mycotoxigenic *Fusarium* species

Mycotoxins produced by various plant pathogenic *Fusarium* species include beauvericin, fumonisins, fusaproliferin, fusaric acid, gibberelic acid and zearalenone all of which were found to contaminate animal and human feed and foods (Marasas *et al.*, 1986; Ritieni *et al.,* 1995; Bacon *et al.,* 1996; Moretti *et al.,* 1996; Rheeder *et al.,* 2002). Rheeder *et al.* (2002), reported eight *Fusarium* species in the *Liseola* section capable of producing fumonisins. Members of the *Fusarium* genus have been reported to infect a range of crop plants including barley, maize and wheat (Jurado *et al.,* 2005). Fumonisin mycotoxins are mainly produced by the largely maize-associated *F. proliferatum* and *F. verticillioides* (Rheeder *et al.*, 2002; Leslie *et al.*, 2004; Shephard *et al.,* 2005). Fumonisin B_1 (FB_1) has been classified by the International Agency for Research in Cancer (IARC; 1993) as a group 2B carcinogen, that is a probable human carcinogen. *F. verticillioides, F. proliferatum* (Section *Liseola*) and *F. nygami* (Section *Dlamini*) are the most proliferative producers of FB_1, though *F. subglutinans, F. anthophilum, F. globsum* (Section *Liseola*), *F. napiform* (Section *Dlamini*), *F. oxysporum* var. *redolens* (section *Elegans*) and *F. poliphialidicum* (Section *Anthrosporiella*) produce FB_1 in smaller amounts (Rheeder *et al.,* 2002). *F. oxysporum* (Leslie & Summerell, 2006) *F. graminearum, F. sporotrichioides, F. poae* and *F. culmorum,* are reported as producers of trichotecenes (Placinta *et al.,* 1999). *F. equisiti* produces zearalenone (ZEN), fusarochromanones, (D'Mello *et al.,* 1997) beauvericin and trichothecenes such as T-2 toxin, nivalenol (Leslie & Summerell, 2006). On the other hand some strains of *F. chlamydosporum* produce moniliformin and various secondary metabolites. *F. avenaceum, F. oxysporum* and *F. proliferatum* are known to produce beauvericin, fusarin C and moniliformin and *F. solani* and *F. subglutinans* fusaric acid, moniliformin and various other toxins (Leslie & Summerell, 2006).

2.2.4.2 Fumonisin mycotoxins

Fumonisins, a family of food-borne mycotoxins, were first isolated in 1988 from cultures of *F. verticillioides* (Sacc.) Nirenberg, then known as *F. moniliforme* (Gelderblom *et al.,* 1988). Fumonisins are important because they are potent inhibitors of sphingolipid biosynthesis (Wang

et al., 1991). Fumonisin biosynthesis is suspected to occur through condensation of amino acid to a acetate-derived precursor (Branham & Plattner, 1993, Blackwell *et al.,* 1994). Chemical structures of different classes of fumonisins were subsequently elucidated (Bezuidenhout *et al.,* 1988). Over the past few decades 28 fumonisin analogues have been characterized and are separated into four main groups: Fumonisin A (FA), Fumonisin B (FB), Fumonisin C (FC) and Fumonisin P (FP; Rheeder *et al.,* 2002). Fumonisins are chemically recognized by a 20-carbon backbone; TCA groups, hydroxyl groups and amino groups. The TCA chains may be removed by alkaline hydrolysis (Beier *et al.,* 1996). Figure 2.2 shows seven naturally occurring fumonisins (FA_1, FA_2, FB_1, FB_2, FB_3, FB_4, and FC_1; Branham & Plattner 1993).



	R_1	R_2	R_3	R_4
FB_1	OH	OH	H	CH_3
FB_2	H	OH	H	CH_3
FB_3	OH	H	H	CH_3
FB_4	H	H	H	CH_3
FA_1	OH	OH	CH_2OH	CH_3
FA_2	H	OH	CH_2OH	CH_3
FC_1	OH	OH	H	H

Figure 2.2 - Chemical structure of fumonisin (Branham & Plattner 1993, Beier *et al.*, 1996).

2.2.4.3 Health consequences of dietary, FB_1 exposure

Chronic dietary exposure to FB_1 is suspected as an environmental factor contributing to the occurrence of neural tube defects (NTDs; Sadler *et al.,* 2002; Bennett & Klich, 2003). Animal studies have demonstrated that dietary FB_1 exposure produce neurodegenerative effects in mice (Osuchowski *et al.*, 2005), pulmonary edema in pigs, leukoencephalomalacia in horses (Marasas *et al.,* 1988) and rabbits (Bucci *et al.*, 1996), nephrotoxicity and liver cancer in rats (Gelderblom *et al.,* 1991), immunodepressive effects in turkey poultry (Li *et al.,* 2000), vitamin A reduction in chicks (Hall *et al.,* 1995), atherosclerosis in monkeys (Fincham *et al.,* 1992; Soriano & Dragacci, 2004). Gelderblom *et al.* (1991), demonstrated cancer promoting properties of FB_1, while

epidemiological studies have linked FB_1 contamination of maize to the occurrence of oesophageal cancer in regions of Transkei (South-Africa), China and North-East Italy (FDA, 2000). In India, a foodborne disease outbreak in 27 villages was suspected of having been caused by the consumption of fumonisin-contaminated corn and sorghum (Bhat *et al.,* 1997). To protect humans and animals from health-damaging effects of FB_1, the American Food and Drug Administration (FDA) determined the maximum allowable fumonisin level in maize at between 2 and 4 ppm for humans and 5-100 ppm for animals (FDA, 2001).

According to Soriano *et al.* (2005), fumonisin B_1 structure resembles the sphingoid base backbone of sphingolipids. Sphingolipids are major components of membranes in eukaryotic cells where they have critical functions (Merrill *et al.*, 1997). FB_1 was shown to inhibit ceramide synthase and blocks the biosynthesis of complex sphingolipids causing sphinanine / sphingosine to accumulate. The accumulation of sphingoid bases is an suspected cause of the fumonisin B_1 toxicity (Merrill *et al.*, 1997). Sphinganine accumulation is closely associated with liver and kidney toxicity (Merrill *et al.,* 2001).

2.2.5 Genetic aspects of fumonisin producing *Fusarium* species.

Fumonisins are polyketide-derived molecules synthesized through a multi-step biosynthetic pathway by enzymes encoded by a coregulated cluster of genes on chromosome I (Butchko *et al.*, 2006). There are 15 *FUM* genes thought to be clustered on chromosome 1 that are involved in fumonisin biosynthesis whereas *FUM* 1 is thought to be one of them because its expression precedes the toxin accumulation and deletion of the gene disrupts fumonisin B1 production at a 99 % level. (Proctor *et al.*, 2003). The presence of *FUM* 1 is used for identification of genes directly responsible for fumonisin biosynthesis in toxigenic *Fusarium* species (Bluhm *et al.*, 2002). According to Sánchez-Rangel *et al.* (2005), the presence of the *FUM* 1 gene can be used to distinguish fumonsin - producing strains from non fumonisin- producing strains and species.

2.2.6 Fumonisin detection

Fumonisins are typically analysed by chromatographic methods such as thin layer chromatography (TLC), liquid chromatography (LC), liquid chromatography- mass spectrometry (LC–MS), gas chromatography–mass spectrometry (GC–MS), and high performance liquid chromatography (HPLC). These methods are reliable, but require expensive instrumentation,

complicated derivatization, extensive clean up and purification, and trained personnel required in operation. For an estimation of fumonisins in a large number of samples methods based on antibodies such as enzyme-linked immunosorbent assay (ELISA) are used. For this study we used ELISA to screen *morogo* samples for the presence of fumonisin contamination and fumonisin B-positive samples were subjected to HPLC analysis to determine relative levels of FB_1, FB_2 and FB_3.

2.2.7 Mycoses

In addition to diseases resulting from dietary, respiratory or dermal exposure to toxic fungal metabolites known as mycotoxicoses, some fungi may grow on animal host tissues producing diseases collectively referred to as mycoses. While primary fungal pathogens affect otherwise healthy individuals, most fungi would cause opportunistic infections by taking advantage of immune-compromised hosts (Bennett and Klich, 2003). In most case studies mycoses were caused by inhalation of spores from an environmental reservoir (Cocuroccia *et al.,* 2003). According to Dignani and Anaissie (2004), mortality rates as a result of fusarial infections among immunocompromised patients range from 50 – 80 %. *F. oxysporum* is considered a serious emerging pathogen of humans exhibiting a broad resistance to available antifungal drugs and together with *F. verticillioides*, *F. dimerum* and *F. solani* are responsible for practically all cases of invasive fusariosis in humans (Guarro and Gene, 1995; Vismer *et al.,* 2002). Invasive fusarial infections in neutropenic patients caused by *F. chlamydosporum* have also been reported (Segal *et al.,* 1998). Fusariosis also affect individuals suffering from leukaemia or allogeneic bone marrow and solid-organ transplant recipients (Lionakis *et al.,* 2003). *F. solani, F. oxysporum* and *F. verticillioides* have been implicated in cases of keratitis, endophtlalmitis (a destructive intra-ocular infection), fungaemia, skin lesions and nail infections (Lieberman *et al.,* 1979; Vismer *et al.,* 2002; Ferrer *et al.,* 2005). Anandi *et al.* (2005), reported a case of breast abscess for which *F. solani* was responsible. On the other hand *F. equiseti* is known to be allergenic (Verma and Gangal, 1994) and have been recovered as a human pathogen (Goldschmied *et al.,* 1993).

2.3 Morphological characteristics of the genus *Fusarium*

Morphological *Fusarium* species identification is complicated by similarity in colony appearance and characteristics of microscopic structures (Nelson *et al.,* 1983). Differentiation of *Fusarium*

species is typically based on macroscopic and microscopic morphological characteristics of single-spore cultures. Macroscopic features include colony growth characteristics, colour and texture of colonies on Potato Dextrose Agar (PDA), while cultures growing on PDA, Synthetic Nutrient Agar (SNA) and Carnation Leaf Agar (CLA) plates are microscopically examined to determine the presence and shape of macro- as well microconidia, phialides and/ or chlamydospores (Nelson *et al.*, 1983). Figure 2.2 schematically illustrates morphological characteristics of the microscopic structures used in *Fusarium* species differentiation.

2.3.1 *Fusarium* colony morphology

For *Fusarium* species identification, colony morphology is observed on 10-14 day old single-spore cultures respectively grown on SNA, CLA and PDA plates. *Fusarium* species vary among themselves from fast-growing to very slow-growing types producing colonies that vary from woolly to slimy yeast-like colonies on PDA at 25°C. Colony color may range from white-creamy to a dark purple shade from the upper side, and from the bottom of the agar plate from colourless to dark purple (Nelson *et al.,* 1983). Since these colony characteristics are species-specific, careful observation and notation thereof is used to differentiate between *Fusarium* species.

2.3.2. Microscopic features

Fusarium species produce three types of spores namely: macroconidia, microconidia and chlamydospores (Nelson *et al.,* 1994) which can be observed and distinguished microscopically (Nelson *et al.,* 1983). Figure 2.3 illustrates microscopic feature of fusarial conidia and spores to be discussed in subsequent paragraphs.

2.3.2.1 Macroconidia

The production of macroconidia (Figure 2.3a-l) mainly in sporodochia, from unbranched phialides (Figure 2.3, z) or branched conidiophores (Figure 2.3y) is a central feature of *Fusarium* species. Macroconidia are observed for their shape, size, septa and shape of basal and apical cells. Basal cells can either be papillate, notched, or even foot-shaped, while apical cells are hooked, nipple-like or conical (Nelson *et al.,* 1983).

2.3.2.2 Microconidia

Differing from macroconidia in size and shape, microconidia (Figure 2.3m-t) are generally smaller, contain up to three septa and vary from kidney to oval shape. (Nelson *et al.*,1983). *Fusarium* species produce microconidia on aerial mycelia and not in sporodochia. An important diagnostic feature is the presence or absence of microconidia occurring in chains on false heads borne on mono - (Figure 2.3u-v) or polyphialides (Figure 2.3w-x; Nelson *et al.*, 1983).

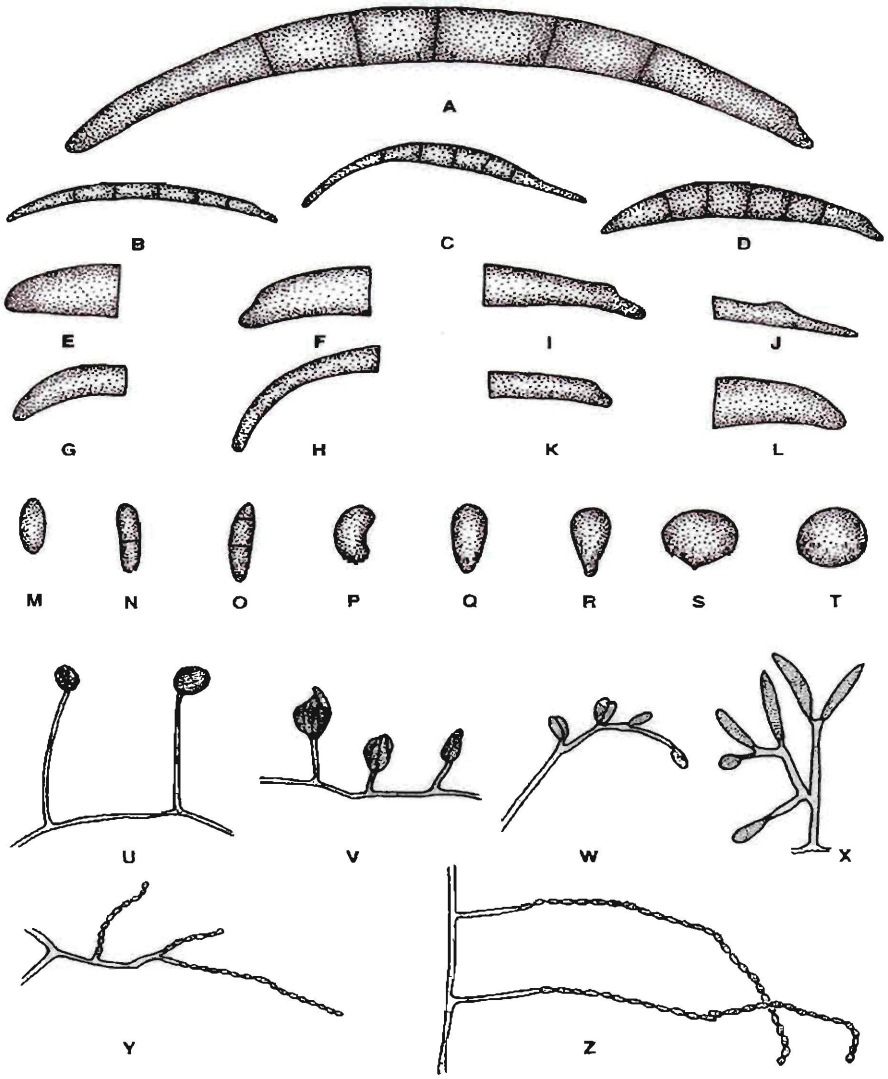


Fig 2.3 - Schematic illustration of the microscopic characteristics used to differentiate species of the genus *Fusarium*. A-L, macroconidia; M-T, microconidia; U-X, Phialides; X-Z, chains (Leslie & Summerell, 2006).

2.3.2.3 Phialides

Phialides are conidiogenous cells that produce macro - and microconidia. Two types of phialides are observed in *Fusarium* species namely monophialides and polyphialides. Polyphialides are usually found in aerial mycelium of 4-7 day old cultures. Polyphialides have no septum and form spores at more than one locus. A monophialide can either be branched or unbranched and has a septum forming cells at a single locus where spores are produced (Nelson *et al.*, 1983).

2.3.2.4 Chlamydospores

Chlamydospores are known as the survival structures of *Fusarium* species and are therefore usually thick-walled rough structures (Figure 2.4, j-l). When chlamydospores are present they could either occur as single spores (Figure 2.4, a,b,g), in pairs (Figure 2.4, f,h) or in short chains (Figure 2.4 j-l) on somatic hyphae, sometimes terminally (Nelson *et al.*, 1983). The appearance and different arrangements of chlamydospores are illustrated in Figure 2.4.

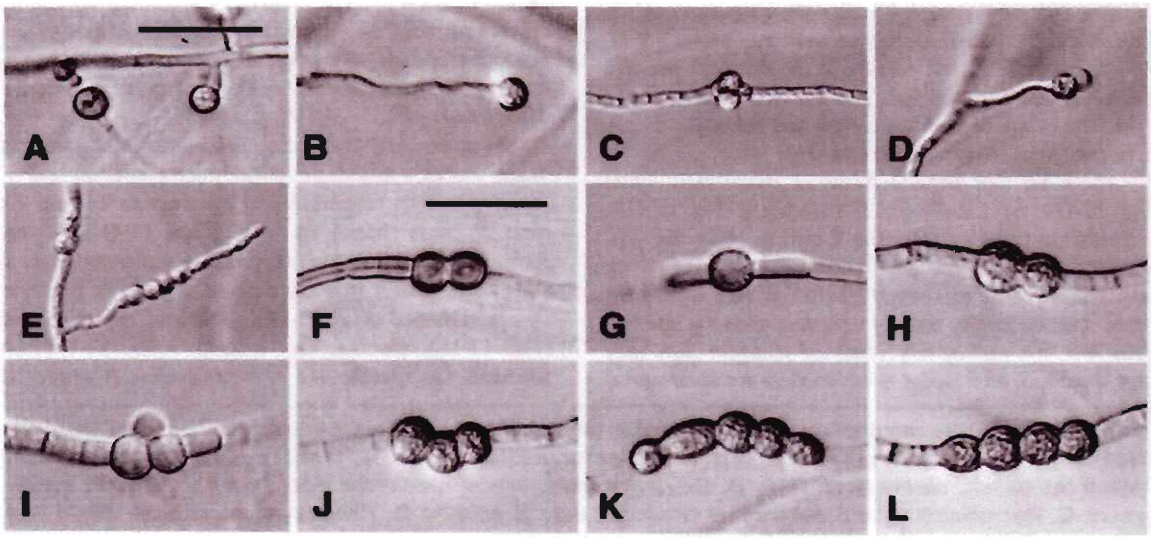


Figure 2.4 - Photographic image of chlamydospore types in *Fusarium* and their different arrangements (Leslie & Summerell, 2006).

2.4. Differentiation of *Fusarium* species relevant to the present study

A number of *Fusarium* species are recognized for their mycotoxgenic potential and/ or ability to cause human cancer or infection, particularly in immunocompromised individuals such as those with HIV / AIDS (Vismer *et al.*, 2002). Traditionally, growth characteristics and microscopic examination of morphological structures according to procedures described in a reliable laboratory manual formed the basis for identification and differentiation of *Fusarium* species. Molecular methods are now also used to confirm microscopically-based species identification (Leslie & Summerell, 2006). Important macro–and microscopic features used for species identification of *Fusarium* isolates will be discussed for some strains relevant to the present study

2.5. Molecular methods

Molecular methods in biodiversity are to determine phylogenetic relationships, taxonomic ranks, diagnostic applications, epidemiology and population genetics (Guarro *et al.*, 1999). They are also rapid, reliable and universally applicable (Guarro *et al.*, 1999). The PCR technique allows the amplification of a target sequence form any amount of DNA up to microgram quantities (Hillis *et al.*, 1996). Primers used for the analyses of taxonomic relationship between species include: β-tubulin, translation-elongation factor 1 (TEF–1α) Histone *H3* or portions of the nuclerar or mitochondrial ribosomal RNA coding region (Leslie & Summerell, 2006). *FUM1* identifies the presence of fumonisin–producing strains (Seo *et al.*, 2001). According to Bezuidenhout *et al.* (2006), 18S, TEF–1α and *FUM1* primers can be used for the amplification of targeted sequences, differentiating between potential fumonisin– and nonfumonisin-producing funsarial strains.

2.6. *Morogo*

Edible wild leafy plants or cultivated crops that are consumed as green vegetables are termed *morogo* (Van Wyk & Gericke, 2003). These leafy vegetables may be obtained in various ways: harvested from the wild or from cultivated fields (Jansen van Rensburg *et al.*, 2007). Species of the genera *Amaranthus* and *Cleome* are two of the many edible plants growing as weeds in tropical to sub-tropical areas of Africa, South America and Asia and are utilised as green leafy vegetables in Southern Africa. Some of these vegetables are lately also cultivated non -

commercially on small scale (Schippers, 2002). Studies indicated that traditional *morogo* play a significant role in nutrition, food security and income generation (Chweya & Mnzava, 1997). Different amaranth species are utilized either as grain crops or leafy vegetables (Schippers, 2002).

Amaranths are herbaceous annual growing plants. Leaves are long-stalked with whitish veins. Flowers vary greatly depending on the species. *Amaranthus hybridus* and *Amaranthus thunbergii* are two commonly occurring species widely utilised as green leafy vegetables in traditional African settings (Mposi, 1999). In addition to their value as food plants, medicinal applications of amaranths have also been reported (Van Wyk & Gericke, 2003). *Cleome gynandra* is a wild growing semi-cultivated tropical leafy vegetable in many parts of sub-Saharan Africa, especially in eastern and southern Africa (Schippers, 2002). The genus *Cleome*, commonly known as Cat's whiskers or African cabbage, is an erect herbaceous annual herb. Depending on environmental conditions it can grow up to 1.5 m tall. Throughout Africa, the tender leaves and flowers are eaten boiled and served as a pot herb, tasty relish, stew or side dish. Leaves are bitter and for this reason cooked with other vegetables such as cowpea (*Vigna* spp), amaranth (*Amaranthus* spp) or black nightshade (*Solanum nigrum L.*). *Cleome* is a rich source of nutrients, especially vitamins (A and C) and minerals (calcium and iron). It also contains some protein (Chweya & Mnzava, 1997). According to Jansen van Rensburg *et al.* (2007), there are seven important groups of leafy vegetables in South Africa which include: Amaranth, spider flower, Chinese cabbage, nightshade, Jews mallow, cowpeas and pumpkins, melons and balsam pear.

2.7. The scope of the present study

In rural areas of South Africa *morogo* vegetables are utilised as a major component of traditional African diets and represent an important source of nutrition. However, many of these plants grow as weeds in maize lands where they might be exposed to contamination with *Fusarium* spores. Members of plant pathogenic *Fusarium* species have been reported to contaminate maize, a natural host plant for fumonogenic stains such as *F. verticillioides* and *F. proliferatum* (par. 2.2.4.1). Relatively high levels of *F. verticilllioides* and *F. proliferatum* could also be present in field grass surrounding cultivated maize lands (par. 2.2.2). In addition to the risk of dietary fumonsin exposure, a number of *Fusarium* species have been implicated in invasive and opportunistic infections, particularly in immune-compromised individuals (par. 2.2.7). Statistics for 2006

showed that almost 10.9 % of S.A population was HIV infected, the larger proportion reported among the black population (Stats SA, 2006). Exposure of rural communities to either dietary fumonisin B_1 or mycotic fusaria associated with food-production environments, was a concern and, was the subject of investigation in the present study.

2.8. Research aim and objectives

The aim of this study was to determine the occurrence of potential mycotoxigenic and mycotic *Fusarium* spp in traditional *morogo* and the food-production environment in a peri-urban residential area in the Rustenburg District, North-West Province. The objectives were the following:

- The use of questionnaires to obtain information on *morogo* cultivation and utilisation from communities in the study area;
- Botanical species identification of plants utilized as *morogo* in Rustenburg;
- Isolation and morphological identification of potentially mycotoxigenic and mycotic *Fusarium* species from traditional *morogo* and other components of the environment;
- Molecular confirmation of morphologically - based identifications;
- Evaluation of the relative distribution of the respective *Fusarium* species in different components of the agro-environment namely *morogo* plants, soil, air and maize;
- Determination of the mycotoxigenic potential of isolates employing molecular techniques to verify the presence of fumonisin encoding genes;
- Screening *morogo* samples for the presence of fumonisin-B group toxins using ELISA;
- Determination of FB_1, FB_2 and FB_3 levels with HPLC.

Chapter 3

DESCRIPTION OF STUDY AREA, *MOROGO* TYPES AND INDIGENOUS KNOWLEDGE

3.1 Introduction

Unique non-documented knowledge, usually of ethnic value, handed down from generation to generation within a society or population is known as indigenous knowledge (Warren 1991). Indigenous Knowledge (IK) encompasses skills, experiences and insights of people applied to maintain or improve their livelihood and forms the information base for a society, facilitating communication and decision making (Flavier *et al.*, 1995). As their main asset to invest in health and food production, IK can be viewed as the social capital and therefore most important for the local community in which the bearers of such knowledge live. Therefore, any interaction with local communities should recognize and appreciate their IK. It is important to realise that indigenous knowledge forms part of a global knowledge system from which it can not be excluded. In this context, IK has value and relevance in itself and should be preserved, transferred, or adopted and adapted in situations where its value is applicable. Before incorporating IK in developmental approaches, it should be understood and critically validated against the usefulness for the intended strategically objectives (World Bank., 2006).

Collection, cultivation and utilization of traditional food–plants in Sub–Saharan countries are deeply imbedded in African culture and IK (Schippers, 2002; Jansen van Rensburg *et al.*, 2007). Many people consider indigenous vegetables, irrespective of whether they originated in Africa or not, as food eaten by local people (Schippers, 2002). In the context of the present study the term 'indigenous' is applied to edible plants that originated in South Africa as well as those that became "naturalised" through use, though they had originated elsewhere (Jansen van Rensburg *et al.*, 2007). *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 or small non-commercial schemes (Van der Walt *et al.*, 2006). These indigenous leafy vegetables form an important component of traditional African diets and are mainly used to supplement grain-based staples, most often maize, with plant protein, vitamins

and minerals (Jansen van Rensburg *et al.*, 2007). An information survey indicated *morogo* plant species are well known and appreciated as vegetables in the rural areas as well as townships of the Rustenburg District in the North-West Province.

3.2. Study Area

The present study was conducted in the Rustenburg area of the North-West Province, which falls within the Summer Rainfall Zone of South Africa. The area is warm to hot with variable rainfall ranging from 450 to 750 mm per year. January has the highest precipitation (an average of 134 mm) whereas July the lowest (an average of 2 mm). Temperature ranges from 16 °C to 31°C during summer months, and during winter months between 3°C and 24 °C (South African Weather Services). A number of ecological regions are distinguished in Rustenburg District, namely mountainous areas, wetlands, streams and river courses, dams, indigenous woodland and grassland floral communities (Ecological and Environmental Consultants., 2003). Rural households of the Rustenburg District who participated in the present survey were situated on the farm Zuurplaat 337 and in the rural township of Phokeng. The Global Position System (GPS) indicated the Zuurplaat 337 farm at coordinates S28°35,688' and E27°08,726' and Phokeng at S25°46,809' and E27°23,100'.

3.3 Description of the rural community

On the Zuurplaat 337 farm lives a family of three consisting of a father, mother and a baby son. As the only breadwinner, the father is employed at the farm, working hard and long hours. He often receives help from his wife with the gardening. The family is not encouraged to have a vegetable garden on the farm and, therefore, naturally growing *morogo* is an important source of nutrition. Naturally-growing *morogo* included thepe (amaranth) and lerotho (African cabbage). They did however cultivate *morogo* known as "boontjies" *morogo* (cowpea). The cowpea *morogo* is watered often once it is grown and protected from the scorching summer sun. Though relatively drought resistant, once watered *morogo* would be available for longer periods. The family maintains their small land of *morogo* with borehole water. Vegetables such as carrots, tomatoes, potatoes, onions, cabbage etc. are bought from the city or nearby shops. Meat is eaten mainly at the end of the month when money is available. Phokeng, outside of Rustenburg, consists of an average of 60 villages, with each village being home to approximately 4200

people. Categorized according to ethnicity and socio-economic status, living conditions of people in these villages varies considerably. Rich educated and high-salary earning people stay in areas characterized by lavish houses, sports cars and stunning gardens. Household members are also minimized here. In the middle-class suburb area people work hard to earn a basic salary but can maintain a relatively comfortable lifestyle. Families without a breadwinner or with one breadwinner employed as a part - time gardener or domestic worker earning a basic salary of R200-R400 monthly are residents in the poor section of Phokeng. Families consist of an average of 7 people living in a two bedroom house. In each of the sixty villages there is at least one shebeen (pub), a small shop selling vegetables, meat, milk, groceries and sweets and a small tuck shop selling ice lollies or ice or cold drink. The above mentioned information was obtained through questionnaires (Appendix A).

3.4. Food types, preparation and eating habits

According to questionnaire information food types, preparation thereof and eating habits vary from household to household, depending on ethnicity and socio-economic status of people.

3.4.1 General

Breakfast, consisting mainly of soft maize porridge and/ or bread and tea is eaten by majority of households. There is no lunch, as everyone returns home in the evenings. Supper is the main meal of the day usually, consisting either of maize with milk, or potatoes and *morogo*. Maize is the staple food. During weekends when everyone is at home fresh vegetable packs consisting of pumpkin, potatoes, tomatoes, carrots, cabbage and onions are bought from grocery stores in town or local shops nearby. Sundays after church, preparations of the meal consisting of meat with rice and vegetables begins. Rain is very important for rural families because without rain there would be neither maize nor *morogo*, which means no food. During the growing season they rely solely on rain.

3.4.2 Preparations of *morogo*

The way in which *morogo* is prepared differs and depends on the preference of the households. Usually *morogo* is cooked with oil, salt, tomato, potato and some spices. Cooking time varies from 10 minutes for sun-dried *morogo* to 20 minutes for freshly picked *morogo* leaves. To store

morogo for the cold and dry winter months, young leaves are first cooked and then rolled into small balls for either drying in direct sunlight or in the shade depending on the household. Dried *morogo* is sealed in an air tight container until needed. Prior to cooking dried *morogo* is first soaked in water. Thepe and lerotho are both prepared in the same manner, and often these types are mixed together as a vegetable relish. *Morogo* is appreciated in most households whereas in others it is eaten only when there is no other option, for it is free or at least less expensive than other vegetables. Between the two types of *morogo* investigated in the present study, it is generally agreed that lerotho is the more preferred one with the sweeter taste. Younger leaves are usually eaten as they are more tasteful, while larger leaves might be mouldy and are thrown away. There are also various fruits growing wild in the field which are consumed by both children and adults. Examples include *marula, mmupudu, mmilo* and *trokvlei.*

3.5 Plants utilised as *morogo*

Morogo is the green leafy parts of a variety of edible plants utilized as traditional vegetables in rural settings of South Africa. A number of *morogo* plants consumed by families in villages of the Rustenburg District were sampled for botanical species identification.

3.5.1 Botanical identification

Ten different plant genera were distinguished among *morogo* commonly consumed by African communities in the Rustenburg District. Species identification by the South African National Botanical Institute (SANBI) is shown in Table 3.1

3.5.2 Occurrence and uses of *morogo* types

Morogo vegetables most commonly used by both Tswanas and Shangaan speaking people in the Rustenburg District include thepe, lerotho, lefe, leswa, dinawa, mchan, delela, motsakubele and mushigi (Table 4.1).

3.5.2.1. Leswa (*Pentarrhinum insipidum* E. Mey)

Also known as leswa (Sesotho), the leaves and young fruits of this plant species are important components of the rural traditional diet in Southern Africa. The young leaves are harvested and

cooked as *morogo*. Leswa contains high levels of calcium, iron and riboflavin, whereas the young fruits have high magnesium and copper (Van Wyk & Gericke., 2003).

3.5.2.2. Thepe (*Amaranthus hybridus* L. subsp. *hybridus* var. *hybridus*)

The genus *Amaranthus* consists of more then 20 species indigenous to Southern Africa where they are widely used as traditional "spinach" (Schippers, 2002). Thepe grows naturally as an annual herb in fields and are often found as weeds growing among maize. These food-plants occur under a wide variety of environmental conditions but are known to tolerate high temperatures and grow in various soil types, although mostly sandy and loamy types. *Morogo* is available in the summer months 2 - 3 weeks after the first rains (Van Wyk & Gericke., 2003). Branches are practically picked bare within two months as the young leaves are tastier than older ones. During severe droughts and winter months *morogo* dries up. On average the protein content of different amaranth types varies between 26 and 30 percent, iron is five times the recommended daily allowance, the calcium content double the recommended daily allowance and the vitamin A no less than 20 times the recommended daily allowance (Van Wyk & Gericke., 2003).

3.5.2.3. Lerotho (*Cleome gynandra* L)

Lerotho is generally considered tastier than thepe, and grows as a weed in a wide range of soil types. It is also found among maize. Lerotho belongs to the family *Capparaceae* and is an annual erect herb plant ready for consumption within 4-6 weeks of the first rains. Although lerotho is not cultivated in South Africa, it has good potential for development as a crop. In other African countries *Cleome* spp are produced commercially (Fox & Norwood Young, 1982). Leaves, stems, young fruits and flowers of the *Cleome* plants are used for cooking (Van Wyk & Gericke., 2003; Jansen van Rensburg *et al.*, 2007). Cleome is sun-dried and preserved for winter. *Cleome gynandra* has been shown to be rich sources of magnesium and iron. Lerotho also has medicinal applications and is used for headaches, pneumonia and to restore blood supply. It is also used before and after giving birth and during breast feeding (Van Wyk & Gericke., 2003).

Table 3.1 - *Morogo* types utilized in the Rustenburg District South Africa.

Traditional name	English name [a]	Scientific name [b]
Thepe (Setswana) Nboya (Shangaan)	Common pigweed	*Amaranthus hybridus* L. subsp.*hybridus var. hybridus.*
Lerotho (Setswana)	African cabbage / Spiderplant	*Cleome gynandra* L.
Lefe (Setswana) Kaka (Shangaan)	-	*Pentarrhinum insipidum* E. Mey
Lefe (Setswana) Ngakaka (Shangaan)	Balsam pear	*Momordica balsamina* L.
Dinawa (Setswana)	Cowpea	*Vigna unguiculata*
Mchan (Shangaan)	-	*Brassica juncea* L. Czern. & *Coss*
Delela (Shangaan)	-	*Corchorus schimperi* Cufod.
Motsakubele (Shangaan)	Lambsquarter	*Chenopodium murale* L.var. *murale*
Mbagala (Shangaan)	-	*Sonchus wilmsii* R.E.Fr
Mushigi (Shangaan) Mushidzi (Tshivenda)	Black Jack	*Bidens pilosa* L.

[a] Van Wyk and Gericke, 2003.
[b] Botanical species identification according to South African national Botanical Institute (SANBI)

3.5.2.4. Lefe (*Momordica balsamina* L.)

Balsam pear, also known as mohodu (Sesotho), or nkaka (Xitsonga), is a hairy perennial herb with slender stems, lobed leaves and tendrils for climbing. Growing in Africa's dry to wet areas with a rainfall of 200-1200 mm annually this plant consists of edible flowers, fruit and seeds (Welman, 2004). The yellow flowers are followed by fruits that turn orange to red when matured. Leaves and young fruit are cooked and used as vegetables. The leaves are a source of vitamin C

and the seeds containing bright red arils taste like watermelons (Van Wyk & Gericke., 2003; Welman, 2004).

3.5.2.5. Dinawa (*Vigna unguiculata*)

Vigna unguiculata is an annual herb, containing fresh seeds which may also be boiled and eaten. However, the dry beans form a favourite foodstuff (Van Wyk & Gericke., 2003). This plant species is rich in carbohydrates, proteins, minerals (potassium, magnesium and phosphorus) and have a low fat content (Davis *et al.*, 1991, Iqbal *et al.*, 2006). Leaves of *Vigna* species are preserved by sun-drying and cooked as a relish to be eaten with maize. Varieties of Dinawa may also be gathered from the field.

3.5.2.6. Mchan (*Brassica juncea* L. Czern. & *Coss*)

This leafy vegetable also known as leaf mustard / rape, grows widely distributed over large part of Southern Africa though it is not indigenous to Africa. Leaves need to be well cooked because of the presence of glucoside sinigrin, and for this reason during the cooking process the boiled water needs to be replaced with fresh water. Sinigrin causes bitterness and the older the plant the worst the taste (Schippers, 2002).

3.5.2.7. Delela (*Corchorus schimperi* Cufod.)

Delela (Shangaan) / Leshwe, (Setswana) is one of approximately 30 *Corchorus* species and are found in dry to semi-arid areas of Africa. Known as Jute mallow, *corchorus* is an edible perennial crop growing up to 2 m high. They have yellow flowers and straight fruit terminating in a beak (Schippers, 2002). The leaves are cooked and eaten as a relish with maize. Boiled water of the delela is used to lower high blood pressure and for the relief of arthrithus. However, *Corchorus* spp. are not a preferred *morogo* type among the Tswana speaking people in Rustenburg.

3.5.2.8. Motsakubele (*Chenopodium murale* L.var. *murale*)

Motsakubele is also known as goosefoot / imbilikicane in isiXhosa and isiZulu. This plant species is a widespread weed distributed in Southern Africa and is said to be one of the most popular wild growing *morogo* plants. The young twigs are eaten either with other foods or on their own (Van Wyk & Gericke., 2000).

3.5.2.9. Mushigi (*Bidens pilosa* L.)

Also known as mokolonyane (Sesotho) and umhlabangubo (isiXhosa) and mushidzi (Tshivenda; Van Wyk & Gericke., 2003), mushigi is thought to have originated in South America and is commonly found in the tropics (Schippers, 2002). *Bidens pilosa* (also referred to as black jack), an erect annual herb with its well known black fruits clinging to clothing, is considered a troublesome weed. This weed grows very fast and can be harvested within 3 weeks after the first rains. Young leaves of black jack are mostly eaten as older leaves have an unpleasant taste. Leaves are cooked with salt, oil, tomatoes and potatoes and eaten with maize. Water used for soaking of black jack leaves is used as a medicinal drink for hangovers and stomach aliments (Personal communication).

Summary

Morogo is best described as plants utilised as leafy vegetables in traditional African diets. Since these plants are either indigenous or naturalised in the area where they grow, they are generally well adapted for growth in the local environment. As a major component of their traditional diet, African populations seem to possess knowledge of where to find wild growing *morogo*, how to prepare and preserve them. Such knowledge apparently is maintained as part of the African food culture and is passed on from generation to generation. IK also include applications of the plants for medicinal purposes. Wild-growing *morogo* is readily accessible during raining seasons, or can be easily and inexpensively cultivated by resource-limited rural and urban African families to be utilised as an important source of nutrition. It would therefore be of great value to preserve knowledge pertaining to Africa's unique food culture. Two of the most widely consumed *morogo* species, namely wild-growing lerotho and thepe, were collected for mycological investigation in the present study.

Chapter 4

MATERIALS AND METHODS

The present study was conducted in two rural villages in the Rustenburg District in South Africa. Questionnaires were used to extract indigenous knowledge (IK) pertaining to *morogo* utilisation in the study area from which household *morogo* was sampled. Plants consumed as *morogo* were sampled for botanical species identification. Samples of maize plants, *morogo* plants, soil and air were subjected to mycological analysis. The isolated fusaria were identified on basis of morphological as well as molecular characteristics. The fumonigenic potential of isolates was determined and *morogo* samples analysed for presence of fumonisin B group of toxins.

4.1 Sampling sites

The present study was carried out in household food-gardens of a peri-urban community in Phokeng outside the town of Rustenburg. A farm-worker household on the nearby Zuurplaat farm was also included in the study. Four sampling localities were identified, each including a site where *morogo* was growing near maize and a site away from maize. Three sampling localities were in Phokeng and one on the Zuurplaat farm.

4.2 *Morogo* sampling

Samples of two types of traditional *morogo* namely thepe (amaranth) and lerotho (African cabbage or spider plant) were collected from each sampling locality during the maize-growing season in February 2006, April 2006 and February 2007. Ten leaves were picked randomly from two different plants of each *morogo* type at a site where they were growing among maize as well as at a site away from maize. Leaves from different plants were placed separately into marked Ziploc bags indicating the following relevant information: locality, plant name, site number, date of collection and household number. At the time of leaf sampling, environmental samples were also taken at each sampling site. Soil was dug up with a spade at three random positions around each of the sampled plants. The soil was immediately transferred to appropriately marked Ziploc bags. Air was sampled by exposing three Pentachloronitrobenzene (PCNB) agar plates for 3 minutes at different positions around each of the sampled plants. PCNB is a selective culture

medium for *Fusarium* and contains bacteria-inhibiting substances (Appendix B). After 3 minutes of exposure air plates were closed, parafilmed immediately and marked according to the site. A maize cob with hair as well as maize leaves were picked at each site where maize was growing and sealed in marked Ziploc bags. Samples were transported to the laboratory in an icebox. Different types of *morogo* obtained from selected households were used for fumonisin analysis.

Fresh as well as traditionally dried *morogo* leaves from 3 different households were also collected for subsequent fumonisin analysis.

4.3 Botanical species identification of *morogo* plants.

Herbarium specimens of each plant were prepared according to guidelines provided by South African National Botanical Institute (SANBI). Specimens consisted of the stem, flowers, roots and leaves of the *morogo* plant. Herbarium specimens were subsequently sent to SANBI in Pretoria for botanical species identification. Results are reported in Table 3.1.

4.4 Isolation, enumeration and identification of *Fusarium*.

Morogo leaves and environmental samples (maize, soil and air) were subjected to standard mycological procedures for the isolation and identification of *Fusarium* to establish the species were occurring in association with *morogo* and its immediate environment.

4.4.1 Isolation of *Fusarium* from external leaves surfaces.

To remove *Fusarium* colonizing external surfaces of *morogo* leaves, the washing procedure described by Medina-Martinez and Martinez (2000) was followed. Each leaf was transferred aseptically from the Ziploc bag to sterile 99mL peptone water followed by shaking for 10 minutes at 200 rpm at room temperature (25 °C). A volume of 1 mL of the 99 mL peptone water containing washed-off surface colonizers, was aseptically removed and transferred to 9 mL sterile peptone water to prepare a 10^{-3} dilution of the washing. The sample was subsequently aseptically diluted up to 10^{-5}. From each dilution a volume of 0.1 mL was used to prepare 10^{-4} to 10^{-6} PCNB agar spread plates (Nelson *et al.*, 1983). Inoculated agar plates were incubated at 25 °C for a minimum of 7 to 21 days.

4.4.2 Isolation of *Fusarium* from soil samples

Upon arrival at the laboratory, three soil samples from each sampling locality were combined and mixed thoroughly to form a composite sample of soil where the *morogo* plants were growing. From the composite soil sample 1g was transferred to 9 mL sterile distilled H_2O to prepare a 10^{-1} dilution. The 10^{-1} dilution was subsequently used for preparation of a 10^{-2} to 10^{-6} dilution series of the soil sample. From each dilution a volume of 0.1 mL was transferred to and spread out on PCNB Agar plates representing10^{-3} to 10^{-7} dilutions. Inoculated agar plates were incubated at 25 °C for a minimum of 7 to 21 days (Nelson *et al.*, 1983).

4.4.3 Isolation of *Fusarium* from maize plants

Maize kernels, hair strands of the cob and maize leaves were used in the following procedures to isolate *Fusarium* colonizing internal and / or external surfaces of these structures. Five maize kernels were first surface sterilized before they were aseptically crushed and each kernel placed indivually on the surface of a PCNB Agar plate. Three hair strands were also placed separately on the surface of separate PCNB Agar plates. Another five maize kernels were washed in 99 mL peptone water according to the method described by Medina-Martinez and Martinez (2000). From the 10^{-2} dilution a dilution series of 10^{-3} to 10^{-6} was prepared. Aliquots of 0.1 mL were aseptically transferred and spread onto PCNB Agar plates 10^{-3} to 10^{-7} which were subsequently incubated at 25 °C for a minimum of 7 to 21 days (Nelson *et al.*, 1983).

4.4.4 Enumeration of *Fusarium* colonies.

After incubation, colonies with a typical appearance on PCNB Agar plates were examined under the stereo-as well as light-microscopes to distinguish *Fusarium* isolates from possible contaminants. Isolates determined as *Fusarium* based on colony characteristics were counted and subsequently transferred to culture media specified for purification and subsequent identification of *Fusarium* species (Nelson *et al.,* 1983).

4.4.5 Purification of *Fusarium* isolates.

Colonies determined as *Fusarium* based and colony morphology (Nelson *et al.*, 1983) were aseptically transferred from PCNB Agar plates to Potato Dextrose Agar (PDA, Appendix B) and

Carnation Leaf Agar (CLA, Appendix B) plates. Inoculated plates were incubated for 4-7 days at 25 °C.

4.4.6 Preparation of single spore cultures

After incubation, PDA and CLA plates were examined for colonies with typical features of *Fusarium* (Nelson *et al.*, 1983). To purify *Fusarium* and prepare a single spore culture for each colony, a small scrape of the *Fusarium* material from CLA was added to a 10 mL sterile water blank and mixed well to suspend conidia. The suspension of conidia was poured over a 2 % Water Agar (WA, Appendix B) plate to cover the entire surface and the excess was discarded. Inoculated plates were incubated in an inclined position at room temperature 25 °C for 16-24 hours. After incubation plates were examined under the stereomicroscope to distinguish *Fusarium* colonies from contaminants (Nelson *et al.*, 1983). Small squares of the agar containing a single germinating conidium were carefully cut out and used to inoculate a PDA, Synthetic Nutrient Agar (SNA) and CLA Agar plate. Agar plates were subsequently incubated at room temperature (25 °C) for 7-10 days, which were exposed to a 12:12-hour light / dark cycle.

4.5 Species identification of *Fusarium* isolates.

Species identification was performed on single spore *Fusarium* cultures of isolates using macroscopic and microscopic characteristics according to prescribed procedures (Nelson *et al.*, 1983; Leslie & Summerell, 2006). Colony and microscopic features were examined and carefully recorded for subsequent species differentiation according to descriptions and identification keys provided in Nelson *et al.* (1983) and Leslie & Summerell. (2006).

4.5.1 Macroscopic identification

Macroscopic identification involved growing *Fusarium* isolates on the three types of media PDA, SNA and CLA plates. Colonies growing on PDA were used for macroscopic observations colony morphology and colony color. CLA plates were used for development of sporodochia. Cultures grown on SNA (Appenidix B) were used to examine the type of microconidia or chlamydospores formed (Nelson *et al.*, 1983; Leslie & Summerell, 2006).

4.5.2 Microscopic identification

Using lactophenol as mountant microscopic slides were prepared and examined based on the following characteristics: macroconidia, microconidia, phialides and chlamydospores (par. 2.3.2.). Macroconidia were observed for their size, shape, number of septa and the length of the apical and basal cells. The smaller microconidia were examined to determine their size, shape, formation (e.g. chains, false heads) and position on condiogenous cells (e.g. mono-or polyphialides). The presence or absence of chlamydospores were observed as well as whether these occurred single, in pairs, chains or even clumps. Chlamydospores are either thick or thin walled (Nelson *et al.*, 1983). Observations were carefully recorded and used for identification of isolates according to identification keys of Nelson *et al.* (1983).

4.6 Molecular analysis

A few isolates representative of potential fumonisin-producing *Fusarium* species were selected for molecular analysis. Standard procedures according to Saghai-Maroof *et al.* (1984) for the extraction of DNA and PCR amplification of appropriate DNA fragments were applied to verify *Fusarium* species identification and determine the presence of fumonisin-encoding genes.

4.6.1 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 cm^2 of each culture was transferred from PDA plate to 9 ml Yeast Peptone Dextrose (YPD) broth (Appendix B). 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 it was used. 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.

4.6.2 DNA extraction procedure

DNA isolation was carried out according to a modified procedure described by Saghai-Maroof *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 (1µl v/v; Appendix 4) was added just prior to it being used. A volume of 500 µl hot (65°C) CTAB isolation buffer, 100 µl 5 % PVP solutions and 30 µl 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) 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 (Mini Eppendorf, Germany) and the aqueous phase containing the extracted DNA transferred to new sterile microfuge tubes. After measuring the volume of the aqueous phase, it was re-extracted with equal volume of TE Buffered Chloroform: Isoamyl alcohol (24:1) 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 µl of 5M NaCl 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 the 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.

4.6.3 DNA amplification with Polymerase Chain Reaction (PCR) procedure

Using the appropriate primers, the targeted DNA fragments were amplified by polymerase chain reaction (PCR) using the ICycler. The total volume of 25 µL for each reaction was made up of single strength PCR master mix containing of Supertherm Taq polymerase, bovine serum albumin (BSA; 50 NG), DNase / RNase-free distilled water, primer mix (eg: TEF 1-α) and lastly the template DNA. The PCR master mix employed was double concentrated and composed of

0.05 units.μL^{-1}Taq DNA polymerase, 4mM $MgCl_2$ and dNTP mix (dATP, dCTP, dGTP, dTTP O.4 m M). PCR conditions began with an initial denaturation step of 5 minutes at 95 °C. This step was followed by 30 cycles of denaturing (95 °C for 30 seconds), primer annealing (55 °C for 30 seconds) and primer extension (72 °C for 60 seconds). Amplification was terminated by a final extension period of 72 °C for 5 minutes. Agarose gel electrophoresis was employed to confirm DNA amplification. The primers used are indicated in Table 4.1.

Table 4.1 - Primers used for PCR detection.

Reference	Primer	Sequence	Gene Target	Product Size (bp)
1	FUM-1 F	5'GTCGAGTTGTTGACCACTGCG3'	Polyketide Synthase (PKS)	800
	FUM-1 R	5'CGTATCGTCAGCATGATAGC3'		
2	EF-1	5'ATGGGTAAGGAGGACAAGAC3'	Translation elongation factor 1-α	700
	EF-2	5'GGAAGTACCAGTGATCATGTT3'		
3	BT-1	5'AACATGCGTGAGATTGTAAGT3'	Beta-Tubulin	650
	BT-2	5'TAGTGACCCTTGGCCCAGTTG3'		

References: (1) Bluhm *et al.,* 2002; (2) Geiser *et al.,* 2004; (3) Leslie & Summerell, 2006.

4.6.4 Confirmation of DNA amplification

Electrophoresis was conducted on 5 µLof PCR product through Agarose gel (1.5 % w/v; Roche, Germany) containing 10 µL ethidium bromide (10 % w/v; BioRad, UK). Each gel was loaded with a DNA molecular weight standard (Molecular Weight Marker XIV; Roche Diagnostics, Germany) to which the intensities of the template DNA bands could be compared. Electrophoresis was performed for 105 minutes at 80 V using 1x TAE buffer.

4.6.5 Gel imaging

Gel images were captured using a Gene Genius Bio Imaging System (Sungene, Synoptics, UK) and GeneSnap (version 6.00.22) software.

4.6.6 DNA sequencing

The *Fusarium* species sequencing were outsourced to Inqaba biotech (South Africa). Blast search software (http://inqaba.ifinch.com) was to view the sequences and to perform BLAST searches in GenBank for sequence identity.

4.7 Detection and determination of fumoninsins.

ELISA technique was used to screen *morogo* samples for the presence of fumonisin B-group toxins. Fumonisin B-positive samples were subsequently subjected to HPLC analysis to determine the relative levels of FB_1, FB_2 and FB_3 in *morogo*.

4.7.1.1 ELISA screening for fumonisins in *morogo*

An ELISA kit (Corporation, Lansing, MI 48912) was employed. A volume of 100 µL of filtrate of each sample was diluted with fumonisin diluents included in the kit. An equal volume of 100 µL of fumonisin conjugate solution was added to the red mixing well. A total of five standards and the diluted samples were added to their appropriate wells. The red wells were mixed thoroughly and 100 µL was transferred to the antibody wells and incubated for 10 minutes at room temperature. The remaining substrate was discarded and rinsed with distilled water. This process was repeated 5 times. The excess water that remained in wells was dried on paper towel. The substrate volume of 100 µL was transferred to antibody wells and a reaction was achieved in 10 minutes. A volume of 100 µL sulphuric acid was added to the antibody well to stop the reaction. A micro-well reader was used to determine the results.

4.7.2 HPLC determination of FB_1, FB_2 and FB_3 in *morogo*

The HPLC method according to Shephard *et al.* (1990), was used to determine fumonisin B_1, B_2 and B_3 levels in *morogo* samples An extract of each *morogo* sample was prepared followed by a clean-up procedure prior to HPLC analysis.

4.7.2.1 Preparation of plant extracts.

A ground freeze-dried sample of each *morogo* plant (2g) and sodium chloride (0.4g) was homogenized in a 30 mL mixture of methanol / water (8:2) for 5 min. The extract was filtered through Whatman No. 4 filter paper and the filtrate collected in a clean vessel.

4.7.2.2 Cleanup procedure for fumonisins determinations.

The filtered extract (10 mL) was diluted with a 40 mL solution of phosphate-buffered saline (PBS; pH 7.0) containing 0.5% Tween 20 solution. The extract was filtered through a micro-fiber filter (Schleicher & Schuell) and the filtrate transferred into a polypropylene syringe barrel, which was attached to the FumoniTest immunoaffinity (IA) column (Vicam). The extract was passed through the IA column according to the producer's instructions at a rate of about 1-2 drops / sec until air passed through the column. Thereafter, PBS (15 mL) was passed through the column at a rate of 1-2 drops / sec. Fumonisins was eluted from the IA column under gravity by passing HPLC grade methanol (3 mL) through the column at a rate of 1 drop / sec. The eluate was collected into a glass vial to be dried under a stream of nitrogen at 60 °C and to concentrate it at the base of the vial with a capacity of 4 mL.

4.7.2.3 HPLC analysis for quantification of fumonsins B – group toxins.

To determine the respective concentrations of FB_1, FB_2 and FB_3, cleaned–up extracts of *morogo* samples, indicated as fumonisin–positive by ELISA were first subjected to derivatisation of fumonisin B.

4.7.2.3.1 Preparation of reagent, standards and extracts

Prior to HPLC analysis, reagents, fumonisin B standards and cleaned-up *morogo* extracts were prepared accordingly to prescribed procedures to quantify relative levels of FB_1, FB_2 and FB_3.

4.7.2.3.2 Preparation of derivatization reagent

Standard solutions of FB_1, FB_2 and FB_3 were prepared by dissolving $1 mg.mL^{-1}$ in acetonitrile / water (1:1). The working standard solutions were prepared from standard stock solutions by transferring 100 µL aliquots of each solution to clean glass vials and adding 200 µL acetonitirle-

H_2O (1:1) to yield working standard solution containing three fumonisin analogs. A amount of 40 mg of *o*-phthaldialdehyde (OPA) were dissolved in 1 mL methanol and diluted with 4 mL disodium tetraborate (0.1 M) before 50 µL 2-mercaptoethanol was added. The reagent solution was stored in capped amber coloured or an aluminum foil-covered vial for no longer than 1 week at room temperature in the dark.

4.7.2.3.3 Preparation of derivative standards

Fifty µL (50 µL) of a FB_1, FB_2 and FB_3 working standard solution (10-100 $\mu g.mL^{-1}$) were transferred to the base of a small vial where it was mixed with 225 µL of the OPA reagent. Within 1 min. 10 µL of the fumonisin-OPA mixture was injected into the HPLC. It is critical to adhere to reproducible time between addition of OPA reagent and injection into HPLC system, since fluorescence of OPA-fumonisin mixture begins to decrease after 2 min.

4.7.2.3.4 Derivatisation of fumonisin B in *morogo* extracts

The purified dry film residue of the sample extract was dissolved in 200 µL methanol. A volume of 50 µL of this extract was transferred to the base of a small vial before 225 µL OPA reagent was added and mixed. Ten µL (10 µL) derivative was injected to HPLC within 2 min of adding OPA reagent.

4.7.2.3.5 HPLC determination.

HPLC method described by Shephard *et al.* (1990), was used for quantification of fumonisin B-group toxins in *morogo* samples. The procedure was carried out under the following chromatographic conditions: The waters HPLC system used was equipped with model 600 delivery system (model Waters 474) fluorescence detector with Excision 338 nm and Emission 455 nm. Data were recorded by Millennium Chromatography Manager software 2010 (Waters Milford MA USA). The separation was carried out on a stainless steel 250 mm internal diameter (id.), 4 μm, Nova pack column (Waters, MA) using a mobile phase of methanol.0.1^{-1} M sodium phosphate buffer (75:25; pH 3.35) pumped at a flow rate of 1 $mL.min^{-1}$.

4.7.2.3.6 Quantitation

The mixed solution containing the standard or sample extract after it was derivatised were filtered through a 0.22 µm membrane filter before loading a volume of 10 µL into a 200 µL injection

loop. The concentration of FB_1, FB_2 and FB_3 in *morogo* was calculated from chromatographic peak areas produced by the respective *morogo* samples using the standard curve as reference.

4.8 Statistical methods

Fusarium isolates from the various sources were subjected to two-way and three-way analyses of variance (ANOVA), using Statsitica 7.1 in terms of the following: counts for each species from each environmental source in localities near and distant from maize, respectively, were log-transform (i.e. by taking the logarithm to base 10 of the count + 1) to establish normality of data to a greater extent. This was used as the dependent variable with regard to three factors, namely, species, source and distance from maize. Statistically significant effects at the 5%; level for each of theses factors could be concluded whenever there were no significant interactions and the P-values were smaller than 0.05. where appropriate, Turkey post hoc comparisons were performed between mean values of the different components and species. In cases of significant interaction effects with distance from maize, Students t-tests (assuming unequal variances) were applied to compare means of distance with means for each species or each source.

Results produced by applying these procedures are presented in Chapter 5. Results obtained from mycological analysis are recorded in Appendix C and graphically illustrated in Figure 5.1. Data were statistically analysed (ANOVA) to determine trends in the relative occurrence and distribution of *Fusarium* in *morogo* and components of the peri-urban food-garden environment (Tables 5.1-5.3; Figures 5.2 and 5.3). Results of ELISA screening and HPLC analysis of *morogo* for the presence of fumonisins are recorded in Table 5.4. Figures 5.4-5.6 show images of PCR amplified products of ß-tubulin and translation elongation factor 1-α (TEF1-α) and DNA sequence similarities of these amplified fragments with GenBank DNA sequnces are shown in Tables 5.5 -5.6.

Chapter 5

RESULTS

Mycological results are presented to indicate trends in the occurrence and distribution of toxigenic and mycotic *Fusarium* populations in the various environmental compartments, namely maize, soil, air and two types of *morogo* (lerotho and thepe). Since some of the *Fusarium* species isolated are known fumonisin producers, results of fumonisin analysis of *morogo* samples are included. Results of molecular techniques used to confirm the morphological identification of these species and their ability to produce fumonisins, are shown.

5.1 Mycological results

Figure 1 compares the combined results of the three sampling opportunities (February and April 2006 and February 2007) at the four sampling localitites (three in Phokeng and one on the Zuurplaat farm). Total numbers of each of the various *Fusarium* species isolated from the various environmental components at sites respectively close to and away from where maize was growing, are illustrated.

Logarithms of isolate numbers illustrated in Figure 5.1 indicate all nine species were isolated in higher numbers from sites close to maize (M+) than from those away from maize (M-). The highest number of isolates from M+ and M- sites respectively is shown for *F. verticillioides* (130, 34), *F. proliferatum* (107, 19), *F. solani* (89,14) and *F. oxysporum* (61, 11). *F. equiseti* was isolated in the lowest number from M+ sites (6). *F. semitectum, F. chlamydosporum* and *F. equiseti* were not isolated from M- sites. Statistical analysis of the number of *Fusarium* isolated from M+ and M- sites respectively, shows that on a 5% level, *Fusarium* was retrieved in significantly higher numbers from air (P=0.00005), soil (P=0.00005), lerotho (P=0.00005) and thepe (P=0.00005) of M+ sites that from M- sites (Table 5.1). Comparing mean values of isolate numbers from the various environmental components indicated a significantly higher number of *Fusarium* was isolated from air (0.27) compared to lerotho (0.17) and thepe (0.09). *Fusarium* was isolated in significantly lower numbers from thepe (0.09, 0.01) than from lerotho (0.17, 0.05) or soil (0.23, 0.05) of M+ and M- sites respectively.

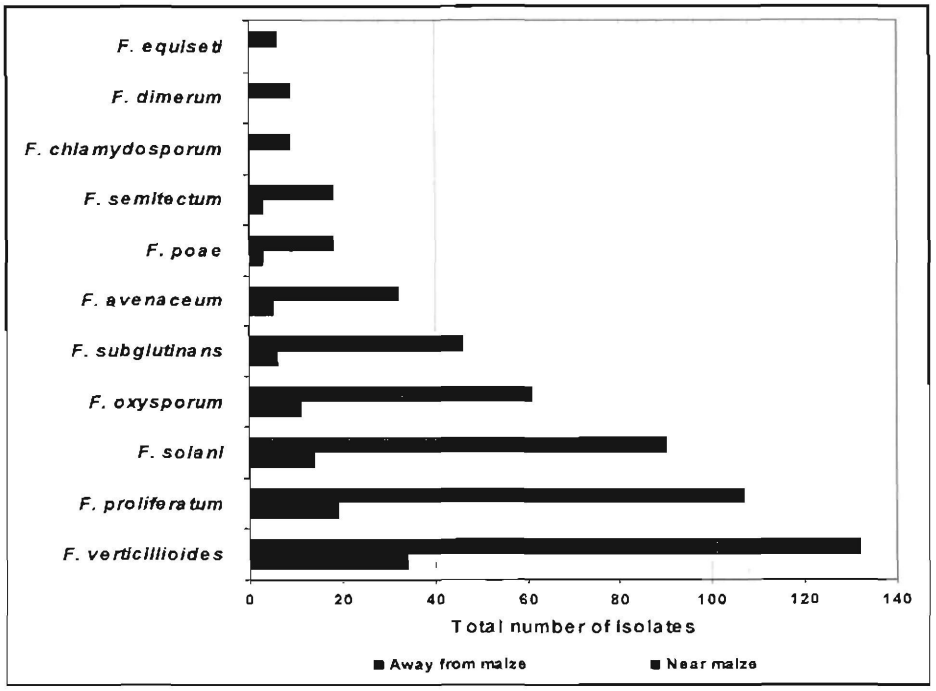


Figure 5.1 - Comparison of the total number of isolates of each of the various *Fusarium* species from localities respectively near and away from where maize was growing

Table 5.1 – Logorithms of *Fusarium* numbers at localities respectively near and away from maize

Environmental component	Near maize Mean (±SE)	Away from maize Mean (±SE)	t	df	P
Air	0.27 ± 0.03^{b}	0.06 ± 0.01^{a}	6.37	189	<0.00005
Soil	$0.23\pm0.03^{a,b}$	0.05 ± 0.01^{a}	5.85	185	<0.00005
Lerotho	0.17 ± 0.02^{a}	0.05 ± 0.01^{a}	5.45	209	<0.00005
Thepe	0.09 ± 0.01^{c}	0.01 ± 0.01^{b}	5.21	163	<0.00005

Superscripts a, b, c, d and e (column-wise): means with the same symbol differ not significantly on a 5% -level.

All nine species identified among the *Fusarium* isolates were retrieved in statistically significant higher numbers from M+ sites compared to M- sites (Table 5.2). P values ranged from <0.00005 (*F. proliferatum*) to 0.0036 (*F. equiseti*). Comparing mean values of isolate numbers, data presented in Table 5.2 show that on a 5% level *F. verticillioides* (0.36) and *F. proliferatum* (0.34) were isolated in significantly higher numbers from M+ sites compared to the other species, and *F. solani* (0.23), *F. oxysporum* (0.19) and *F. subglutinans* (0.15) in significantly higher numbers that *F. avenaceum* (0.11), *F. poae* (0.1), *F. semitectum* (0.08), *F. dimerum* (0.05), *F. equiseti* (0.03) or *F. chlamydosporum* (0.03). The three species mentioned last were not isolated from M- sites.

Table 5.2 – Logorithms of *Fusarium* numbers at localities respectively near and away from maize

	Near maize Mean (±SE)	Away from maize Mean (±SE)	t	df	P
F. chlamydosporum	0.03 ± 0.01^{a}	0^{a}	3.10	83	0.0013
F. equiseti	0.03 ± 0.01^{a}	0^{a}	2.75	83	0.0036
F. dimerum	0.05 ± 0.01^{a}	0^{a}	3.67	83	0.0002
F. semitectum	$0.08\pm0.02^{a,b}$	0.01 ± 0.01^{a}	3.10	130	0.0012
F. poae	$0.1\pm0.02^{a,b,c}$	0.02 ± 0.01^{a}	3.41	119	0.0005
F. avenaceum	$0.12\pm0.02^{a,b,c}$	0.03 ± 0.02^{a}	3.10	129	0.0012
F. subglutinans	$0.15\pm0.03^{b,c,d}$	$0.04\pm0.02^{a,b}$	3.61	120	0.0002
F. oxysporum	$0.19\pm0.03^{c,d}$	$0.06\pm0.02^{a,b}$	3.50	129	0.0003
F. solani	0.23 ± 0.03^{d}	$0.07\pm0.02^{a,b}$	4.0	130	0.00005
F. proliferatum	0.34 ± 0.04^{e}	$0.11\pm0.03^{b,c}$	5.31	130	<0.00005
F. verticillioides	0.36 ± 0.04^{e}	0.15 ± 0.04^{c}	3.99	123	0.00005

Superscripts a, b, c, d and e (column-wise): means with the same symbol differ not significantly on a 5% -level.

Figure 5.2 illustrates the relative numbers of the various *Fusarium* species isolated from air, soil, lerotho and thepe respectively at localities away from where maize was growing. *F. verticillioides* was isolated in significantly higher numbers from the air than from lerotho and thepe. *F. proliferatum* was retrieved in highest numbers from soil and in a higher number from lerotho than from air of thepe. *F. oxysporum* was not isolated from soil or thepe, but in relatively high numbers from lerotho and air, while *F. solani* was retrieved in highest numbers from soil and air. The highest number of *F. poae* and *F. semitectum* were retrieved from lerotho.

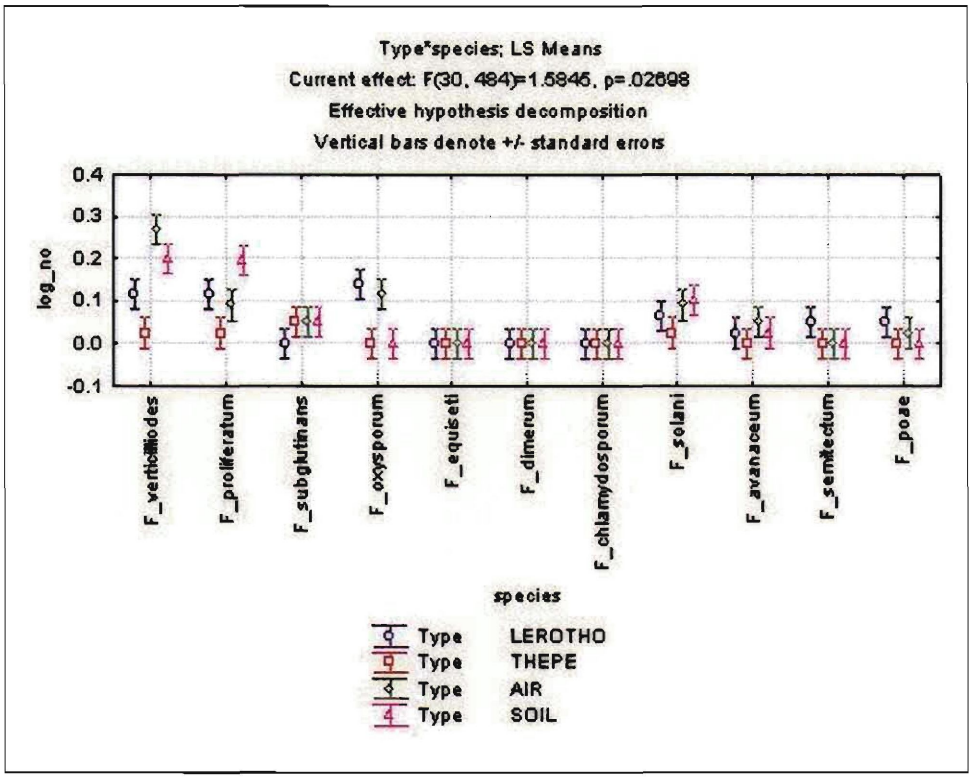


Figure 5.2 - Illustration of the relative distribution of the various *Fusarium* species in the different environmental compartments at M- sites

Mean values of isolate numbers further indicated *F. verticillioides* (0.26) and *F. proliferatum* (0.25) were isolated in significantly higher numbers from maize compared to the other species (Table 5.3). Mean log values of other species ranged from 0.01 (*F. chlamydosporum* and *F. equiseti*), 0.03 (*F. dimerum*), 0.06 (*F. avenaceum* and *F. semitectum*), 0.08 (*F. poae* and *F. subglutinans*) to 0.1 (*F. solani*) and 0.11 (*F. oxysporum* Table 5.3).

Table 5.3 - Comparison of mean log values of the various *Fusarium* species isolated from maize

F. chlamydosporum	0.01[a]
F. equiseti	0.01[a]
F. dimerum	0.03[a]
F. semitectum	0.06[a]
F. avenaceum	0.06[a]
F. poae	0.08[a]
F. subglutinans	0.08[a]
F. solani	0.1[a]
F. oxysporum	0.11[a]
F. proliferatum	0.25[b]
F. verticillioides	0.26[b]

Superscripts a and b (column-wise): means with the same symbol differ not significantly on a 5% -level.

Figure 5.3 illustrates the relative distribution of the various *Fusarium* species isolated from maize leaves, seed (kernels) and hair (silk). *F. verticillioides, F. proliferatum* and *F. oxysporum* were isolated in significantly higher numbers from maize kernels and silk than from leaves, *F. solani* and *F. poae* in significantly higher numbers from kernels and *F. semitectum* from silk compared to isolates from the leaves. *F. equiseti* and *F. avenaceum* were isolated in slightly higher numbers from kernels and *F. dimerum* and *F. semitectum* from silk.

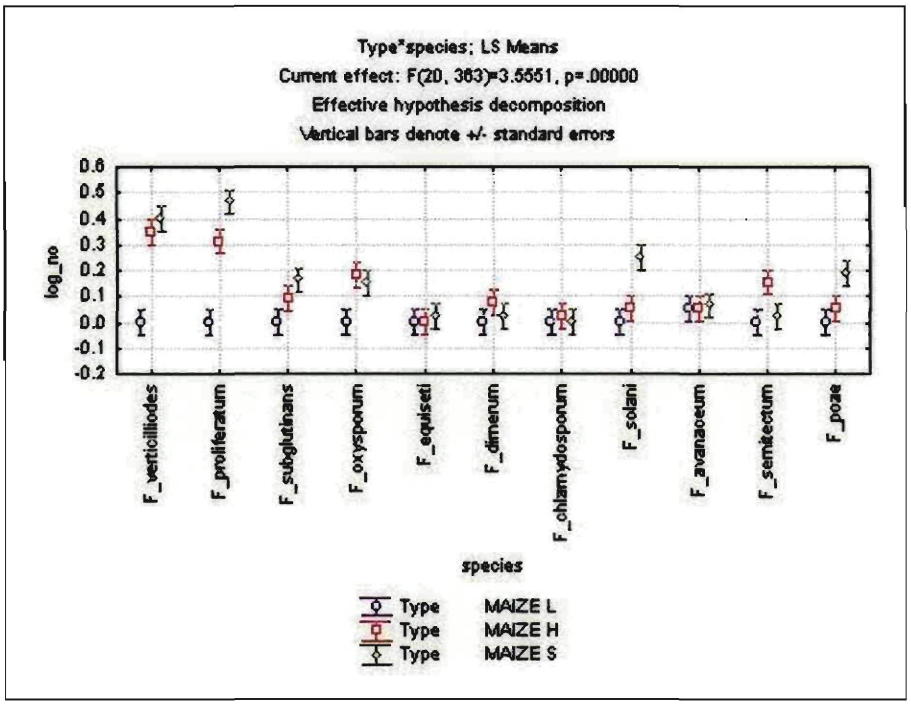


Figure 5.3 - Illustration of the relative distribution of the various *Fusarium* species in maize leaves, seed and hair

5.2 Fumonisin results

Table 5.3 show results of ELISA screening of *morogo* samples for the presence of fumonisins and the concentrations of the fumonisin B-group toxins determined by HPLC. ELISA detected total fumonisins in one of the thepe samples (34.1 $ng.g^{-1}$) and in lerotho (44.8 $ng.g^{-1}$). Fumonisin B_1 (FB_1) concentration in these samples measured 44.8 $ng.g^{-1}$ and 58.7 $ng.g^{-1}$ respectively. One of the combined samples containing all the different *morogo* types measured 114 $ng.g^{-1}$ FB_1.

Table 5.4 - Fumonisin levels in samples of household *morogo* from the Rustenburg District detected by ELISA and HPLC

Morogo type	Total fumonisins by ELISA ($ng.g^{-1}$)	Fumonisin B-group toxins by HPLC ($ng.g^{-1}$)		
		FB_1	FB_2	FB_3
*Thepe (1)	34.1	44.8	Nd	Nd
*Thepe (2)	Nd	Nd	Nd	Nd
Lerotho	44.8	58.7	Nd	Nd
Leswa	Nd	Nd	Nd	Nd
Mushigi	Nd	Nd	Nd	Nd
Pumpkin	Nd	Nd	Nd	Nd
Lefe	Nd	Nd	Nd	Nd
Dinawa	Nd	Nd	Nd	Nd
#Combined sample (1)	Nm	114	Nd	Nd
#Combined sample (2)	Nm	Nd	Nd	Nd

Nd – not detected; Nm – not measured

* Thepe 1 and 2 were sampled from different localities

Senescing *morogo* samples were combined in one sample for HPLC analysis. Samples 1 and 2 were from different localities

5.3 Molecular results

Fusarium isolated in the present study were identified based on colony morphology an microscopic structures according to Nelson *et al.* (1983). Isolates from strains suspected of being fumonisin producers, were subsequently subjected to molecular analysis to confirm their morphologically-based species identification. This was based on the DNA sequences of their translation elongation factor 1-α (TEF1-α) and ß-tubulin genes, and to verify the presence of the *FUM 1* gene required for fumonisin production. Primers and procedure for PCR amplification of these DNA segments and sequencing thereof are described in Chapter 4 (par. 4.6). DNA was

extracted from individual isolates selected to represent those identified morphologically as *F. oxysporum, F. proliferatum, F. solani* and *F. subglutinans*. A 800 bp amplicon was obtained each species after PCR amplification mixes containing *FUM 1* primers were resolved on ethidium bromide stained gels. Based on work done by Flaherty and Woloshuk (2004), such a result indicated the potential of these isolates from *morogo* to produce fumonisins. The goal is to draw a correlation the presence and levels of *F. oxysporum, F. proliferatum F. solani* and *F. subglutinans* in the *morogo* of the study area, the PCR detection of the *FUM 1* gene in these isolates and the detection of fumonisins in *morogo* samples.

5.3.1 PCR amplification products

For molecular confirmation of the morphologically-based identification of *F. oxysporum, F. proliferatum F. solani* and *F. subglutinans*, DNA was extracted from fresh, freeze-dried as well as frozen mycelial samples. The best PCR results were obtained with DNA extracted from fresh mycelia of a 5 - 7 day old culture on Potato dextrose Agar (PDA) plates. PCR-amplified products using β-tubulin, TEF1-α and *FUM1* as primers, are shown in Figures 5.11 - 5.13. Composite gels were used for the figures however negative controls are not shown.

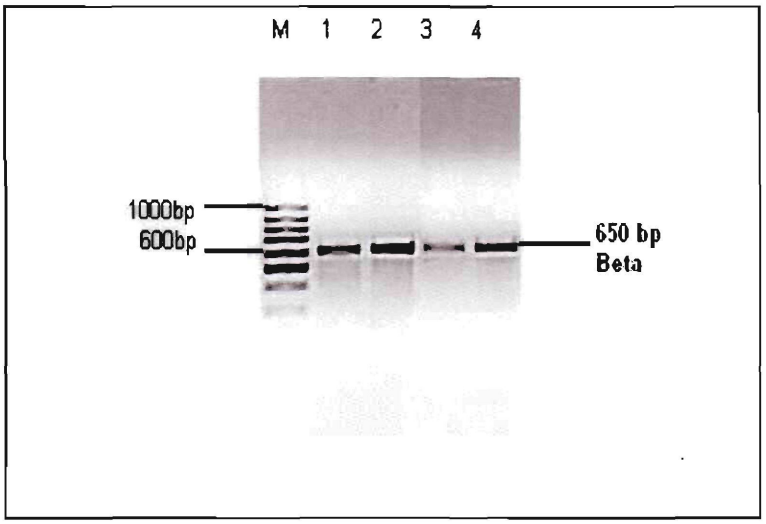


Figure 5.4 - A negative image of an ethidium bromide stained gel showing the successful PCR amplification of an isolate DNA fragment using a beta-tubulin primer. M, marker with 1000 bp; Lane 1-4, DNA of *F. oxysporum, F. proliferatum, F. solani* and *F. subglutinans*.

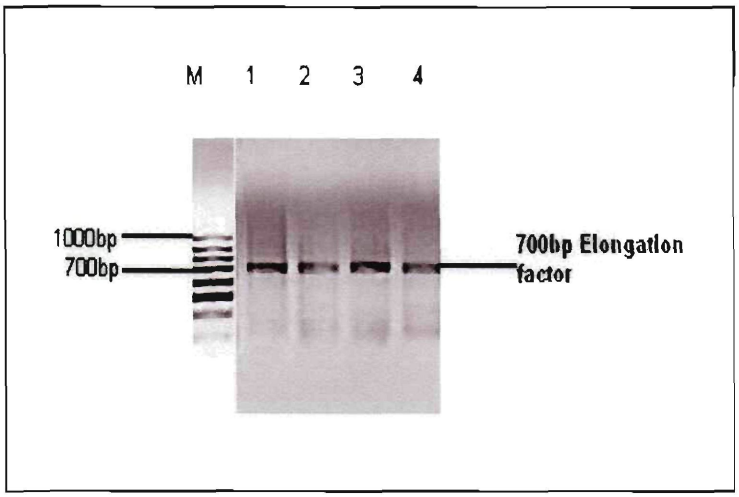


Figure 5.5 - A negative image of an ethidium bromide stained gel showing the successful PCR amplification of an isolate DNA fragment using an TEF1-α primer set. M, marker with 1000 bp; Lane 1-4, DNA of *F. oxysporum, F. proliferatum, F. solani* and *F. subglutinans.*

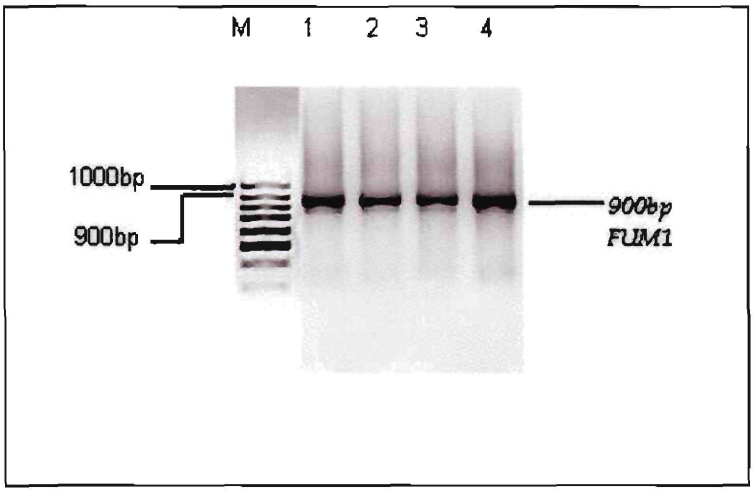


Figure 5.6 - A negative image of an ethidium bromide stained gel showing the successful PCR amplification of an isolate DNA fragment using a *FUM1* primer. M, marker with 900 bp; Lane 1-4, DNA of *F. oxysporum, F. proliferatum, F. solani* and *F. subglutinans.*

5.3.2 Sequencing and BLAST search results

Sequencing of the PCR amplification products of β-tubulin and translation elongation factor 1-primers was done by Inqaba Biotech (SA). The sequences were viewed in Geospiza Finch software (http://inqaba.ifinch.com) and analysed by BLASTN search through GenBank using the same software. BLASTN results are shown in Tables 5.5 and 5.6.

Based on β-tubulin and translation elongation factor1-α DNA sequences, the GenBank species identification of the various *Fusarium* isolates are indicated in Tables 5.5 and 5.6. Despite some noise, DNA sequence results for *F. proliferatum* (S7 BT1; Appendix D) indicated a 99% sequence similarity existed between the β-tubulin sequence of this isolate and that of *F. proliferatum* of the GenBank. When these β-tubulin fragment from the same isolate was re-amplified and sequenced again, the resultant sequence (S8 BT1; Appendix D) was without much noise and a similar identification result was obtained (Table 5.5). Moreover, 99 % similarity was found between the GenBank β-tubulin DNA sequence and that of another isolate from the present study which was morphologically identified as *F. proliferatum*. Comparison of the Genebank DNA sequences with the PCR-amplified product of the TEF 1-α supported the identification of *F. proliferatum* based on morphological characteristics and β-tubulin DNA sequence completely (Table 5.6). Morphological identification of *F. proliferatum* isolates from the present study can thus be considered accurate.

Some conflicts existed between the morphologically-based and molecular species identification of *F. solani* and *F. subglutinans* (Tables 5.5 and 5.6). DNA sequence analysis of *F. solani* from the same *morogo* sample indicated sequence similarity with Genebank strains of *F. oxysporum*. There was considerable noise in the sequence of both PCR amplified *F. solani* sequences Appendix E) and in one case only 289 of the 350 bp was used for Genebank determination of DNA sequence similarity which was only 91% (Table 5.5). The TEF α-1 sequence data could be used to identified the sample only to genus level (Table 5.6). However, when DNA extracted from a morphologically identified *F. solani* isolate retrieved from another *morogo* sample was amplified by PCR and the DNA sequenced, β-tubulin (S1 BT1) and TEF1-α (S1 EF1) DNA sequences had no background noise and showed 95% similarity with that of *F. solani* DNA sequences in GenBank. S1 BT1 and S1 EF1 DNA sequences are shown in Appendix D.

Table 5.5 - Table providing GenBank identification when ß tubulin sequences were used. The laboratory codes and morphological identified species names are given in the first column. The codes are provided in brackets and explanations of these are given below. Original sequence chromatograms in which only the codes are used could be found in Appendix D. Also indicated are whether there was noise, percentage similarity, number of sequences selected (second column) and number useful for Genbank (last column). E-values were smaller then 0.0.

Code used and Morphological identification	Nucleotides Used	Noise		Genebank ID	% Similar	Seq. ID
		No	Yes			
Fusarium proliferatum (S7 BT)	50-550		✓	*Fusarium proliferatum* strain NRRL22944	99	498 / 503
Fusarium proliferatum (S8 BT)	50-550	✓		*Fusarium proliferatum* NRRL 31071	96	433 / 449
Fusarium proliferatum (P2 BT)	1-480	✓		*Fusarium proliferatum* NRRL 31071	99	475 / 476
Fusarium solani (S BT)	50-400		✓	*Fusarium oxysporum* f. sp. *melonis*	91	263 / 289
Fusarium solani (S5 BT)	10-480		✓	*Fusarium oxysporum* f. sp. *conglutinans*	92	370 / 401
Fusarium solani (S1 BT1)	10-480	✓		*Fusarium solani*	95	417 / 436
Fusarium subglutinans (BBT)	50-580		✓	*Fusarium oxysporum* f.sp. *radicis-lycopersici*	98	523 / 531

Table 5.6 - A table providing Genbank identification when translation elongation factor 1-α sequences were used. The laboratory codes and morphological identified species names are given in the first column. The codes are provided in brackets and explanations of these are given below. Original sequence chromatograms in which only the codes are used could be found in Appendix D. Also indicated are whether there was noise, percentage similarity, number of sequences selected (second column) and number useful for Genbank (last column). E-values were smaller then 0.0.

Code used and Morphological identification	Nucleotides Used	Noise		Genebank ID	% Similar	Seq. ID
		No	Yes			
Fusarium proliferatum (SS EF1)	50-580		✓	*Fusarium proliferatum* strain NRRL22944	96	413 / 430
Fusarium proliferatum (P E1F)	10- 620	✓		*Fusarium proliferatum*	99	332 / 333
Fusarium solani (S5 EF1)	10- 630	✓		*Fusarium* sp.	99	329 / 330
Fusarium solani (S1 EF1)	10-600	✓		*Fusarium solani* strain FCC4633	95	267 / 280
Fusarium subglutinans (B EF1)	10-150	✓		*Fusarium oxysporum* f. sp. *melonis*	90	518 / 570
Fusarium oxysporum (U EF1)	10-640	✓		*Fusarium oxysporum* f. sp. *melonis*	98	599 / 611

Conflict also arisen between the morphological and molecular identification of *F. subglutinans* (Tables 5.5 and 5.6). DNA sequence similarity of β-tubulin and TEF α -1 (98% and 90%, respectively) was in both instances closest to that of *F. oxysporum* of Genebank. This conflict could be explained by morphological resemblances between *F. subglutinans* and *F. oxysporum* when grown on PDA plates (Nelson *et al.*, 1983; Leslie and Summerell, 2006). Common features of these two species include their oval to single shaped microconidia produced false heads and abundantly-produced thin-walled sickle-shaped macroconidia. It is thus possible that identification of some of the isolates, either as *F. subglutinans* or *F. oxysporum*, were not correct. DNA was subsequently extracted from another *F. oxysporum* isolate retrieved in the present study and PCR amplification of ß-tubulin and elongation factor α-1 repeated. Microscopic identification was also repeated and the presence of chlamydospores was specifically observed. In this case, the translation elongation factor 1- α DNA sequence confirmed the morphological identification. Geiser *et al.* (2004) created a database 'FUSARIUM ID version 1.0' based on partial translation elongation factor α-1 data to correctly identify fumonsin-producing *Fusarium* species and relate the species name more accurately to species toxicity and pathogenicity.

Results suggest trends with respect to the occurrence and distribution of the various *Fusarium* species isolated from a peri-urban food production environment. Environmental factors that might influence *Fusarium* population distribution patterns and possible implications for the community will be discussed.

Chapter 6

DISCUSSION

The genus *Fusarium* comprises a group of mycotoxigenic fungi many of which are pathogenic to a wide range of plants causing disease under diverse environmental conditions (Doohan *et al.*, 2003). According to Leslie and Summerell (2006), *Fusarium* species occur ubiquitous in soil, on plants and other organic substrates. In the present study *Fusarium*, isolated from various components of the environment of peri-urban food gardens in the Rustenburg District of South-Africa, represented 11 different species (Figure 5.1). Some of these species, notably *F. verticillioides, F. proliferatum* and *F. subglutinans* are associated world-wide with maize and the production of fumonisins in commercial corn (Munkvold and Desjardins, 1997; Doohan *et al.*, 2003). *Fusarium* diseases in commercial maize could cause notable losses in yields and therefore, are of major economic importance (Parry *et al.,* 1995). In addition, phytopathogenic *Fusarium* species are responsible the pre- and / or post-harvest production of potent mycotoxins in various cereal grains (Placinta *et al.,* 1999; Edwards, 2004; Soriano and Dracacci, 2004).

The present study investigated the presence of *Fusarium* in the environment of peri-urban food gardens where maize are grown, in some cases in combination with traditional vegetables. Results illustrated in Figure 5.1 indicates that *Fusarium* was isolated in higher numbers from sites close to maize (M+ sites) than from sites away from maize (M- sites). It was observed that even species not typically associated with maize, e.g. *F. solani, F. semitectum* and *F. poae,* were isolated in larger numbers from M+ sites than from M- sites. Three species, namely *F. avenaceum, F. chlamydosporum* and *F. equiseti* were isolated from M+ sites, but not from M-sites. This trend suggests that maize plays a role in the occurrence of *Fusarium* in the food-garden environment. Food production ecosystems are artificial and most suitable habitats for plant pathogens. Disease epidemics are established in host plant communities that are genetically and spatially relatively uniform while human activities advance the dissemination of spores (Burdon *et al*,1989, Zeller *et al.*, 2004).

Statistical analysis of results indicates *Fusarium* was isolated in significantly higher numbers from air, soil, lerotho as well as thepe from M+ sites compared to M- sites (Table 5.1). The highest number of isolates was obtained from air and, also in a relatively high number, from soil. Species such as *F. equiseti, F. oxysporum, F. proliferatum, F. subglutinans* and *F. verticillioides* are soil-borne fungi generally associated with maize ecosystems (Doohan *et al.*, 2003; Nesci *et al.*, 2006). Windels and Kommendahl (1974) reported *F. solani* and *F. oxysporum* as the predominant species isolated from root sysems and soils of grass and cornfields. Sanchez-Rangel *et al.* (2005) isolated of *F. avanaceum, F. oxysporum* and *F. semitectum* from maize in Mexico. According to Fandohan *et al.* (2003), *F. proliferatum* and *F. verticillioides* were most frequently isolated from commercial maize of Benin in West Africa, and Kpodo *et al.* (2000) reported the isolation of *F. chlamydosporum, F. equiseti* and *F. semitectum* from commercial maize of Ghana. In South Africa, *F. proliferatum* and *F. verticillioides* were found in home-grown maize and maize-based products of the Eastern Cape Province such as in traditionally brewed maize beer (Shephard *et al.*, 1996; Rheeder *et al.*, 2002; Shephard *et al.*, 2005).

Various environmental factors influence the survival and dispersal of fusarial spores. Climatic conditions most likely to play a role in the incidence of *Fusarium* in agro-environments include temperature, humidity, light intensity and wind (Hörberg, 2002; Doohan *et al.*, 2003). According to Doohan *et al.* (2003), the role of climate in the survival of fusaria in an ecosystem could be either direct or indirect. Cotton and Munkvold (1998) demonstrated that *Fusarium* can survive in maize stalk residue for up to 2 years. Serving as an inoculum for infection of new plants, *Fusarium* spores have been shown to be dispersed from the soil and plant debris into the air by human activity, rain and irrigation (Hörberg, 2002; Edwards, 2004; Nesci *et al.*, 2006). Air currents facilitate spore dissemination which would be enhanced by wind, while water availability and temperature influence fungal mode of reproduction and spore germination. Climate also exerts indirect effects on fungal populations by determining soil quality and, therefore, the nature and health of the vegetation (Doohan *et al.*, 2003; Jones & Harrison., 2004).

Fusarium was isolated in relatively low numbers from *morogo* vegetables (lerotho and thepe) growing close to maize (Figure 5.2). *Morogo* plants seemed healthy at the time of sampling and disease symptoms typical of fusarial infection, were not observed. *F. verticillioides* and *F. proliferatum* are known to establish symptomless associations with host plants (Munkvold and Desjardins, 1997). Furthermore, *Fusarium* species have been isolated from a wide range of grasses and broad-leaved weeds in the vicinity of commercial grain crops (Edwards, 2004). The possibility of endophytic associations between *Fusarium* and *morogo* plants was not investigated and can thus not be excluded. Another explanation for the occurrence of *Fusarium* in association with *morogo* vegetables is that vegetable leaf surfaces trap fusarial spores from the air. *Fusarium* was isolated from thepe in significantly lower numbers than from lerotho, which might reflect differences in leaf surface characteristics.

Compared to the other nine species, typically maize-associated *F. proliferatum* and *F. verticillioides* were isolated in significantly higher numbers from samples collected at sites near maize (Table 5.2). Mean values further indicated that, even from sites away from maize, these species were isolated in significantly higher numbers compared to species such as *F. semitectum, F. avanaceum* and *F. poae*. The last-mentioned two species are mainly associated with *Fusarium* head blight (FHB) in wheat, while *F. equiseti, F. proliferatum* and *F. verticillioides* are reported to cause ear rot in maize (Torres *et al.*, 2001; Brennan *et al.*, 2003; Doohan *et al.*, 2003).

Figure 5.2 illustrates the relative distribution of the various *Fusarium* species in air, soil, lerotho and thepe. Species isolated in notable numbers from soil and / or air included *F. oxysporum F. proliferatum, F. solani* and. *F. verticillioides*. Cotten and Munkvold (1997) found that the survival of *F. proliferatum*, *F. subglutinans* and *F. verticillioides,* is enhanced by maize residue lying just underneath the soil surface where microconidia survived for as long as 900 days without loss of viability. The authors suggested moisture and temperature conditions probably were more favourable under than above the soil's surface and concluded the maize residue facilitated the production of inoculum that secured the survival of *Fusarium* in the agro-environment. The formation of conidia in maize residue and debris was shown to continue for as long as moist conditions prevailed

and the temperature was favourable (Cotten and Munkvold, 1997; Doohan *et al.,* 2003; Jones & Harrison, 2004). Wind, rain, irrigation and insects contribute to the dissemination of the *Fusarium* inoculum, sometimes over long distances (Doohan *et al.,* 2003; Roháčik and Hudec, 2005). Fernando *et al.* (1997) actually demonstrated the displacement of macroconidia and ascospores downwind from where they were released. Results illustrated in Figure 5.2 further indicate that species predominantly isolated from air and soil, namley *F. oxysporum F. proliferatum, F. solani* and. *F. verticillioides* were also isolated in higher numbers from lerotho than from thepe. These observations seem to support the interpretation that, in combination with factors enhancing the production and dispersal of spores into the air, leaf characteristics of lerotho plants may play a prominent role in trapping *Fusarium* spores more effectively from the air.

Results from the present study seem in accordance with findings of Cotten and Munkvold (1998), suggesting that maize is a major factor in the occurrence of *Fusarium* in food production environments. Results in Table 5.3 show *F. proliferatum* and *F. verticillioides* were isolated in significantly higher numbers from maize components, i.e. leaves, hair and seeds, than the other nine species. *F. proliferatum, F. subglutinans* and *F. verticillioides* are reported the most commonly isolated species from maize plants (Cotten and Munkvold, 1998; Doohan *et al.* 2003). Figure 5.3 illustrates *F. proliferatum, F. poae F. solani, F. subglutinans* and *F. verticillioides* were predominantly isolated from maize seeds and *F. dimerum, F. oxysporum,* and *F. semitectum* from maize hair (silk). Studies elsewhere reported maize silks to be infected by airborne or water-splashed conidia of *F. proliferatum* and *F. verticillioides,* species isolated from symptomatic as well as asymptomatic maize seeds (Munkvold and Desjardins, 1997; Fandohan *et al.* 2003).

In addition to causing plant diseases, *F. proliferatum* and *F. verticillioides* are prolific producers of fumonisin toxins (Rheeder *et al.*, 2002; Hinojo *et al.*, 2006) and are most commonly found in diseased and asymptomatic maize seeds (Bacon and Hinton, 1996; Nelson *et al.* 1993). Fumonisins are natural contaminants of cereals and are mostly found in corn and corn-based products (Shephard *et al.,* 1996). Of the different fumonisin classes, fumonisin B_1 (FB_1) is the most abundant (Norred, 1993). However, Van der Walt *et al.* (2006) also detected high levels of FB_1 in household *morogo* samples from the

Limpopo Province. In the present study, HPLC analysis of household *morogo* revealed low levels of FB_1 in one sample each of lerotho and thepe, as well as in a combined sample of different *morogo* types (Table 5.4). Based on these findings, molecular techniques were subsequently applied to a selected number of isolates obtained from *morogo* samples firstly, to verify correct morphologically-based species identification and secondly to determine the presence or not of genes encoding fumonisin biosynthesis.

PCR amplification and subsequent DNA sequencing of ß-tubulin (Figure 5.4) and translation elongation factor 1-α (TEF1-α; Figure 5.5), established a sufficient degree of similarity between *F. proliferatum, F. solani, F. subglutinans* and *F. oxysporum* and Genebank (Tables 5.5 and 5.6) and FUSARIUM ID Version 1.0 (Geiser *et al.* 2004) DNA sequences of these species to accept their morphological identification as accurate. The PCR primer *FUM 1* was used to amplify and identify fumonsin-encoding genes in *Fusarium* isolates from *morogo* vegetables. Species lacking the *FUM 1* DNA region for fumonsin biosynthesis is not capable of producing fumonisins (Seo *et al.*, 1991; Bluhm *et al.*, 2004). Results illustrated in Figure 5.6 show *FUM 1* amplified in *F. proliferatum, F. solani, F. subglutinans* and *F. oxysporum*, which confirmed the capacity of these isolates to produce fumonisins should environmental factors be conducive. Warm (15 to 30 °C) and humid (a_ω = 0.98) conditions favour fumonisin production by *Fusarium* (Doohan *et al.* 2003). Rustenburg area falls within the Summer Rainfall Zone of South Africa where the average monthly precipitation during the maize-growing months of November until April ranges from 38 to 105 mm and the average daily temperatures varies between 20 °C to 26°C (par. 3.2). Dilkin *et al.* (2002) found 24.5 °C the ideal temperature for fumonisin production, while other studies indicated FB_1 is optimally produced at 30 °C (Marin *et al.*, 1999). Peri-urban food-gardens are likely to be watered in which case moisture for fumonisin production would be available. Fumonisin B1 has been classified by the International Agency for Research in Cancer (IARC; 1993) as a probable human carcinogen. Dietary FB_1 is reported to produce diverse biological effects largely due to its disruption of sphingolipid metabolism (Soriano *et al.*, 2005). Chronic ingestion of fumonisins may lead to organ toxicity (Bennet and Klich, 2003), mutagenesis as a result

of DNA damage (Lerda *et al.,* 2005), oxidative injury to cellular macromolecules (Domijan *et al.,* 2007) and immune-suppression (Ferrante *et al.,* 2002).

Mycotic risks posed by the presence of *Fusarium* in a prei-urban environment are a great concern. Opportunisitic infections caused by *Fusarium* are a worldwide problem largely affecting immune-suppressed or immunocompromised individuals and cancer patients, particularly those with haematological cancers (Boutati and Anaissie, 1997; Guarro *et al.,* 2000). *Fusarium* is considered the second most common mold pathogen after *Aspergillus fumigatus* (Flemming *et al.*, 2002). Mycotic *Fusarium* species isolated in the present study include *F. chlamydosporum, F. dimerum, F. oxysporum, F. proliferatum, F. solani, F. subglutinans* and *F. verticillioides.* Infections caused by *Fusarium* affect the eyes, skin, heart and lungs (Guarro & Gene, 1995; Austen *et al.*, 2001; Badar *et al.*, 2003; Cuero, 1980). Mortality from opportunistic fusarial infections is estimated between 70 – 80 % of those affected because of fusarial drug resistance (Guarro *et al.,* 2000) and treatment being further complicated by the spread of the organism through the blood to other organs (Dignani and Anaissie, 2004). The HIV prevalence in the North-West Province is estimated at 10.3%. The overall HIV prevalence in urban formal settings of South Africa is indicated as 11.9%, but in informal urban settings as 21.6% (Connolly *et al.,* 2004). These estimates suggest that the poorer sector of urban societies is disproportionately affected by HIV. Population density in peri-urban settings exposes more individuals to inhalation of, and skin contact to spores in the air, the two most common routes of entry for *Fusarium* pathogens (Dignani and Anaissie, 2004) thus enhancing the risk of life-threatening opportunistic infections in HIV-positive individuals.

In summary, results from the present study show various mycotoxigenic and mycotic *Fusarium* species are present in the peri-urban environment. Results suggest home-grown maize in household food gardens could be a major source of *Fusarium* which was also isolated from traditional *morogo* vegetables growing in the same environment, air and soil. Among those species prolific fumonisin-producers, i.e. *F. verticillioides* and *F. proliferatum,* were recovered in the highest numbers from maize, soil, air and *morogo* samples. These results suggested a general contamination of the environment with

fumonisin-producing *Fusarium*. Furthermore, fumonisin B_1 was detected in some of the *morogo* samples. *Fusarium* species retrieved from food-plants grown in home gardens and those that were present in the air, are all associated with opportunistic infections in immune-compromised individuals. *F. verticillioides* and *F. proliferatum* and *F. solani* were isolated in the highest numbers. *F. solani* is indicated in literature as the most dangerous human pathogen after *A. fumigatus*. Dietary fumonisin B_1 and opportunistic fusarial infections have important health consequences for HIV-positive individuals.

CONCLUSION AND RECOMMENDATIONS

Phokeng, where the present study was conducted, consists of approximately 60 villages with each village being home to about 4200 people. These figures suggest a relatively high population density in Phokeng. In the poor section of Phokeng, the average family consists of 7 persons per family living in a two bedroomed house. In many cases, families are without a breadwinner and subsist on an income of R200-400 per month. These food-insecure households are dependent on home-grown food to supplement their meagre diet (questionnaire information). According to Bourne *et al.* (2002), the poor sector of society is also disproportionately affected by the HIV/AIDS epidemic. In 2001 the HIV prevalence (%) in the North-West Province was indicated as 23.7±2.8 with a projected doubling time of 13.4±2.5 months (Williams and Gouws, 2001). Based on projections of the Actuarial Society of South Africa (ASSA), an estimated 667000 people in the North-West Province were HIV-positive in 2007 and 104000 were living with AIDS (Dorrington *et al.*, 2002).

From the afore-mentioned, it could be expected that in the poor section of Phokeng, a notable proportion of food-insecure residents could be affected by HIV and AIDS. Results from the present study demonstrated maize could be a significant source of fumonisin-producing *Fusarium* species in food-gardens of Phokeng, putting resource-poor families living there at risk of dietary fumonisin exposure. The high levels of mycotic *Fusarium* isolated from maize, *morogo* and the air add to the additional risk for those infected by HIV and suffering AIDS to contract opportunistic fusarial infections though ingestion, inhalation or skin contact with *Fusarium* spores. Urban activities are expected to enhance the dissemination of fusarial spores over a wide area where the population density in relatively high. The present study concludes that growing maize in peri-urban food gardens introduces a significant health risk to the population of Phokeng.

Based on results from the present study, it is recommended that maize planting be avoided in food-gardens of peri-urban households. However, maize is viewed as an accessible staple food for resource-poor families who do not have sufficient income to buy from urban stores. Risks of *Fusarium* in the environment could be reduced by

substituting maize with other staples, e.g potatoes, sweet potatoes or even a traditional crop such as amadumbe. These crops would add nutritional value to the restricted diet of food-insecure families. Where maize is grown, residue should not remain in the gardens, but be removed and either destroyed by burning them or burying them deep in the ground to prevent spores from being disseminated to the environment. Strategies to inform residents of risks and how to avoid it should be developed and implemented. Governmental programmes to support peri-urban home-garden schemes aimed at the cultivation of nutritious food, i.e vegetables in stead of maize, are strongly recommended. In collaboration with ARC-VOPI, the cultivation of traditional African green leafy vegetables (*morogo*) in peri-urban food-gardens should have priority.

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APPENDIX A

APPENDIX A

QUESTIONNAIRE 1

Scheme number:

Name and surname:

Current season:

---

1. What types of *morogo* do you plant?
2. Do you use seeds? Yes ☐ No ☐
3. Where do you get the seeds from?

   Previous years crop ☐ Relatives ☐

   Bought ☐

   Further comment:
4. How many times a year do you plough?
5. Do you rely only on rain for water? Yes ☐ No ☐

   Further comment:
6. If not, what method of watering do you employ?

   Flooding ☐ Sprinkler ☐ Other ☐

7. How often do you water the plants?

Summer Winter

Further comment:

8. How do you sell the *morogo*?

Washed ☐ Unwashed ☐

Roots intact ☐ Leaves only ☐

9. Who do you supply the *morogo* to?

Villagers ☐ Rustenburg market ☐ Other ☐

Further comment:

10. Have you noticed any of the plants going mouldy or exhibiting brown spots?

Yes ☐ No ☐

11. If yes, how often do you notice this?

12. Does it affect your harvest? Yes ☐ No ☐

13. What have you done / are going to do about this problem?

14. Would you like us to arrange talks / consultations with an agricultural institute?

Yes ☐ No ☐

APPENDIX B

APPENDIX B

MEDIA USED FOR *FUSARIUM* CULTIVATION AND IDENTIFICATION

Pentachloronitrobenzene (PNCB) Agar

15.0g Difco peptone

1.0g KH_2PO_4

0.5g $MgSO_4.7H_2O$

20.0g Agar

1.0g Pentachloronitrobenzene (Terraclor)

1.0 liter distilled water.

The pH was adjusted to between 5.5 and 6.5. The mixture was autoclaved. After autoclaving and allowing the mixture to cool to ± 55°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,
- Chloromphenicol 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

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

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.

Carnation Leaf Agar (CLA)

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

1 litre distilled water

Sterilized carnation leaf pieces

20g of the Bacteriological agar was added to 1 litre of distilled water and the mixture autoclaved. Following autoclaving, the agar was dispensed into 9mm 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.

Synthetic Nutrient Agar (SNA)

1g KH_2PO_4

1g KNO_3

0.5g $MgSO_4.7H_2O$

0.5g KCl

0.2g Glucose

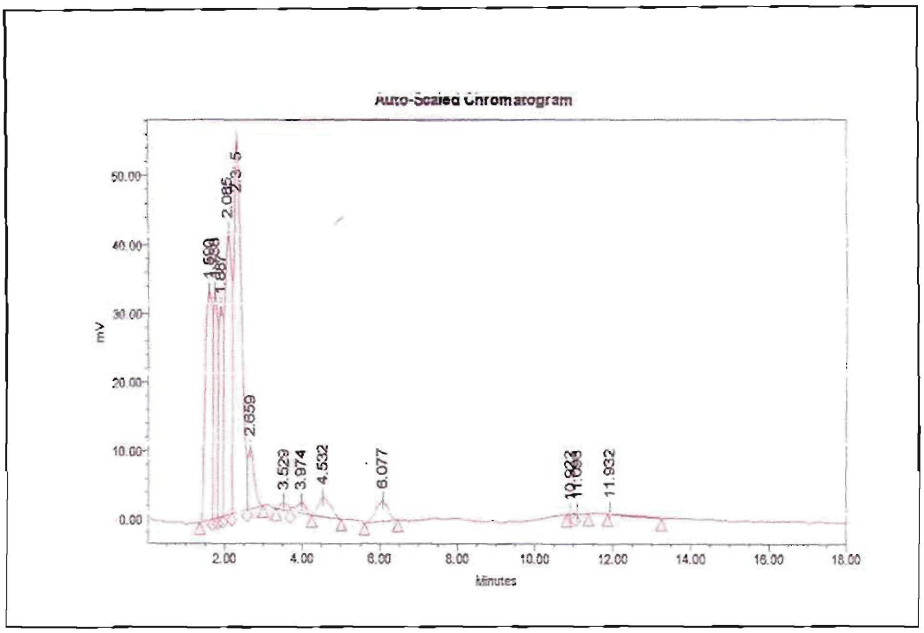
0.2g Saccharose

20g Agar

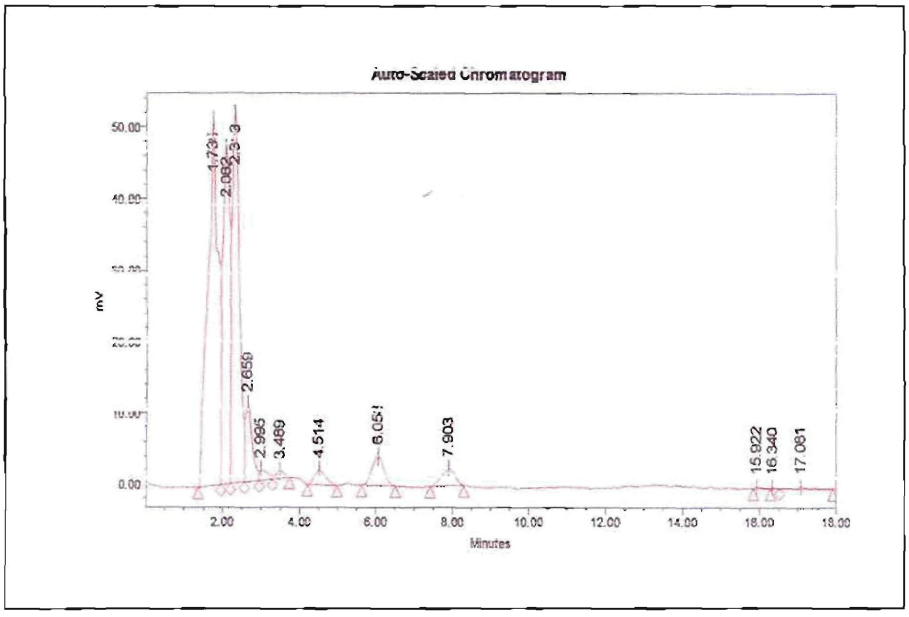
1000 ml dH_2O

All the above was added to 1 litre of distilled water and the mixtures autoclaved. Following autoclaving, the agar was dispensed into 9 mm sterile Petri dishes. One piece of filter paper was aseptically put in the centre of each agar plate. The contents were allowed to solidify.

APPENDIX C



Chromatogram of HPLC analysis of a lerotho sample from Phokeng, Rustenburg District, North-West Province, South Africa.



Chromatogram of HPLC analysis of a thepe sample from Phokeng, Rustenburg District, North-West Province, South Africa.

APPENDIX D