FUSARIUM IN SUBSISTENCE AGRO-ENVIRONMENTS, AFRICAN DARK GREEN LEAFY VEGETABLES (*MOROGO*) AND CONSUMER HEALTH: AN ECOLOGICAL APPROACH

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The LORD your GOD said:

Each of you is to take up a stone on his shoulder to serve as a sign.

In the future, when asked what the stone means, tell them:

The LORD your GOD did this so that all the peoples of the earth might know

the hand of the LORD is powerful.

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Adapted from Chapter 4 in the book of Joshua
New International Version of the Holy Bible

TABLE OF CONTENTS

.....continue on next page

.....continue on next page

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Having concluded documentation of this research leaves me with an overwhelming sense of humbleness. Throughout this project I was constantly aware of GOD's guidance and directions, HIS careful provision of personal qualities, material means, opportunities and persons at the right time at the right place. May outcomes of this research grow into actions that will serve GOD's compassion to so many in Africa who are distressed by chronic hunger and disease.

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LIST OF FIGURES

.....continue on next page

LIST OF TABLES

.....continue on next page

ABSTRACT

Traditionally, the African diet consists of grain-based staples supplemented with a variety of pulses, tubers and green leafy vegetables. In the northern parts of South Africa, the term *morogo* is used with reference to a collection of dark green leafy vegetables either grown for subsistence, or gathered from the field. Derived from wild African varieties, *morogo* crops are well adapted to local growing conditions and require low water and agrochemical inputs. Some *morogo* plants appear spontaneously in soil disturbed by ploughing, while others are grown as soil cover in maize lands.The accessibility of *morogo* vegetables, whether by means of simple cultivation or collection, is a unique advantage to resource-limited rural and peri-urban households depending on home-grown food for sufficient nutrition.

Though maize is less drought resistant than the traditional African grains (i.e. millet varieties, sorghum, signal and bushman grasses), it gained preference as subsistence crop because of larger yields produced under favourable conditions. However, *Fusarium* infestation of maize and contamination of human food with their toxins have globally become a major health and economic concern. Fumonisins, a group of potent structurally-related secondary metabolites produced by various *Fusarium* species, have also been reported in home-grown maize in South Africa. Dietary fumonisins have diverse biological effects that have been linked with organ toxicity, carcinogenesis and immune suppression. A number of fumonogenic *Fusarium* species have furthermore been identified as causative agents of opportunistic infections in immune compromised individuals. In South African, an estimated 10.9% of the population was HIV-positive in 2006. The rural sector is disproportionately affected by the pandemic. The presence of *Fusarium* in subsistence agro-environments thus has aggravating health implications for rural families, who in many instances are food-insecure and in addition affected by chronic diseases, HIV infection and AIDS.

The present study investigated the incidence of toxigenic *Fusarium* in rural subsistence agro-environments and food gardens of peri-urban households. The most prolific fumonisin-producers, *F. proliferatum* and *F. verticillioides*, were predominantly isolated from various components of the environment. Other fumonigenic species included *F. oxysporum* and *F. subglutinans*. *Fusarium* was isolated in significantly higher numbers from environmental samples of localities where maize was also growing. The genetic predisposition of isolates for fumonisin production was demonstrated. Moreover, fumonisin B_1 was detected in samples of household *morogo*. Results indicate maize as the most likely source of *Fusarium*

contamination in subsistence food production environments, including of *morogo* vegetables growing in close proximity. *Fusarium* species profiles of soil and air samples suggest maize residues and debris on the soil surface may play a crucial role in the survival and dissemination of toxigenic *Fusarium* in rural subsistence agro-environments. Isolates retrieved from the air samples included the following mycotic species: *F. chlamydosporum, F. equiseti, F. oxysporum, F. proliferatum, F. solani F. subglutinans* and *F. verticillioides*. Of these infectious species, *F. solani,* is considered the most dangerous for immunocompromised individuals after pathogenic *Aspergillus fumigatus*.

Though compositional data on *morogo* vegetables are severely limited, they are expected to possess health-protective and immune-strengthening properties similar to that indicated for commercial dark green leafy vegetables. In the present study the nutrient and phytochemical composition of the most commonly consumed *morogo* species were determined and some health-protective properties evaluated *in vitro*. Wild-growing varieties of thepe (*Amaranthus* spp), lerotho (*Cleome gynandra*) and dinawa *(Vigna uiguiculata*) from different geographical regions in the Limpopo and North-West Provinces of South Africa were analysed. Results indicated high concentrations of minerals and micronutrients (i.e. Ca, Mg, Fe, Zn and Se), folate, alpha-linolenic acid (omega-3 polyunsaturated fatty acid) and antioxidant phytochemicals (i.e. polyphenols, carotenoids and β-carotenes). Consuming vegetables and fruit containing these nutrients and phytochemicals in large amounts, is claimed in literature to reduce risks of chronic diseases and strengthen immunity. Provided consumer safety is improved, it seems likely that a degree of health protection could be derived from *morogo* consumption. Antioxidant, antileukemic and anticaricnogenic activities were demonstrated *in vitro* for the following six wild-growing *morogo* species: ditaka (*Lagenaria siceraria*), thepe (*Amaranthus thunbergii*), dinawa (*Vigna unguiculata*), mushidzhi (*Bidens pilosa*), lefe (*Pentarrhinum insipidum*) and lerotho (*Cleome gynandra L.*). This finding is important considering the fact that dietary fumonisin B_1, chronic HIV infection as well as a diet deficient in folate and omega-3 fatty acids (e.g Westernised diet), in al three instances, would contribute towards oxidative stress and tissue damage. Excessive and / or persistent oxidative stress elicits inappropriate inflammatory responses now recognised as a key-event in the onset of chronic diseases. These include cardio- and cerebrovascular diseases (CVDs), diabetes Type 2 and cancer that have high incidences in AIDS patients as well as Westernised societies.

Recommendations for further research include the following aspects: (i) mycological surveys to determine factors contributing towards maintaining *Fusarium* epidemics in environments

of subsistence agriculture and food gardens of peri-urban households; (ii) post-harvest conditions affecting the dietary safety of *morogo* vegetables, amongst others of fumonisin production; (iii) nutritional analysis of *morogo* vegetables aimed at broadening the nutritional and phytochemical database on traditional African dark green leafy vegetables, (iv) *in vitro* studies to accurately determine the health beneficial qualities of *morogo* vegetables and (v) epidemiological studies to accurately access the human health effects as a result of regular *morogo* consumption.

Research outcomes might indicate a need to adapt some traditional food production practices and post-harvest food handling to lower consumer risks of fumonsin exposure and opportunistic fusarial infections. With consumer safety as a prerequisite, the availability and utilisation of *morogo* vegetables should be encouraged in rural as well as urban evironments. The long-term aim of this research is aptly described in the International Fund for Agricultural Development (IFAD) definition of household food security: food adequacy complying with nutrient and safety requirements as well as cultural preferences.

OPSOMMING

Tradisioneel bestaan die Afrika dieet uit graan-gebaseerde stapelvoedsel aangevul met 'n verskeidenheid van peulvrugte, knolle en blaargroentes. In die noordelike dele van Suid Afrika word die term *morogo* gebruik as verwysend na 'n vesameling donkergroen blaargroentes wat vir onderhoud verbou of uit die veld versamel word. Aangesien hulle van wilde Afrika variëteite afgelei is, is *morogo* gewasse goed by plaaslike groeitoestande aangepas en verg die verbouing daarvan lae insette van water en landbou chemikalieë. Sommige *morogo* plante verskyn spontaan in die grond wat deur ploeg versteur is, terwyl ander as grondbedekking in mielielande geplant word. Die toeganklikheid van *morogo* as voedsame groente, hetsy deur eenvoudig verbouing of versameling, is 'n unieke voordeel vir hulpmiddel-gestremde plattelandse en halfstedelike huishoudings vir wie voldoende voeding afhanklik is van tuis verbouing.

Hoewel mielies minder droogtebestand is as die tradisionele Afrika grane (d.i. verskillende grasvariëteite, graansorghum en boesmangras), het dit voorkeur as bestaansgewas verwerf terwille van die groter opbrengs wat dit lewer onder gunstige toestande. *Fusarium* besmetting van mielies en toksien kontaminasie van menslike voedsel het egter wêreldwyd 'n groot gesondheids- en ekonomiese probleem geword. Fumonisiene, 'n groep gevaarlike struktureel-verwante sekondêre metaboliete wat deur verskeie *Fusarium* spesies geproduseer word, is reeds in tuis-verboude mielies in Suid Afrika geraporteer. Fumonisiene in die dieet het uiteenlopende biologiese effekte tot gevolg wat verbind word met orgaantoksisiteit, karsinogenese en immuun-onderdrukking. 'n Aantal fumonigeniese *Fusarium* spesies is ook geïdentifiseer as agente van opportunistiese infeksies in immuun-gekompromiteerde individue. In Suid Afrika was 10.9% van die bevolking na raming MIV-positief in 2006. Die plattelandse sektor word disproporsioneel deur die pandemie geraak. Die teenwoordigheid van *Fusarium* in bestaansboerdery omgewings het dus verswarende implikasies vir plattelandse families, wie in baie gevalle ook nie voedselsekuriteit het nie en boonop deur chroniese siektes, MIV infeksie en VIGS geteister is.

Die huidige studie het ondersoek ingestel na die voorkoms van toksigene *Fusarium* in plattelandse bestaansboerdery omgewings en voedseltuine van halfstedelike huishoudings. Die oorvloedigste fumonisien produseerders, *F. proliferatum* en *F. verticillioides*, is hoofsaaklik uit verskeie komponente van die omgewing geïsoleer. Ander fumonigeniese

spesies het ingesluit *F. oxysporum* en *F. subglutinans*. *Fusarium* is in betekenisvolle hoër getalle geïsoleer uit omgewingsmonsters van lokaliteite waar mielies ook gegroei het. Die genetiese predisposisie van isolate om fumonisiene te produseer, is gedemonstreer. Verder is fumonisiene ook in verskeie monsters van huishoudelike *morogo* uit al die studiegebiede opgespoor. Resultate dui daarop dat mielies die mees waarskynlike bron van *Fusarium* kontaminasie in bestaansboerdery omgewings is, insluitend van *morogo* wat in die nabyheid daarvan groei. *Fusarium* profiele in grond- en lugmonsters toon aan dat mieliereste en oorblyfsels op die grondoppervlak 'n beslissende rol het in die oorlewing en verspreiding van toksigene *Fusarium* in bestaansboerdery omgewings. Isolate uit lugmonsters sluit die volgende mikotiese spesies in: *F. chlamydosporum, F. equiseti, F. oxysporum, F. proliferatum, F. solani, F. subglutinans* en *F. verticillioides*. Val hierdie infektiewe spesies word *F. solani* naas patogeniese *Aspergillus fumigatus*, as die gevaarlikste vir immuun-gekompromiteerde individue gereken.

Alhoewel samestellingsdata vir *morogo* groente uiters beperk is, word verwag dat dit gesondheidsbeskermende en immuun-versterkende eienskappe besit gelykstaande aan dít wat vir kommersiële groente aangedui word. In die hierdie studie is die voedingskundige en fitochemiese samestelling van die mees algemeen verbruikte *morogo* spesies bepaal en sekere gesondheids-beskermende eienskappe *in vitro* geëvalueer. Wild-groeiende variëteite van thepe (*Amaranthus* spp), lerotho (*Cleome gynandra*) en dinawa *(Vigna uiguiculata*) uit verskillende geografiese streke van die Limpopo en Noordwes Provinsies van Suid Afrika is ontleed. Resultate dui op hoë konsentrasies minerale en mikrovoedingstowwe (d.i. Ca, Mg, Fe, Zn and Se), folaat, alfa-linoleniese suur (omega-3 poli-onversadigde vetsuur) en antioksidant fitochemikalieë (d.i. polyphenols, carotenoids and β-carotenes). In die literatuur word daarop aanspraak gemaak dat die eet van groente en vrugte wat hierdie voedingstowwe en fitochemikalieë in groot hoeveelhede bevat, beskerming verleen teen chroniese siektes en die immuniteit versterk. Indien verbruikersveiligheid verbeter word, lyk dit waarskynlik dat 'n mate van gesondheidsbeskerming verkry kan word deur *morogo* groentes te eet. Die antioksidant, antileukemiese en antikarsinogeniese aktiwiteite van die volgende ses wild-groeiende *morogo* plante is *in vitro* gedemonstreer: ditaka (*Lagenaria siceraria*), thepe (*Amaranthus thunbergii*), dinawa (*Vigna unguiculata*), mushidzhi (*Bidens pilosa*), lefe (*Pentarrhinum insipidum*) en lerotho (*Cleome gynandra L.*). Hierdie bevinding is belangrik wanneer die feit in gedagte gehou word dat fumonisien B_1 in die dieet, chroniese HIV infeksie sowel as 'n dieet wat tekort is aan folaat en omega-3 vetsuur (bv. die verwesterse dieet), in al drie gevalle sou bydra tot oksidatiewe stres en weefselbeskadiging. Oormatige en / of

volgehoue oksidatiewe stres ontlok onvanpaste inflammatoriese response wat nou as 'n sleutelgebeurtenis in die onstaan van kroniese siektes gereken word. Dit sluit in kardio- en serebrovaskulêre siektes (KVSe), diabetes Tipe 2 en kanker wat 'n hoë voorkoms in VIGS pasiënte sowel as verwesterse samelewings.

Aanbevelings vir verdere navorsing sou die volgende aspekte insluit: (i) mikologiese opnames om vas te stel watter faktore daartoe bydrae om *Fusarium* epidemies in stand te hou in die omgewing van bestaansboerderye en voedseltuine van halfstedelike huishoudings; (ii) omgewingsfaktore wat na die oes die dieetkundige veiligheid van *morogo* groentes sou beïnvloed, o.a. fumonisien produksie; (iii) voedingkundige ontleding van *morogo* groentes met die oog daarop om die voedingskundige en fitochemiese databasis van donkergroen Afrika blaargroentes te verbreed; (iv) *in vitro* studies om die gesondheidsvoordelige kwaliteite van *morogo* groentes noukeurig te bepaal; (v) epidemiologiese studies om noukeurig vas te stel wat die uitwerking op menslike gesondheid is wanneer *morogo* gereeld geëet word.

Uitkomstes van navorsing mag 'n noodsaaklikheid aantoon vir aanpassings in sommige tradisionele voedselproduksie praktyke en voedsel hantering na die oes ten einde risiko's vir fumonisien blootstelling en opportunistiese fusariële infeksies te verlaag. Met verbruikersveiligheid as 'n voorvereiste, behoort die beskikbaarheid en verbruik van *morogo* groentes aangemoedig te word in plattelandse sowel as stedelike omgewings. Die langtermyn doelwit van hierdie navorsing word gepas beskryf in die Internasionale Fonds vir Landboukundige Ontwikkeling (IFLO) se definisie van huishoudelike voedselsekuriteit: toereikende voedsel wat voldoen aan voedingskundige en veiligheidsvereistes sowel as kulturele voorkeure.

Chapter 1

INTRODUCTION

1.1 The biological systems approach

Knowledge of the human genome brought understanding of how environmental factors and nutrients modulate genetically-controlled mechanisms involved in disease development (Kaput, 2004). Aimed at predicting human health and disease outcomes, the biological systems approach is essentially an ecological interpretation of environment-gene-nutrient interactions (Desiere, 2004). In this context, human dependence on, and the continuous exposure to food throughout a lifetime, renders diet probably the most important environmental factor challenging the human biological system (Ordovas & Corella, 2004). Pathogens in the nutritional environment establish detrimental interactions with humans and contribute to disease, either through infection or by way of harmful metabolites secreted in food. Health-injuring toxic effects exerted at a molecular and cellular level cause tissue and organ damage that manifest in chronic disease (Nelson *et al.*, 1994; Bennett and Klich, 2003).

1.2 *Fusarium*, fumonisins and the subsistence food-production environment

Over the last decades, research in various fields of science focused on plant pathogenic fungi, their toxins in commercial food commodities and biochemical mechanisms mediating their harmful effects in humans (Bennett & Klich, 2003). Mycotoxins vary widely in their chemistry and toxicology and when ingested, adverse biological effects produced in exposed individuals could range from antinutritional and immune-suppressive to teratogenic, mutagenic, genotoxic and carcinogenic (Riley *et al.*, 2001; Baumrucker & Prieschl, 2002; Carratù *et al.*, 2003; Sudakin, 2003; Mulè *et al.*, 2004). Species of the genus *Fusarium* are ubiquitous in agricultural environments and have been studied extensively for the damage they cause in commercial crops and fusarial toxins contaminating human food (Sudakin, 2003; Soriano & Dragacci, 2004). Most notable among these toxins are the fumonsins, a class of potent structurally-related toxins (Rheeder *et al.*, 1992; Rheeder *et al.*, 2002) classified by the International Agency for Research on Cancer as Group 2B carcinogens, i.e. substances probably carcinogenic to humans (IARC, 1993).

Fusarium species have a widespread occurrence worldwide (Marasas, 1996) and are prevalent in pre- as well as post-harvest maize and maize ecosystems (Fandohan *et al.*, 2005; Nesci *et*

al., 2006; Samapundo *et al.*, 2007). In contrast with natural environments, agricultural ecosystems are artificial and as such most suitable habitats for phytopathogenic fungi. In agricultural fields host plant communities are abundant and genetically as well as spatially relatively uniform. Anthropogenic activity provides an effective mechanism for the dissemination of fungal spores (Burdon *et al.*, 1989). Maize ecosystems, therefore, comprise ideal environments for toxigenic *Fusarium* species to establish and perpetuate disease epidemics in their plant hosts (Munkvold & Desjardins, 1997). Cotton and Munkvold (1998), furthermore demonstrated that fumonigenic *Fusarium* species survived in maize stalk residue and continued to produce macroconidia for over 2 years. Air-borne and splash dispersal of fusarial spores from plant residue and debris lying on the soil surface contribute towards maintaining *Fusarium* populations in a wide area where other co-existing plants also become contaminated (Hörberg, 2002; Doohan *et al.*, 2003; Leslie *et al.*, 2004).

These reports are of particular interest for subsistence families dependent on growing their own food. Vegetarian foods utilised in the traditional African diet include tubers, legumes and a variety of green leafy vegetables collectively called *morogo* in local African languages (Khumbane, 1997; Modi *et al.*, 2006). In subsistence settings *morogo* vegetables often grow in a mixed system with maize (Jansen van Rensburg *et al.,* 2007). Some *morogo* plants emerge spontaneous as pioneer plants after soil has been disturbed by ploughing (Odhav *et al.*, 2007), while others are planted among the maize for soil cover (Modi *et al.*, 2006). Potential fumonisin-producing *Fusarium* in the homegrown maize (Rheeder *et al.,* 1992; Rheeder *et al.*, 2002; Shephard *et al.*, 2005) contaminate a wide area with their spores (Hörberg, 2002; Nesci *et al.*, 2006) which most likely also include the *morogo* vegetables growing in area.

1.3 Consequences of dietary fumonisin exposure

Ingestion of fumonisin-contaminated food cause irreversible damage to human cells through biochemical mechanisms that produce oxidative, inflammatory, carcinogenic and / or immune-suppressive effects (Riley *et al.*, 2001). Animal studies indicated that biochemical processes responsible for cellular detoxification of FB_1 deplete splenic and hepatic glutathione (GSH; Atroshi *et al.*, 1999). Depletion of endogenous GSH reportedly gives rise to oxidative stress (OS) and the production of pro-inflammatory cytokines or other mediators of inflammation, such as cyclooxygenase 2 (COX-2; Sharma *et al.*, 2003). Philpott & Ferguson (2004), maintain that inflammation and elevated COX-2 levels are factors in the initiation of carcinogenesis. Toxic effects exerted by fumonisins mostly ascribe to the ability

of these fungal metabolites to disrupt sphingolipid metabolism (Riley *et al.*, 2001; Carratù *et al.*, 2003; Van der Westhuisen *et al.*, 2004). Imbalances in sphingolipid intermediates are suggested to form the basis of immune-suppression due to fumonisin exposure (Baumrucker and Prieschl, 2002). Sphingolipids are major structural components of eukaryotic membranes where they function as signalling molecules in processes that control cell proliferation, differentiation, growth, senescence and apoptosis (Colombaioni & Garcia-Gil, 2004; Kacher & Futerman, 2006). Corrier (1991), considers the immune system particularly vulnerable to toxic insults. The author based this view on the fact that immunity is effectively accomplished through the continual and rapid proliferation of immune cells, their differentiation and migration to appropriate sites of action, as well as interactions between various types of immune cells. These complex interactive events are regulated and coordinated through intricate inter- and intracellular cell-signalling networks in which various sphingolipid intermediates have vital roles.

Perturbations in cell-signalling processes adversely affect immune cell responsiveness and weaken immune defence against invading pathogens and cancer initiation (Baumrucker & Prieschl, 2002). Dietary exposure to FB1 also affects immune cell proliferation and responsiveness indirectly through its inhibition of folate uptake (Courtemanche *et al.*, 2004). Cellular folate has a crucial role in the biosynthesis of DNA and proteins (Carratù *et al.*, 2003) both of which are in high demand by fast proliferating immune cells (Baumrucker & Prieschl, 2002). Following intestinal absorbtion, products of folate metabolism are taken up by cell by endocytosis, a process mediated by GPI-anchored folate receptors localised in membrane regions enriched with sphingolipids (Wolf, 1998). FB_1-induced depletion of structural sphingolipids destabilises membrane folate receptors by which folate uptake is effectively blocked (Stevens & Tang, 1997). Insufficient cellular folate, causing nucleotide imbalances, inhibits the proliferation of $CD8^+$ T-lymphocytes, i.e. the immune cells responsible for the destruction of virally-infected and cancerous cells (Courtemanche *et al.*, 2004). Courtemanche *et al.* (2004), ascribe the increased misincorporation of uracil during DNA synthesis because of a folate deficiency, to the onset of cardiovascular diseases and certain cancers. These findings could explain the observation by Boudes *et al.* (1990), namely that folate deficiency contributed to faster disease progression after HIV infection of T-lymphocytes.

By further compromising immunity, dietary FB1 enhances the vulnerability of the exposed HIV-positive individuals to opportunistic infections. This seems particularly important in view of the prevalence of HIV infection and AIDS in the South African (Statistics South

Africa, 2006) and the fact that fumonigenic fusaria are also implicated as agents of life-threatening opportunistic infections and disseminated fusariosis in immunocompromised individuals (Nelson *et al.*, 1994; Pujol *et al.*, 1997; Wanke *et al.*, 2000). Opportunistic fusarial infections seem almost unavoidable in subsistence settings with high levels of airborne fusarial spores. According to Dignani & Anaissie (2004) the most common routes for spores of mycotic fusaria to gain entrance to the body is either through inhalation or contact with skin lesions. Mortalities in these cases range between 50-80%, mainly because effective treatment of infections is complicated by a combination of the fusarial agent's resistance to drugs and its unique capacity to spread to other organs through the blood (Boutati & Anassie, 1997).

The excessive and persistent overproduction of free radicals in response to chronic infections, consumes the body's endogenous antioxidant capacity (Torre *et al.*, 2002). The resultant oxidative stress (OS) elicits pro-inflammatory immune responses that precipitate chronic diseases, notably cardio- and cerebrovascular diseases (CVDs), type II diabetes and cancer (Dröge, 2002; Philpott & Ferguson, 2004). These OS-related chronic diseases have high incidences in patients manifesting clinical AIDS (Greist, 2002; Spano *et al.*, 2002; Torre *et al.*, 2002), but are also collectively referred to as 'diseases of lifestyle' for their association with health-injuring factors in the Westernised urban lifestyle and diet (Simopoulos, 2002; Suresh & Das, 2003). Urbanisation of black South Africans is typically accompanied by a shift from the low-fat and plant protein-rich traditional rural diet towards a high animal fat- and protein-rich Westernised diet (Steyn, 2006). Epidemiological studies in South Africa suggest a close connection between the rise in CVDs and diabetes Type 2 in urbanised black populations and their adoption of a Westernised diet (Vorster, 2002), the atherogenicity of which was shown to become significantly enhanced with prolonged urban exposure (Bourne *et al.*, 2002).

1.4 The health-protective value of African dark-green leafy vegetables (*morogo*)

Appropriate nutrition could prevent, amend or ameliorate disease outcomes that are consequences of the interaction between negative factors in the human nutritional environment and the human biological system. At a molecular level, certain nutrients and phytochemicals have crucial roles in the normal immune response to infection and the protection against OS-induced cellular damage and the onset of chronic disease (Field *et al.*, 2002; Olinski *et al.*, 2002; Courtemanche *et al.*, 2004). Fruit and vegetables, in particular dark green leafy vegetables, contain large amounts antioxidant compounds (Das, 2003; Surh &

Ferguson, 2003; Lako *et al.*, 2007). In plants toxic free radicals are continuously formed from cellular structures involved in photosynthesis and respiration. Plant leaves, therefore, are naturally equipped with antioxidant molecules that form vital components of enzymatic and non-enzymatic detoxification systems for protection against photochemically-induced OS (Zimmermann & Zentgraf, 2005). Green leafy vegetable consumption could lower the risk of chronic disease by providing substantial amounts of antioxidant nutrients and phytochemicals. These molecules may act either directly as reducing equivalents, or substitute for endogenous antioxidants that have been consumed by the persistent overproduction of free radicals as a result of chronic infection (Glew *et al.*, 1997; Rodriguez-Amaya, 1997; Van Duyn *et al.*, 2000; Simopoulos, 2001; Tapiero *et al.*, 2001). *Morogo* vegetables have been shown to be rich sources of antioxidant molecules (Reddy *et al.*, 2003; Mnkeni, 2007; Odhav *et al.*, 2007).

In summary, maize ecosystems most likely are contributing towards maintaining fusarial epidemics in subsistence agro-environmens. The presence of *Fusarium* in subsistence agro-environments could put rural human populations at risk of chronic dietary exposure to fumonisins. Interacting with the human biological system at a molecular level, fumonisin toxins initiate chronic diseases, including cancer, and weaken immune defences against infections. The high incidence of *Fusarium* in subsistence food-production environments furthermore constitutes an aggravated risk of opportunistic infections in HIV-positive individuals. Chronic infection generates excessive amounts of endogenous free radicals that enhance oxidative stress (OS) and inflammation that are key events in the development of chronic disease. However, consumption of dark-green leafy vegetables, such as *morogo*, provides substantial amounts of antioxidant molecules that counter OS pro-actively. Antioxidant protection against OS-related injury of cellular macromolecules could lower risks of OS-induced chronic diseases and slow down FB1-induced deterioration of crucial immune functions.

1.5 Hypothesis

Based on the afore-mentioned information, the following hypothesis was formulated for investigation:

Although consumer safety is generally assumed, food produced on a household level for family subsistence is neither grown nor stored under the conditions that secure the consumer safety of commercial foods. Growing vegetables in a mixed system with maize, the host plant of various fumonigenic *Fusarium* species, imposes a serious health risks on subsistence families. In addition to the risk of *Fusarium* contamination and pre- or postharvest fumonisin

production in *morogo* vegetables, maize-associated *Fusarium* species are also agents of complicated opportunistic infections in immunecompromised individuals. However, traditional African dark-green leafy vegetables (*morogo*) are nutritious and equivalent in their health-protective value as the conventional dark-green leafy vegetables consumed in the Western diet. Health-protective value of dark-green leafy vegetable consumption ascribes to the substantial amounts of minerals, micronutrients, folate and antioxidant phytochemicals they possess and their contribution to a low-fat plant-protein rich diet.

1.6 Outline of the thesis

Chapter 2 of the thesis considers consumer risks and benefits *morogo* consumption and other traditional plants based on two small-scale unrelated studies. Discussion of results from these investigations is presented in the following peer-reviewed article:

Title 1: Indigenous African food plants - sources of protection or vehicles of disease?
First author: A.M. van der Walt (responsible for manuscript preparation)
Co-authors: K.S.A. Mossanda, S.D. Jivan, W.J. Swart and C.C. Bezuidenhout
Journal: *Indilinga* (2005), 4: 270-279.

Mycological risks that are postulated were investigated in four ecologically different areas respectively in the Limpopo and North-West Provinces. The incidence of mycotoxigenic *Fusarium* species in various components of rural subsistence agro-environments, i.e. maize, *morogo* plants, soil and air and the presence of fumonisins in *morogo* vegetables were investigated. The investigation in study areas of the Limpopo Province are presented in Chapter 3, and findings of the study in a peri-urban subsistence food-production environment in the Rustenburg District, in Chapter 4. Two peer-reviewed articles on the findings of these studies are titled:

Title 1: Fumonisin-producing *Fusarium* strains and fumonisins in traditional African vegetables (*morogo*)
First author: A.M. van der Walt (responsible for manuscript preparation)
Co-authors: E. Van der Linde, M. Alberts, P. Modjadji, S.D. Jivan and C.C. Bezuidenhout
Journal: *South African Journal of Science* (2006), 102: 151-155.

Title 2: *Fusarium* in the household food garden environment of a peri-urban community.
First author: A.M. van der Walt (responsible for manuscript preparation)
Co-authors: M.I.M. Ibrahim, H.S. Steyn and C.C. Bezuidenhout.
Journal: *South African Journal of Science* (2007), 103: 504-508

A molecular study was conducted to investigate the use of a multiplex PCR method for the rapid detection of fumonisin-positive *Fusarium* in *morogo* vegetables. Results of this study are presented and discussed in Chapter 5 and a peer-reviewed article titled (Appendix 1):

Title: Multiplex PCR-based detection of potential fumonisin-producing *Fusarium* in traditional African vegetables

First author: C.C. Bezuidenhout (responsible for manuscript preparation)

Co-authors: M. Prinsloo and A.M. van der Walt

Journal: *Environmental Toxicology* (2006), 21: 360-366

Morogo species, most commonly consumed by both urban and rural populations in the study areas, were analysed to determine the folate contents, fatty acid profiles and concentrations of dietary minerals, trace elements and antioxidant phytochemicals all of which are claimed in literature to have health-protective properties. Presented in Chapters 6 and 7, results of the chemical analyses of *morogo* vegetables are also reported in the following peer-reviewed articles:

Title 1: Alpha-linolenic acid and folate in wild-growing African dark green leafy vegetables (*morogo*)

First author: A.M. van der Walt (responsible for manuscript preparation)

Co-authors: Du T. Loots, M.I.M. Ibrahim and C.C. Bezuidenhout

Journal: *Public Health Nutrition* (2008). In press

Title 2: Minerals, trace elements and antioxidant phytochemicals in wild-growing African dark-green leafy vegetables (*morogo*).

First author: A.M. van der Walt (responsible for manuscript preparation)

Co-authors: Du T. Loots, M.I.M. Ibrahim and C.C. Bezuidenhout

Journal: Submitted for publication to the *South African Journal of Science* on 18 April 2008

To investigate the health-protective qualities the vegetables, extracts of six *morogo* species, including those analysed for folate, fatty acid composition and antioxidant phytochemicals, were evaluated *in vitro* for antioxidant, antileukemic and anticarcinogenic activity. This was a pilot study because only small amounts of *morogo* were available towards the end of the 2006/2007 growing season. Results are presented in Chapter 8 titled:

In vitro antioxidant, antileukemic and anticarcinogenic activity of African dark green leafy vegetables (*morogo)* extracts.

The pilot study indicates aqueous extracts of *morogo* vegetables might have radical scavenging activities that could contribute towards inhibiting the cytoviability of leukaemic and carcinoma cells. Sufficient amounts of these *morogo* plants have since been acquired for more comprehensive testing which is currently in progress.

In Chapter 9, a summary of findings is given from which relevant conclusions are drawn. Possible adaptations in rural agricultural practices in order to diminish *Fusarium* levels and the risk of *morogo* contamination with fumonisins, are proposed. The chapter concludes with meaningful suggestions for future research in this field. Copies of the published articles are appended.

Chapter 2

INDIGENOUS AFRICAN FOOD PLANTS: VEHICLES OF DISEASE OR SOURCES OF PROTECTION?

(Indilinga – African Journal of Indigenous Knowledge Systems; 2005, 4(1):270-9)

2.1 Introduction

It is estimated that 901 million people will be living in sub-Saharan Africa by 2010, a number that could grow to 1.32 billion by 2025. Of these, 67% will be rural and the majority of them poor. (http://www.geography.ccsu.edu/kyem/GEOG466.../POPULATION_GEOGRAPHY.htm). According to Medaglini & Hoeveler (2003), endemic HIV/AIDS, malaria and tuberculosis are both cause and consequence of poverty and account for more than half of all deaths in countries of sub-Saharan Africa. HIV/AIDS, however, is distinguished by the fact that infections are highest amongst adults aged between 20 and 40 and this has a pronounced influence on the income, expenditure pattern, food production and coping strategies of rural households (SARPN, 2003; SADC FANR, 2003). The cumulative effect of HIV/AIDS, causing loss of labour and knowledge of traditional farming for subsistence, has contributed to the present situation of reduced crop and dietary diversity, widespread malnutrition, general micronutrient deficiencies and decreased human resistance to infections in rural regions of Sub-Saharan Africa (SARPN, 2003; Mbaya, 2003; SADC FANR, 2003; Wiggins, 2003).

In rural settings of Southern Africa, traditional diets of black communities consist largely of corn- or grain-based staples served with cooked traditional *morogo* that is either cultivated for subsistence or collected from the field where they grow as members of the natural flora. Home-grown groundnut and beans serve as a valuable source of plant protein and also add variety to the basic diet of grain and vegetables. For centuries these traditional crops have been cultivated in the practice of subsistence farming without chemical fertilisers or pesticides. Because indigenous plants are well adapted to local growing conditions their requirements for soil fertility, plant protection and water are modest and subsistence farmers benefit from low production inputs (http://www.ipgri.cgiar.org/Institue/fact_leafyveg.htm).

However, fungi and their spore are ubiquitous in the environment and all crops are at risk for fungal infestation and subsequent mycotoxin contamination. Mycotoxins are "natural" food contaminants and their formation is often unavoidable, even in commercial crops (Bennett & Klich, 2003). According to Barrett (2000), mycotoxin exposure is more likely to occur in parts of

the world where poor methods of food handling and storage are common and where few regulations exist to protect exposed populations. As opposed to commercial crops and retail food products, home-grown foodstuffs are not subjected to quality control to ensure their dietary safety. Consumers might therefore unknowingly be exposed to health-injuring levels of dietary toxins. Human health risks associated with mycotoxin exposure could significantly add to the existing burden of disease of consumer populations in rural regions of Southern Africa. Chronic dietary exposure to mycotoxins is associated with the occurrence of various types of cancer, kidney toxicity and immune suppression (Bennett & Klich, 2003; Ferguson *et al.*, 2004).

However, traditional vegetables such as *morogo*, bean plants and groundnuts are expected to be equal in nutritional value and probably possess phytochemicals with health-protecting or health-supporting qualities similar to green vegetables and leguminous crops used in developed countries. A number of epidemiological and laboratory studies reviewed by Suhr & Ferguson (2003) demonstrated that some edible plants of Western and Asian diets contain substances with health-protecting properties. However, scientific data to substantiate the dietary safety or health-pertaining benefits of edible plants used in traditional African diets does not exist.

A small-scale study was conducted to verify the dietary pattern and plant types used as traditional *morogo* in the Gyani district of the Limpopo Province of South Africa. The mycological quality and folic acid contents of these plants were determined. In another study extracts of Bambara groundnut, rooibos tea, devil's claw and cancer bush were evaluated for antimutagenic and anticarcinogenic properties.

2.2 Materials and Methods

Questionnaires were used to determine dietary patterns of rural households in the rural district of Gyani in the Limpopo Province of South Africa. Samples of fresh and dried *morogo* were collected from four rural households, a small cultivation scheme and a town stall. These included leaves and flowers of calabash (*Lagenaria siceraria*), *ligushe* leaves (*Corchorus tridens)* and cowpea leaves (*Vigna subterranean*). Samples of cooked *morogo* were prepared by mixing these plants according to the traditional custom before it was cooked and sampled. For mycological analysis fresh, dried and cooked samples were subjected to standard isolation and purification methods and microscopic identification up to genus level was carried out according to prescribed techniques (Nelson *et al*, 1983; Samson & Pitt, 1985; Simmons, 1996). The folic acid content of dried cowpea leaves, cooked calabash leaves mixed with the flowers and cooked *ligushe* leaves were determined by the Standards South Africa using the standard microbiological assay (Barton-Wright, 1961). Extracts of black and white Bambara groundnut (*Vigna subterranean*), rooibos tea

(*Aspalathus linearis*), cancer bush (*Sutherlandia fructescens*) and devil's claw (*Harpagophytum procumbens*) were subjected to a modification of the *Salmonella* antimutagenicity test using daunomycin as mutagen (Mossanda *et al,* 2001). The anticarcinogenic potential of these plants was evaluated in animal model using mouse skin. Inhibition of tetradecanoyl-phorbol-3 acetate (TPA) induced expression of the COX-2 gene through suppression of NF-κB activation was determined in the presence of plant extracts. COX-2 gene expression levels were measured using Western blotting assays (Na *et al*, 2004).

2.3 Results and Discussion

Data reported in questionnaires indicated that in a typical Gyani household an adult would normally consume three meals per day, each of which could consist of about 200 g of maize in the form of thick porridge or *putu* and 150 g of cooked vegetables. Vegetables represent different types of traditional *morogo*, often mixed together and flowers sometimes added for a tasty cooked dish. This preparation might include or could be substituted with groundnut.

The relative numbers of fungal isolates from fresh, dried and cooked samples were reported in Table 1. The highest number of surface-colonising fungi were isolated from uncooked fresh *morogo* namely *ligushe* (121) and fresh *calabash* (95), compared to those from cooked samples of *ligushe* (8) and calabash (6). A relatively small number of isolates were obtained from uncooked dried cowpea (15) and none from cooked cowpea.

Table 2.1: The total number of isolates and relative numbers (percentages) of surface-colonising species of *Penicillium Aspergillus, Fusarium and Alternaria* from fresh and cooked samples of traditional *morogo*

Sample	No. of isolates	*Penicillium*	*Aspergillus*	*Fusarium*	*Alternaria*
Ligushe (fresh)	121	4(>3)	1(<1)	88 (>72)	4(>3)
Ligushe (cooked)	8	0	0	8(100)	0
Calabash (fresh)	95	10(>10)	1(>1)	44(>46)	0
Calabash (cooked)	6	0	0	6(100)	0
Cowpea (dried)	15	7(>46)	3(20)	0	2(>13)
Cowpea (cooked)	0	0	0	0	0

This data suggest that food processing, including cooking and/or drying, notably lowered the level fungi associated with external surfaces of *morogo* plants. *Fusarium spp* was the dominant group among surface fungi isolated from both fresh (84%) and cooked (100%) ligushe, as well as fresh (46%) and cooked (100%) calabash, but was absent from both dried and cooked cowpea. In the case of cowpea, almost half the isolates from dried cowpea were *Penicillium spp* (47%), followed by *Aspergillus spp* (20%) and *Alternaria spp* (13%). Members of these genera occurred in relatively low numbers in fresh *ligushe* (2%, 2% and 4% respectively) and calabash (11%, 1% and 0 respectively). The mitosporic genera, *Aspergillus, Penicillium* and *Fusarium* all have representative species in soil (Carlile *et al,* 2001), and it is likely that these isolates represented post-harvest contaminants. In addition, many species of *Penicillium* and *Aspergillus* are xerotolerant and can grow at very low water potentials (Carlile *et al,* 2001), which might explain their relatively high numbers in dried cowpea.

Although these surface-colonising fungi could be harmless saprophytes or post-harvest contaminants, it should also be recognised that post-harvest environmental stresses potentially could induce some strains of these genera to produce toxigenic secondary metabolites (mycotoxins) known or suspected of causing human disease (Carlile *et* al, 2001; Bennett & Klich, 2003). For instance, not all *Fusarium spp* produce fumonisins and the presence of these moulds does not necessarily mean that their toxins are also present (Bennett & Klich, 2003). However, the high level of *Fusarium spp* (>84%) detected among the external colonisers in human food is important because of thei toxigenic potential of some species. These moulds are associated with the production of a range of potent toxins, including thrichothecenes and fumonisins such as fumonisin B1. The International Agency for Research on Cancer has classified FB1 as group 2B (probably carcinogenic) implying that dietary exposure could have important health consequences in humans (Bennett & Klich, 2003). In cell culture and animal studies FB1 has been reported to exhibit immuno-suppressive effects (Nair, 1998; Oswald *et al,* 2003). *Aspergillus* and *Penicillium* also contain species that are known producers of potent toxins such as aflatoxins, ochratoxins, citrinin and patulin, all of which are associated with human disease (Bennett & Klich, 2003). In addition, life-threatening secondary infections caused by strains of filamentous fungi, including *Fusarium spp* has become a difficult clinical challenge, particularly in the treatment of immunocompromised patients (Segal *et al*, 1998; Guarro *et al*, 2000; Dignani & Anaissie, 2004). Results depicted in Table 2 showed that a total number of 52 isolates were associated with the leaf interior of *ligushe,* compared to 13 in fresh calabash. Dried cowpea was not subjected to surface sterlisation. *Alternaria* spp dominated internal colonising fungi of both *ligushe* (67%) and *calabash* (46%).

Table 2.2: The total number of isolates and relative numbers (percentages) of *Penicillium Aspergillus, Fusarium and Alternaria* from internal structures of fresh samples of traditional *morogo*

Sample	No. of isolates	*Penicillium*	*Aspergillus*	*Fusarium*	*Alternaria*
Ligushe (fresh)	52	2(4)	1(2)	11(21)	35(>56)
Calabash (fresh)	13	2(>15)	2(>15)	3(23)	6(>46)

These results seem to be in agreement with findings of Blodgett *et al.* (2000) who demonstrated that *Alternaria* spp comprised the main component of endophytic fungi isolated from asymptomatic leaves and petioles of *Amaranthus.hybridus*. Secondary metabolites produced by *Alternaria alternata* have been shown to be involved in phytotoxicity of this fungus in a range of crop and weed species (Abbas & Boyette, 1992; Abbas *et al*, 1995). Many plant products used in human diets have been reported to frequently been infected by species of *Alternaria spp* capable of toxin production. Abbas & Riley (1996) demonstrated that plant pathogenic *Alternaria alternata lycopersici* causing stem cancer in tomato plants is capable of producing both AAL toxin and fumonisin B_1 (FB_1) *in vitro*. According to Bennett & Klich (2003), the presence of potential mycotoxin-producing fungi in crops could lead to mycotoxin problems particularly when handling and storage practices are conducive to mould growth. FB_1 and AAL toxin are structurally related and have been shown to cause disruption of sphingolipid metabolism in plant systems, cell culture and animal studies (Abbas *et al*, 1994; Turner *et al*, 1999). Data from animal and cell-culture studies suggested a role for the disturbance in the equilibrium of sphingolipid intermediates in the occurrence of mycotoxin-related carcinogenesis and certain immunosuppressing effects (Riley *et al*, 2001; Berek *et al*, 2001; Baumrucker & Prieschl, 2002). Members of the genera *Aspergillus*, *Penicillium* and *Fusarium* occurred in varying degrees among isolates obtained from internal structures of both fresh *ligushe* and *calabash* leaves.

Table 2.3: The folate contents of ligushe, calabash and cowpea

Crop	Folic acid ($\mu g.100mg^{-1}$)
Dried cowpea leaves (*Vigna unguiculata*)	107
Cooked calabash leaves mixed with flowers (*Lagenaria siceraria*)	52
Cooked fresh ligushe leaves (*Corchorus tridens*)	27

On the positive side it was found that some of these traditional *morogo* plants contained relatively high levels of folate. The highest folate content was reported in Table 3 for dried uncooked cowpea leaves (107μg.100mg^{-1}), while in cooked *calabash* leaves (mixed with flowers) folate measured 52μg.100mg^{-1} and in cooked *ligushe* 27 μg.100mg^{-1}. Although this was not determined, these values seem to indicate that cooking probably affects the folate content of *morogo*. Traditionally these plants are combined in one dish, and based on questionnaire information the daily intake of about 450mg of *morogo* could supply the adult consumer with more than 250μg folate. To decrease the risk for birth defects a daily supplementation of 400 μg folic acid is recommended for woman of child-bearing age. Folate has a critical role in the biosynthesis of DNA. Sufficient levels of cellular folate are therefore essential during the formation of new cells, including immune cells, and the maintenance of genomic stability (Kim, 1999; Ferguson *et al,* 2004). In sufficient quantities, dietary folate is associated with a decreased risk for heart disease and thrombosis (Tapiero *et al*, 2001; Das, 2003). According to Rampersaud *et al* (2002) there is also convincing evidence that fruit and vegetables, common sources of food folate in the diet, decrease the risk for many cancers. Other constituents commonly found in folate rich foods, including vitamin C, fibre and potassium, may also work alone or in conjunction with folate to decrease risk for chronic diseases. Food folates are concentrated in foods such as dried beans and peas, peanuts and dark green leafy vegetables. Based on these findings, it seems likely that from a staple diet consisting of *morogo*, beans and groundnuts, rural communities would derive some health benefits that are now associated with folate-rich foods.

Table 2.4: Percentage inihibition of daunomycin mutagenic activity in the presence of plant extracts

Plant extract	Daunomycin mutagenicity inhibition (%)
White bambara groundnut (*Vigna subterranean*)	47.5
Black Bambara groundnut (*Vigna subterranean*)	44.5
Rooibos tea (*Aspalathus linearis*)	81.4
Devil's claw (*Harpagophytum procumbens*)	97.6
Cancer bush (*Sutherlandia fructescens*)	48.8

Results depicted in Table 4 indicated that certain indigenous foods and medicinal plants widely used in Southern Africa might contain phytochemicals that exert antimutagenic and anticarcinogenic activity. Mutagens have been shown to act in initiating early stages of cancer development (Ferguson, 2002). Contamination of agricultural products with toxins produced by

strains of *Aspergillus, Penicillium,* and *Fusarium* has been identified as a significant mutagenic and carcinogenic hazard (Ferguson, 1999). Daumomycin mutagenicity was shown to be inhibited in the presence of plant extracts from devil's claw (97.6%), rooibos tea (81.4%), cancer bush (48.8%), and white and black Bambara groundnut (47.5% and 44.5% respectively). Figure 1 illustrates the effect of those plant extracts on tetradecanoyl-phorbol-3 acetate (TPA) induced expression of the COX-2 gene in mouse skin.

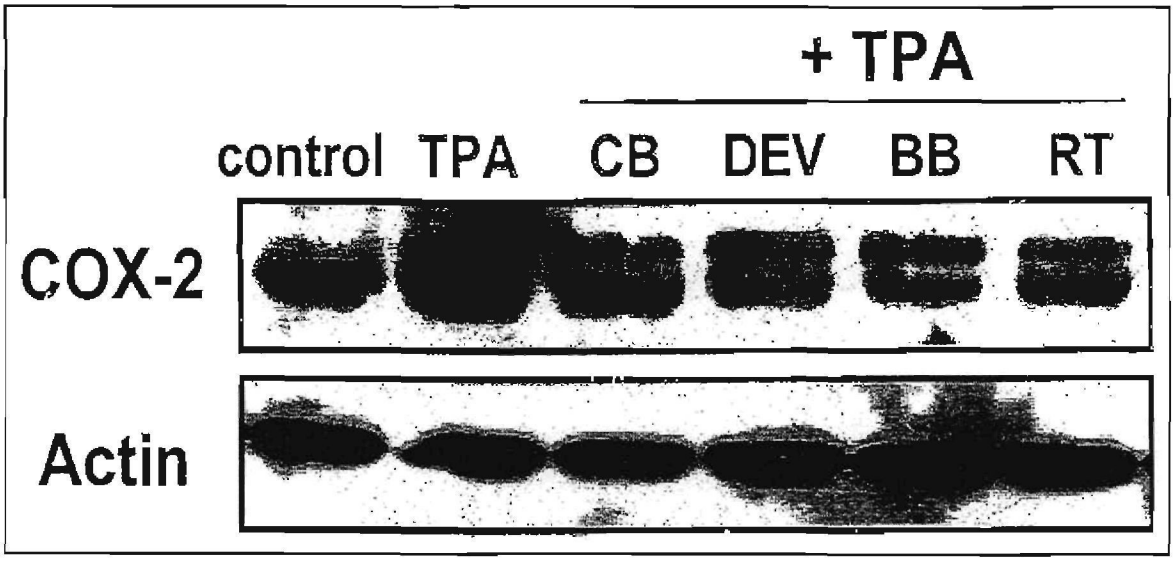

Figure 2.1 - Image of the Western blot gel illustrating the effect plant extracts on tetradecanoyl-phorbol-3 asetate (TPA) stimulated Cox-2 expression in mouse skin. CB – Cancer Bush; DEV – Devil's Claw; BB – Bambara Groundnut; RT – Rooibos tea

Mossanda and co-workers (2001) has already elucidated the molecular mechanism of the anti-carcinogenic and anti-inflammatory effects for two African traditional plants, namely cancer bush (*Sutherlandia fructescens*) and devil's claw (*Harpagophytum procumbens*). Used as a beverage (tea), these plants find application in the traditional treatment of cancer and inflammatory diseases respectively. Suhr and Ferguson (2003) reviewed a number of recent studies reporting on the chemopreventive potential of a variety dietary and medicinal antimutagens.

2.4 Concluding remarks

Dietary exposure to toxins produced by strains of *Aspergillus, Penicillium, Fusarium* and *Alternaria* could enhance the risk of cancer and influence immune cell functioning. In HIV/AIDS individuals these impacts could have important consequences. In addition to enhancing consumer resistance to disease, African food plants appear to possess nutrients and/or phytochemicals that could counteract some of the health-injuring effects of harmful dietary substances. In view of the

devastating outcome of chronic poverty, food insecurity and chronic disease in Africa, rural communities could reap long-term benefits from research supporting indigenous knowledge pertaining to the use of dietary safe, health-protecting and/or immune-strengthening indigenous food plants and promote the cultivation of such crops in cultural subsistence farming.

Mycological data reported above suggest that rural families employing traditional methods of vegetable cropping might be at risk of chronic dietary exposure to mycotoxins. Chapter 3 reports on the investigation of these risks in rural subsistence food production settings of the Limpopo Province.

Chapter 3

FUMONISIN-PRODUCING *FUSARIUM* SPECIES AND FUMONISINS IN TRADITIONAL AFRICAN VEGETABLES (*MOROGO*)

(South African Journal of Science, 2006, 102:151-155)

3.1 Introduction

Rural African communities generally supplement grain-based staple diets with traditional vegetables. Referred to in local African languages as *morogo,* traditional leafy vegetables constitute a diverse range of food-plants, some of which are cultivated for subsistence, while others may either grow as weeds in cultivated lands or as members of the natural field flora (Van Wyk & Gericke, 2000). Investigation revealed the presence of fumonisin-producing fusaria and indicated variable degrees of fumonisin contamination in household *morogo* of rural communities dependent on subsistence food production. In such settings, *morogo* usually occur in close proximity of homegrown maize which can be considered a likely source of fusarial contamination in the fields

The common and world-wide occurrence of toxigenic strains of *F. verticillioides*, *F. proliferatum*, *F. oxysporum* and *F. subglutinans* in commercial maize is well documented and African countries are no exception in this respect (Marasas, 1996; Kpodo *et al.*, 2000; Gamanya & Sibanda, 2001; Rheeder *et al.*, 2002; Fandohan *et al.*, 2003; Fandohan *et al.*, 2005). Globally distributed in different environments, these fungal species are recognised for their capacity to produce a range of potent fumonisin toxins in various agricultural crops (Placinta *et al.*, 1999; Soriano & Dragacci, 2004). Rheeder and co-workers (1992) reported the occurrence of fumonisin B-producing *Fusarium moniliforme* (now *F. verticillioides)* strains in homegrown maize in the Transkei-region of the Eastern Cape, South Africa. Epidemiological, animal and cell culture studies have linked dietary fumonisin B produced by grain-associated *Fusarium* strains to various human diseases, including various forms of organ toxicity and cancer (Ferguson, 2002; Carratù *et al.*, 2003). Other studies reported fusarial strains as aetiological agents of secondary infections in immunocompromised individuals (Guarro *et al.*, 2000; Vismer *et al.*, 2002; Dignani & Anaissie, 2004). According to Munkvold and Desjardins (1997), toxigenic *F. verticillioides* strains produce abundant airborne microconidia (spores) in

crop residue from where they are likely to become widely disseminated. Reviewing the problem of dietary mycotoxins, Bennett and Klich (2003) reported that mycotoxin exposure is more likely to occur in parts of the world where inadequate methods of food handling and storage are common and where there are few regulations to protect exposed populations. Subsistence foods are not subject to quality control legislation, and traditional methods of food storage in resource-limited rural communities could enhance dietary risks associated with microbiological contamination of food. Generally not too fastidious about food quality in times of food shortages, chronically poor rural households might be exposed to dietary microbiological risks that would further add to their burden of disease.

The present study reports on the occurrence of fumonisin-producing *Fusarium* strains and varying amounts of fumonisin B in traditionally dried as well as freshly collected household *morogo* from three geographically separate study areas in the northern parts of South Africa.

3.2 Materials and Methods

Sample Collection. For mycological analysis, four plants of two different types of traditional *morogo* were collected from each of five collection sites, namely, a stall[4] in town, a scheme farmer[5], two subsistence[6] farmers and a village household[7] in Giyani, situated in north-eastern region of the Limpopo province in South Africa. At the collection site, samples of *Corchorus* cf. *trilocularis* (*ligusha*) and cf. *Lagenaria siceraria* (*calabash*) were transferred to plastic bags, sealed and transported to the laboratory in a cooled container. To isolate internal as well as external fungal colonisers, five leaves were randomly selected from each plant. Samples for fumonisin analysis included traditionally dried household *morogo*[8] from the Dikgale Demographic Surveillence Site (DDSS; Capricorn District, Limpopo Province) as well as freshly collected oven-dried *morogo*[9] from the Nzhelele Valley (Thohonyandou district, Limpopo), DDSS and Rustenburg (North-West province). *Morogo* samples for fumonisin analysis consisted either of a single plant species, a traditional mixture[10] of two plant species or a laboratory-combined mixture of more than two plant species.

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[4] Informal selling points of traditional food
[5] Traditional food cultivated in small schemes employing irrigation, pest control and chemical soil improvement
[6] Traditional food for household use cultivated without irrigation, pest control and chemical soil improvement
[7] Traditional food produced in a village garden for household use
[8] Freshly harvested household *morogo* cooked prior to being sun-dried on open surfaces, e.g corrugated iron.
[9] Freshly collected *morogo* oven-dried upon arrival at laboratory
[10] Different types of traditional *morogo* mixed and cooked together, sometimes including flowers

Plant identification. Fresh and herbarium-prepared specimens of *morogo* plants used in the present investigation were sent to the South African National Biodiversity Institute (SANBI) for scientific species identification.

Fungal isolation and identification. Standard procedures were employed for the isolation of *Penicillium, Aspergillus, Fusarium and Alternaria* strains from *morogo* samples. To optimise the isolation of fungi from internal leaf structures, fresh *Corchorus* cf. *trilocularis* and cf. *Lagenaria siceraria* leaves from the rural district of Giyani were subjected to surface sterilisation (Osono & Takeda, 1999; Suryanarayanan *et al.*, 2003) after which leaves were allowed to dry in sterile containers. Four pieces of approximately $2mm^2$ each were aseptically cut from each of the surface-sterilised leaves and transferred to Corn Meal Agar plates (Oxoid, South Africa) containing $150mg.L^{-1}$ tetracycline (ICN, Tetracycline hydrochloride, Separations, South Africa). For the isolation of surface-associated fungi, a standard mycological washing procedure with sterile 1% peptone water (Biolab, Merck, South Africa) containing 0.01% Tween 80 was employed to remove fungi from the external leaf surfaces (Medina-Martínez & Martínez, 2000). Following 10 minutes of shaking at 25°C to wash off any surface colonisers into the diluent, serial dilutions were prepared using the same diluent. Subsequent surface plating was carried out in duplicate using 0.1 ml aliquots from each dilution onto each of the following culture media to which bacteria-inhibiting substances were added in the concentration indicated: Potato Dextrose Agar (Biolab, Merck, South Africa) containing 5% sodium chloride (UniLAB, Saarchem, South Africa) and 40 $mg.L^{-1}$ oxytetracycline (ICN, Oxytetracycline hydrochloride, Separations, South Africa), Malt Extract Agar plates (Biolab, Merck, South Africa) containing $150mg.L^{-1}$ tetracycline (ICN, Tetracycline, hydrochloride, Separations, South Africa) and Rose Bengal Chloramphenicol Agar plates (Biolab, Merck, South Africa). Inoculated agar plates were incubated at 25±1°C for a minimum of 7 days. After incubation, mould colonies on plates of the three highest dilutions of each sample were separately transferred to Potato Dextrose Agar plates (Biolab, Merck, South Africa). Single spore cultures of each colony were subsequently prepared on 1,5% water agar (Agar Bacteriological, Biolab, Merck, South Africa). Isolates were identified to genus level by microscopic observation using the taxonomic keys of Nelson *et al.* (1983) Isolates identified as members of the genus *Fusarium* were submitted to the Biosystematics Division of the Plant Protection Research Institute of the Agricultural Research Institute (ARC- PPRI) for species identification.

Fumonisin analysis. Dried *morogo* samples were submitted to [‡]PROMEC for quantification of fumonisins by HPLC-FD and subsequent confirmation by liquid chromatography-mass spectophotometry.

3.3 Results and Discussion

The South African National Biodiversity Institute (SANBI) identified the following plant species used as traditional *morogo* by rural households in the respective study areas: (i) *Corchorus* cf. *trilocularis* L.; *Corchorus asplenifolius* aff. Burch; (ii) cf. *Lagenaria siceraria*; (iii) *Cleome gynandra* L; (iv) *Amaranthus hybridus* L subsp. *hybridus* var. *hybridus*; *Amaranthus thunbergii* Moq; *Amaranthus thunbergii* sensu Suess. & Podlech; (v) *Chenopodium morale* L. var. *morale*; (vi) *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*; (vii) *Sonchus wilmsii* R.E.Fr.; *Sonchus oleraceus* L.; (vii) *Brassica rapa*; *Brassica juncae* (L.) Czern. & Coss.; (viii) *Bidens pilosa* L.; *Bidens biternata* (Lour.) Mer. & Sheriff; (ix) *Pisum savitum* L. subsp. *savitum*; (x) *Malva parviflora* L. var. *parviflora*; (xi) *Curcurbita* species. According to information gained by questionnaire, some of these plant species are cultivated for subsistence, while others grow as weedy plants in cultivated lands or as members of the natural field flora of the region. Different parts of plants may be consumed. However, leafy parts (sometimes of different species mixed together often also including some flowers) are cooked as a tasty green vegetable relish to be served with maize porridge or cooked legumes.

The washing procedure yielded 79 *Fusarium* isolates that were subsequently demonstrated be the dominating genus among surface-associated isolates which also included *Aspergillus niger* (3), *Aspergillus sydowii* (1), *Penicillium aurantiogreseum* (2) and *Alternaria* sp (3). *Fusarium* species were absent those fungi retrieved from internal leaf structures that predominantly *Alternaria* species (34), although a *Aspergillus* sp. (1) and *Penicillium* sp. (2) also occurred.

Of the 79 *Fusarium* isolates retrieved from Giyane *morogo*, 65 were eventually identified by the ARC-PPRI. The 14 other original isolates either did not grow after sub-culturing, mutated in the process or subsequently formed atypical structures that made morphologically based identification impossible. Identified isolates were assigned PPRI accession numbers and are being preserved in the National Collection of Fungi for subsequent studies. Of interest were

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results illustrated in Figure 1 showing that species most frequently recovered from the *morogo* were *F. proliferatum* (21 isolates) and *F. verticillioides* (19).

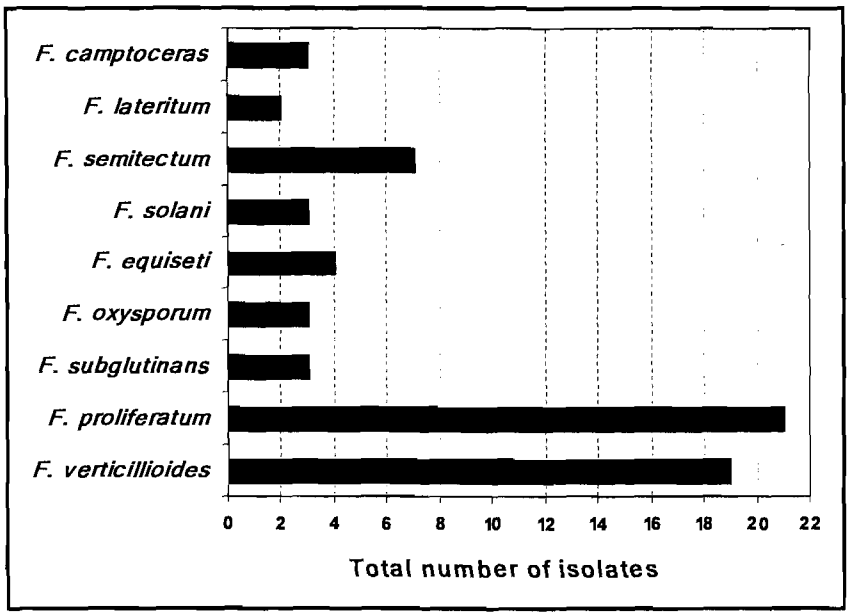

Figure 3.1: Relative numbers of the respective *Fusarium* species isolated from *morogo*

Widely distributed and occurring frequently in maize, *F. verticillioides* and *F. proliferatum* are listed by Rheeder *et al.* (2002) as the most important producers of fumonisins. Occurrence of *F. verticillioides*, *F. proliferatum*, *F. subglutinans* (3), and *F. oxysporum* (3) in association with traditional *morogo* is noteworthy for the fact that these fumonisin-producing strains constituted 71% of the total number of isolates. Scientific data regarding fungal populations associated with traditional *morogo* and the occurrence of toxigenic *Fusarium* species in traditional vegetables is severely lacking. Though this study was conducted on a small scale, our results raise questions, firstly about the dietary safety of traditional staple vegetables for rural consumer populations dependent on subsistence vegetable crops, and secondly, about the source of fusarial contamination.

Results depicted in Figure 2 illustrate that *F. verticillioides*, *F.subglutinans* and *F. oxysporum* occurred more frequently in association with *ligusha*, whereas *F. proliferatum* .strains were predominantly recovered from *calabash.*

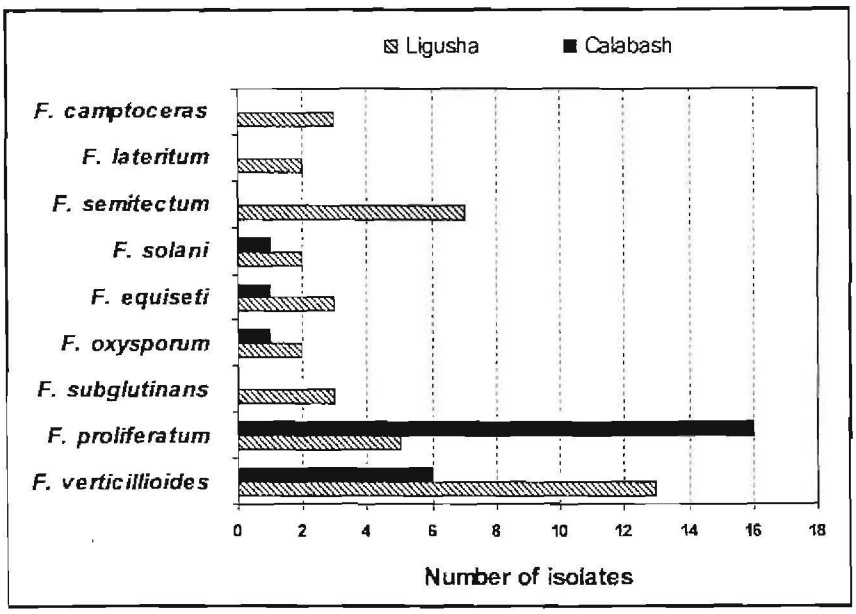

Figure 3.2: The relative distribution of different *Fusarium* species in ligusha and calabash respectively

Since these toxigenic fusaria apparently occurred in a non-pathogenic relationship with *morogo* plants (they were recovered fro surfaces of leafs without disease symptoms), the relative distribution of the different species might be meaningful in terms of the possible source of vegetable contamination. *Fusarium* infection and post-harvest contamination of maize reportedly constitute a worldwide problem causing crop loss and deterioration of stored food (Marasas *et al.*, 1979; Logrieco and Moretti, 1995; Kedera *et al.*, 1999; Soriano & Dragacci, 2004). Reviewing fumonisin contamination of maize, Munkvold and Desjardins[16] described *F. moniliforme* (now *F. verticillioides*) as an almost constant companion of maize plants and seeds, commonly found colonising symptomless maize plants. In a recent study by Fandohan and co-workers (2005) found *F. verticillioides* and *F. proliferatum* to be the two most commonly occurring *Fusarium* species in pre-harvested as well as stored maize of small-scale farmers in four agro-ecological zones of Benin. Closer to home, Rheeder *et al.* (1992) reported the frequent occurrence of *F. verticillioides* (formerly *F. moniliforme*) in 'visibly nonmouldy' homegrown maize of rural communities in the Transkei region.

Figure 3 is a photograph of one of the Giyane sampling sites showing *ligusha* growing among dry homegrown maize (*Zea mays L.*) on land where vegetables are collected either for consumption or storage after being processed.

Figure 3.3: *Ligushe* (indicated by arrows) growing as weeds in maize fields of a rural community of Gyani, Limpopo Province

F. verticillioides reported to occur ubiquitously in soil and maize crop residue (Munkvold & Desjardins, 1997), in this case might be the source of vegetable contamination when airborne spores spread to agricultural lands. Though a cultivated crop, in rural subsistence settings *calabash* usually grow in close proximity of maize lands. Leslie *et al.* (2004) reported that the range of *Fusarium* species found in native prairie grasses in Kansas, paralleled that which were typically recovered from maize or sorghum growing in the adjacent areas. Among these were *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. Furthermore, these grass-associated fusaria were found to generate toxins in quantities similar to those produced by isolates of the same species recovered from agricultural hosts. According to Rheeder *et al.* (2002), *F. verticillioides* and *F. proliferatum* are the most important producers of fumonisins because of their overall high levels of toxin production, their wide geographical distribution and frequent occurrence on maize.

In another study Bezuidenhout and co-workers (2006) evaluated the application of a multiplex PCR-based method for the rapid detection of potential fumonisin-producing *Fusarium* in traditional African vegetables using six *Fusarium* isolates from the Giyane *morogo*, namely four *F. verticillioides* (PPRI 7363, PPRI7367; PPRI7370, PPRI 7372) and one *F. subglutinans*

(PPRI 7365). A fumonisin-positive MRC *Fusarium verticillioides* (MRC 4319) strain was included as a reference culture. Primer sets employed for the purpose of the study targeted the following conserved gene fragments: (i) the polyketide synthase *FUM1* gene involved in fumonisin biosynthesis; (ii) the translation elongation factor 1-alpha (TEF 1-α) of *Fusarium* spp and (iii) a conserved region of fungus 18S mRNA. These fragments were all of distinct sizes and products of the multiplex PCRs could be resolved using 2 % (w/v) agarose gels. These molecular results confirmed strains retrieved from Giyane *morogo* did belong to the genus *Fusarium* and also that isolates possessed the polyketide synthase *FUM1* gene for fumonisin biosynthesis. However, the expression of genes encoding fumonisin production and regulation is expected to be influenced by complex interactions of biotic and abiotic environmental factors. In this respect, predisposing conditions influencing fumonisin biosynthesis and accumulation in traditional leafy vegetables have not been investigated or verified and it is as yet unclear whether such conditions exist pre- or post-harvest. Since they are secondary metabolites, mycotoxins are formed towards the end of exponential growth stage. In rural settings, households generally lack refrigeration facilities for food storage and so it is expected that post-harvest senescence of fusarial-contaminated crops and subsequent storage would be risk factors for fumonisin production and accumulation in traditional crops.

This seems to be confirmed by results of chemical analysis of household *morogo* sampled from different study areas reported in Table 1. Results in Table 2 compare fumonisin B1 levels in sun-dried household *morogo* and freshly collected oven-dried (in the laboratory) samples from four villages of the Dikgale Demographic Surveillence Site (DDSS) in the Limpopo province. The fumonisin B1 concentration in sun-dried *Cleome gynandra* L. from Madiga was three times that of the Moduane sample. In the fresh *Cleome gynandra* L. of Moduane the fumonisin B1 concentration was 3.4 times that of the sun-dried vegetable collected from the same site. In *Amaranthus thunbergii* Moq. sample of Mantheding this observation was reversed and fumonisin B1 was not detected in the fresh sample. Nevertheless, fumonisin contamination of *morogo* samples is indicated but apparently was neither related to plant species nor to geography. An adult is reported to consume on average about 300g *morogo* per meal which is on a daily basis when *morogo* is available (unpublished questionnaire information). In addition, subsistence maize probably is contaminated as well. The exposure risk implied by this finding would have important consequences in poor rural communities dependent on subsistence staple vegetables.

Table 3.1: Fumonisin B1 in traditional household *morogo* from different regions

Sample	Collection site	Fumonisin B1 ($ng.g^{-1}$)
[1]Combined sample	DDSS	224
[2]Combined sample	Rustenburg	114
[3]Combined sample	Rustenburg	n.d
Amaranthus cruentus	Rustenburg	45
Cleome gynandra	Rustenburg	59
Cucurbita sample	Nzhelele Valley	66
Brassica rapa	Nzhelele Valley	n.d

[1] Oven-dried senescent sample of *Amaranthus thunbergii, Corchorus tridens* and *Cleome gynandra*

[2] Oven-dried senescent sample of *A. hybridus* subsp. *cruentus, C. gynandra, Momordica balsamina* and *Pentarrhinum insipidum*

[3] Oven-dried freshly collected sample of *Chenopodium album, Sonchus wilmsii* R.E.Fr. and *Corchorus schimperi* Cufod.

Table 3.2: Levels of fumonisin B-group toxins in sun-dried and freshly sampled oven-dried household *lerotho* and *thepe* growing close to maize

Sample	Village	Fumonisin B1 ($ng.g^{-1}$)
[1]*Cleome gynandra* L. *and Amaranthus thunbergii* sensu Suess & Podlech.	Sefateng	25.0
[1]*Cleome gynandra* L.	Madiga	46.2
[1]*Amaranthus thunbergii* Moq.	Mantheding	53.0
[2]*Amaranthus thunbergii* Moq.		nd
[1]*Cleome gynandra* L. *and Amaranthus thunbergii* Moq.	Moduane	26.4
[1]*Cleome gynandra* L.		14.5
[2]*Cleome gynandra* L.		48.8

[1] Sun-dried household samples

[2] Oven-dried freshly collected sample

Epidemiological evidence and animal studies have linked dietary fumonisin B_1 (FB_1) to various human disease conditions. FB_1 was evaluated as a Group 2B carcinogen indicating that this mycotoxin is probably carcinogenic to humans (International Agency for Research on Cancer, 1993). Studies by Lerda *et al.* (2005) suggested that increased oxidative stress induced by FB_1 exposure can damage DNA indirectly, thus acting as a mechanism for FB_1-associated hepatotoxicity. Furthermore, reported FB_1-induced lipid peroxidation that affects cellular membranes, mitochondria and DNA synthesis, as well as FB_1 disruption of the metabolism of sphingolipids that are important components of all eukaryotic cellular membranes, collectively constitute mechanisms through which this agent might impair cellular viability (JECFA, 2001; Van der Westhuizen *et al.*, 2004). Sphingolipid intermediates, as well as complex sphingolipids, have been shown to be important as signal-transducing molecules involved in control of cell proliferation, survival, differentiation and apoptosis (Colombaioni & Garcia-Gil, 2004). Evidence that interference with sphingolipid metabolism disrupted membrane receptor sites involved for cellular uptake of folate also exists (Kim *et al.*, 1997; Carratù *et al.*, 2003). Because folate is essential for DNA biosynthesis and methylation (Wagner, 1995), folate deficiency might result in DNA damage and instability as well as aberrant patterns of DNA methylation, all of which are considered to be factors in carcinogenesis (Kim *et al.*, 1997).

Disruption of sphingolipid metabolism also influences immune cell proliferation and essential intra- and intercellular communication between various types of immune cells, thereby affecting their activation status (Baumrucker & Prieschl, 2002). Animal and cell culture studies have indicated that other immune functions may also be affected by FB_1. Processes involved in the destruction of infecting bacteria, such as phagocytosis and oxidative burst in neutrophils, were shown to be regulated by sphingolipid intermediates (Baumrucker & Prieschl, 2002; Theumer *et al.*, 2003). Moreover, a number of *Fusarium* species, including *F. verticillioides*, *F. oxysporum* and *F. solani* have been implicated as causative agents of secondary infections in immuno-suppressed individuals (Dignani & Anaissie, 2004; Dornbusch *et al.*, 2004).

3.4 Concluding remarks

Based on the 2004 survey, the Department of Health estimated the prevalence of HIV infection among adults and children of the Limpopo and North-West Provinces at 9.8% and 10.3% respectively, and among antenatal attendees 19.3% and 26.7% (Department of Health, 2005; Centre for Actuarial Research, 2005). Given these statistics, the presence of harmful fusarial

strains in rural subsistence agro-environments is an aggravating health risk factor in poor communities dependent on traditional vegetables. This is so whether these fungi occur as food-related producers of mycotoxins exerting diverse biochemical effects, or as aetiological agents of secondary infections. The sources and routes of mycotoxigenic and mycotic *Fusarium* contamination in poor rural areas, as well as predisposing environmental factors for fumonisin production in traditional crops and vegetables, are being investigated by the *Morogo* Research Program (MRP).

The incidence of *Fusarium* in subsistence food-production environments and the mycotoxigenic and mycotic risks associated with their presence, were also investigated in a peri-urban environment where many resource-poor households resort to home-gardening to augment their food supplies. Chapter 4 reports on findings of this investigation.

Chapter 4

FUSARIUM POPULATIONS IN THE HOUSEHOLD FOOD-GARDEN ENVIRONMENT OF A PERI-URBAN COMMUNITY

(South African Journal of Science, 2007, 103:504-508)

4.1 Introduction

The genus *Fusarium* includes various phytopathogenic species of economic importance, mainly for the damage they cause to commercial crops (Munkvold & Desjardins, 1997). Invasion of plant hosts is often linked with the capacity of the fungal pathogen to produce toxins (Bennett & Klich, 2003). Dietary exposure to fusarial toxins causes irreversible tissue damage through biochemical mechanisms that produce pro-oxidative, pro-inflammatory, carcinogenic and / or immune-suppressive effects at a cellular level (Baumrucker & Prieschl, 2002; Gelderblom *et al.*, 2004; Kouadio *et al.*, 2005; Domijan *et al.*, 2007). Some toxigenic *Fusarium* species have furthermore been implicated as causative agents of life-threatening opportunistic infections in immune-suppressed individuals (Nelson *et al.*, 1994). Mortalities ranged between 50 – 80% in these cases, mainly because effective treatment of infection was complicated by drug resistance by the *Fusarium* pathogens and their blood-borne spread to various organs of the body (Dignani & Anaissie, 2004).

Peri-urban families living on a severely restricted budget in South Africa often resort to growing food at home to augment their food supply. Maize grown as the traditional staple in these situations is supplemented with green leafy vegetables (called *morogo*), often collected from the field (Jansen van Rensburg *et al.*, 2007). Some *morogo* species also appear spontaneously in disturbed soils and consequently are found growing among the maize (Modi *et al.*, 2006; Odhav *et al.*, 2006). Maize ecosystems naturally harbour several toxigenic *Fusarium* species, however, notably those producing fumonisin toxins (Munkvold & Desjardins, 1997; Fandohan *et al.*, 2003; Fandohan *et al.*, 2005). *Fusarium* where food is grown has important health implications, particularly for food-insecure populations that, according to Bourne *et al.* (2002), are disproportionately affected by disease because of their unsatisfactory nutritional status.

We conducted a pilot study in a peri-urban community in the Rustenburg district of South Africa to determine the occurrence of nine *Fusarium* species in the vicinity of household food

gardens and the influence of home-grown maize nearby. We considered the factors that may contribute towards sustaining *Fusarium* species in such environments and discuss the possible health implications for local communities.

4.2 Materials and Methods

Sample collection. Samples were collected from food gardens of selected households at four localities in the peri-urban township of Phokeng near Rustenburg during the maize-growing season in February and April 2006 as well as February 2007. Two naturally-growing *morogo* types commonly utilised in the study area, namely thepe (*Amaranthus hybridus*) and lerotho (*Cleome gynandra*) were sampled at 4 localities where *morogo* vegetables were growing with maize on the same plot of land, and four localities where *morogo* plants were growing in the field at a distance away from maize. At each locality 10 leaves were collected from two separate plants each of thepe and lerotho. Five leaves of a maize plant as well as a maize cob were also sampled. At each locality samples were taken of the top soil layer at three random positions around each of the plants. Three agar plates containing pentachloronitrobenzene (PCNB) medium selective for *Fusarium* were exposed at three random positions around each plant for three minutes to trap *Fusarium* spores from the air. Air plates were immediately closed and secured with parafilm. Thepe, lerotho, soil and maize samples were transferred separately to 'ziplock' plastic bags. Samples were transported to the laboratory on ice and upon arrival immediately processed for mycological analysis.

Cutlure media for isolation and morphological identification of *Fusarium*. A culture medium selective for the isolation of *Fusarium* containing Peptone PCNB (Terraclor, Sigma, South Africa) to which were added the following antibiotics for inhibition of bacterial growth: benzylpenicillin (Fresenius, South Africa), pendistrep (Virbac Animal Health, South Africa) and chloramphenicol Pure (Pharmachemie, South Africa). For the purification and preparation of single spore cultures of *Fusarium* isolates the following growth media were used: Water Agar plates (WA; Agar Bacteriological, Biolab, Merck, South Africa) and Carnation Leaf Agar (CLA; consisting of a Water Agar plate with a piece of carnation leaf γ-sterilised by Isostar, South Africa, placed on the surface). For species identification, single spore cultures were subsequently transferred to the following culture media: for observation and distinction of colony morphology to Potato Dextrose Agar (PDA; Biolab, Merck, South Africa); for observation of distinctive microscopic structures to Carnation Leaf Agar (CLA) plates and Synthetic Nutrient Agar (SNA; Sigma, South Africa). Culture media were prepared as described in the laboratory manual of Nelson *et al.* (1983).

Isolation of *Fusarium*. For the isolation of *Fusarium* colonising external surfaces of *morogo* leaves, maize leaves and kernels, the washing procedure described by Medina-Martinez & Martinez (2000) was applied. Each leaf and kernel was separately added to 99 mL of sterile 1% peptone water (Biolab, Merck, South Africa) containing 0.01% Tween 80 and shaken for 10 min on a rotary shaker at room temperature to remove spores from leaf surfaces. Diluents containing the rinsed-off *Fusarium* spores were subsequently diluted (10^{-3} to 10^{-5}) and 0.1 mL aliquots of each dilution were used for spread plating onto PCNB agar. Internal colonising *Fusarium*, was isolated by sterilising leaf and kernel surfaces in 1% hydrogen peroxide for 1 min. followed by aseptical rinses in distilled water three consecutive times (Jivan, 2006). Employing aseptic procedures, 5 squares of approximately 1 cm^2 cut from each *morogo* leaf, and 10 pieces from each maize leaf, were transferred separately to the surface of a separate PCNB agar plate. After surface sterilisation according to similar procedure, ten maize kernels from each maize cob were each placed on a separate PCNB agar plate. The three soil samples from each sampling site were thoroughly mixed at the laboratory into a composite soil sample of which 1 g was carefully weighed and transferred to 9 mL sterile distilled water (10^{-1} dilution). A dilution series of 10^{-2} to 10^{-5} was subsequently aseptically prepared and 0.1 mL aliquot of each dilution used for surface plating onto PCNB agar. All plates (including parafilmed air plates) were subsequently incubated at 25°C for a minimum of 7 days.

Purification of *Fusarium* colonies. After incubation, plates were examined under a stereo-microscope. Colonies suspected of being *Fusarium* were selected on basis of characteristics described by Nelson *et al.* (1983) and purified according to procedures described in this manual. A 1 cm^2 piece of PCNB agar containing the selected colony was aseptically cut from the PCNB plate and transferred to a CLA plate. After incubation of 7 to 10 days at 25°C during which time plates were exposed to a 12:12 hour light/dark cycle, single-spore cultures were prepared by flooding each plate with 9 mL of sterilised distilled water, and pouring the mixture aseptically over the surface of a WA plate. The WA plates were carefully rotated and the excess water was drained off before being incubated at 25°C in an inclined position. After 16-24 hours of incubation, WA plates were examined under stereomicroscope and a piece of agar with a single germinating spore was aseptically transferred to the surface of each of a PDA, CLA and SNA plate. Inoculated plates were incubated at 25°C for 7-14 days.

Morphological identification of *Fusarium* isolates. After incubation, single spore cultures of *Fusarium* isolates growing on the PDA, SNA and CLA were used for morphological identification based on colony and microscopic characteristics according description of the illustrated laboratory manuals of Nelson *et al.* (1983) and Leslie & Summerell (2006).

Macroscopic observations of single-spore colonies growing on PDA plates included colony morphology, colony colour, as well as the presence and position of the sporodochia. Using lactophenol for slide preparation, microscopic observations were based on characteristics (e.g. position, size, shape, arrangement) of microconidia, macroconidia and chlamydospores of single spore cultures on CLA plates. Characteristics were carefully recorded and used for identification according to identification keys in Nelson *et al.* (1983) and Leslie & Summerell (2006).

Statistical methods. Statistica 7.1 was employed to subject numbers of *Fusarium* isolated from the various environmental components to two-way and three-way analyses of variance (ANOVA) using the following: counts for each species from each environmental component in localities respectively near maize and away from maize, were log-transformed (i.e. 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 regards to three factors namely species, component and distance from maize (i.e. near or away from maize). Statistically significant effects on a 5 % level for each of these factors can be concluded whenever no significant interactions existed and the P-values are smaller than 0.05. Where appropriate, Tukey post hoc comparisons were performed between mean values of the different components and species. In cases of significant interaction effects with distance from maize, Student-t tests (assuming unequal variances) were applied to compare means of distance with means for each species or for each component (Snedcor & Cochran, 1980).

4.3 Results

Figure 4.1 illustrates the combined isolate number of each *Fusarium* species retrieved from samples collected during three sampling occasions from four localities at sites near and away from maize respectively. Results show all nine species occurred in various degrees in localities where maize was growing with the number of isolates ranging from 6 (*F. equiseti*) to 130 (*F. verticillioides*). In localities away from where maize was growing, the number of isolates was notably lower varying between 3 (*F. poae*) and 34 (*F. verticillioides*). *F. equiseti, F. chlamydosporum* and *F. semitectum* were neither retrieved from localities away from maize.

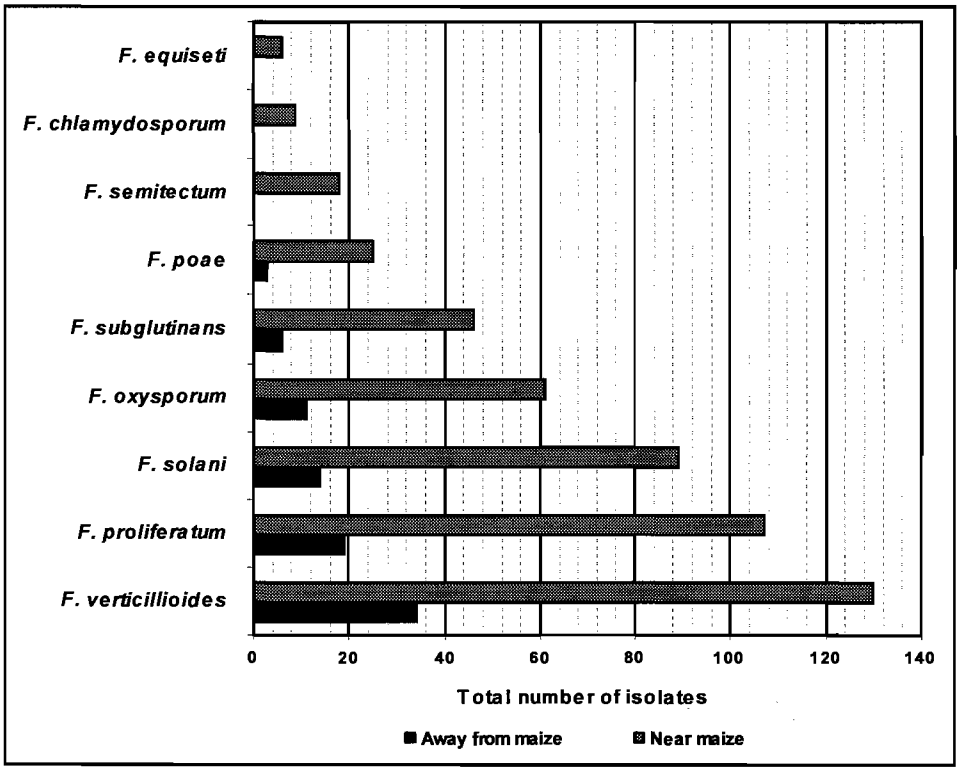

Figure 4.1: *Fusarium* species distribution among the total number of isolates respectively retrieved from localities near and away of maize

Results in Table 4.1 also show this trend for isolates retrieved from air (P<0.0001), soil (P<0.0001), lerotho (P=0.0005) and thepe (P=0.0001), with the number of *Fusarium* isolates from localities near maize significantly higher in comparison with localities away from maize.

Table 4.1: Relative occurrence of *Fusarium* at localities respectively near and away from maize

Environmental component	Near maize Mean (±SE)	Away from maize Mean (±SE)	t	df	P
Air	0.58±0.1	0.15±0.05	4.49	56.5	<0.0001
Soil	0.51±0.08	0.12±0.05	4.31	56.7	<0.0001
Lerotho	0.41±0.06	0.14±0.04	3.70	62.5	0.0005
Thepe	0.26±0.05	0.04±0.02	4.42	46.2	0.0001

Mean log-numbers of *Fusarium* isolated from localities near maize were highest in air (0.58±0.1) and soil (0.51±0.08) and lower in lerotho (0.04±0.06) and thepe (0.26±0.05). Indicating the relative numbers of the various *Fusarium* species, results in Table 4.2 show that, on a 5 % level, all species were isolated in significantly higher numbers from localities near maize in comparison with localities away from maize. Mean log numbers further indicate that *F. verticillioides* (0.85±0.9) and *F. proliferatum* (0.78±0.09) were the predominant species retrieved from localities near maize, followed by *F. solani* (0.69±0.1), *F. oxysporum* (0.52±0.1) and *F. subglutinans* (0.4±0.11). Away from maize, mean log-numbers for the respective *Fusarium* species ranged from 0.04±0.04 (*F. semitectum*) to 0.24±0.07 (*F. proliferatum*) and 0.3±0.1 (*F. verticillioides*).

Tukey post hoc comparison of *Fusarium* species numbers respectively retrieved from each of the different environmental components is shown in Table 4.3. Means of species log-numbers indicate, on a 5 % level, that *F. verticillioides* (0.83), *F. proliferatum* (0.66) and *F. solani* (0.61) were isolated from the air in numbers significantly higher than *F.poae* (0.16), *F. equiseti* (0.13) and *F. chlamydosporum* (0.08). *F. semitectum* (0) were not retrieved from air samples. Similar results are shown for species means of *F. verticillioides* (0.71) and *F. proliferatum* (0.68) isolated from the soil. *F. equiseti* were retrieved from neither soil nor lerotho. No significant difference was found for species means of isolates from lerotho or thepe. However, in comparison with thepe, mean log numbers indicate *F. verticillioides* (0.83) and *F. oxysporum* (0.43) were retrieved in significantly higher numbers from air. For the remainder of the species, no significant difference was found.

A total of 150 *Fusarium* isolates were retrieved from the silk (69) and kernels (81) of maize cobs sampled from the four localities. Two-way analysis of variance (ANOVA) was conducted to determine interaction between maize cob components (i.e. leaves, silk and kernels) and *Fusarium* species (Table 4.4). No *Fusarium* was isolated from maize leaves and, on a 5 % level, no significant interaction (P = 0.2491) was obtained for kernels, silk and the *Fusarium* species isolated from them. However, means of species log numbers indicate that *F. semitectum* (0.23), *F. equiseti* (0.04) and *F. chlamydosporum* (0.04) were retrieved from maize kernels and silk in numbers significantly lower in comparison with *F. proliferatum* (0.79) and *F. verticillioides* (0.75). Of the maize-associated species, *F. subglutinans* (0.32) was isolated from kernels and silk in numbers significantly lower than *F. proliferatum.*

Table 4.2: Relative *Fusarium* species distribution at localities respectively near and away from maize

Fusarium species	Near maize Mean (±SE)	Away from maize Mean (±SE)	t	df	P
F. verticillioides	0.85±0.9	0.3±0.1	4.18	29.5	0.0001
F. proliferatum	0.78±0.09	0.24±0.07	4.57	28.7	<0.0001
F. solani	0.69±0.1	0.15±0.07	4.33	27.6	0.0002
F. oxysporum	0.52±0.1	0.14±0.06	3.15	25.1	0.0021
F. subglutinans	0.4±0.11	0.09±0.05	2.60	20.7	0.0085
F. poae	0.25±0.08	0.05±0.03	2.21	19.8	0.0194
F. semitectum	0.22±0.07	0.04±0.04	2.28	22.5	0.0162
F. chlamydosporum	0.15±0.05	0	2.88	15.0	0.0057
F. equiseti	0.11±0.04	0	2.53	15.0	0.0115

Results show trends of a significant higher number of *Fusarium* isolates from localities near maize (Figure 4.1). Mean log numbers of isolates respectively retrieved from air, soil, thepe and lerotho indicate the same trend (Table 4.1) which is demonstrated in Table 4.2 for each of the nine *Fusarium* species. Relative to *F. poae, F. equiseti, F. chlamydosporum* and *F. semitectum*, the number of isolates of the maize pathogenic species *F. verticillioides* and *F. proliferatum* were significantly higher in air and soil (Table 4.3) and, when compared to numbers of *F. equiseti, F. chlamydosporum* and *F. semitectum*, also in maize cobs (Table 4.4).

Table 4.3: Relative *Fusarium* species distribution respectively in air, soil, lerotho and thepe

Component	Spec*Dist interact*	*F. verticillioides*	*F. proliferatum*	*F. solani*	*F. oxysporum*	*F. subglutinans*	*F. poae*	*F. semitectum*	*F. equiseti*	*F. chlamydosporum*
Air	0.0280	$^{d}0.83^{a}$	$^{d}0.66^{a}$	$^{d}0.61^{a,b}$	$^{d}0.43^{a,b,c}$	$^{d}0.4^{a,b,c}$	$^{d}0.16^{b,c}$	$^{e}0^{c}$	$^{d}0.13^{c}$	$^{d}0.08^{c}$
Soil	0.0514	$^{d,e}0.71^{a}$	$^{d}0.68^{a}$	$^{d}0.45^{a,b}$	$^{d}0.45^{a,b}$	$^{d}0.28^{a,b}$	$^{d}0.11^{b}$	$^{d,e}0.09^{b}$	$^{e}0^{b}$	$^{d}0.06^{b}$
Lerotho	0.7722	$^{d,e}0.5^{a}$	$^{d}0.42^{a,b}$	$^{d}0.38^{a,b}$	$^{d,e}0.41^{a,b}$	$^{d}0.12^{a,b}$	$^{d}0.23^{a,b}$	$^{d}0.33^{a,b}$	$^{e}0^{b}$	$^{d}0.06^{a,b}$
Thepe	0.8088	$^{e}0.26^{a}$	$^{d}0.27^{a}$	$^{d}0.23^{a}$	$^{e}0.04^{a}$	$^{d}0.17^{a}$	$^{d}0.1^{a}$	$^{d,e}0.1^{a}$	$^{d,e}0.08^{a}$	$^{d}0.1^{a}$
Comp*Dist interact**		0.6992	0.2849	0.4895	0.0298	0.6155	0.9981	0.4023	0.0204	0.9583

* *Fusarium* Species*Distance from maize interaction P-values; ** Environmental component*Distance from maize interaction P-values
Superscripts a, b and c (row-wise): means with the same symbol differ not significantly on a 5% -level.
Superscripts d and e (column-wise): means with the same symbol differ not significantly on a 5% -level.

Table 4.4: Relative *Fusarium* species distribution in maize cobs (silk and kernels)

Species*maize cob	*F. proliferatum*	*F. verticillioides*	*F. solani*	*F. oxysporum*	*F. subglutinans*	*F. poae*	*F. semitectum*	*F. equiseti*	*F. chlamydosporum*
0.3950	0.79^{a}	$0.75^{a,b}$	$0.38^{a,b,c}$	$0.36^{a,b,c}$	$0.32^{b,c}$	$0.29^{b,c}$	0.23^{c}	0.04^{c}	0.04^{c}

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

4.4 Discussion

With the exception of *F. poae*, *Fusarium* species isolated from the environment of peri-urban food gardens have been reported to be capable of causing opportunistic infections in immune-suppressed individuals (Pujol *et al.*, 1997). Moreover, all species targeted for detection in the present survey produce mycotoxins (Logrieco *et* al., 1998; Rheeder *et* al., 2002) that could compromise immune functioning (Baumrucker & Prieschl, 2002; Berek *et al.*, 2001; Hymery *et al.*, 2006). Results illustrated in Figure 4.1 suggest these mycotic and mycotoxigenic *Fusarium* species are common members of autochthonous microbial populations in the environment of peri-urban food gardens. *Fusarium* species were retrieved in significantly higher numbers from localities in the proximity of home-grown maize in contrast with localities away from maize (Table 4.1). Though true of all nine *Fusarium* species, the observation was most pronounced for *F. verticillioides, F. proliferatum, F. subglutinans* and *F. oxysporum* (Table 4.2). Commonly associated with commercial maize ecosystems (Kpodo *et al.*, 2000; Fandohan *et al.*, 2003; Nesci *et al.*, 2006), the first three species are maize plant pathogens causing seedling disease, root and crown rot, stalk rot and ear rot (Cotton & Munkvold, 1998). Apart from the study of Sreenivasa and co-workers (2006) in which *F. solani* was detected in freshly harvested maize, the presence of this species in maize ecosystems is not often reported.

The isolation of *F. solani* in significant numbers (P=0.0002) is of particular interest in view of the high incidence of HIV infection in South Africa (Statistics South Africa, 2006). Pujol *et al.* (1997) describe *F. solani* as the most dangerous filamentous fungus for immunocompromised patients after *Aspergillus fumigatus*, whereas Boutati and Anaissie (1997) report it as the species predominantly isolated from fatal cases of disseminated fusarioses in patients with hematologic malignancies. *Fusarium poae*, usually connected with destructive diseases of wheat (Edwards, 2004; Roháhčik & Hudec, 2005), was also retrieved in significant numbers (P=0.0194) from localities near maize. *F. equiseti* and *F. chlamydosporum* were not isolated from localities away from maize, but in relatively small numbers near these plants. Relative to *F. verticillioides,* which was isolated as the predominant species, *F. equiseti* and *F. chlamydosporum* represented a small percentage of *Fusarium* isolated from commercial maize in Ghana (Kpodo *et al.*, 2000).

Figure 4.1 and Tables 4.1 and 4.2 indicate that maize may play a role in maintaining *Fusarium* species in the environment of peri-urban home gardens. Table 4.3 reveal significant

interaction of maize-associated *F. verticillioides* and *F. proliferatum* with air (P=0.0280) and soil (P=0.0514). Cotton and Munkvold (1998) found *F.verticillioides* and *F. proliferatum* multiplied rapidly during the growing season on maize leaf surfaces as well as in rainwater trapped in leaf sheaths, and subsequently survived for up to two years in soil and maize plant residue on the soil surface. Mean species log-numbers depicted in Table 4.4 show that *F. verticillioides* (0.75) and *F. proliferatum* (0.79) isolated from maize cobs were primarily associated with maize kernels and silk, which might indicate a pathogenic relationship. *Fusarium* establishes infection when spores in the environment land on the silk, germinate and enter the ear after pollination, according to Cardwell *et al.*, (2000). 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 plant debris on the soil surface. Rossi *et al.* (2002) found that, under humid field conditions, fusarial spores in crop debris germinate and continue to produce macroconidia. These spores become airborne by splash dispersal during rain showers or irrigation and are disseminated over substantial distances by air currents (Hörberg, 2002; Maiorano *et al.*, 2007).

It thus seems likely that in the home-gardens, *F. verticillioides, F. proliferatum* and possibly *F. subglutinans* and *F. oxysporum*, occurring in association with the home-grown maize, are maintained in the environment by survival in maize residues and debris on the soil surface. Dispersed from plant debris, and depending on spore and leaf surface characteristics, airborne spores are trapped in differing degree by *morogo* plants growing in association with maize. This scenario might explain why, in the same environment, *F. verticillioides* was retrieved in higher numbers from thepe than from lerotho, while *F. oxysporum, F. poae* and *F. semitectum* were isolated in greater numbers from lerotho than from thepe (Table 4.3).

The multiplex PCR method described by Bezuidenhout *et al.* (2006) and employed by Jivan (2006) confirmed the presence of the *FUM 1* gene in *F. oxysporum, F. proliferatum, F. solani* and *F. subglutinans* isolated from *morogo* plants. Furthermore, HPLC analysis detected 44.8 $ng.g^{-1}$ fumonisin B_1 in thepe and 58.7 $ng.g^{-1}$ in lerotho (Alli, 2007). Similar findings were reported from investigations in maize-based subsistence agro-environments in the Limpopo Province (Van der Walt *et al.*, 2006).

Fusarium species in peri-urban home-garden environments have public-health implications. Urban activities are expected to enhance dissemination of fusarial spores, while human population density of peri-urban settings put more individuals at risk of opportunistic fusarial infections. Several case studies identified *F. verticillioides, F. proliferatum, F. solani* and *F.*

oxysporum (Boutati & Anaissie, 1997; Summerbell *et al.*, 1988; Segal *et al.*, 1998; Guarro *et al.*, 2000; Ortoneda *et al.*, 2004) as causative agents for disseminated fusariosis in immunocompromised individuals, in most instances with fatal consequences (Nucci & Anaissie, 2002; Dignani & Anaissie, 2004). The prevalence of *Fusarium* infections of the skin, the upper respiratory tract and eyes suggests these organs serve as portals of entry, eventually leading to multiple organ infection (Boutati & Anaissie, 1997; Dornbusch *et al.*, 2004). In the present survey these pathogens were isolated in considerable numbers from air, indicating an unavoidable risk for residents of inhalation, skin contact, or eye exposure to fusarial spores. *Fusarium* spores in the environment enhance the risk of HIV-positive individuals contracting secondary fusarial infections which, according to Pujol *et al.* (1997) and Dignani and Anaissie (2004), are most difficult to treat. Common features of opportunistic *Fusarium* infections include the presence of fusaria in the blood stream, the high frequency of skin lesions and a high mortality rate in patients with suppressed immunity as a result of pathogen drug resistance (Bodey *et al.*, 2002).

All the *Fusarium* species targeted for isolation in the present study are producers of potent toxins including beauvericin (Logrieco *et al.*, 1998), fumonisins (Rheeder *et al.*, (2002) and trichothecenes (Sudakin, 2003). Dietary mycotoxins may produce any of a range of biological effects depending on which of the following properties they possess: antinutritional (Carratù *et al.*, 2003), oxidative (Kouadio *et al.*, 2005), pro-mutagenic (Domijan *et al*, 2007), pro-inflammatory (Osuchowski *et al.*, 2004), tumorgenic (Soriano *et al.*, 2005), genotoxic (Lerda *et* al., 2005) and/or immune-suppressive (Baumrucker & Prieschl, 2002; Theumer *et al.*, 2003; Hymery *et al.*, 2006).

4.5 Concluding remarks

Food gardens play a vital role in providing resource-limited families with nutrition on household level (Modi *et al.*, 2006; Jansen van Rensburg *et al.*, 2007). The present study suggests, however, that home-grown maize in food gardens may play a cardinal role in maintaining harmful *Fusarium* species in the peri-urban environment. The findings reported here invite further attention, in view of public health implictions. A database on possible sources and mechanisms of dissemination of toxigenic and mycotic *Fusarium* in peri-urban food production settings should serve future strategies aimed at safe subsistence food-production for vulnerable populations.

The purification and morphological identification of the *Fusarium* isolates were laborious and extremely time consuming. Some isolates were lost during subculturing, an essential step in *Fusarium* identification. Furthermore, correct identification based on microscopic features requires specialised training and experience. Since mycological safety of subsistence food is equally important as that of commercial commodities, a rapid molecular method to detect fumonigenic *Fusarium* in *morogo* was evaluated. Chapter 5 reports on the use of a multiple PCR-based method.

Chapter 5

MULTIPLEX PCR-BASED DETECTION OF POTENTIAL FUMONISIN-PRODUCING *FUSARIUM* IN TRADITIONAL AFRICAN VEGETABLES

(Environmental Toxicology, 2006, 21(4):360-366)

5.1 Introduction

Leafy wild vegetables (*morogo*) have been part of the diet of African people for many generations (Khumbane, 2002). These are unrelated plants and are known by various names in different parts of the African continent. The plants are commonly used to supplement staple diets of maize. Besides the high nutritional value of these plants, they also possess substances with health protecting properties (Van Wyk *et al.*, 2000; Van der Walt *et al.*, 2006). However, recent mycological analysis of *morogo* from rural areas in the Limpopo Province of South Africa indicated that a considerable proportion of fungal isolates belonged to the genus *Fusarium* (Van der Walt *et al.*, 2006). Several of the *Fusarium* sp. isolates were *F. verticillioides*, *F. proliferatum*, *F. subglutinans* (Van der Walt *et al.*, 2006). These are generally pathogens of grains and cereals, but have also been implicated as opportunistic pathogens that cause dermal, blood, respiratory and other ailments, particularly in immuno-compromised individuals (Hue *et al.*, 1999; Dornbusch *et al.*, 2004; O'Donnell *et al.*, 2004). A further concern was that these species also produce fumonisins, mycotoxins that were demonstrated to cause birth defects, alimentary canal irritations, as well as a number of other diseases in humans and animals (Rheeder *et al.* 2002). Furthermore, the fumonisin group of toxins, has, amongst others, mutagenic and thus carcinogenic properties (Galvano *et al.*, 2002). Of particular concern in the study of Van der Walt *et al.* (2006) was the large number of *Fusarium* isolated from cooked, fresh and stored *morogo* samples. It is thus important that the levels of these fungi are determined in foods in general, but in particular in the foods of rural African communities that may have a large number of immuno-compromised individuals among them, i.e. babies, HIV positive individuals and the elderly.

The identification of *Fusarium* sp. in food crops is problematic. This is due to a number of factors including diversity among the *Fusarium* spp, problems with clear morphological characteristics that separates the species, variation and mutation of isolates in culture (Bluhm *et al.*, 2002). Present species differentiation is based on morphological characteristics such as

shape and size of macroconidia, presence or absence of macroconidia and colony morphology (Bluhm *et al.*, 2002). These limitations have had serious implications on the taxonomy of toxigenic and pathogenic species (Geiser *et al.*, 2004). Extensive training and expertise are required to isolate and identify these species. The process is laborious and time-consuming. Rapid and reliable methods to identify toxigenic *Fusarium* sp. in food sources would be an important step towards the protection of humans and animals from the effects of the toxins.

Polymerase chain reaction (PCR) assays were developed to identify *Fusarium* species in samples (Grimm & Geisen, 1998). Conserved segments of structural and functional genes have been characterised and used in PCR assays to identify toxigenic *Fusarium* spp (Grimm & Geisen, 1998; Hue *et al.*, 1999; Bluhm *et al.*, 2002). These include internal transcribed (ITS) regions (between ribosomal genes). Several candidate primers based on the ITS were developed for the detection and identification of *Fusarium* sp. in blood, environmental and food samples (Grimm & Geisen, 1998; Hue *et al.*, 1999; Bluhm *et al.*, 2002). Conserved segments of genes that are directly involved in the biosynthesis of toxins are also useful for PCR assays. Bluhm *et al,* (2002; 2004) developed a multiplex PCR assay for the simultaneous detection of fumonisin (using polyketide synthase, *FUM1* gene) and trichothecene producing *Fusarium* sp. in cornmeal. A recent phylogenetic study of the polyketide synthase gene was conducted by Kroken *et al.* (2003) who demonstrated that within the subphylum Pezizomycotina of the phylum Ascomycota to which the genus *Fusarium* belong, polyketide synthase can be coded for by between 7 and 25 different genes. This could have implications for reliability of the use of the *FUM1* gene. The data base for this particular gene is, however, limited and thus requires further investigation. Other candidate genes should also be surveyed for the development of PCR based assays. Particularly useful should be the translation elongation factor 1-α (TEF) gene (Geiser et al., 2004). Conserved primers were developed and a database (FUSARIUM-ID v. 1.0) is available on a local BLAST server. Thus from multiplex PCRs, the fragment for TEF could be isolated, re-analysed by sequencing to determine the identity of the species present in the sample. Genes regulating the biosynthetic pathway (*FCC1* and *PAC1*) were genetically characterized and sequenced (Shim & Woloshuk, 2001; Flaherty *et al.*, 2003). These sequences could be used to construct primers to increase the sensitivity of such a PCR based detection of potential fumonisin producing *Fusarium* sp. The reliability of using these particular genes for identification is, however, untested.

The aim of this study was to develop a PCR-based diagnostic test for simultaneous detection and identification of potentially fumonisin producing *Fusarium* spp. The objectives were to

(i) test existing primers for detection and identification of *Fusarium* spp, i.e translation elongation factor primers (TEF); (ii) test existing primers for determining the potential of isolates to produce fumonisin B1, i.e. (*FUM1*) and (iii) combine the various individual PCR steps into a single diagnostic PCR assay, i.e multiplex-PCR.

5.2 Materials and Methods

Sample Preparation. *Fusarium verticilliodes* reference strain (MRC 4319) was obtained from PROMEC, Medical Research Council, South Africa. The *Fusarium* spp isolates (Table 5.1) were obtained as described by Van der Walt *et al.* (2006) and identified by the South African Agricultural Research Council's Plant Protection Research Institute (PPRI), National Collection of Fungi.

Table 5.1: A list of the *Fusarium* spp isolates used in this study.

PPRI Number	*Fusarium* sp.	PPRI Number	*Fusarium* sp.
7363	*Fusarium verticillioides*	7370	*Fusarium verticillioides*
7365	*Fusarium subglutinans*	7372	*Fusarium verticillioides*
7367	*Fusarium verticillioides*		

Single spore purified isolates and reference cultures were used for DNA isolation and subsequent amplification. Approximately $2mm^2$ of each isolate was inoculated, in duplicate, into yeast peptone dextrose (YPD) broth. Each sample was shake-incubated at 200 rpm for 48 hours at room temperature. After the incubation period, 45mL samples were collected and centrifuged at 4000 rpm for 5 minutes. The supernatant was then carefully discarded. TE buffer (15mL) was added to remove as much of the growth medium as possible. The sample was centrifuged for a second time at 4000 rpm for 5 minutes and the supernatant discarded. The portion that remained was frozen at -65°C for 2 hours in preparation for the subsequent freeze drying process. The dried material obtained was regarded as the sample from which extraction of DNA was performed.

DNA Extraction Procedure. A CTAB-PVP DNA extraction procedure was used to extract DNA from 50mg of freeze-dried fungal material. The weighed sample was transferred to 2mL microfuge tubes. Double strength CTAB isolation buffer, heated to 65°C and to which 0.2% (v/v) β-mercaptoethanol was added immediately prior to use, together with a 5% PVP solution and proteinase K (20mg/mL) was added to each sample tube. All tubes were

incubated in a 65°C water bath for 30 minutes with gentle inversion of tubes every 3 minutes. An equal volume of TE buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and extraction of the aqueous phase was allowed to transpire at room temperature for 10 minutes. Sample tubes were then centrifuged at 13 400rpm in a bench-top centrifuge (MiniSpin Eppendorf, Germany) for 5 minutes at room temperature. A measured volume of the aqueous phase was transferred to a new microfuge tube and was re-extracted with an equal volume of TE buffered chloroform:isoamyl alcohol (24:1) for 10 minutes at room temperature. The tubes were centrifuged at 13 400rpm for a further 5 minutes after which the aqueous phase was transferred to a clean microfuge tube. The DNA was allowed to precipitate at -80° for a minimum of 1 hour in NaCl (5M) and 99.5% ice cold ethanol. In order to pellet the precipitated DNA, the tubes were centrifuged at 13 400rpm for 5 minutes at 4°C. After pouring off the supernatant, the NaCl was removed by washing the pellet with ice cold 70% ethanol. Following a brief spin, the supernatant was poured off and the pellet dried under vacuum. The DNA was reconstituted by suspending it in 50µl of TE buffer for 1 hour at 65°C. All DNA samples were stored at 4°C until required.

DNA Amplification. DNA was amplified by polymerase chain reaction (PCR) using an ICycler (BioRad, UK) thermal cycler. The total volume for each reaction was 25µl and constituted single strength PCR master mix, Supertherm *Taq* polymerase (1U; JM Holdings, UK), bovine serum albumin (BSA; 50ng), DNase/RNase-free distilled water, primer mix (Table 2) and template DNA. The PCR master mix employed was double concentrated and contained 2.5U *Taq* polymerase in 20mM Tris-HCl, 100mM KCl, 3.0mM $MgCl_2$, Brij 35, 0.01% (v/v), dNTP mix (dATP, dCTP, dGTP, dTTP each 0.4mM) with a final pH of 8.3 (at 20°C; PCR Master; Roche, Germany).

The following protocol was used for all PCRs. Single primers also used in multiplex are indicated in Table 5.2. Denaturing of DNA was conducted as follows: the initial denaturation at 95°C for 5 min was followed by 29 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 seconds) and extension at 72°C for 60 seconds. Amplification was terminated after a final extension period of 72°C for 5 minutes. This protocol was optimized by determining the common annealing temperature at which each of the primers indicated in Table 5.2 amplified the target genes without any non-specific fragment amplification. Furthermore, annealing temperatures between 50 and 62°C were tested and for the multiplex PCR, determining primer concentrations in the reaction was critical. Various primer concentrations of each of the primer sets were tested for successful amplification of fragment

of the expected size. Results of the various optimization experiments are not shown. It was also essential to add 1 U of *Taq* to each reaction.

Table 5.2: Primers used in this study. The expected product sizes are ideal for multiplex diagnostic PCR assay. SSU 0017F contained the GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC (Muyzer et al., 1996)

Ref	Primer name	Sequence	Gene Target	Product Size (bp)
1	FCC-1 F	5'-CGGTCCGACGACAAATGACTGG-3'	Fusarium Cyclin C-1	1500
	FCC-1 R	5'-CGACACAATGTCGCTTCTGG-3'		
2	SSU 0017F	5'- AGT AGT CAT ATG CTT GTC-3'	18S Small Sub-unit	570
	SSU 0583R	5'-TCT GGA CCT GGT GAG TTT CC-3'		
3	FUM-1 F	5'-GTCGAGTTGTTGACCACTGCG-3'	Polyketide Synthase (PKS)	800
	FUM-1 R	5'-CGTATCGTCAGCATGATAGC-3'		
4	EF-1	5'-ATGGGTAAGGAGGACAAGAC-3'	Translation elongation factor 1-α	700
	EF-2	5'-GGAAGTACCAGTGATCATGTT-3'		

1, Shim & Woloshuk (2001); 2, Kowalchuk et al., (1997); 3, Bluhm *et al.*, (2002); 4, Geiser *et al.*, (2004).

Confirmation of DNA Amplification. Electrophoresis was conducted on 5µl of PCR product through agarose gels (2% w/v; Roche, Germany) containing 0.5 µg/mL ethidium bromide (BioRad, UK). Each gel was also loaded with a DNA molecular weight standard (100 bp Molecular Weight Marker; BioRad, UK) to which the sizes and intensities of the template DNA bands could be compared. Electrophoresis was performed for 105 minutes at 80V using 1x TAE buffer. Gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software.

Sequence analysis. Amplified DNA fragments were sequenced by Inqaba Biotec, South Africa. Blastn searches (http://www.ncbi.nlm.nih.gov/BLAST for *FUM1* or http://fusarium.cbio.psu.edu for TEF) were used to confirm the identity of the amplified sequences.

5.3 Results

The four agarose (1% w/v) gels depicted in Figure 5.1 show the sizes of the four gene fragments targeted in this study. The DNA was from *F. verticillioides* MRC 4319 and the sizes of the fragments were as expected (Table 5.2).

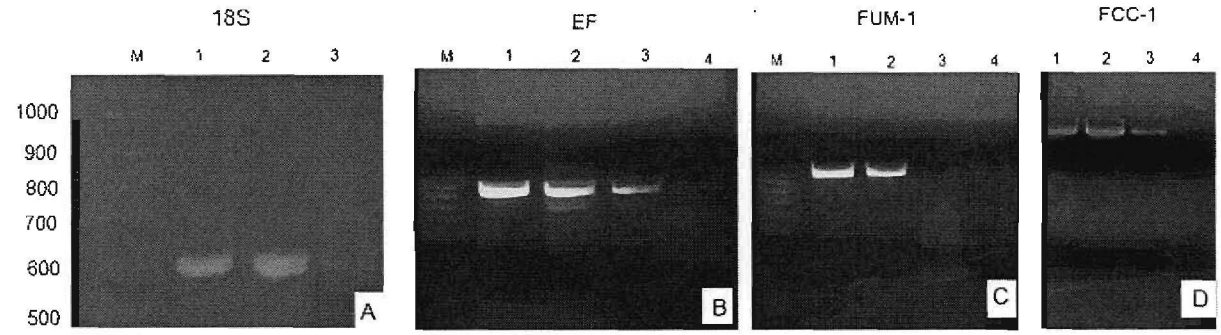

Figure 5.1: PCR reactions showing the size of the various fragments. Serial dilutions of genomic DNA are demonstrated for B, C and D. In the latter three cases electrophoresis was for 30 minutes at 80V. For A the electrophoresis was for 105 minutes. The letters A, B, C and D on the gel indicates the 18S rRNA gene fragment, elongation factor (EF), polyketide synthase gene (*FUM1*) and fusarium cyclin C-1 (*FCC1*) respectively. Numbers 1 to 4 in gels B, C and D indicate 1000, 100, 10 and 1 ng of the target DNA. The numbers 1, 2 and 3 in A indicate DNA

Gels B, C and D also demonstrate that up to 1ng of *Fusarium* DNA could be detected by the individual *EF*, *FUM1* and *FCC1* primers. Bluhm *et al.* (2002) defined detection limits as the minimal amount of DNA template that yielded a clearly visible product on agarose gels containing ethidium bromide. The detection limit in our study was less sensitive than a previous study (Bluhm et al., 2002) that showed PCR detection limits between 100 pg and 1 ng of *Fusarium* DNA. This lower detection limit in our study could have been due to the lower $MgCl_2$ concentrations that we used in our PCRs. Bluhm *et al.* (2002) used 2.5 mM whereas we used 1.5 mM in each of our reactions. The goal of our study was to optimize the multiplex PCR instead of only optimizing PCR detection of individual genes.

From Figure 5.1 it is evident that agarose concentrations greater than 1% (w/v) and electrophoresis times longer than 105 minutes should be used in the multiplex PCR. The primers were combined and various annealing temperatures as well as primer concentrations were also tested. From the results it was evident that annealing temperature of 55°C worked the best. Another aspect that had to be considered was optimising the PCR for the 18S rRNA

primers. The 18S primers were selected since they could amplify a spectrum of fungal species and was useful in fungal dynamics studies. These primers would thus act as positive controls i.e. they should amplify the 550 bp fragment of any fumonisin negative as well as non *Fusarium* species. When we attempted to use the 18S rRNA gene primers, we consistently obtained three fragments (1500, 1200 and the expected 550 bp). However, when a GC clamp, typically used in PCR denaturing gradient gel electrophoresis (DGGE) was added to the 5' end of the SSU 0017F primer, only one fragment was obtained (Figure 5.1 gel A).

Another aspect to determine was the optimum concentration for the various primers. We found that it was also important to combine the *FUM* and EF primers at equal concentrations and then to adjust the 18S primers (containing the GC clamp) to a 1:2 ratio with respect to the *FUM1* and EF primer combination. The concentration of the latter two primers was thus 25 pmol per set and the concentration of the 18S primer 12.5 pmol. The *FUM1* optimized primer concentration in the multiplex PCR was consistent with findings of Bluhm *et al.* (2002). No information was available for the 18S and EF primers. Unfortunately the *FCC1* primers did not amplify in the multiplex and was thus left out for further analysis. The composite agarose gel in Figure 5.2 shows several Fusarium DNA samples at 100 ng that were amplified by multiplex PCR. These include the *F. verticillioides* MRC 4319 strain as well as all the identified isolates listed in Table 5.1.

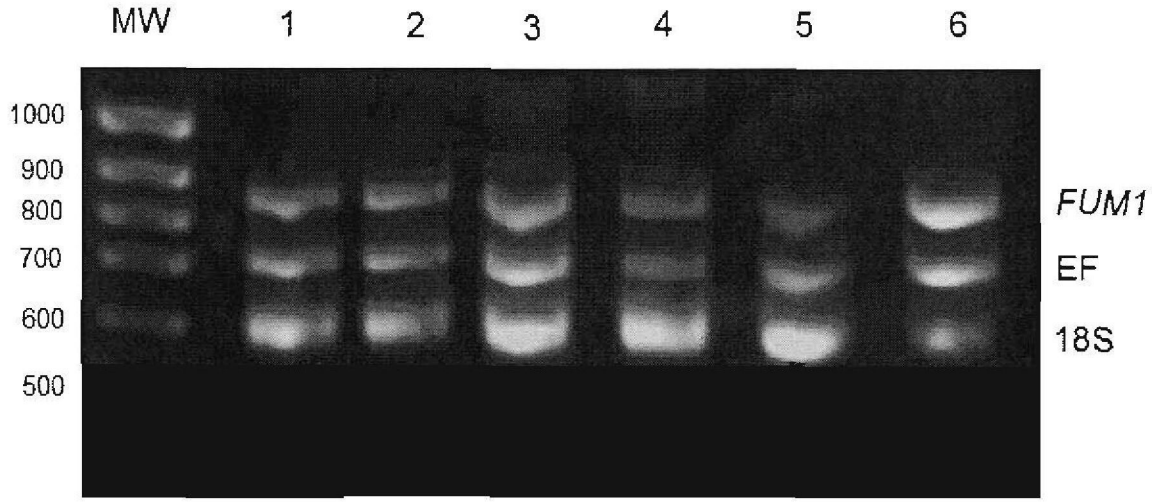

Figure 5.2: A 2% (w/v) agarose gel depicting the successful multiplex PCRs in which *FUM1*, EF and 18S primers were used. The DNA concentration 100ng per individual reaction and presented *F. verticillioides* MRC 4319 and the identified isolates listed in Table 5.1.

It is evident from Figure 5.2 that these primers could be utilized for detection of fumonisin positive *Fusarium* sp. when 100 ng of target DNA is present in the PCR mixture. Detection limits of this multiplex primer combination was evaluated and the results depicted in Figure 5.3.

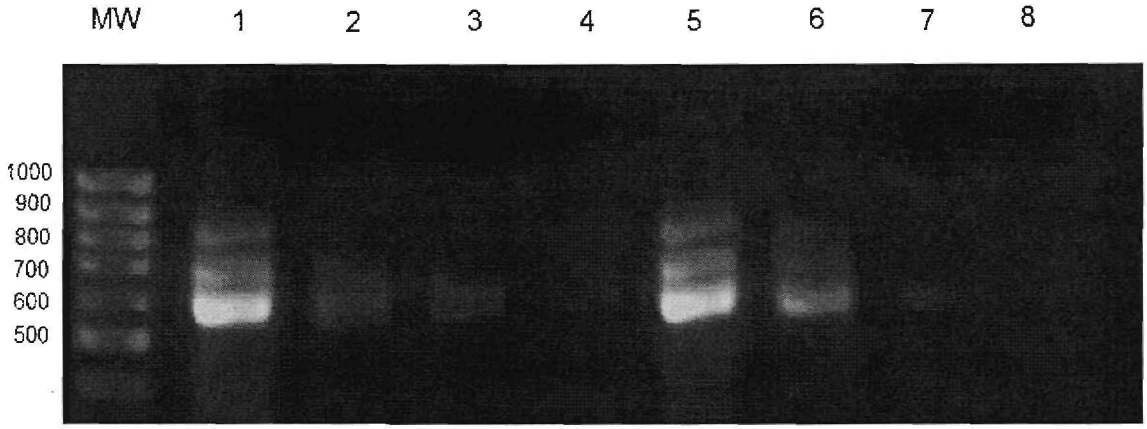

Figure 5.3: A 2% (w/v) agarose gel depicting the successful multiplex PCRs in which *FUM1*, EF and 18S primers were used. The DNA was from *F. verticillioides* (lanes 1 to 4) and *F. subglutinans* (lanes 5 to 8). DNA concentrations in the various reactions were 100, 10, 1 and 0.1 ng in each of the Lanes 1 to 4 and 5 to 8 respectively.

At 1 and 0.1 ng of target DNA the 18S fragments were weakly amplified. When the amount of target DNA was above 1 ng then all three PCR products were observed. Although the detection limit of the primer set for detection the fumonisin biosynthesis gene (*FUM1*) was lower than previously described (Bluhm *et al.*, 2002), this approach may have certain advantages over conventional methods. It is relatively fast and combining them with quantitative PCR and/or DNA fingerprinting methods could be powerful. Quantitative PCR methods would allow for determining levels of contamination by *Fusarium* spp. and DNA fingerprinting methods for determining the species diversity of this genus in the sample.

5.4 Discussion

The selected primers amplified the targeted fragments. Fragment identities were confirmed by sequence analysis. When the primers were combined in multiplex PCRs, three fragments of appropriate sizes were obtained. This demonstrates the potential of using these fragments in a multiplex PCR that aims at the detection of fumonisin positive *Fusarium* species. In diagnostic multiplex PCR processes it is essential that the target sequences are well selected to ensure good sensitivity, but also that PCR products yielded by the various primers are of

distinct sizes. The primers used in this study targeted three types of sequences: (i) one that is specific for detection of a gene involved in fumonisin biosynthesis (*FUM1)* (ii) a sequence of the elongation factor (EF) of *Fusarium* spp. and (iii) a sequence of conserved fungus 18S regions. These were all of distinct sizes and products of the multiplex PCRs could be resolved using 2 % (w/v) agarose gels. To eliminate the limitation of potential false negatives that could be associated with general PCR failure, we included the 18S primer set as an internal control. PCR failure could be associated with PCR inhibiting compounds that are co-extracted with the DNA (Fortin *et al.*, 2004). An internal control, as the one used in this study, is thus essential in multiplex PCRs. Although the primer sets yielded the expected PCR fragments in multiplex PCRs, further careful optimization of this process is essential to improve the detection limits of the primer sets. Besides determining the optimum primers concentrations in the PCR mix, it will also be important to determine the detection limit in the presence of related and unrelated fungal genera, as well as host plant, DNA.

Assays that could detect toxigenic *Fusarium* spp. *in situ* were previously described by Bluhm et al. (2002; 2004). In these cases ITS (internally transcribed spacers region) primers were used as internal controls. The ITS primers were specific for *Fusarium* spp. In our study the transcription elongation factor 1-α (TEF 1-α) was used as a control for *Fusarium* spp. This gene has the additional advantage that when detection of *Fusarium* spp directly from plant material is used, the opportunity exist to cut the fragments from the agarose gels, re-analyze them by PCR-DGGE and then followed by sequencing, one could rapidly determine which species are present. Such results would also be informative about the diversity of the *Fusarium* species present in such plant crops. Yergeau *et al.* (2005) used EF primers in PCR-DGGE application to assess the diversity of *Fusarium* spp. in asparagus crops. They showed the usefulness of these primers in such an application but moreover, demonstrated the added advantage of using the elongation factor primers i.e. that a well established database is available for identification of *Fusarium* sp. obtained by such as study.

This multiple PCR method can thus be combined with the PCR-DGGE technique to determine the dynamics of fungal species, particularly in stored food crops. The 18S rRNA fragment primer combination used in this study could be evaluated for this purpose when DNA from food crops are analysed. Subsequent to multiplex PCR and agarose electrophoresis the band representing the 18S fragments could be excised, re-amplified by the same primer set and subjected to DGGE. The DNA profiles could then be analysed and individual bands excised and sequenced. The sequence data could be analysed by GeneBank or ribosomal data bases. Moreover, the system could also be optimized for quantitative multiplex PCR. This

could increase the sensitivity of the method. Further advantage of the latter technique is that it would further reduce the analysis time and give a quantitative measurement of the amount of target DNA fragments present in the sample. Schnerr *et al.* (2001) used several approaches ranging from hotstart PCR, inclusion of pyrophosphatase, and Uracil DNA glycosylase to increase the quantitative PCR detection limits, specificity of the reaction and reduce the risk of PCR carryover. These approaches could thus be useful to explore.

A need exist for the accurate and sensitive detection of pathogenic fungi from environmental, crop and clinical samples due to the increase in fungal infections (Diaz and Fell, 2004; Dornbusch *et al.*, 2004). New opportunistic pathogenic fungal infections are also increasing and may emerge as new species within genera (Diaz and Fell, 2004). This scenario worsens in the case of immuno-compromised individuals. Rural African communities may consist of a large proportion of such individuals. Food sources that were mycologically analysed by Van der Walt *et al.* (2006) contained potential opportunistic pathogens such as *F. verticillioides* and *F. proliferatum* (Dornbusch *et al.*, 2004; O'Donnell et al., 2004). These species are also known fumonisin producers. It is thus important that methods are evaluated and optimized for rapid and accurate detection of these and other mycotoxigenic fungi in the food sources of these communities. Such information could be used to recommend improved storage methods and thus decreasing the risk of exposure to the fungal species and their toxins.

5.5 Concluding remarks

In summary, this study provide evidence that primers *FUM1*, EF and 18S described could be useful tools for rapid detection and identification of mycotoxigenic fungi in food samples. The primers could also be useful to study the dynamics of the fungal colonisers in food crops and additionally could be useful to construct fungal rDNA libraries for DGGE analysis. Information from such studies will additionally provide information to discuss the scope of the functional roles. Studies are underway to further optimise the multiplex PCR and to test the detection limits in the presence of competing DNA.

The health-protective value of vegetable consumption is widely acclaimed. However, such information on traditional African vegetables is scarce and, where it exists, gives a rather fragmented picture. Three of the most commonly-consumed *morogo* vegetables were analysed to determine their folate contents and fatty acid profiles - constituents that could influence the harm caused by dietary fumonisin exposure. Data and discussion on possible health benefits are presented in Chapter 6.

Chapter 6

ALPHA-LINOLENIC ACID AND FOLATE IN WILD-GROWING AFRICAN DARK-GREEN LEAFY VEGETABLES (*MOROGO*)

(Public Health Nutrition, 2008 – In Press)

6.1 Introduction

Chronic lifestyle-related cardio- and cerebrovascular diseases (CVDs) and diabetes type 2 are becoming more pronounced in urbanised black South Africans (Voster, 2002). Levitt and Mollentze (1995) predicted type 2 diabetes could affect over 3 million South Africans by 2010. The occurrence of these lifestyle-related chronic diseases apparently does not discriminate on a socio-economic basis (Panz & Joffe, 1999; Van der Merwe *et al.*, 2000; Vorster, 2002). Currently, dietary transition from a traditional low-fat, plant protein-rich rural diet towards a high-fat, animal protein-rich Westernised diet receives much attention as a factor contributing to the increased occurrence of chronic diseases of lifestyle in urbanised black South Africans (Bourne *et al.*, 2002; Steyn, 2006). In rural diets, a variety of cultivated and / or wild-growing African green leafy vegetables (*morogo*), supplements traditional maize-based staples (Jansen van Resnburg *et al.*, 2007). Concerning micronutrient levels, *morogo* vegetables compare well with spinach, swiss chard and cabbage (Kruger *et al.*, 1998; Odhav *et al.*, 2006; Mnkeni *et al.*, 2007). Dark green leafy vegetables (DGLVs) are also indicated as rich sources of folate (Kalter, 2000; Das, 2003) and 18 : 3*n*-3 (Simopoulos, 1991; Singh *et al.*, 2004; Innis, 2007). Nutritional data on cultivated varieties of traditional African vegetables are fragmentary and almost non-existent for wild-growing *morogo* species. The present study reports on the fatty acid profiles and folate contents of three wild-growing *morogo* species: cowpea (*munawa*), vegetable amaranth (*thepe*) and *spider flower (*lerotho*).

6.2 Materials and Methods

Sample collection and preparation. Sampling sites were situated in three geographically separated and climatically distinct areas of South Africa. One fresh field sample of each *morogo* type, namely amaranth, spider flower and leafy cowpea were collected from rural villages respectively in the Rustenburg District (North-West Province), as well as Vhembe and Capricorn Districts (Limpopo Province). One traditionally sun-dried household sample of

* Also known as African cabbage or cat's whiskers

spider flower was obtained from a rural family in the Capricorn District. Samples were transported to the laboratory in Ziploc plastic bags on ice. Upon arrival at the laboratory fresh samples were freeze-dried and stored at -20 °C until analysis. Finely ground freeze-dried sample material and the traditionally sun-dried were subsequently used for fatty acid analysis. Samples for folic acid determinations were accurately weighed before oven-drying at 105 °C for approximately 1 hour until oven-dried weight remained constant. Moisture loss was recorded for use as dry : wet mass conversion factor in calculations of folate concentrations in fresh leaves.

Chemicals. All organic solvents used were of GC grade and, together with fatty acid standards, were purchased from Sigma (USA).

Folic acid analysis. Freeze-dried *morogo* samples were sent to the South African Bureau of Standards (SABS) in Pretoria for folic acid analysis. A standard method Microbiological Assay of Folic Acid in Foods and Pharmaceutical Products was employed (Barton-Wright, 1961; AOAC, 1999). *Streptococcus faecalis* (ATCC 8043) was the test organism. Materials included: USP folic acid standard (Merck), Difco Bacto-folic AOAC medium (Code 0967), Oxoid M.R.S. agar (Code CM 361) as culture media and Oxoid M.R.S. agar (Code CM 359) to prepare the inoculum. Folic acid detection limit was $0.0005\mu g.mL^{-1}$. Dry mass concentrations were converted for expression as $\mu g.100g^{-1}$ wet mass.

Fatty acid determinations. Heptadecanoic acid (72 mM), as an internal standard, was added to 25mg of lyophilized sample followed by 100µL of a 45mM solution of butylated hydroxytoluene and 2mL methanolic HCl (3N). The mixture was subsequently vortexed before incubation at 90°C for 4 hours. Following cooling to room temperature, the sample was extracted twice with 2mL of hexane, dried under a nitrogen stream and finally re-suspended with 100 µL of hexane, 1µL of which was injected onto the GC-MS via splitless injection. An Agilent 6890 GC ported to a 5973 Mass Selective detector (California, USA) was used for identification and quantification of individual fatty acids. For the acquisition of an electron ionization mass spectrum, an ion source temperature of 200°C and electron energy of 70 eV was used. The gas chromatograph was equipped with a SE-30 capillary column (Chemetrix, USA), a split/splitless injection piece (250°C) and a direct GC-MS coupling (260°C). Helium (1 ml/min) was used as the carrier gas. An initial oven temperature of 50°C was maintained for 1.5 minutes and then allowed to increase to 190°C at a rate of 30°C / min. The oven temperature was maintained at 190°C for 5 minutes and then allowed to increase at a rate of 8°C per min to 220°C this temperature being maintained for 2 min. Finally, ramped at a rate of

3°C per min, the oven temperature was maintained at 230°C for 24 min. All samples were analysed in triplicate and reported as the mean concentration in mg/100g ± standard deviation.

Statistical analysis. Data of fatty acid analysis were processed using StatSoft, Inc. (STATISTICA Data Analysis Software System, Version 7.1, 2006). Statistical evaluation of measured folic acid concentrations employed statistical techniques included in computerised data processing programme of the South African Bureau of Standards (SABS), Pretoria, South Africa.

6.3 Results

Botanical species identification. Amaranth (*thepe*) samples were identified as *Amaranthus hybridus L. subsp. cruentus* (*L.*) *Thell.* (Rustenburg), *Amaranthus hybridus L. subsp. hybridus var hybridus* (Vhembe) and *Amaranthus thunbergii Moq.* (Capricorn). Spider flower (*lerotho*) from Rustenburg and Capricorn were in both instances identified as *Cleome gynandra L.* and cowpea (*munawa*) as *Vigna unguiculata* (*L.*) *Walp.* susp. *unguiculata* (Table 6.1).

Folate contents. Results of the folic acid determinations (Table 6.1) indicated considerable variation in folic acid concentrations in *morogo*: 217μg.100g^{-1} (spider flower, Capricorn), 154μg.100g^{-1} (leafy cowpea, Vhembe), 122μg.100g^{-1} (amaranth, Vhembe), 130μg.100g^{-1} (amaranth, Capricorn), and 72μg.100g^{-1} fresh weight (amaranth, Rustenburg).

Table 6.1: Folic acid contents and botanical species identification of wild-growing amaranth, spider flower and cowpea

Plant species	Study area	Folic acid contents (μg.100g^{-1} fresh sample)
Amaranthus hybridus L. subsp. cruentus (*L.*) *Thell.* (amaranth / *thepe*)	Rustenburg	72
Amaranthus hybridus L. subsp. hybridus var hybridus (amaranth / *thepe*)	Vhembe	122
Amaranthus thunbergii Moq. (amaranth / *thepe*)	Capricorn	130
Cleome gynandra L. (Spider flower / *lerotho*)	Capricorn	217
Vigna unguiculata (*L.*) *Walp.* susp. *unguiculata* (cowpea / *dinawa*)	Vhembe	154

Fatty acid profiles. Total measured fatty acid concentrations (Table 6.2) ranged between 1610.2mg.100g^{-1} in Capricorn amaranth to as high as 2941.6mg.100g^{-1} dry mass in spider flower of Rustenburg. The major polyunsaturated fatty acids (PUFAs) detected in all samples included linolenic acid (LNA; 18 : 3*n*-3) ranging from 753.8 (SD=48.6) to 1629.7 (SD=77.1) and linoleic acid (LA; 18 : 2*n*-6) from 110.8 (SD=7.1) to 506.3 (SD=47.2) mg.100g^{-1} of dry mass. Palmitic acid, the predominant saturated fatty acid (SFA), varied between 420.6 (SD=83.2) and 662.0 (SD=21.2) mg.100g^{-1} and the only mono-unsaturated fatty acid (MUFA), palmitoleic acid between 34.7 (SD=0.3) and 79.0 (SD=9.3) mg.100g^{-1} dry mass. Table 6.3 indicates the relative percentages of SFAs and PUFAs in terms of the total measured fatty acid concentrations. SFAs : PUFAs ratio's ranged from between 1 : 1.4 to as high as 1 : 2.8 and PUFAs contents from between 51.5% (Vhembe amaranth) to 73.5% (Vhembe cowpea leaves). Notable variations were observed in measured concentrations of both 18 : 2*n*-6 and 18 : 3*n*-3 in samples of the same plant genus but different species or from different localities. The highest dry mass concentrations of PUFAs (Table 6.2) are reported for Rustenburg samples of amaranth and spider flower with values indicated for 18 : 2*n*-6 respectively as 506.3 (SD=47.2) and 385.7 (SD=9.7) and for 18 : 3*n*-3 respectively as 1183.9 (SD=114.9) and 1554.3 (SD=59.4) mg/100g^{-1}. Expressed in percentages of total measured fatty acids (Table 6.3), ratio's of 18 : 2*n*-6 : 18 : 3*n*-3 PUFAs in Vhembe amaranth (1 : 9), spider flower (1 : 4-5) and Rustenburg amaranth and Vhembe cowpea leaves (1 : 3.4) were in favour of 18 : 3*n*-3 PUFAs.

Table 6.2: Saturated fatty acid profiles of some traditional leafy vegetables consumed in South Africa

	Measured fatty acid concentrations (mg/100g dry mass; n = 3)																	
	Myristic Acid (14 : 0)		Palmitic Acid (16 : 0)		Stearic Acid (18 : 0)		Arachidic Acid (20 : 0)		Behenic Acid (22 : 0)		Lignoceric Acid (24 : 0)		Palmitoleic Acid (16 : 1)		Linoleic Acid (18 : 2)		Linolenic Acid (18 : 2)	
Vegetable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Total fatty acids
Amaranth*	5.8	0.5	552.0	40.7	73.8	5.9	13.6	3.3	16.4	2.4	60.2	6.5	79.0	9.3	506.3	47.2	1183.9	114.9	2491.2
Amaranth**	18.0	1.2	570.6	40.2	192.5	15.4	83.5	5.4	12.7	2.4	29.1	2.8	40.2	1.7	110.8	7.1	964.7	60.5	2081.5
Amaranth***	9.5	0.2	422.1	16.5	50.6	21.5	14.1	1.3	13.0	11.0	37.6	3.6	58.1	14.3	217.4	74.7	753.8	48.6	1610.2
Spider flower*	8.6	0.3	662.0	21.2	127.7	2.5	38.3	3.6	35.6	3.0	38.0	1.9	91.4	0.5	385.7	9.7	1554.3	59.4	2941.6
Spider flower***	16.6	5.2	420.6	83.2	70.0	13.0	23.6	4.7	0.7	0.3	24.2	4.4	45.3	9.7	171.0	33.7	899.1	159.0	1696.8
[1]Spider flower ***	11.2	7.4	593.8	26.3	107.1	1.0	24.3	0.8	0.4	0.01	25.2	0.04	65.1	5.9	379.4	2.1	1629.7	77.1	2864.7
[2]Cowpea**	5.2	0.1	576.4	3.5	75.9	1.6	7.0	0.01	0.4	0.01	24.6	0.5	34.7	0.3	474.1	2.8	1599.8	26.3	2817.5
[2]Cowpea**	10.6	0.4	450.6	13.5	45.4	21.0	10.1	2.3	5.3	5.7	38.2	4.0	68.8	16.6	243.0	74.6	822.3	85.3	1728.5

Sample from Rustenburg*, Vhembe** and Capricorn***

[1] Traditionally sun-dried household sample

[2]Collected from two different villages in the Nzhelele Valley

Table 6.3: The relative amounts of the various fatty acids (%) measured in samples of wild-growing amaranth, spider flower and cowpea

Vegetable	Percentage fatty acid (%)													
	Myristic Acid	Palmitic Acid	Stearic Acid	Arachidic Acid	Behenic Acid	Lignoceric Acid	Palmitoleic Acid	Linoleic Acid	Linolenic Acid	LA : LNA	SFAs	MUFAs	PUFAs	SFA : PUFAs
Amaranth*	0.23	22.1	3.0	0.55	0.65	2.4	3.1	20.3	47.5	1 : 2.3	28.9	3.1	67.8	1 : 2.3
Amaranth**	0.86	27.4	2.9	4.0	0.61	1.40	1.9	5.2	46.3	1 : 8.9	37.2	1.9	51.5	1 : 1.4
Amaranth***	0.59	26.2	3.1	0.87	0.8	2.33	3.6	13.5	46.8	1 : 3.5	33.9	3.6	60.3	1 : 1.8
Spider flower*	0.29	22.5	4.3	1.3	1.2	1.3	3.1	13.1	52.8	1 : 4.0	31.9	3.1	65.9	1 : 2.1
Spider flower***	0.98	24.8	4.1	1.4	0.04	1.4	2.7	10.1	52.9	1 : 5.2	32.7	2.7	63.0	1 : 1.9
[1]Spider flower ***	0.39	20.7	3.7	0.85	0.01	0.88	2.3	13.2	56.9	1 : 4.3	26.5	2.3	70.1	1 : 2.6
[2]Cowpea**	0.18	22.0	2.7	0.25	0.01	0.87	1.2	16.8	56.7	1 : 3.4	26.0	1.2	73.5	1 : 2.8
[2]Cowpea**	0.61	26.1	2.6	0.58	0.30	2.2	4.0	14.0	47.5	1 : 3.4	32.4	4.0	61.5	1 : 1.9

Sample from Rustenburg*, Vhembe** and Capricorn***

[1]Traditionally sun-dried household sample

[2]Collected from two different villages in the Nzhelele Valley

6.4 Discussion

Steyn (2006) describes the low-fat plant protein-rich diet of rural black South Africans as most prudent. Wild-growing dark green leafy vegetables (DGLVs), such as amaranth, spider flower and cowpea, feature prominently in this type of diet (Schippers, 2002; Jansen van Rensburg *et al.*, 2007). DGLVs are important sources of food folate (Kalter, 2000; Das, 2003). Table 6.1 indicate folic acid concentrations of amaranth varied between 72 – 130μg.100g^{-1}, measured 217μg.100g^{-1} in spider flower and 154μg/100g^{-1} in cowpea. South African Food Composition Data (SAFCOD) tables (Kruger *et al.*, 1998) report the folic acid concentrations in raw samples of these vegetables respectively as 64, 346 and 141μg.100g^{-1}. The folic acid concentrations in raw leaves of other *morogo* vegetables such as amadumbe, black jack and nightshade are respectively 126, 351 and 404 μg.100g^{-1} and that of raw commercial Swiss chard, 52 μg.100g^{-1} (Kruger *et al.*, 1998). Boiled, amaranth measured 5μg.100g^{-1} folic acid, cowpea leaves 60μg.100g^{-1}, sweet potato leaves (also consumed as *morogo*) 124μg.100g^{-1} and commercial spinach 146μg.100g^{-1} (Kruger *et al.*, 1998). Values reported from the National Food Consumption Survey (Labadarios *et al.*, 2005) for the percentage energy respectively derived from plant and animal sources, indicate rural populations in South Africa consume a larger amount plant foods than their urban contemporaries. Families in the Mopani District of the Limpopo Province indicated that, when available, *morogo* is eaten on a daily basis (Van der Walt *et al.*, 2005). Results from the present study and information provided in by SAFCOD suggest that, consumed on a regular basis in normal amounts, *morogo* vegetables could be an important source of dietary folate.

In addition, DGLVs generally contain small amounts of fat predominantly in the form of polyunsaturated fats (Kruger *et al.*, 1998). In the present study, six saturated fatty acids (SFAs), one monounsaturated fatty acid (MUFA) and two polyunsaturated fatty acids (PUFAs), 18 : 2*n*-6 and 18 : 3*n*-3, featured in differing ratio's in the fatty acid profiles of the three types of *morogo* (Table 6.2). In all samples, palmitic acid was the predominant SFA measured in concentrations higher than 18 : 2*n*-6 but lower than 27% of 18 : 3*n*-3. The SFAs to PUFAs ratio's in wild-growing *morogo* vegetables ranged between 1 : 1.4 – 2.8 (Table 6.3). Calculated from the SAFCOD tables (Kruger *et al.*, 1998) SFAs to PUFAs ratio's of other *morogo* and commercial vegetables were of the same order (1 : 1.1 – 2.5).

The following percentages of energy from total fat are indicated for various groups of adult black South African males: rural areas – 22.9%, informal settlements – 24.3%, urban middle class – 26% and urban upper class – 30.6% (Bourne & Steyn, 2000; Steyn, 2006). Steyn *et al.* (2001) found a rural black population derives 3.7 – 4.4% of energy from SFA as opposed to 8.5 – 9.2% of an urban black population. The following consumption pattern is indicated for black South Africans in terms of the percentage of the population consuming a food item respectively in rural and urban settings: meat – 46 and 74%; full cream milk and products – 10 and 60%; vegetable fats and oils – 26 and 62% (Steyn, 2006). The adoption of a Westernised diet due to urbanisation (Bourne & Steyn, 2000; Bourne *et al.*, 2002), appears to be linked with a propensity among black South Africans to consume greater amounts of fat. Moreover, the fat content of their diet seems to increase with the degree affluence. Simopoulos (2001) describes the Western diet as 'deficient' in 18 : 3*n*-3 mainly because of the high intakes of cereals and vegetable oils rich in 18 : 2*n*-6, and low intakes of fruits, nuts and DGLVs containing large amounts of 18 : 3*n*-3. Sanders (1999) maintains that, consumed in normal amounts, DGLVs would contribute little to the dietary intake of 18 : 3*n*-3 because of the overall low fat content. In rural settings, *morogo* vegetables are prepared mixed with other plant foods, including groundnuts and traditional legumes such as cowpeas, which are expected to contribute to the overall 18 : 3*n*-3 intake (Kruger *et al.*, 1998; Simopoulos, 2001; Van der Walt *et al.*, 2005; Faber *et al.*, 2007; Jansen van Rensburg *et al.*, 2007).

Adequacy in dietary 18 : 3*n*-3 is essential for the biosynthesis of docosahexaenoic acid (DHA) – the long-chain PUFA derivative with vital membrane, brain and cardiac functions (Sanders, 1999; Innis, 2007). In all the *morogo* vegetables 18 : 3*n*-3 was the predominant PUFA with 18 : 2*n*-6 to 18 : 3*n*-3 ratio's ranging between 1 : 2.3 to 8.9 (Table 6.3). Eicosapentanoic acid (EPA), an intermediate in the synthesis of DHA, and DHA are both absent from plant foods and very low in ruminant fats such as milk and dairy products[14]. Since the synthesis of DHA from 18 : 3*n*-3 via EPA occurs at a slow rate in humans, EPA and DHA sufficiency is dependent on dietary intake from sources such as oily fish and seafood (Innis, 2007). With limited access to oily fish and seafood, poultry and eggs would serve as an alternative, though less efficient, source of EPA and DHA, provided animals are not fed on grain concentrates high in 18 : 2*n*-6 which competitively inhibits the conversion of EPA to DHA (Innis, 2007). Dietary studies in South Africa indicate the following relevant consumption pattern in rural and urban black South Africans respectively: grain-based food (maize porridge, sorghum and bread) – 98.6 and 98.5%, fish (canned) – 3.2 and 10.6%, eggs and egg products – 7.4 and 16.2% and vegetables – 48.1 and 42.8% (Steyn, 2006). Rural and

urban black South Africans thus have equally high 18 : 2*n*-6 intakes from grain staples, and seem to derive EPA and DHA primarily from poultry, eggs and canned fish. However, raised on commercial corn mixtures, poultry and eggs purchased from urban retail outlets are expected to have a higher 18 : 2*n*-6 content (Sanders, 1999; Simopoulos, 2001) than similar items consumed by rural farm-based families. Furthermore, the percentage urban black South Africans consuming vegetable fats and oils is 2.4 times the rural subjects (Steyn, 2006) implying a higher overall 18 : 2*n*-6 intake by urban populations. Rural populations furthermore consume a wider range of vegetables, including DGLVs (*morogo*) which is absent from the vegetable list of their urban contemporaries (Steyn, 2006).

By comparison, urban black South Africans seem more likely to consume a diet high in SFAs and 18 : 2*n*-6 and lower in folate than rural families in subsistence settings. This dietary trend could relate to findings of epidemiological studies suggesting the high fat content of their Westernised diet might be a prominent factor in the rise of Western lifestyle diseases in urbanised black South Africans (Levitt & Mollentze, 1995; Van Rooyen *et al.*, 2000; Vorster *et al.*, 2000; Vorster, 2002). Notwithstanding underlying genetic factors, obesity in black populations is attributed to over-nutrition and linked with the consumption of a Westernised diet (Joffe & Seftel, 1994; Van Rooyen *et al.*, 2000). In conjunction with other negative factors (e.g. excessive alcohol use, cigarette smoking and a sedentary lifestyle) obesity enhances risks of chronic diseases among adults (Bourne & Steyn, 2000; Vorster *et al.*, 2000; Puoane *et al.*, 2002; Steyn, 2006). Biological functions of long-chain fatty acids respectively derived from 18 : 2*n*-6 and 18 : 3*n*-3 differentially modulate risks of CVDs (Simopoulos, 1991; Simopoulos, 2001; Das, 2003), degenerative and inflammatory diseases (Innis, 2007; Lombardo & Chicco, 2006) as well as diabetes type 2 (Suresh & Das, 2003). Proposed antioxidant and anti-inflammatory actions of folate (Das, 2003) may further enhance the health protective value of *morogo* consumption. In combination with tetrahydrobiopterin and insulin, folate suppresses superoxide anion generation and increases endothelial nitrooxide (eNO) and prostacyclin production, both of which are potent platelet anti-aggregators and vasodilators (Lombardo & Chicco, 2006). The inhibiting effect exerted by dietary 18 : 3n-3 intake on the clotting activity of platelets (Renaud *et al.*, 1986), appears to act complementary to mechanisms by which folate lowers risks of CVDs. Mandatory folic acid fortification of various food items, and national strategies aimed at increasing fruit and vegetable intake and reducing saturated fat consumption in Western countries, emphasise the importance of folate and 18 : 3*n*-3 in disease prevention (Mills, 2000; Das, 2003).

6.5 Concluding remarks

The present study underscores the value of *morogo* vegetables as low-fat food items that could contribute notable amounts of folate and provide 18 : 3*n*-3 in excess of 18 : 2*n*-6. Lowering fat intakes and including *morogo* vegetables could adjust the diet of urbanised black South Africans to a more prudent one. Reporting the health beneficial qualities of *morogo* could improve the image of these valuable vegetables and enhance its marketability in the wider society and in urban centers. Future research should focus on expanding the nutritional data base, including possible antinutritional properties. Epidemiological studies could accurately assess the role of *morogo* vegetables in health and disease.

Plants absorb minerals from the soil and use these in diverse anabolic reactions to synthesise organic molecules, including an array antioxidant compounds to protect their exposed structures against oxidative solar damage. Consumers can therefore expect to derive substantial amounts of these antioxidant molecules from consuming dark-green leafy vegetables. The *morogo* vegetables above were also analysed to establish the levels of specific minerals, micronutrients and phytochemical that reportedly play a role in the detoxification of tissue-damaging free radical species.

Chapter 7

MINERALS, MICRONUTRIENTS AND ANTIOXIDANT PHYTOCHEMICALS IN WILD-GROWING AFRICAN DARK-GREEN LEAFY VEGETABLES (*MOROGO*)

(Manuscript submitted to the South African Journal of Sciences)

7.1 Introduction

The leaves of local wild and cultivated plants feature prominently as vegetables in the traditional starch-based African diet (Smith & Eyzaguirre, 2007). Supplementary medicinal uses categorise some African green leafy vegetables as functional foods for having health benefits beyond basic nutrition (Van Wyk & Gericke, 2003. The dietary value of plant leaves pertains to their position as primary producers in the human food chain. Green plants absorb the minerals they need for diverse anabolic processes from the soil. The complex organic compounds thus manufactured in plant leaves include antioxidant molecules for protection against oxidative solar radiation (Zimmerman & Zentgraf, 2005). Leafy vegetables are, therefore, important dietary sources of minerals, micronutrients and phytochemicals with health-protective and immune-strengthening properties. Molecular evidence suggest micronutrients and antioxidant molecules in green leafy vegetables lower risks of cancer and cardiovascular diseases through mechanisms that modulate free radical attack on nucleic acids, proteins and polyunsaturated fatty acids (Borek, 2003; Philpott & Fergson, 2004; Lako *et al.*, 2007). Lako *et al.* (2007) demonstrated that leafy vegetables have a higher antioxidant capacity than either fruits or root crops.

The Setswana/Sesotho group of African languages use the term *morogo* collectively in reference to the aerial parts of edible plants consumed as green leafy vegetables (Van der Walt *et al.*, 2006). Plants utilised as *morogo* are either indigenous or naturalised in the areas where they grow and many are accessible by collection from the wild (Jansen van Rensburg *et al.*, 2007). Traditional African dark-green leafy vegetables (DGLVs) are underutilised and neglected where people consider them inferior to commercially-produced conventional vegetables of the westernised diet (Smith & Eyzaguirre, 2007). The general lack of information on the chemical composition, nutritional value and health benefits of traditional African DGLVs may also influence peoples' perceptions of *morogo* consumption negatively.

The present study reports on the mineral and phytochemical composition of wild-growing varieties of three types of *morogo* widely consumed in different geographical regions of South Africa. The mineral and micronutrient contents, carotenoid and polyphenol levels in *morogo* are compared with that of conventional and commercially-grown dark-green leafy vegetables. The nutritional benefits and potential health-protective value of *morogo* consumption are briefly discussed.

7.2 Materials and Methods

Sample collection and preparation. Sampling localities were situated in three geographically separated and climatically distinct areas of South Africa. Fresh leaves of the following naturally-occurring *morogo* vegetables were collected from rural villages in the Rustenburg District of North-West Province and Vhembe and Capricorn Districts in Limpopo Province: *Amaranthus hybridus* L. subsp. *hybridus, Amaranthus thunbergii* Moq., *Cleome gynandra* L. and *Vigna unguiculata* (leafy variety). Plants collected from various sampling sites were immediately transferred to "zip-lock" plastic bags, transported to the laboratory on ice and freeze-dried immediately upon arrival. Freeze-dried samples were stored at -20 °C until analysis. Finely ground freeze-dried samples were used in subsequent chemical analyses.

Chemicals. All standards were purchased from Sigma (USA). All organic solvents used were of HPLC grade purchased from Burdick and Jackson (USA). All other reagents were purchased from Merck (Darmstadt, Germany).

Mineral and trace element analysis. A 100mg freeze-dried sample of each plant was accurately weighed and carefully heated in 1mL of nitric acid (70%) until clarity is achieved. After cooling, 3mL of water was added and heating resumed for a further 10 min. Finally the solution was cooled and made up to 10mL with deionised water. The mineral composition of each sample was determined with an Agilent 7500c inductively coupled argon plasma mass spectrometer (ICP-MS). Calibrations were performed using external standard prepared from 1000ppm single stock solution made up with 2% nitric acid. The external calibrations were run in the same analytical sequence as the samples. Samples were analysed in triplicate and values are reported as mean ± standard deviation in $mg.100g^{-1}$ dry mass.

Total phenolics analysis. The extraction of total phenolics in samples was carried out in triplicate according the modified method of Kähkönen *et al.* (1999). Ground dry plant material (25mg) was extracted with 5 x 1mL of 80% aqueous methanol using Ultra Turrax mixer for 5 min. Samples were centrifuged at 3000rpm for 10 min and extracts collected

extracts were taken to dryness under nitrogen. Residues were dissolved in 1mL aqueous methanol 80%. The amount of total phenolics in extract samples was determined as previously described (Loots *et al.*, 2006) using Folin-Ciocalteu's procedure (Singleton & Rossi, 1965). Volumes of 200µL of extract were transferred to test tubes to which 1mL Folin-Ciocalteu's reagent was added. The mixture was allowed to stand for 8 min at room temperature. A volume of 0.8mL sodium-carbonate (7.5%) was subsequently added, mixed and allowed to stand for 30 min. Absorption was measured at 765nm (Shimadizu UV-1601 Spectrophotometer). Total phenolic content was expressed as a mean ± standard deviation as gallic acid equivalents (GAE) in mg.100g^{-1} dry mass.

Total carotenoids analysis. Total carotenoids content was determined in triplicate as decribed by Edwards *et al.* (1998) with slight modifications. Approximately 25mg of sample was weighed into a 10mL centrifuge tube with 3g of glass beads then mixed with 5mL DMSO, and placed in a pre-heated water bath at 45°C for 30 min. The tube was vortexed for 15 sec every 10 min. After incubation, samples were centrifuged at 4000rpm for 5 min. The supernatant was collected in a 25 mL volumetric flask. The samples were re-extracted with 5mL acetone until the absorbance of the centrifuged layer was less than 005. Acetone was added to the collected supernatant such that a final volume of 25 mL was achieved. The absorbance was determined at 477 nm (Shimadizu UV-1601 Spectrophotometer). Total carotenoid conent is described as a mean ± standard deviation in mg/100g dry mass.

Beta-carotene extraction and HPLC analysis. A modification of the procedure described by Lakshiminarayana *et al.* (2005) was subsequently applied to 50mg of each sample for extraction of β-carotenes in triplicate. Extraction was carried out in triplicate in ice cold acetone and the procedure was repeated until the extract was colourless. An aliquot of 1.5mL of each extract was dried under a stream of nitrogen. The residue was redissolved in 250µL of acetone before being filtrated by a 0.45µm nylon membrane filter. Sample filtrates were analysed by HPLC to determine the β-carotene contents and reported as a mean ± standard deviation in mg.100g^{-1} dry mass.

HPLC analysis. Analytical separations were performed according to a modified procedure of De Ancos *et al.* (2000) using a Hewlett-Packard System Series Model 1100 with a UV-visible detector. The column was a 4.6mm x 250mm Nova-Pack C18 4µm Waters (Waters Corporation). Solvents were HPLC grade methanol and ethyl acetate. A gradient system was used involving two mobile phases. Mobile phase A consisted of methanol/water (75:25v/v) and mobile phase B of ethyl acetate. A gradient was run starting from 0% B to 70% B in 10

min, followed by 70% B to 100% B in 4 min. Sample eluted at approximately 15 min. At the end of the gradient the column was requillibrated under the initial conditions by a new gradient condition beginning at time 14 until 20 min with a final composition of eluent B at 0%. Standard curves of β-carotene (95%) were constructed by plotting HPLC peak absorbance area versus concentration of the β-carotene in the injected sample.

7.3 Results

Mineral and trace elements (Table 7.1). Indicated in $mg.100g^{-1}$ dry weight, Ca concentrations in fresh samples ranged between 1722.2±59.7 (*V. unguiculata*) and 3100.0±94.7 (*C. gynandra*; Rustenburg). Ca concentration in fresh *A. hybridus* (Rustenburg) was considerably higher than that of *A. hybridus* (Vhembe). Mg concentrations ranked highest in the Rustenburg District samples of *A. hybridus* (1400.0±0.3) and *C. gynandra* (1311.4±45.). *A. thunbergii* contained notably lower Mg (520.0±18.0) and *V. unguiculata* the lowest (392.3±13.6). Concentrations of Fe were exceptionally high in *A. thunbergii* (236.8±8.2) while in other samples it ranged between 14.8±0.5 (*A. hybridus*; Rustenburg) to 97.9±3.4 (*V. unguiculata*). Fe in *C. gynandra* was notably higher in the Capricorn sample (89.7±3.1) than in the Rustenburg sample (38.1±1.3). Zn concentrations varied between 0.6±0.2 (*A. hybridus*; Rustenburg) and 43.7±15.2 (*C. gynandra*; Rustenburg). Comparing these values, Zn was higher in the Vhembe sample of *A. hybridus* (4.2±1.5) and in *A. thunbergii* (12.7±4.4) and lower in the Capricorn *C. gynandra* (8.4±2.9). The highest Se concentrations were measured again in the Rustenburg samples of *A. hybridus* and *C. gynandra* (0.8±0.3 and 0.5±0.01 respectively).

Table 7.1: Mineral elements in three traditional African leafy vegetables sampled from different geographical regions in South Africa

Plant species	District	Mineral element concentration (mg/100g dry mass)[a]				
		Ca	Mg	Fe	Zn	Se
Amaranthus hybridus	Rustenburg	2700.0±70.2	1400.0±0.3	14.8±0.5	0.6±0.2	0.8±0.3
Amaranthus hybridus	Vhembe	1772.2±61.4	871.7±30.2	94.9±3.3	4.2±1.5	0.3±0.01
Amaranthus thunbergii	Capricorn	1932.3±66.9	520.0±18.0	236.8±8.2	12.7±4.4	0.2±0.01
Cleome gynandra	Rustenburg	3100.0±94.7	1311.4±45.4	38.1±1.3	43.7±15.2	0.5±0.01
Cleome gynandra	Capricorn	1943.7±67.3	847.6±29.3	89.7±3.1	8.4±2.9	0.2±0.01
Vigna unguiculata	Vhembe	1722.2±59.7	392.3±13.6	97.9±3.4	6.1±0.1	0.2±0.01

[a] Mean ± SD (n=3)

Total phenolics concentrations (Table 7.2). Expressed as mg GAE/100g dry mass, total phenolics concentrations ranged from 1057.3±61.9 to 2905.9±94.5, this highest value indicated for *V. unguiculata*. Comparing the amaranth samples, the highest concentration of total phenolics were indicated for *A. hybridus* of Rustenburg (2181.2±30.20), followed by *A. thunbergii* from Capricorn (1137.7±41.9), with the lowest concentration in *A. hybridus* of Vhembe (1057.3±61.9). Rustenburg *C. gynandra* was also higher in total phenolics (1923.9±87.2) compared to the sample obtained from Capricorn (1659.1±30.0).

Total carotenoid concentrations (Table 7.2). Expressed as $mg.100g^{-1}$ dry mass, the highest total carotenoids concentration was measured in *Vigna unguiculata* (194.9±5.0). In *C. gynandra* again the Rustenburg sample showed higher amounts (162.3±1.1) of total carotenoids concentrations than in the Capricorn sample (93.9±3.9). In amaranth samples the total carotenoids concentration was lowest in *A. thunbergii* (88.6±10.7) and highest in the Vhembe sample of *A. hybridus* (131.3±10.7).

Beta-carotene concentrations (Table 7.2). Indicated as mg/100g dry mass, β-carotene concentrations ranged from 0.4±0.1 (Rustenburg, *C. gynandra*) to 18.4±1.5 (Rustenburg, *A. hybridus*). The concentration of β-carotene was considerably lower in the Vhembe sample of *A. hybridus* (1.6±1.1) and *A. thunbergii* (1.6±0.2). In *C. gynandra* the β-carotene concentration was notably higher in the Capricorn sample (1.7±0.6). *V. unguiculata* measured 3.8±0.3 mg/100g β-carotenes.

Table 7.2: Total phenolic compounds, total carotenoids and β-carotenes in three traditional African green leafy vegetables sampled from different geographical sites

Plant species	District	Total phenolics[a] (mg GAE/100g dry mass)	Total carotenoids[a] (mg/100g dry mass)	β-carotene[a] (mg/100g dry mass)
Amaranthus hybridus	Rustenburg	2181.2±30.2	113.6±9.3	18.4±1.5
Amaranthus hybridus	Vhembe	1057.3±61.9	131.3±10.7	1.6±1.1
Amaranthus thunbergii	Capricorn	1137.7±41.9	88.6±10.7	1.6±0.2
Cleome gynandra	Rustenburg	1923.9±87.2	162.3±1.1	0.4±0.1
Cleome gynandra	Capricorn	1659.1±30.0	93.9±3.9	1.7±0.6
Vigna unguiculata	Vhembe	2905.9±94.5	194.9±5.0	3.8±0.3

[a] Mean ± SD (n=3)

7.4 Discussion

Toxic radical molecules are continuously generated from plant cellular structures that are involved in photosynthesis and respiration. For protection, plants manufacture organic detoxification molecules in which Fe, Zn and Se feature as essential structural components. Ca^{2+} cations play a vital role in regulating cellular transmembrane trafficking of elements and molecules.[4] Dark-green leafy vegetables (DGLVs) are, therefore, primary sources of minerals, trace elements, and antioxidant molecules such as polyphenols and carotenoids, all of which function in enzymatic and/or non-enzymatic-mediated plant defences against radiation-induced oxidative stress (Rodriguez-Amaya, 1997; Van Duyn & Oivonka, 2000). In the present study, leaves of wild-growing varieties of two amaranth species (*A. hybridus, A. thunbergii*), two samples of cat's whiskers (*C. gynandra*) and one of cowpea (*V. unguiculata*) were analysed for their mineral, micronutrient, total phenolics, total carotenoid and β-carotene contents. Concentrations of the minerals Ca and Mg, and micronutrients Fe, Zn and Se varied in samples of the same plant species, but from different geographical localities (Table 7.1). This could be expected when considering the mineral and micronutrient content of plant leaves is a function of the environment and in leafy vegetables would be strongly influenced by the chemical composition of the soil and the climate (Mnkeni *et al.*, 2007; Modi, 2007). The highest concentrations of Ca (2700 and 3100 $mg.100g^{-1}$) and Mg (1400 and 1311 $mg.100g^{-1}$) were respectively measured in *A. hybridus* and *C. gynandra* both sampled from the Rustenburg area. Interestingly, these samples respectively contained the lowest and highest Zn concentrations (0.6 and 44 $mg.100g^{-1}$) suggesting Zn was differentially absorbed by these two plant species. Odhav *et al.* (2007) reported the following mineral and micronutrient concentrations respectively in *A. hybridus* and *A. spinosis*: Ca – 2365 and 3931 $mg.100g^{-1}$, Mg – 1317 and 1166 $mg.100g^{-1}$, Fe - 21 $mg.100g^{-1}$ (in both species) and Zn - 18 $mg.100g^{-1}$ (in both species). These plants were also wild-growing and sampled from assorted habitats, i.e. disturbed land, roadside and field in Kwazulu-Natal. The South African Food Composition Data (Kruger *et al.*, 1998) reported the following concentrations in $mg.100g^{-1}$ fresh sample of an unspecified amaranth species: Ca – 2378, Mg - 1323, Fe - 21, Zn - 4.9 and Se - 4.1. Measured in $mg.100g^{-1}$ fresh samples of spinach and swiss chard contain these elements in the following respective concentrations: Ca – 832 and 1182, Mg – 664 and 788, Fe – 30 and 44, Zn – 4.5 and 7.4, Se – 8.4 and 12 (Kruger *et al.*, 1998). The afore-mentioned values suggest that conventional DGLVs have a higher content of Fe and Se, but contain Ca and Mg in lower concentrations than the wild-growing *morogo* varieties. This information is particularly important for households that are not in a position to access conventional vegetables, whether for economic or demographic reasons.

Carotenoids, pigment molecules responsible for the colour of many fruits and vegetables, have important functions in photosynthesis and are abundant in plant leaves. Beta-carotenes, for instance, are prevalent in photosytem I. (Bergquist, 2006). About 10% of carotenoids in vegetables have provitamin A activity (Khan *et al.*, 2007). A study by Faber (2002) links the high incidence of vitamin A, Ca, Fe and Zn deficiencies to the low nutrient-dense diet consumed by the majority of children aged between 2 and 5 years of age. Another study found that for children in rural areas, consuming *morogo* contributed significantly to their intake of calcium and iron, but the biggest nutrient contribution of *morogo* consumption was towards the total intake of vitamin A (Faber *et al.*, 2007). Carotenoid and β-carotene concentrations measured in the three species of wild *morogo* are shown in Table 7.2. The total carotenoid concentration in *A. hybridus* (131mg.100g^{-1}), *C. gynandra* (162mg.100g^{-1}) and *V. unguiculata* (195mg.100g^{-1}) compared well with that of commercial baby spinach (140mg.100g^{-1}) reported by Bergquist (2006). The β-carotene content of the Rustenburg sample of *A. hybridus* (18mg100g^{-1}), and that of the unspecified amaranth species (16mg.100g^{-1}), reported by Kruger *et al.* (1998) in the SAFCOD tables, were markedly higher than that of in *A. thunbergii* (1.6mg.100g^{-1}), *C. gynandra* (0.4 and 1.7mg.100g^{-1}) and *V. unguiculata* (3.8mg/100g). In commercially-grown *A. tricolor* levels of total carotenoids (251mg.100g^{-1}) and *β*-carotene (39mg.100g^{-1}) were much higher, and in *A. gangeticus* and *A. viridis* the *β*-carotene concentrations of 52.9 and 291 mg.100g^{-1} were respectively reported (Gupta *et al.*, 2005; Lako *et al.*, 2007). According to De Pee and Bloem (2007) the bioavalability of carotenoids in DGLVs is reduced by the leaf matrix. Notwithstanding this limitation and distinct from being vitamin A precursors, carotenoids also exhibit considerable antioxidant capacity based on its symmetrical linear 40-carbon tetraterpene structure which features alternating double and single carbon-carbon bonds (Rodriquez-Amaya, 1997; Khan *et al.*, 2007; Lako *et al.*, 2007).

Polyphenols are another class of phytochemicals contributing considerably towards the total antioxidant capacity of DGLVs (Duthie *et al.*, 2000). Grouped together on the basis of their structures having aromatic rings, antioxidant activities of polyphenols are mainly through the donation of hydrogens (Rahman *et al.*, 2006). Results in Table 7.2 indicate that wild-growing varieties of *Amaranthus*, *Cleome* and *Vigna* spp contained phenolic compounds in amounts comparable to that of conventional and commercially-grown non-conventional vegetables. Total phenolics in *A. hybridus*, *C. gynandra* and *V. unguiculata* respectively measured 2181, 1924 and 2960mg.100g^{-1} and in commercial spinach, 2100mg.100g^{-1} (Pandjaitan *et al.*, 2000). Lako *et al.* (2007) reported total phenolics concentrations of 2000mg.100g^{-1} in leaves of commercially produced *Ipomaea batata* which is also eaten as *morogo* in South Africa. Odhav (2007)

demontrated that methonolic plant extracts (100mg.mL^{-1}) prepared from wild-growing varieties of *A. hybridus*, *A. spinosus* and *Cleome monophylla* respectively exhibited radical scavenging capacities of 90%, 88% and 84% relative to the 100% of the positive control, flavanoid rutin. Polyphenol-rich plant extracts have been demonstrated to protect against atherogenesis by inhibiting oxidation of low density lipoproteins (LDL) in endothelial cells and macrophages (Diplock, 2000; Duthie *et al.*, 2000). Collins (2001) linked the decreased cancer incidence following dietary β-carotene supplementation to antioxidant protection enhancing resistance to ROS-induced DNA strand breaks. Other studies ascribed the protective properties of dietary polyphenols and carotenoids against chronic diseases to the ability of these compounds to quench singlet oxygen or scavenge ROS thus interrupting the transfer of radical reactions from one cell to adjacent cells (Duthie *et al.*, 2000; Rahman *et al.*, 2006; Spiteller, 2006).

Consumption of dark-green leafy vegetables containing considerable amounts of minerals, micronutrients and antioxidants, could be particularly important in resource-poor households who are most likely to suffer nutrient deficiencies and, because of their unsatisfactory nutritional status, are more vulnerable to infection and chronic diseases (Bourne *et al.*, 2002). Nutrient profiles of the wild-growing *morogo* varieties reported in this study, support the view of South African authors who consider *morogo* cropping a feasible strategy for resource-poor populations to access a more diverse, nutrient-dense diet (Labadarios *et al.*, 2005; Faber *et al.*, 2007; Faber & Wenhold, 2007). Moreover, in a joint publication on *Plant Diversity, Sustainable Livelihoods and the HIV/AIDS Crisis* UNDP/FAO, Gari (2004) expresses the view that wild-growing food plants are an affordable and practical source of nutrition to improve the nutritional status of rural HIV-affected households. The author base this view on the fact that wild edible plants represent an inexpensive, labour-responsive means of improving the micronutrient quality of poor diets. *Morogo* crops, because they derive from indigenous African edible plant species that are adapted to local environmental conditions, grow on soils of limited fertility, are drought tolerant and can be harvested in a short period of time (Jansen van Rensburg *et al.*, 2007).

7.5 Concluding remarks

The utilisation of wild-growing *morogo* species seems in line with the ecohealth approach advocated by Lebel (2003) namely that managing biodiversity could have an important role in dietary diversification, improved nutrition and the betterment of human health. Wild *morogo* varieties gathered from the field are readily accessible and the consumption thereof could improve the nutrient density of poor diets. Home-garden cropping of *morogo* vegetables seems

another feasible strategy for resource-poor populations to enrich their starch-based diets with health-protective minerals, micronutrient and antioxidant phytochemicals.

Since *morogo* plants have been demonstrated to contain substantial amounts of important health-protective constituents, a pilot study was conducted to investigate the radical scavenging capacities and ability of some vegetables to inhibit the cytoviability of leukaemic and carcinoma cells *in vitro*. Findings of this preliminary study are reported and discussed in Chapter 8.

Chapter 8

RADICAL SCAVENGING CAPACITIES OF SIX *MOROGO* EXTRACTS AND THEIR INHIBITION OF CYTOVIABILITY IN ACUTE MYELOID LEUKAEMIA AND CARCINOMA CELLS – A PRELIMINARY INVESTIGATION

8.1 Introduction

Sustained overproduction of free radicals gives rise to persistent oxidative stress (OS) and pro-inflammatory immune responses both recognised as key events in the development of various cancers, including leukemia (Dröge, 2002; Torre *et al.*, 2002). Dietary constituents that counter OS, either by scavenging endogenously-generated free radicals or substituting endogenous antioxidant molecules that have been consumed, lower cancer risks by preventing oxidative damage to DNA or interrupting pro-inflammatory events that lead to the onset of chronic diseases (Dröge, 2002; Das, 2003). The majority of vegetarian foods are natural sources of various classes of antioxidant compounds (Izevbigie, 2003; Reddy *et al.*, 2003). which Lako *et al.* (2007) have shown to be present in larger amounts in leafy vegetables compared to other plant parts. Plant species utilised as non-conventional leafy vegetables, including members of the Amaranthaceae family and *Bidens pilosa,* are rich sources of dietary antioxidants (Izevbigie, 2003; Chiang *et al.*, 2004; Sani *et al.*, 2004; Dasgupta & De, 2007). Regardless of the chemical nature of individual antioxidant compounds, the capacity of a plant extract to scavenge free radicals can be determined as a measure of its antioxidant activity (Cai *et al.*, 1998). Since dark-green leafy vegetables have been reported to contain substantial amounts of antioxidant phytochemicals such as carotenoids and polyphenols (Lako *et al.*, 2007), traditional African leafy vegetables (*morogo*) are expected to exhibit a high degree of antioxidant activity.

With only small amounts of the *morogo* available towards the end of the growing season, a pilot study was conducted to evaluate *in vitro* the radical scavenging capacity (RSA) of aqueous extracts of six *morogo* vegetables, four of which are wild-growing varieties. Since high antioxidant activity is often linked to protection against cancer, the capacity of these plant extracts to inhibit the cytoviability of leukaemic and carcinoma cells, were also examined. Results reported are briefly discussed in terms of possible health benefits of *morogo* consumption.

8.2 Materials and methods

Extract preparation. Fresh leaves of six plant species utilised in South Africa as traditional *morogo* were frozen and freeze-dried upon arrival at the laboratory and stored at -20 °C until analysis. The following plant species, indicated by their African names, were evaluated: ditaka (*Lagenaria siceraria*), thepe (*Amaranthus thunbergii*), dinawa (*Vigna unguiculata*), mushidzhi (*Bidens pilosa*), lefe (*Pentarrhinum insipidum*), lerotho (*Cleome gynandra L.*). Botanical species identification was done by the South African National Botanical Institute (SANBI). To prepare aqueous extracts, 200mg of the lyophilised sample of each plant species was boiled for 10 min in 2mL distilled water. Following centrifugation at 3000rpm for 10 min, supernatants were collected and used to prepare the following concentrations for each of the extracts: 0.2, 0.4 and 0.8mg dry weight per mL hot water.

Radical scavenging capacity. The DPPH$^{\bullet}$ photometric assay described by Cai et al. (2003) was employed to measure the radical scavenging capacity of aqueous extracts of the six *morogo* plants respectively at concentrations of 2, 4 and 8mg.mL^{-1}. A volume of 0.1 mL of each extract concentration was mixed with 3.9mL of an 80% ethanolic 0.6mM DPPH$^{\bullet}$ solution (Aldrich). Tubes were vortexed for 15 sec. and allowed to stand for 180 min at room temperature. Most test compounds should have reacted completely during this period. Used as the positive standard, the reaction time of vitamin C (Sigma) is less than 1 min. due to its fast oxidation. Ethanol (80 %) was used as blank and DPPH$^{\bullet}$ solution without test sample (3.9mL DPPH$^{\bullet}$ + 0.1mL 80% ethanol) served as negative control. The test was performed in triplicate. The decrease in the absorbance of DPPH$^{\bullet}$ at 515nm was measured by a Hewlett Packard UV-Vis Spectrometer (Palo Alto, CA) at different time intervals until the reaction reached a plateau. Absorbance decreased because of a colour change from purple to yellow as the radical is scavenged by antioxidant compounds through the donation of hydrogen to form a stable DPPH-H molecule (Aruoma *et al.*, 1997). Inhibition of DPPH was calculated using the average values of the triplicate readings in the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100$$

Cytoviability inhibition of acute myeloid leukemia (AML) cells. The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was employed to evaluate the cytoviability of three lines of Acute Myeloid Leukemia (AML) cells after incubation with aqueous *morogo* extracts used in concentrations of 2, 4 and 8mg.mL^{-1}. The

MTT assay evaluates metabolic competence by assessing mitochondrial performance with regards to the conversion of yellow MTT to the purple formazan derivative by mitochondrial succinate dehydrogenase (enzyme) in viable cells (Selvakumaran *et al.*, 2003). A decrease in cellular MTT reduction could be an index of cell damage (Abe & Matzuki, 2000). Three types of AML cells were employed: AML-M3, AML-S and AML-F (National Cancer Institute, Cairo University, Cairo, Egypt). Cell density was calculated using a heamocytometer (Neubauer). AML cells were plated in 96-well plates at a concentration of 3×10^4 cells in 200µL medium per well. The RPMI 1640 (Sigma) medium contained 10 % (v/v) fetal calf serum, 100µg.mL^{-1} streptomycin and L-glutamine 2nM. After 24 hour incubation at 37°C in a humidified 5% CO_2 / 95% air, the medium in the wells was replaced with fresh medium containing *morogo* plant extracts in the different concentrations. The assay was run in triplicate. Cells were incubated for another 24 hours before the supernatant was removed. A volume of 100µL of 5mg.mL^{-1} MTT solution (Sigma), pH 4.7 modified in 0.9% NaCl, was added to each well and incubated for another 4 hours. The supernatant was subsequently removed and 100 µL dimethyl sulfoxide (DMSO; Aldrich) added to each well before plates were placed on a shaker for 15 min. Absorbance was measured at 550nm with a microplate reader (Bio-Rad, Richmond, CA). Wells containing untreated cells served as controls and wells containing DMSO but no cells, as blanks. The effect of each extract on the proliferation of AML cells is expressed as the % cytovialability (AML cells having remained viable in the presence of *morogo* extracts) using the average values of the triplicate readings in the following formula:

$$\% \text{ Cytoviability} = \frac{100 - \text{Absorbance}_{550} \text{ of treated cells}}{A_{550} \text{ of control cells}} \times 100$$

Cytoviability inhibition of Ehrlich Ascite carcinoma cells (EACCs). To evaluate *in vitro* effect of aqueous *morogo* extracts on the cytoviability of carcinoma cells, transplanted Ehrlich Ascites Carcinoma Cells (EACCs) from animals were obtained from the National Cancer Institute, Cairo University, Cairo, Egypt. EACCs were washed three times with PBS and cell counts adjusted to 10^5 cells per 0.1mL of media (3×10^4 per 30µL medium). Purification methods and treatment protocols described above were used to determine the effect of each aqueous *morogo* extract on the viability of EACCs. Seeded in RPMI 1640 (Sigma), EACC cells were incubated with *morogo* extracts (in concentrations described above) at 37°C in a humidified 5 % CO_2 / 95 % air for 12 hours. Wells containing untreated cells served as

controls. Cell viability was determined using a modified version of the cytotoxic trypan blue exclusion technique described by Bennett *et al.* (1976).

8.3 Results

Results from this preliminary investigation show the radical scavenging capacities (RSAs) of the various *morogo* plant extracts were enhanced as the concentration increased from 2 to 8mg.mL^{-1}. Results further indicate that the extracts of six plants varied in their capacity to scavenge free radicals. The following relative RSA percentages are reported respectively for the lowest (2mg.mL^{-1}) and highest (8mg.mL^{-1}) concentrations of the plant extracts: ditaka – 30% and 73% (Figure 7.1); dinawa – 18% and 55% (Figure 7.2); lefe – 39% and 75% (Figure 7.3); lerotho – 25% and 64% (Figure 7.4); mushidzi – 47% and 69% (Figure 7.5); thepe – 23% and 58% (Figure 7.6). The highest RSA at an extract concentration of 2mg.mL^{-1} was exhibited by mushidzi (47%), and the at an extract concentration of 8mg.mL^{-1} by lefe (75%). During incubation with aqueous *morogo* extracts, cytoviabilities (CVs) of both AML cells and EACCs decreased as the RSAs increased with increased plant extract concentration. The relative CV percentages indicate the amount of cells that remained viable after incubation with plant extracts illustrate the four cell lines exhibited differential sensitivities towards the inhibitory effects of the various *morogo* plant extracts. At the 8mg.mL^{-1} concentration, the highest CV percentages are shown for the AML-M3 cell line after incubation with ditaka (77%), dinawa (61%), lerotho (56%) and mushidzi (58%), and the lowest for EACCs with ditaka (33%; Figure 7.1) and lefe (34%; Figure 7.3), for AML-2 with lefe (24%; Figure 7.3) and for AML-3 and AML-2 with mushidzi (38%; Figure 7.5). At the 8mg.mL^{-1} extract concentration, notwithstanding relatively low RSAs of respectively 55%, 64% and 58%, the CVs of the four cell lines after incubation with dinawa ranged between 50 – 61% (Figure 7.2), with lerotho between 47 – 64% (Figure 7.4) and with thepe between 56 – 66% (Figure 7.6).

In summary, after incubation with the 8mg.mL^{-1} concentrations of the respective *morogo* extracts, CVs of AML-M3, AML-2 and EACC cell lines showed the most pronounced reduction with lefe that also exhibited the highest RSA of the six *morogo* plants. The CV of the EACC cell line was reduced to the same extent by the ditaka extract which also measured a high RSA. The CVs of AML-3 and AML-2 cell lines were also notably reduced by the mushidzi extract for which a relatively high RSA is indicated. The dinawa, lerotho and thepe extracts exhibited lower RSAs compared to the other three *morogo* extracts and reduced the CVs the four cell lines with almost equal percentages.

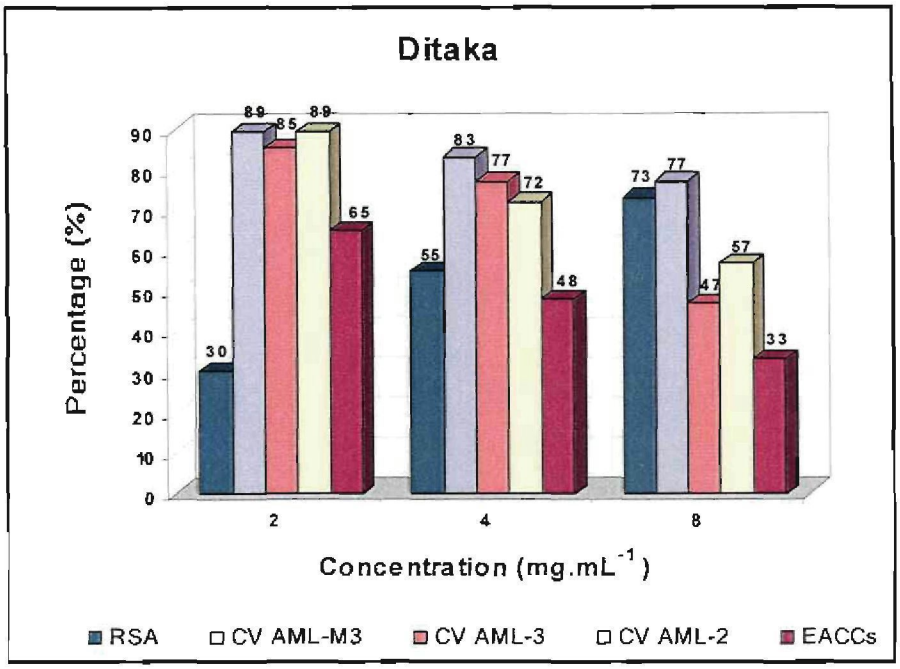

Fig. 8.1: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia cells denoted AML-M3, AML-3, AML-2 and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of ditaka extract

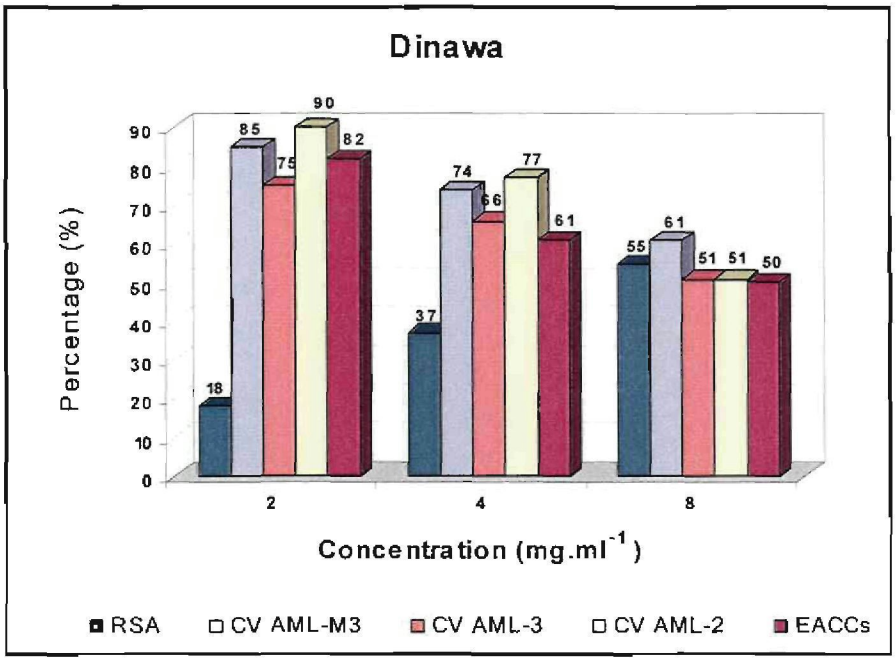

Fig. 8.2: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia denoted AML-M3, AML-3, AML-2 cells and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of dinawa extract

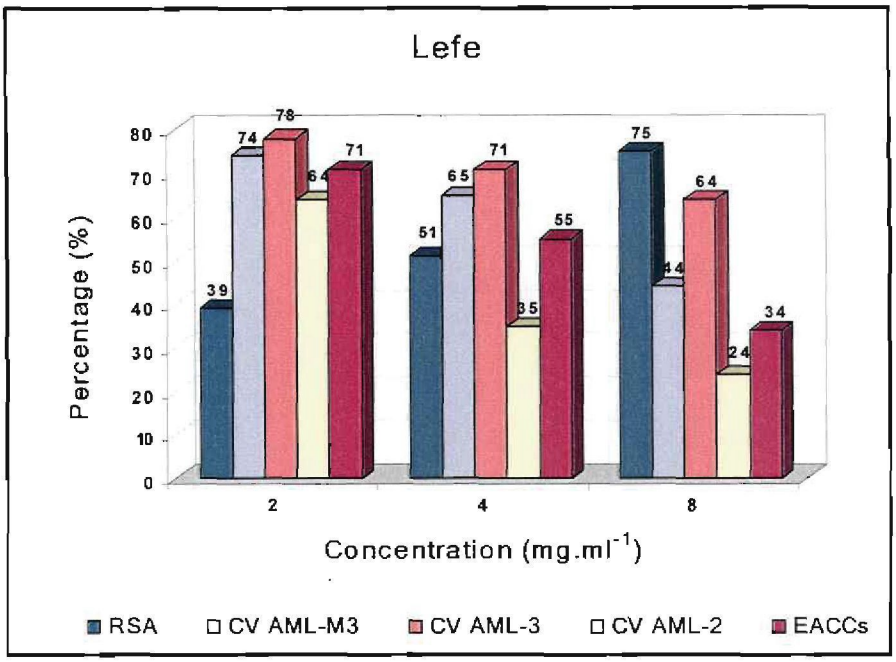

Fig. 8.3: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia denoted AML-M3, AML-3, AML-2 cells and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of lefe extract

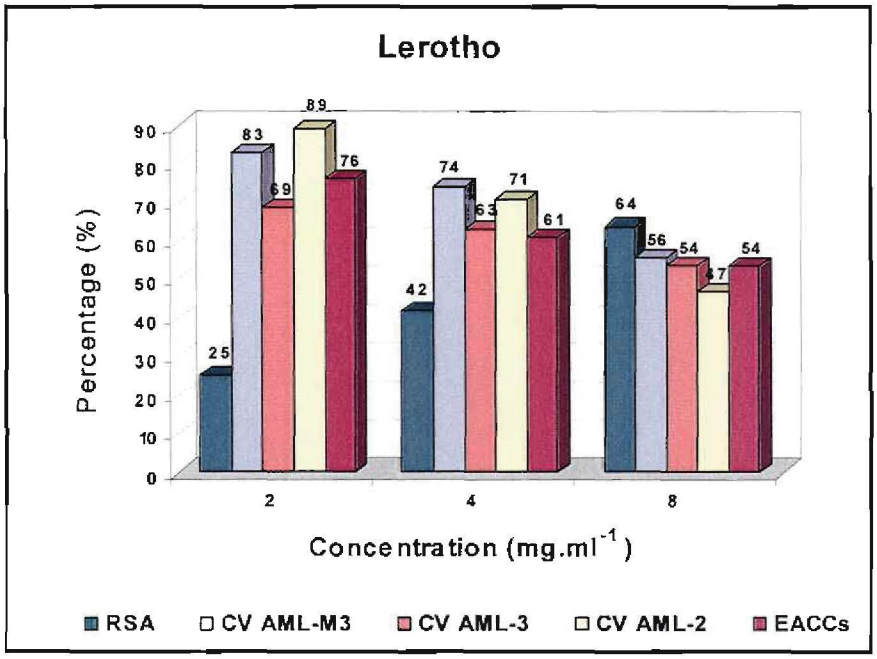

Fig. 8.4: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia denoted AML-M3, AML-3, AML-2 cells and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of lerotho extract

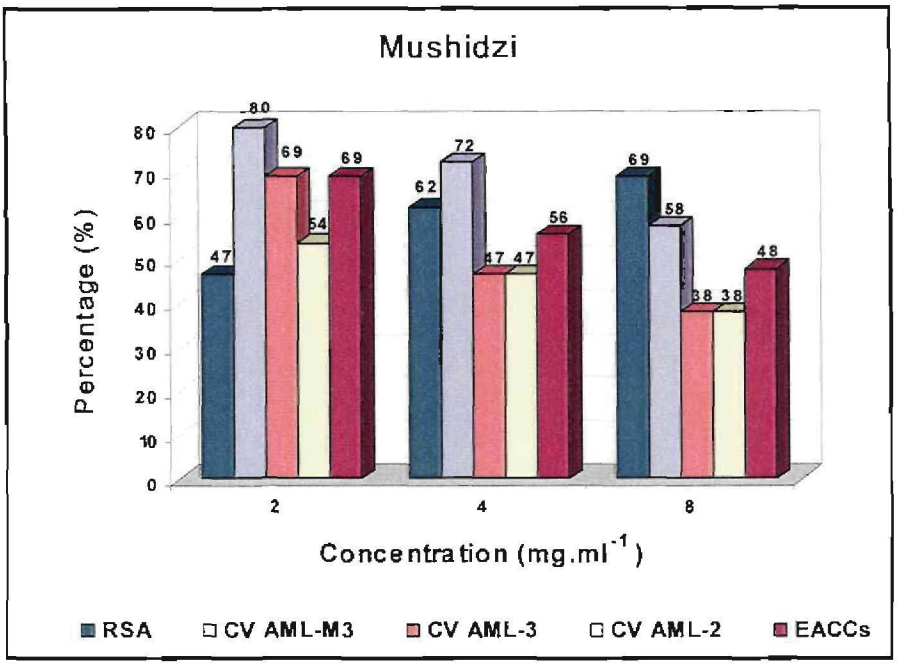

Fig. 8.5: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia denoted AML-M3, AML-3, AML-2 cells and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of mushidzi extract

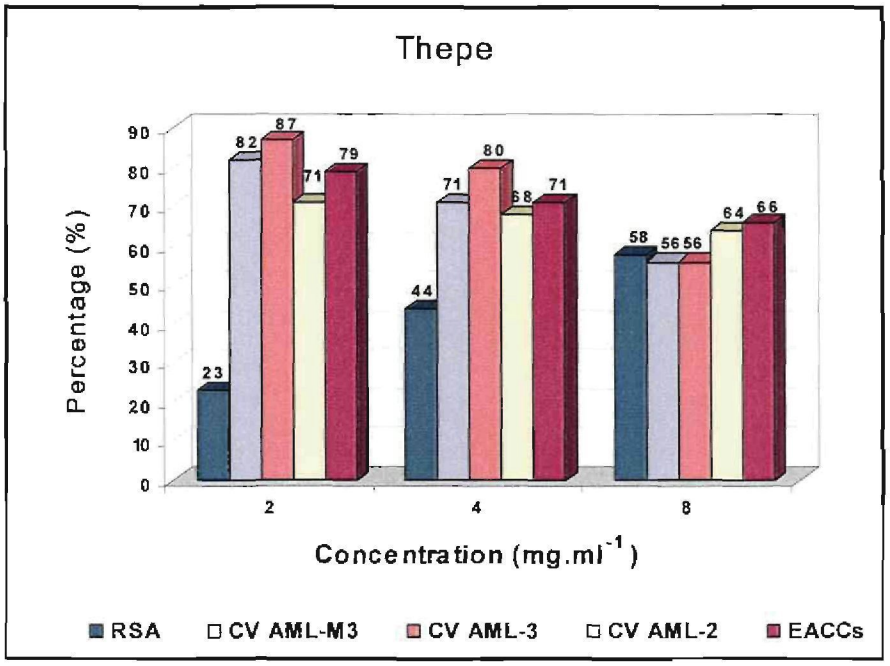

Fig. 8.6: Percentage the radical scavenging activity (RSA) and cytoviability (CV) of three types of acute myeloid leukaemia denoted AML-M3, AML-3, AML-2 cells and Ehrlich Ascite Carcinoma cells (EACCs) remaining after incubation with three different concentrations of thepe extract

8.4 Discussion

Figures 7.1 to 7.6 illustrate the relative radical scavenging activities (RSAs) and the capacities of the six *morogo* plants to inhibit the cytoviablity (CV) of leukaemic and carcinoma cells *in vitro*. The RSAs of the aqueous *morogo* extracts were enhanced at higher extract concentrations. At a concentration of 8mg.mL^{-1}, lefe (*Pentirrhinum insipidum*), ditaka (*Lagenaria siceraria*) and mushidzi (*Bidens pilosa*) exhibited higher RSAs than the other three extracts. In literature, high antioxidant capacities are also reported for other plant species utilised as dark-green leafy vegetables (DGLVs), including *Amaranthus viridis* (thepe), *Ipomoea batata* leaves, *Colocasia esculenta* (taro), *Chenopodium album* (Chiang *et* al., 2007; Lako *et al.*, 2007), all of which are eaten as *morogo*. These plants are often termed 'non-conventional leafy vegetables' for their association with traditional diets in regions where they grow (Smith & Eyzaguirre, 2007). Chiang *et al.* (2004) characterised six phytocompounds with DPPH radical scavenging activity in *Bidens pilosa* aqueous extracts which Yang *et al.* (2006) could demonstrate protected normal human erythrocytes *in vitro* against oxidative damage. Plant leaves are rich sources of antioxidant phytochemicals such as polyphenols, carotenoids and β-carotene (Bergquist, 2006) which the majority of reports suggest contributed significantly to antioxidant activity of dark-green leafy vegetables (Reddy *et al.*, 2003; Sani *et al.*, 2004; Yang *et al.*, 2006; Lako *et al.*, 2007).

In the present study, notwithstanding the relatively low RSAs exhibited by dinawa (Figure 7.2) and thepe (Figure 7.6) at the highest extract concentration of 8mg.mL^{-1} CVs of the four cell lines respectively ranged between 50 – 61% and 56 – 66% after incubation. The lefe extract, on the other hand, exhibited a high RSA of 75% and reduced the CV of AML-2 CV to as low as 24% and EACCs to 34% (Figure 7.3). The mushidzi (*Bidens pilosa*) extract, which also exhibited a relatively high RSA of 69% reduced the CVs of both AML-3 and AML-2 to 38% and that of EACCs to 48% (Figure 7.5). According to Chih *et al.* (1996) is *Bidens pilosa* a major ingredient of herbal tea of the Far East claimed to prevent inflammation and cancer. Steenkamp and Gouws (2006) reported that *Bidens pilosa* inhibited the proliferation of carcinoma cell lines by 10%. Chang *et al.* (2001) demonstrated hot water extracts of *Bidens pilosa* exhibited antileukaemic activity in five leukaemic cell lines. *In vitro* and *in vivo* studies by Sani *et al.* (2004) linked the protection against hepatocarcinogenisis caused by damage due to free radicals to the high antioxidant capacity of the aqueous extract of *A. gangeticus*.

The respective RSAs exhibited by the various *morogo* extracts and their inhibition of the CVs of the different three leukaemic cell lines and carcinoma cells, varied notably. Results from

the present study suggest an inverse relationship existed between RSAs of the *morogo* extracts and their reduction of the CVs of leukaemic and carcinoma cells, although this relationship was not clearly illustrated for all six the plant extracts. (Dasgupta and De 2007) also found such a correlation did not always exist. According to these authors, some plants with high phenol/flavonoid contents exhibit low antioxidant capacities which, in this study, might be the case with dinawa and thepe. The leukaemic and carcinoma cell lines used in the present study responded to incubation with *morogo* aqueous extracts with differential sensitivities, an observation also mentioned by Steenkamp and Gouws (2006).

8.5 Concluding remarks

In view of information provided in the above-mentioned studies, results from this preliminary investigation suggest a degree of health protection may be derived from *morogo* consumption. Notwithstanding the limitations of the present study because of the small samples that ere available at the time, data presented here warrant further investigation. *Morogo* could be highly accessible as a source of nutritious vegetables in both rural and urban environments. It can be obtained by gathering from the wild or through homestead cultivation that requires relatively low inputs of water and chemical soil improvement (Jansen van Rensburg *et al.*, 2007). A more comprehensive study on the RSAs of the six *morogo* plants and their effects on the CVs of leukaemic and carcinoma cells is currently conducted with samples now available in sufficient amounts after the last growing season.

The summary provided in Chapter 9 of all aspects investigated in the present study, places the potential value of results reported in this chapter in the context of the question considered in Chapter 2: indigenous African food-plants – are they vehicles of disease or sources of protection?

Chapter 9

SUMMARY AND CONCLUSION

9.1 Summary

Rural subsistence agro-environments and peri-urban homestead food gardens usually reflect the composition of the traditional African diet in which maize staples, cooked *morogo*, tuber vegetables and legumes still have common place. Though some *morogo* plants appear spontaneously as pioneer plants in soil disturbed by ploughing, others are specifically grown as soil cover in maize lands. Knowledge of *morogo* species, their cultivation as crops, dietary uses and/or medicinal applications is deeply imbedded in African tradition. Most *morogo* crops derive from plants that are either indigenous or naturalised in the areas where they occur. These traditional African crops are usually better adapted to local environmental conditions than commercial crops and are grown with low inputs of water and agrochemicals. The cultural connectedness and accessibility of *morogo*, irrespective of family socioeconomic status, is a unique advantage.

Notwithstanding the advantages of *morogo* vegetables, findings of the present study strongly suggest that the traditional practice of intercropping vegetables with maize might introduce serious health risks. Results indicate that maize probably serve as a reservoir for fumonigenic and mycotic *Fusarium* species, in both rural and peri-urban food-production environments. Species that were predominantly isolated from these maize ecosystems included prolific fumonisin-producers such as *F. verticillioides* and *F. proliferatum* as well as *F. solani*, a most dangerous opportunistic fungal pathogen causing life-threatening complications in immunocompromised individuals. These dangerous *Fusarium* species are dispersed in the environment through their spores and isolates were obtained from soil, air and *morogo* plants growing among the maize or close by. The present study further confirmed the presence of the *fum 1* gene, one of a cluster of genes encoding fumonisin production, in representative *Fusarium* isolates retrieved from *morogo*. Fumonisin B1 was also detected in some of the *morogo* samples.

Chronic oxidative stress caused by dietary fumonisins, HIV infection of T-lymphocytes and opportunistic infections, is a key event in the initiation of chronic AIDS-related diseases. Narrowing the dietary diversity, the unavoidable consequence of poverty and food insecurity, relates to low intakes of health-supporting nutrients such as omega-3 polyunsaturated fatty

acids and folate. Deficiencies in these critical nutrients are another factor contributing to oxidative stress, tissue injury and the onset of cardio- and cerebrovascular diseases and diabetes type 2. Though characteristic of AIDS, this compendium of diseases is also collectively referred to as 'diseases of lifestyle' for being associated with a westernised diet and urban lifestyle.

The present study has demonstrated that plants utilised as *morogo* contain substantial amounts of minerals, essential trace elements, nutrients and phytochemical with health-protecting and/or immune-strengthening properties. Incorporating *morogo* in the diet could be particularly important for individuals vulnerable to infection because of their low nutritional status. *Morogo* consumption may also benefit the urbanised black population of higher socio-economic status who, because of high intakes of animal fat and low intakes of fruit and vegetables, are predisposed to chronic diseases of lifestyle.

6.2 Conclusion and recommendations

Morogo is an affordable and readily accessible source of nutritious vegetables the consumption of which would contribute towards improving the nutritional status, increasing the resistance to infection and lowering risks of chronic disease. Strategies should be developed and employed to advance the availability of traditional dark green leafy vegetables (*morogo*), not only for rural household subsistence, but also in peri-urban homestead gardens. The creation of novel urban markets for *morogo* vegetables might encourage rural farmers to scale up their production for market supply thus improving their livelihoods. Most importantly, the social image of *morogo* should be improved in the face of the dominant Westernised diet. A vital step in this direction would be to re-align urban black South Africans of the higher socio-economic classes with their traditional African food culture, because their lifestyle and value systems decisively influence the less fortunate sectors of our society.

Notwithstanding their nutritional benefits and health-protecting value, the consumer safety of *morogo* vegetables grown for family subsistence, particularly those intercropped with maize cannot be assumed. Resource-poor families in rural as well as peri-urban settings are adversely compounded by a combination of environmental stresses. Not least among these are the prevalence of disease, food insecurity and the indirect consequences of AIDS on family structure, labour and resources. Fumonigenic and mycotic *Fusarium* species in their food-production environments could add to the burden of disease in vulnerable populations who are

dependent on home-grown food. Since maize seems a likely reservoir of harmful fusaria, strategies to lower the incidence of *Fusarium* and discourage their survival and dissemination in the food-production environments, should be promoted. These could include:

- the cultivation of traditional African grains crops, i.e sorghum or pearl millet that are more resilient to African conditions and, therefore, likely to be more resistant to fungal infestation;
- crop rotation to interrupt the perpetuation of *Fusarium* epidemics;
- growing vegetables away from maize could lower risks of *Fusarium* contamination;
- the removal of maize crop residues from the land as soon as possible to reduce spore generation;
- limiting human and animal activities that cause fusarial spores in soil and plant debris to become airborne Minimum or no tillage is now advised and widely practiced in commercial maize ecosystems specifically aimed at reducing *Fusarium* levels;

The scope for research in this field is wide and some aspects urgent. The following aspects are proposed for investigation:

- the role of maize in maintaining *Fusarium* epidemics in subsistence food-production environments and routes of *Fusarium* distribution in both rural subsistence agro-environments and peri-urban homestead gardens;
- the development of alternative agricultural practices, or appropriate adaptations to existing ones, aimed at limiting *Fusarium* spore production and dissemination in home-grown food environments;
- environmental conditions favouring fumonisin production in *morogo* vegetables pre- or post-harvest, i.e post-harvest plant senescence, food handling and traditional methods of food processing;
- strategies to encourage traditional rural populations to adapt their agricultural behaviour and food processing customs would be an important topic for social science research;
- expanding the existing fragmentary database on the botanical species identification of *morogo* plants, their collection from the wild, their cultivation, uses and processing;
- information on the nutritional properties and phytochemical composition of *morogo* vegetables should be broadened.

- epidemiological studies are required to specifically relate *morogo* consumption and *in vitro* biological effects with health and disease outcomes in rural and urban consumer populations.

Results from the present study have been presented as seventeen oral papers and eight posters at international conferences.

Based on research in the present study the *Morogo* Research Programme (MRP) was founded in 2004 in the School of Environmental Sciences and Development, North-West University (Potchefstroom Campus). The MRP have since realised research collaboration with the School of Physiology, Nutrition and Consumer Sciences of the North-West University, as well as research institutes of the Agricultural Research Council, i.e ARC-VOPI and ARC-PPRI. To further promote research collaboration in South Africa on *morogo* plants, the MRP hosted the IKS/MRP Thematic Workshop in Potchefstroom in 2006. More information at: http://www.puk.ac.za/fakulteite/natuur/soo/mkb/MRP_e.html.

Activities of the MRP were further extended in the Initiative for the Development of Indigenous Food-plants of Africa (IDIFA) – an African network for research collaboration and interaction on indigenous and traditional food-plants utilised on the continent. More information at: http://www.puk.ac.za/fakulteite/natuur/soo/mkb/IDIFA_e.html. As a reuslt of IDIFA networking, the MRP is presently engaged in collaborative research projects with the following groups in other African countries:

- Department of Biochemistry, Faculty of Agriculture, Cairo University, Giza, Egypt (Prof. A.M. Aboul-Enein)
- Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City, Alexandria, Egypt (Prof. M. El-Demellawy)
- International Development Research Centre (IDRC) funded project in collaboration with Kenya and Benin and supervised by Bioversity International, Rome Italy.

"Let food be your medicine and medicine your food"

- Hippocrates -
(460 – 370 BC)

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APPENDIX

INDIGENOUS AFRICAN FOOD PLANTS: VEHICLES OF DISEASE OR SOURCES OF PROTECTION?

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ABSTRACT

The impact of recurring droughts and endemic poverty-related chronic diseases reduces the ability of poor rural households to cope with the demands of providing health-sustaining food for the family. The dietary safety and health-supporting qualities of traditional staples are important factors in consumer health. Because all crops are subject to fungal infestation, mycotoxin contamination of food has become a global problem. Home-grown foods of rural communities are not evaluated for consumer safety, and data pertaining to their health benefits are limited. This study determines the mycological quality of traditional leafy vegetables, commonly referred to as morogo, *and investigates the folate content of such crops, as well as the antimutagenic and anticarcinogenic potential of indigenous rooibos tea and other traditional food and medicinal plants. Results showed that a notable number of fungi isolated from* morogo *belonged to genera with known toxigenic species.* Morogo *plants were shown to be relatively folate-rich, and varying degrees of antimutagenic and anticarcinogenic activity were demonstrated for extracts from white and black Bambara groundnut, rooibos, cancer bush and devil's claw. Possible health-injuring impacts associated with chronic dietary exposure to certain mycotoxins, as well as health-supportive qualities of some functional foods and medicinal plants, are discussed.*

Keywords: Traditional *morogo*; edible plants; mycological stability; health-protecting qualities.

INTRODUCTION

It is estimated that 901 million people will be living in sub-Saharan Africa by 2010, a figure that could rise to 1.32 billion by 2025. Of these, 67% will be rural, and the majority of them poor (CCSU [S.a.]). According to Medaglini and Hoeveler (2003), endemic HIV/AIDS, malaria and tuberculosis are both cause and consequence of poverty and account for more than half of all deaths in sub-Saharan African countries. HIV/AIDS, however, is distinguished by the fact that infections are highest among adults aged between 20 and 40, and this has a marked impact on the income, expenditure pattern, food production and coping

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strategies of rural households (SARPN 2003; SADC FANR 2003). The cumulative effect of HIV/AIDS, causing loss of labour and knowledge of traditional farming for subsistence, has contributed to the present situation of reduced crop and dietary diversity, widespread malnutrition, general micronutrient deficiencies and decreased human resistance to infections in rural regions of sub-Saharan Africa (SARPN 2003; Mbaya 2003; SADC FANR 2003; Wiggins 2003).

In rural settings of Southern Africa, traditional diets of black communities consist mainly of corn- or grain-based staples served with cooked traditional *morogo* that is either cultivated for subsistence or collected from the field where they grow as members of the natural flora. Home-grown groundnut and beans serve as a valuable source of plant protein, adding variety to the basic diet of grain and vegetables. For centuries such traditional crops have been cultivated in the practice of subsistence farming without chemical fertilisers or pesticides. Because indigenous plants are well adapted to local growing conditions, their requirements for soil fertility, plant protection and water are modest, and subsistence farmers benefit from low production inputs (CGIAR [S.a.]). However, fungi and their spore are ubiquitous in the environment, and all crops are at risk of fungal infestation and subsequent mycotoxin contamination. Mycotoxins are "natural" food contaminants, and their formation is often unavoidable, even in commercial crops (Bennett & Klich 2003). According to Barrett (2000), mycotoxin exposure is more likely to occur in parts of the world where poor methods of food handling and storage are common and where few regulations exist to protect exposed populations. Contrary to commercial crops and retail food products, home-grown foodstuffs are not subjected to quality control to ensure their dietary safety. Consumers may therefore unknowingly be exposed to health-injuring levels of dietary toxins. Human health risks associated with mycotoxin exposure could significantly add to the existing burden of disease among consumer populations in rural regions of Southern Africa. Chronic dietary exposure to mycotoxins is associated with the occurrence of various types of cancer, kidney toxicity and immune suppression (Bennett & Klich 2003; Ferguson *et al.* 2004).

However, traditional vegetables such as *morogo*, bean plants and groundnuts are expected to be equal in nutritional value and probably possess phytochemicals with health-protecting or health-supporting qualities similar to green vegetables and leguminous crops used in developed countries. Several epidemiological and laboratory studies reviewed by Suhr & Ferguson (2003) demonstrated that some edible plants of Western and Asian diets contain substances with health-protecting properties. However, there are no scientific data to substantiate the dietary safety or health-pertaining benefits of edible plants used in traditional African diets.

A small-scale study was conducted to verify the dietary pattern and plant types used as traditional *morogo* in the Gyani district of the Limpopo Province of South Africa. The mycological quality and folic acid contents of these plants were determined. In another study extracts of Bambara groundnut, rooibos tea, devil's claw and cancer bush were evaluated for antimutagenic and anticarcinogenic properties.

MATERIALS AND METHODS

Questionnaires were used to determine the dietary patterns of rural households in the rural district of Gyani in the Limpopo Province of South Africa. Samples of fresh and dried *morogo* were collected from four rural households, a small cultivation scheme and a town stall. These included calabash leaves and flowers (*Lagenaria siceraria*), *ligushe* leaves (*Corchorys tridens)* and cowpea leaves (*Vigna subterranean*). Samples of cooked *morogo* were prepared by mixing these plants according to the traditional custom before they were cooked and sampled. For mycological analysis, fresh, dried and cooked samples were subjected to standard isolation and purification methods, and microscopic identification up to genus level was carried out according to prescribed techniques (Nelson *et al.*, 1983; Samson & Pitt, 1985; Simmons, 1996). The folic acid content of dried cowpea leaves, cooked calabash leaves mixed with the flowers and cooked *ligushe* leaves were determined by the Standards South Africa using the standard microbiological assay (Barton-Wright 1961). Extracts of black and white Bambara groundnut (*Vigna subterranean*), rooibos tea (*Aspalathus linearis*), cancer bush (*Sutherlandia fructescens*) and devil's claw (*Harpagophytum procumbens*) were subjected to a modification of the *Salmonella* antimutagenicity test using daunomycin as mutagen (Mossanda *et al.* 2001). The anticarcinogenic potential of these plants was evaluated in animal model using mouse skin. Inhibition of tetradecanoyl-phorbol-3 acetate (TPA) induced expression of the COX-2 gene through suppression of NF-κB activation was determined in the presence of plant extracts. COX-2 gene expression levels were measured using Western blotting assays (Na *et al.* 2004).

RESULTS AND DISCUSSION

Data reported in questionnaires indicated that in a typical Gyani household, an adult would normally consume three meals per day, each of which could consist of about 200 g of maize in the form of thick porridge or *putu* and 150 g of cooked vegetables. Vegetables represent different types of traditional *morogo*, often mixed together, and flowers are often added for a tasty cooked dish. This preparation may include, or could be substituted with, groundnut.

The relative numbers of fungal isolates from fresh, dried and cooked samples are reported in Table 1. The highest number of surface-colonising fungi were isolated from uncooked fresh *morogo*, namely *ligushe* (121) and fresh calabash (95), compared to those from cooked samples of *ligushe* (8) and calabash (6). A relatively small number of isolates were obtained from uncooked dried cowpea (15) and none from cooked cowpea. This data suggest that food processing, including cooking and/or drying, notably lowered the level of fungi associated with external surfaces of *morogo* plants. *Fusarium spp* was the dominant group among surface fungi isolated from both fresh (84%) and cooked (100%) *ligushe*, as well as fresh (46%) and cooked (100%) calabash, but was absent from both dried and cooked cowpea. In the case of cowpea, almost half the isolates from dried cowpea were *Penicillium spp* (47%), followed by *Aspergillus spp* (20%) and

Alternaria spp (13%). Members of these genera occurred in relatively low numbers in fresh *ligushe* (2%, 2% and 4%, respectively) and calabash (11%, 1% and 0, respectively). The mitosporic genera, *Aspergillus*, *Penicillium* and *Fusarium* all have representative species in soil (Carlile *et al.* 2001), and it is likely that these isolates represented post-harvest contaminants. In addition, many species of *Penicillium* and *Aspergillus* are xerotolerant and can grow at very low water potentials (Carlile *et al.* 2001), which might explain their relatively high numbers in dried cowpea.

Although these surface-colonising fungi could be harmless saprophytes or post-harvest contaminants, it should also be recognised that post-harvest environmental stresses could potentially induce some strains of these genera to produce toxigenic secondary metabolites (mycotoxins) known or suspected of causing human disease (Carlile *et al.* 2001; Bennett & Klich 2003). For instance, not all *Fusarium spp* produce fumonisins, and the presence of these moulds does not necessarily mean that their toxins are also present (Bennett & Klich 2003). However, the high level of *Fusarium spp* (>84%) detected among the external colonisers in human food is important because of the toxigenic potential of some species. These moulds are associated with the production of a range of potent toxins, including thrichothecenes and fumonisins such as fumonisin B_1. The International Agency for Research on Cancer has classified FB_1 as group 2B (probably carcinogenic), implying that dietary exposure could have important health consequences in humans (Bennett & Klich 2003). In cell culture and animal studies FB_1 has been reported to exhibit immuno-suppressive effects (Nair 1998; Oswald *et al.* 2003). *Aspergillus* and *Penicillium* also contain species that are known producers of potent toxins such as aflatoxins, ochratoxins, citrinin and patulin, all of which are associated with human disease (Bennett & Klich 2003). In addition, life-threatening secondary infections caused by strains of filamentous fungi, including *Fusarium spp,* have become a difficult clinical challenge, particularly in the treatment of immunocompromised patients (Segal *et al.* 1998; Guarro *et al.* 2000; Dignani & Anaissie 2004).

The results depicted in Table 2 show that a total of 52 isolates were associated with the leaf interior of *ligushe*, compared to 13 in fresh calabash. Dried cowpea was not subjected to surface sterilisation. *Alternaria spp* dominated internal colonising fungi of both *ligushe* (67%) and calabash (46%). These results seem to concur with the findings of Blodgett *et al.* (2000) who demonstrated that *Alternaria spp* comprised the main component of endophytic fungi isolated from asymptomatic leaves and petioles of *Amaranthus hybridus*. Secondary metabolites produced by *Alternaria alternata* have been shown to be involved in phytotoxicity of this fungus in a range of crop and weed species (Abbas & Boyette 1992; Abbas *et al.* 1995). Many plant products used in human diets have been reported to frequently be infected by species of *Alternaria spp* capable of toxin production. Abbas & Riley (1996) demonstrated that plant pathogenic *Alternaria alternata lycopersici* causing stem cancer in tomato plants is capable of producing both AAL toxin and fumonisin B_1 (FB_1) *in vitro*. According to Bennett & Klich (2003), the presence of potential mycotoxin-producing fungi in crops could lead to mycotoxin problems particularly when handling and storage practices are

conducive to mould growth. FB_1 and AAL toxin are structurally related and have been shown to cause disruption of sphingolipid metabolism in plant systems, cell culture and animal studies (Abbas *et al.* 1994; Turner *et al.* 1999). Data from animal and cell-culture studies suggested a role for the disturbance in the equilibrium of sphingolipid intermediates in the occurrence of mycotoxin-related carcinogenesis and certain immunosuppressing effects (Riley *et al.* 2001; Berek *et al.* 2001; Baumrucker & Prieschl 2002). Members of the genera *Aspergillus*, *Penicillium* and *Fusarium* occurred in varying degrees among isolates obtained from internal structures of both fresh *ligushe* and calabash leaves.

On the positive side it was found that some of these traditional *morogo* plants contained relatively high levels of folate. The highest folate content is reported in Table 3 for dried uncooked cowpea leaves (107 μg.100 mg^{-1}), while in cooked *calabash* leaves (mixed with flowers) folate measured 52 μg.100 mg^{-1}, and in cooked *ligushe* 27 μg.100 mg^{-1}. Although this was not determined, these values seem to indicate that cooking probably affects the folate content of *morogo*. Traditionally these plants are combined in one dish, and based on questionnaire information, the daily intake of about 450 mg of *morogo* could supply the adult consumer with more than 250 μg folate. To decrease the risk of birth defects, a daily supplement of 400 μg folic acid is recommended for woman of child-bearing age. Folate plays a critical role in the biosynthesis of DNA. Sufficient levels of cellular folate are therefore essential during the formation of new cells, including immune cells, and the maintenance of genomic stability (Kim 1999; Ferguson *et al.* 2004). In sufficient quantities, dietary folate is associated with a decreased risk of heart disease and thrombosis (Tapiero *et al.* 2001; Das 2003). According to Rampersaud *et al.* (2002), there is also convincing evidence that fruit and vegetables, common sources of food folate in the diet, decrease the risk of many cancers. Other constituents commonly found in folate-rich foods, including vitamin C, fibre and potassium, may also work alone or in conjunction with folate to decrease the risk of chronic diseases. Food folates are concentrated in foods such as dried beans and peas, peanuts and dark green leafy vegetables. Based on these findings, it seems likely that from a staple diet consisting of *morogo*, beans and groundnuts, rural communities would derive some health benefits now associated with folate-rich foods.

The results depicted in Table 4 indicate that certain indigenous foods and medicinal plants commonly used in Southern Africa may contain phytochemicals that exert antimutagenic and anticarcinogenic activity. Mutagens have been shown to act in initiating early stages of cancer development (Ferguson 2002). Contamination of agricultural products with toxins produced by strains of *Aspergillus*, *Penicillium*, and *Fusarium* has been identified as a significant mutagenic and carcinogenic hazard (Ferguson 1999). Daumomycin mutagenicity was shown to be inhibited in the presence of plant extracts from devil's claw (97.6%), rooibos tea (81.4%), cancer bush (48.8%), and white and black Bambara groundnut (47.5% and 44.5%, respectively). Figure 1 illustrates the effect of those plant extracts on tetradecanoyl-phorbol-3 acetate (TPA) induced expression of the COX-2 gene in mouse skin. Mossanda and co-workers (2001) have already elucidated the molecular mechanism of the anti-carcinogenic and anti-inflammatory effects for two African traditional plants, namely cancer bush

(*Sutherlandia fructescens*) and devil's claw (*Harpagophytum procumbens*). Used as a beverage (tea), these plants find application in the traditional treatment of cancer and inflammatory diseases respectively. Suhr & Ferguson (2003) reviewed a number of recent studies reporting on the chemopreventive potential of a variety of dietary and medicinal antimutagens.

SUMMARY AND CONCLUSION

Dietary exposure to toxins produced by strains of *Aspergillus, Penicillium, Fusarium* and *Alternaria* could enhance the risk of cancer and influence immune cell functioning. These impacts could have important consequences in HIV/AIDS individuals. In addition to enhancing consumer resistance to disease, indigenous African food plants probably possess phytochemicals that could counteract the health-injuring effects of harmful dietary substances. In view of the devastating outcome of chronic poverty, food insecurity and chronic disease in Africa, rural communities could reap long-term benefits from research supporting indigenous knowledge pertaining to the use of dietary safe, health-protecting and/or immune-strengthening indigenous food plants, and promote the cultivation of such crops in cultural subsistence farming.

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RESULTS

Table 1: Total number of isolates and relative numbers (percentages) of surface-colonising species of Penicillium Aspergillus, Fusarium and Alternaria from fresh and cooked samples of traditional *marogo*

Sample	No. of isolates	*Penicillium Spp*	*Aspergillus spp*	*Fusarium spp*	*Alternaria spp*
Ligushe (fresh)	121	4(>3)	1(<1)	88 (>72)	4(>3)
Ligushe (cooked)	8	0	0	8(100)	0
Calabash (fresh)	95	10(>10)	1(>1)	44(>46)	0
Calabash (cooked)	6	0	0	6(100)	0
Cowpea (dried)	15	7(>46)	3(20)	0	2(>13)
Cowpea (cooked)	0	0	0	0	0

Table 2: Total number of isolates and relative numbers (percentages) of *Penicillium Aspergillus, Fusarium* and *Alternaria* from internal structures of fresh samples of traditional *marogo*

Sample	No. of isolates	*Penicillium*	*Aspergillus*	*Fusarium*	*Alternaria*
Ligushe (fresh)	52	2(4)	1(2)	11(21)	35(>56)
Calabash (fresh)	13	2(>15)	2(>15)	3(23)	6(>46)

Table 3: Folate contents of *ligushe*, calabash and cowpea

Crop	Folic acid concentration (µg.100mg)
Dried cowpea leaves (*Vigna unguiculata*	107
Cooked calabash leaves mixed with flowers (*Lagenaria siceraria*)	52
Cooked fresh *ligushe* leaves (*Corchorys tridens*)	27

Table 4: Percentage inhibition of daunomycin mutagenic activity in the presence of plant extracts

Plant extract	Daunomycin mutagenicity inhibition (%)
White bambara groundnut (*Vignea* subterranean)	47.5
Black Bambara groundnut (*Vignea* subterranean)	44.5
Rooibos tea (*Aspalathus linearis*)	81.4
Devil's claw (*Harpagophytum procumbens*)	97.6
Cancer bush (*Sutherlandia fructescens*)	48.8

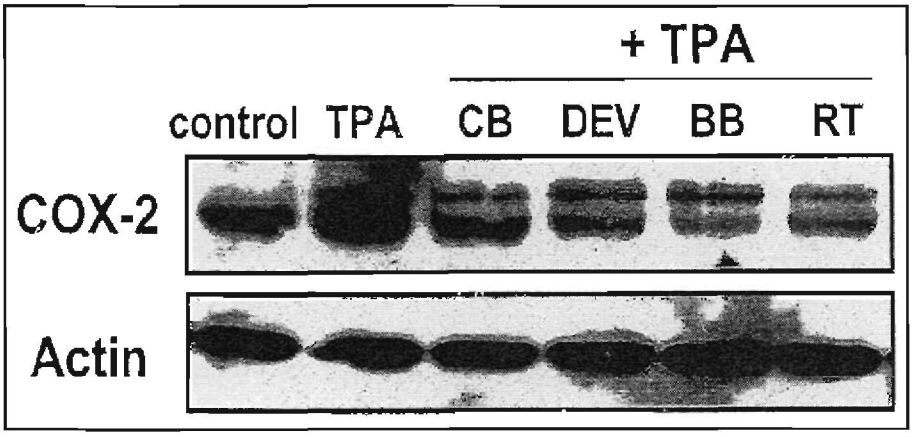

Figure 1: Image of the Western blot gel illustrating the effect of plant extracts on tetradecanoylphorbol-3 asetate (TPA) stimulated COX-2 expression in mouse skin. CB – Cancer Bush; DEV – Devil's Claw; BB – Bambara Groundnut.

Fumonisin-producing *Fusarium* strains and fumonisins in traditional African vegetables (*morogo*)

A.M. van der Walt[a*], E. van der Linde[b], M. Alberts[c], P. Modjajdi[c], S.D. Jivan[a] and C.C. Bezuidenhout[a]

In rural settings, African communities supplement grain-based staples with traditional vegetables collectively known as *morogo*. Mycotoxigenic and mycotic fungi in the agro-environment could have important health consequences for rural communities dependent on subsistence food production. The study reported here used standard techniques for the isolation and morphologically based identification of isolates to investigate the incidence of potential fumonisin-producing fusaria occurring on leaf surfaces of traditional African leafy vegetables (*morogo*). *Fusarium verticillioides*, *F. proliferatum*, *F. subglutinans* and *F. oxysporum* were retrieved in varying numbers from different *morogo* plants. Typically maize-associated, *F. verticillioides* and *F. proliferatum* were the dominant species among isolates from *ligusha*, a weedy plant growing in maize lands. Fumonisin B group toxins were also detected in varying levels in samples of dried *morogo* from study areas in different geographical regions of the Limpopo and North West provinces of South Africa. In addition to their toxigenic and carcinogenic potential, strains of these fungal species reportedly cause secondary infections in immunocompromized individuals. In rural subsistence communities with a relatively high prevalence of AIDS, these findings imply additional burdens on HIV-affected immune systems, enhancing the risk of cancer development and secondary infections.

Introduction

Rural African communities generally supplement grain-based staple diets with traditional vegetables. Referred to in local African languages as *morogo*, traditional leafy vegetables constitute a diverse range of food plants, some of which are cultivated for subsistence, while others may grow either as weeds in cultivated lands or as members of the natural field flora.[1] Investigation revealed the presence of fumonisin-producing fusaria and indicated variable degrees of fumonisin contamination in household *morogo* of rural communities dependent on subsistence food production. In such settings, *morogo* usually grow in close proximity to homegrown maize, which can be considered a likely source of fusarial contamination in fields.

The common and worldwide occurrence of toxigenic strains of *F. verticillioides*, *F. proliferatum*, *F. oxysporum* and *F. subglutinans* in commercial maize is well documented and African countries are no exception in this respect.[2-7] Globally distributed in different environments, these fungal species are recognized for their capacity to produce a range of potent fumonisin toxins in various agricultural crops.[8,5,9] Rheeder and co-workers[10] reported the occurrence of fumonisin B-producing *Fusarium moniliforme* (now *F. verticillioides*) strains in homegrown maize in the Transkei region of the Eastern Cape, South Africa. Epidemiological, animal and cell culture studies have linked dietary fumonisin B produced by grain-associated *Fusarium* strains to various human diseases, including forms of organ toxicity and cancer.[11,12] Other studies reported fusarial strains as aetiological agents of secondary infections in immunocompromized individuals.[13-15]

According to Munkvold and Desjardins,[16] toxigenic *F. verticillioides* strains produce abundant airborne microconidia (spores) in crop residues from where they are likely to become widely disseminated. Reviewing the problem of dietary mycotoxins, Bennett and Klich[17] reported that mycotoxin exposure is more likely to occur in parts of the world where inadequate methods of food handling and storage are common and where there are few regulations to protect exposed populations. Subsistence foods are not subject to quality control legislation, and traditional methods of food storage in resource-limited rural communities could enhance dietary risks associated with microbiological contamination of food. Generally not fastidious about food quality in times of food shortages, chronically poor rural households might be exposed to dietary microbiological risks that would further add to their burden of disease.

The present study reports on the occurrence of fumonisin-producing *Fusarium* strains and varying amounts of fumonisin B in traditionally dried as well as freshly collected household *morogo* from three geographically separate study areas in the northern parts of South Africa.

Materials and methods

Sample collection. For mycological analysis, four plants of two different types of traditional *morogo* were collected from each of five collection sites, namely, a stall[a] in town, a scheme farmer[b], two subsistence[c] farmers and a village household[d] in Gyani, situated in the northeast of Limpopo province. At the collection site, samples of *Corchorus* cf. *trilocularis* (*calabash*) and cf. *Lagenaria siceraria* (*ligusha*) were transferred to plastic bags, sealed and transported to the laboratory in a cooled container. To isolate internal as well as external fungal colonizers, five leaves were randomly selected from each plant. Samples for fumonisin analysis included traditionally dried household *morogo*[e] from the Dikgale Demographic Surveillance Site (DDSS; Capricorn District, Limpopo) as well as freshly collected oven-dried *morogo*[f] from the Nzhelele Valley (Thohoyandou District, Limpopo), DDSS and Rustenburg (North-West Province). *Morogo* samples for fumonisin analysis consisted either of a single plant species, a traditional mixture[g] of two plant species or a laboratory-combined mixture of more than two plant species.

Plant identification. Fresh and herbarium-prepared specimens of *morogo* plants used in the present investigation were sent to

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[a]Informal selling points of traditional food.
[b]Traditional food cultivated in small schemes employing irrigation, pest control and chemical soil improvement.
[c]Traditional food for household use cultivated without irrigation, pest control and chemical soil improvement.
[d]Traditional food produced in a village garden for household use.
[e]Freshly harvested household *morogo* cooked prior to being sun-dried on open surfaces, e.g corrugated iron.
[f]Freshly collected *morogo* oven-dried upon arrival at laboratory.
[g]Different types of traditional *morogo* mixed and cooked together, sometimes including flowers.
[h]Dikgale Demographic Surveillence Site, Capricorn District, Limpopo, South Africa; traditionally dried samples.

the South African National Botanical Institute (SANBI) for scientific species identification.

Fungal isolation and identification. Standard procedures were employed for the isolation of *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria* strains from the *morogo* samples. To optimize the isolation of fungi from internal leaf structures, fresh *Corchorus* cf. *trilocularis* and cf. *Lagenaria siceraria* leaves from the rural district of Gyani were subjected to surface sterilization[18,19] after which leaves were allowed to dry in sterile containers. Four pieces of approximately 2 mm^2 each were aseptically cut from each of the surface-sterilized leaves and transferred to Corn Meal Agar plates (Oxoid) containing 150 mg l^{-1} tetracycline (ICN, tetracycline hydrochloride, Separations, South Africa). For the isolation of surface-associated fungi, a standard mycological washing procedure with sterile 1% peptone water (Biolab, Merck, South Africa) containing 0.01% Tween 80 was employed to remove fungi from the external leaf surfaces.[20] Following 10 minutes of shaking at 25°C to wash off any surface colonizers into the diluent, serial dilutions were prepared using the same diluent. Subsequent surface plating was carried out in duplicate using 0.1 ml aliquots from each dilution placed onto each of the following culture media to which bacteria-inhibiting substances were added in the concentration indicated: Potato Dextrose Agar (Biolab, Merck) containing 5% sodium chloride (UniLAB, Saarchem, South Africa) and 40 mg $^{-1}$ oxytetracycline (ICN, oxytetracycline hydrochloride, Separations), Malt Extract Agar plates (Biolab, Merck) containing 150 mg l^{-1} tetracycline (ICN, tetracycline hydrochloride, Separations) and Rose Bengal Chloramphenicol Agar plates (Biolab, Merck). Inoculated agar plates were incubated at 25 ± 1°C for a minimum of 7 days. After incubation, mould colonies on plates of the three highest dilutions of each sample were separately transferred to Potato Dextrose Agar plates (Biolab, Merck). Single spore cultures of each colony were subsequently prepared on 1.5% water agar (Agar Bacteriological, Biolab, Merck). Isolates were identified to genus by microscopic observation using the taxonomic keys of Nelson *et al.*[21] Isolates identified as members of the genus *Fusarium* were sent to the Biosystematics Division of the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) for species identification.

Fumonisin analysis. Dried *morogo* samples were sent to PROMEC[1] for quantification of fumonisins by HPLC-FD and subsequent confirmation by liquid chromatographymass–spectrometry.

Results and discussion

The South African National Biodiversity Institute (SANBI) identified the following plant species used as traditional *morogo* by rural households in the study areas: (i) *Corchorus* cf. *trilocularis* L.; *Corchorus asplenifolius* aff. Burch; (ii) cf. *Lagenaria siceraria*; (iii) *Cleome gynandra* L. (iv) *Amaranthus hybridus* L subsp. *hybridus* var. *hybridus*; *Amaranthus thunbergii* Moq; *Amaranthus thunbergii* sensu Suess. & Podlech; (v) *Chenopodium morale* L. var. *morale*; (vi) *Vigna unguiculata* (L.) Walp. subsp. *unguiculata*; (vii) *Sonchus wilmsii* R.E.Fr.; *Sonchus oleraceus* L. (vii) *Brassica rapa*; *Brassica juncae* (L.) Czern. & Coss.; (viii) *Bidens pilosa* L.; *Bidens biternata* (Lour.) Mer. & Sheriff (ix) *Pisum savitum* L. subsp. *savitum*; (x) *Malva parviflora* L. var. *parviflora*; (xi) *Curcurbita* sp. According to information gained by questionnaire, some of these plant species are cultivated for subsistence, while others grow as weedy plants in cultivated lands or as members of the natural field flora of the region. Different plant parts may be consumed. However, leafy parts (sometimes of different species mixed together often also including flowers) are cooked as a tasty green vegetable relish to be served with maize porridge or cooked legumes.

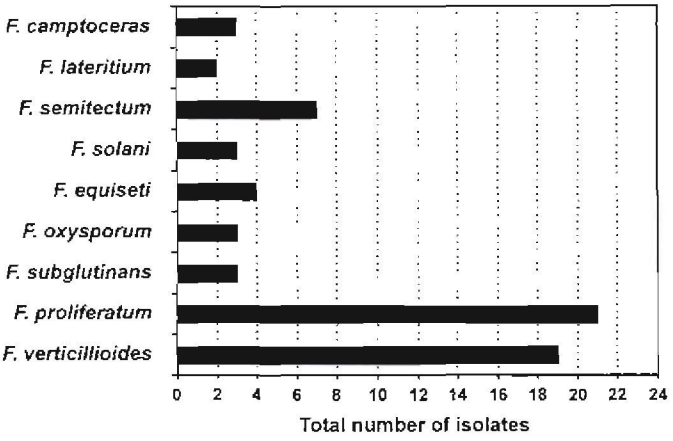

Fig. 1. Relative numbers of the respective *Fusarium* species isolated from traditional *morogo*.

The washing procedure yielded 79 *Fusarium* isolates that were subsequently demonstrated to be the dominating genus among surface-associated fungi which also included *Aspergillus niger* (3), *Aspergillus sydowii* (1), *Penicillium aurantiogreseum* (2) and *Alternaria* sp. (3). However, *Fusarium* species were absent from those fungi retrieved from internal leaf structures that were predominantly *Alternaria* species (34), although *Aspergillus* sp. (1) and *Penicillium* sp. (2) also occurred.

Of the 79 *Fusarium* isolates retrieved from Gyani *morogo*, 65 were eventually identified by the ARC-PPRI. The 14 other original isolates either did not grow after sub-culturing, mutated in the process or subsequently formed atypical structures that made morphologically based identification impossible. Identified isolates were assigned PPRI accession numbers and are being preserved in the National Collection of Fungi for subsequent study. Of interest were results illustrated in Fig. 1 showing that the species most frequently recovered from the *morogo* were *F. proliferatum* (21 isolates) and *F. verticillioides* (19). Widely distributed and occurring frequently in maize, *F. verticillioides* and *F. proliferatum* are listed by Rheeder *et al.*[5] as the most important producers of fumonisins. The occurrence of *F. verticillioides*, *F. proliferatum*, *F. subglutinans* (3) and *F. oxysporum* (3) in association with traditional *morogo* is noteworthy for the fact that these fumonisin-producing strains constituted 71% of the total number of isolates. Scientific data on fungal populations associated with traditional *morogo* and the occurrence of toxigenic *Fusarium* species in traditional vegetables is lacking. Though this study was conducted on a small scale, our results raise questions, firstly about the dietary safety of traditional staple vegetables for rural consumer populations dependent on subsistence vegetable crops, and secondly, about the source of fusarial contamination.

Results depicted in Fig. 2 illustrate that *F. verticillioides*, *F. subglutinans* and *F. oxysporum* occurred more frequently in association with *ligusha*, whereas *F. proliferatum* strains were predominantly recovered from *calabash*. Since these toxigenic fusaria apparently occurred in a non-pathogenic relationship with *morogo* plants (they were recovered from the surfaces of leafs that did not exhibit any disease symptoms), the relative distribution of the different species might be meaningful in terms of the possible source of vegetable contamination. *Fusarium* infection and post-harvest contamination of maize reportedly constitute a worldwide problem causing crop loss and deterioration of stored food.[9,22–24] Reviewing fumonisin

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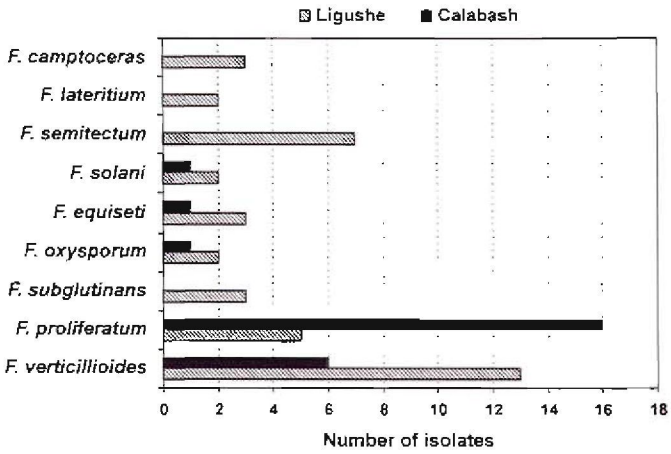

Fig. 2. Relative distribution of different *Fusarium* species in *ligusha* and *calabash*, respectively.

Fig. 3. *Ligusha* (indicated by arrows) growing in maize fields of a rural community of Gyani, Limpopo province.

contamination of maize, Munkvold and Desjardins[16] described *F. moniliforme* (now *F. verticillioides*) as an almost constant companion of maize plants and seeds, commonly found colonizing symptomless maize plants. In a recent study, Fandohan and co-workers[7] found *F. verticillioides* and *F. proliferatum* to be the two most commonly occurring *Fusarium* species in pre-harvested as well as stored maize of small-scale farmers in four agro-ecological zones of Benin. Closer to home, Rheeder *et al.*[11] reported the frequent occurrence of *F. verticillioides* (formerly *F. moniliforme*) in 'visibly nonmouldy' homegrown corn of rural communities in the Transkei region.

Figure 3 is a photograph of one of the Gyani sampling sites showing ligusha growing among dry homegrown maize (*Zea mays*) on land where these vegetables are collected either for consumption or storage after being processed. *F. verticillioides*, reported to occur ubiquitously in soil and maize crop residue,[16] in this case might be the source of vegetable contamination when airborne spores spread onto agricultural lands. Though a cultivated crop, in rural subsistence settings *calabash* usually grows in close proximity to maize lands. Leslie *et al.*[25] reported that the range of *Fusarium* species found in native prairie grasses in Kansas paralleled that which were typically recovered from maize or sorghum growing in adjacent areas. Among these were *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. Furthermore, these grass-associated fusaria were found to generate toxins in quantities similar to those produced by isolates from the same species recovered from agricultural hosts. According to Rheeder *et al.*,[5] *F. verticillioides* and *F. proliferatum* are the most important producers of fumonisins because of their overall high levels of toxin production, their wide geographical distribution and common presence on maize.

In another study, Bezuidenhout and co-workers[26] evaluated the application of a multiplex PCR-based method for the rapid detection of potential fumonisin-producing *Fusarium* in traditional African vegetables using six *Fusarium* isolates from the Gyani *morogo*, namely, four *F. verticillioides* (PPRI 7363, PPRI 7367, PPRI 7370, PPRI 7372) and one *F. subglutinans* (PPRI 7365). A fumonisin-positive MRC *Fusarium verticillioides* (MRC 4319) strain was included as a reference culture. Primer sets employed for the purpose of the study targeted the following conserved gene fragments: (i) the polyketide synthase *FUM1* gene involved in fumonisin biosynthesis; (ii) the translation elongation factor 1-alpha (TEF 1-α) of *Fusarium* spp. and (iii) a conserved region of fungus 18S mRNA. These fragments were all of distinct sizes and products of the multiplex PCRs could be resolved using 2% (w/v) agarose gels. These molecular results confirmed strains retrieved from Gyani *morogo* as belonging to the genus *Fusarium* and showed that these isolates possessed the polyketide synthase *FUM 1* gene for fumonisin biosynthesis. However, the expression of genes encoding fumonisin production and regulation is expected to be influenced by complex interactions of biotic and abiotic environmental factors. In this respect, predisposing conditions influencing fumonisin biosynthesis and accumulation in traditional leafy vegetables have neither been investigated nor verified and it is as yet unclear whether such conditions exist pre- or post-harvest. Since they are secondary metabolites, mycotoxins are formed towards the end of the exponential growth stage. In rural settings, households generally lack refrigeration facilities for food storage and so it is expected that post-harvest senescence of fusarial-contaminated crops and subsequent storage would be risk factors for fumonisin production and accumulation in traditional crops.

This seems to be confirmed by results of chemical analysis of household *morogo* sampled from different study areas reported in Table 1. Fumonisin contamination of these samples apparently was neither related to plant species nor by geography. One sample of traditionally dried *Cleome gynandra* L. from DDSS contained fumonisin B_{1-3} in amounts totalling nearly 47 000 ng g^{-1}. The reported 300 g of *morogo* consumed daily by an adult (unpublished survey information) relates this quantity of fumonisin contamination to an estimated daily exposure of 14.1 mg fumonisin B from vegetable consumption alone. Subsistence maize is probably contaminated as well. Some *morogo* samples contained fumonisins in lower concentrations or not at all. However, the exposure risk implied by this finding has important consequences in poor rural communities dependent on subsistence staple vegetables.

Epidemiological evidence and animal studies have linked dietary fumonisin B_1 (FB_1) to various human diseases. FB_1 was evaluated as a Group 2B carcinogen, indicating that this mycotoxin is probably carcinogenic to humans.[27] Studies by Lerda *et al.*[28] suggested that increased oxidative stress induced by FB_1 exposure can damage DNA indirectly, thus acting as a mechanism for FB_1-associated hepatotoxicity. Furthermore, reported FB_1-induced lipid peroxidation that affects cellular membranes, mitochondria and DNA synthesis, as well as FB_1 disruption of the metabolism of sphingolipids that are important components of all eukaryotic cellular membranes, collectively constitute mechanisms through which this agent might impair cellular viability.[29,30] Sphingolipid intermediates, as

Table 1. Fumonisin B concentrations in traditional household morogo from different regions.

Plant species	Collection site	FB_1 (ng g^{-1})	FB_2 (ng g^{-1})	FB_3 (ng g^{-1})	Total (ng g^{-1})
Amaranthus viridis + tomato	DDSS[1]	n.d.	n.d.	n.d.	n.d.
Amaranthus viridis + tomato	DDSS	143	n.d.	n.d.	143
Cleome gynandra	DDSS	1932	568	n.d.	2500
Cleome gynandra	DDSS	38 860	6511	1565	46 937
Vigna unguiculata	DDSS	n.d.	n.d.	n.d.	n.d.
Vigna unguiculata	DDSS	1601	518	n.d.	2119
Amaranthus viridis + *Cleome gynandra*	DDSS	n.d.	n.d.	n.d.	n.d.
Cleome gynandra + *Vigna unguiculata*	DDSS	n.d.	n.d.	n.d.	n.d.
Vigna unguiculata + pumpkin	DDSS	n.d.	n.d.	n.d.	n.d.
Amaranthus viridis + *Cleome gynandra*	DDSS	661	n.d.	n.d.	661
Combined sample[2]	DDSS	224	n.d.	n.d.	224
Curcubita spp.[3]	Nzhelele Valley[4]	66	n.d.	n.d.	66
Brassica rapa[5]	Nzhelele Valley	n.d.	n.d.	n.d.	n.d.
Combined sample[6]	Rustenburg[7]	114	n.d.	n.d.	114
Combined sample[8]	Rustenburg	n.d.	n.d.	n.d.	n.d.

n.d., not detected.
[1]Freshly collected oven-dried samples of *Vigna unguiculata, Cleome gynandra, Amaranthus viridis, Citrullus lanatus* (Thunb.)Matsum & Nakai, *Chorchorus tridens* L. and *Vernonia fastigiata* Oliv. & Hiern.
[2]Freshly collected senescent samples.
[3]Thohoyandou District, Limpopo.
[4]Freshly collected senescent samples.
[5]Freshly collected oven-dried samples of *Amaranthus hybridus* L. *subsp. cruentus* (L.) Thell. *Cleome gynandra* L., *Momordica balsamina* L., *Penarrhinum insipidum* E. Mey.
[6]Rustenburg District, North-West province, South Africa.
[7]Freshly collected oven-dried samples of *Chenopodium album, Sonchus wilmsii* R.E.Fr. *Chorchorus schimperi* Cufod.

well as complex sphingolipids, have been shown to be important as signal-transducing molecules involved in control of cell proliferation, survival, differentiation and apoptosis.[31] Evidence that interference with sphingolipid metabolism disrupted membrane receptor sites involved in cellular uptake of folate also exists.[12,32] Because folate is essential for DNA biosynthesis and methylation,[33] folate deficiency might result in DNA damage and instability as well as aberrant patterns of DNA methylation, all of which are considered to be factors in carcinogenesis.[32]

Disruption of sphingolipid metabolism also influences immune cell proliferation and essential intra- and intercellular communication between various types of immune cells, thereby affecting their activation status.[34] Animal and cell culture studies have indicated that other immune functions may also be affected by FB_1. Processes involved in the destruction of infecting bacteria, such as phagocytosis and oxidative burst in neutrophils, were shown to be regulated by sphingolipid intermediates.[34,35] Moreover, a number of *Fusarium* species, including *F. verticillioides, F. oxysporum* and *F. solani*, have been implicated as causative agents of secondary infections in immuno-suppressed individuals.[15,36]

Concluding remarks

Based on a 2004 survey, the Department of Health estimated the prevalence of HIV infection among adults and children of the Limpopo and North West provinces to be 9.8% and 10.3%, respectively, and among women attending antenatal clinics as 19.3% and 26.7%.[37,38] Given these statistics, the presence of harmful fusarial strains in rural subsistence agro-environments would be an aggravating health risk factor in poor communities dependent on traditional vegetables. This is so whether these fungi occur as food-related producers of mycotoxins exerting diverse biochemical effects, or as aetiological agents of secondary infections. The sources and routes of mycotoxigenic and mycotic *Fusarium* contamination in poor rural areas, as well as predisposing environmental factors for fumonisin production in traditional crops and vegetables, ase being investigated by the *Morogo* Research Programme.

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Fusarium populations in the household food gardens of a peri-urban community

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Toxigenic *Fusarium* species, studied mainly for the damage they cause to commercial crops, have received scant research attention in the context of small-scale food production. In this regard home gardens are an important source of nutrition for resource-poor urban families in Africa. We have investigated the presence of *Fusarium* in household food gardens in a peri-urban setting in the Rustenburg district of South Africa. Standard techniques were employed for the isolation and morphological species identification of *Fusarium* species from various sources, namely, maize, soil, air and naturally growing *morogo* vegetables, thepe and lerotho. Nine *Fusarium* species with mycotic and mycotoxigenic potential were specifically targeted for detection: *F. verticillioides*, *F. proliferatum*, *F. solani*, *F. subglutinans* and *F. oxysporum* were predominantly isolated from maize, air, soil and *morogo* vegetables. All species were isolated in significantly higher numbers from localities in proximity to maize. *Fusarium chlamydosporum*, *F. semitectum* and *F. equiseti* were not retrieved where maize was absent, whereas *F. verticillioides* and *F. proliferatum* were predominantly isolated from maize cobs. These results have public-health implications. All nine *Fusarium* species retrieved from peri-urban food gardens produce toxins and, except for *F. poae*, have been implicated in opportunistic infections in immune-suppressed individuals.

Introduction

The genus *Fusarium* includes various phytopathogenic species of economic importance, mainly for the damage they cause to commercial crops.[1] Invasion of plant hosts is often linked with the capacity of the fungal pathogen to produce toxins.[2] Dietary exposure to fusarial toxins causes irreversible tissue damage through biochemical mechanisms that produce pro-oxidative, pro-inflammatory, carcinogenic and/or immune-suppressive effects at a cellular level.[3-6] Some toxigenic *Fusarium* species have furthermore been implicated as causative agents of life-threatening opportunistic infections in immune-suppressed individuals.[7] Mortalities ranged between 50–80% in these cases, mainly because effective treatment of infection was complicated by drug resistance by the *Fusarium* pathogens and their blood-borne spread to various organs of the body.[8]

Peri-urban families living on a severely restricted budget in South Africa often resort to growing food at home to augment their food supply. Maize grown as the traditional staple in these situations is supplemented with green leafy vegetables (called *morogo*), often collected from the field.[9] Some *morogo* species also appear spontaneously in disturbed soils and consequently are found growing among the maize.[10,11] Maize ecosystems naturally harbour several toxigenic *Fusarium* species, however, notably those that produce fumonisin toxins.[1,12,13] *Fusarium* where food is grown has important health implications, particularly for food-insecure populations that, according to Bourne *et al.*,[14] are disproportionately affected by disease because of their unsatisfactory nutritional status.

We conducted a pilot study in a peri-urban community in the Rustenburg district of South Africa to determine the occurrence of nine *Fusarium* species in the vicinity of household food gardens and the influence of home-grown maize nearby. We considered the factors that may contribute towards sustaining *Fusarium* species in such environments and discuss the possible health implications for local communities.

Materials and methods

See Appendix.

Results

Figure 1 illustrates the number of isolates of each *Fusarium* species retrieved from samples collected on three sampling occasions from four localities at sites near to, or distant from, maize. All nine species occurred in varying degrees where maize was growing, with the number of isolates ranging from 6 (*F. equiseti*) to 130 (*F. verticillioides*). In localities remote from maize, the number of isolates was notably smaller, varying between 3 (*F. poae*) and 34 (*F. verticillioides*). *Fusarium equiseti*, *F. chlamydosporum* and *F. semitectum* were not retrieved from maize-free localities. Table 1 shows the evidence for *Fusarium* isolates retrieved from air, soil, lerotho and thepe as being relatively high from localities near maize. Mean log numbers of *Fusarium* isolated from where maize grew were highest in air (0.58 ± 0.1) and soil (0.51 ± 0.08) and lower in lerotho (0.04 ± 0.06) and thepe (0.26 ± 0.05). Table 2 shows that, on a 5% level, all species were isolated in significantly higher numbers where maize was nearby. Mean log numbers further indicate that *F. verticillioides* (0.85 ± 0.9) and *F. proliferatum* (0.78 ± 0.09) were

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the predominant species retrieved from localities near maize, followed by *F. solani* (0.69 ± 0.1), *F. oxysporum* (0.52 ± 0.1) and *F. subglutinans* (0.4 ± 0.11). Away from maize, mean log numbers ranged from 0.04 ± 0.04 (*F. semitectum*) to 0.24 ± 0.07 (*F. proliferatum*) and 0.3 ± 0.1 (*F. verticillioides*).

Tukey post hoc comparisons of *Fusarium* species numbers retrieved from each of the different environmental components are shown in Table 3. Means of log numbers indicate that *F. verticillioides* (0.83), *F. proliferatum* (0.66) and *F. solani* (0.61) were isolated from the air in numbers significantly higher than *F. poae* (0.16), *F. equiseti* (0.13) and *F. chlamydosporum* (0.08). *Fusarium semitectum* (0) were not retrieved from air samples. Similar results apply to *F. verticillioides* (0.71) and *F. proliferatum* (0.68) isolated from soil. *Fusarium equiseti* were retrieved from neither soil nor lerotho. No significant differences were found between the contamination of lerotho and thepe. In comparison with thepe, *F. verticillioides* (0.83) and *F. oxysporum* (0.43) were retrieved in significantly higher numbers from air. The other pathogens were not significantly different from each other in this respect.

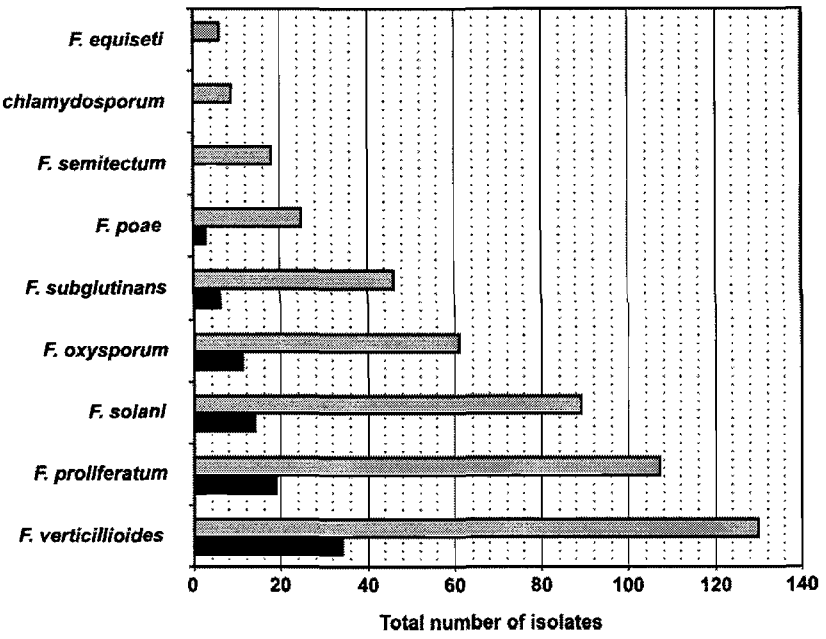

Fig. 1. *Fusarium* species distribution among the total number of isolates respectively retrieved from localities near (grey bars) and away from (black bars) maize.

In total, 150 *Fusarium* isolates were retrieved from the silk (69) and kernels (81) of maize cobs sampled from the four localities. Two-way ANOVA was used to determine interaction between maize cob components (leaves, silk and kernels) and *Fusarium* species (Table 4). No *Fusarium* was isolated from maize leaves and there was no significant interaction ($P = 0.25$) between kernels, silk and the *Fusarium* species isolated from them. In terms of log numbers, however, *F. semitectum* (0.23), *F. equiseti* (0.04) and *F. chlamydosporum* (0.04) were retrieved in significantly smaller numbers from maize kernels and silk than with *F. proliferatum* (0.79) and *F. verticillioides* (0.75). Of the maize-associated species, *F. subglutinans* (0.32) was isolated from kernels and silk in significantly lower numbers than *F. proliferatum*.

Fusarium isolates were retrieved in significantly higher numbers from localities near maize (Fig. 1). Mean log numbers of isolates retrieved from air, soil, thepe and lerotho respectively reflect this result (Table 1), which is demonstrated also in Table 2 for each of the nine *Fusarium* species. The number of isolates of *F. verticillioides* and *F. proliferatum* was significantly higher in air and soil relative to *F. poae*, *F. equiseti*, *F. chlamydosporum* and *F. semitectum* (Table 3) and, compared to *F. equiseti*, *F. chlamydosporum* and *F. semitectum*, also in maize cobs (Table 4).

Table 1. Relative occurrence of *Fusarium* (log numbers) at localities near and far from maize, respectively.

Environmental component	Near maize (mean ± s.e.)	Away from maize (mean ± s.e.)	*t*	d.f.	*P*
Air	0.58 ± 0.1	0.15 ± 0.05	4.49	56.5	<0.0001
Soil	0.51 ± 0.08	0.12 ± 0.05	4.31	56.7	<0.0001
Lerotho	0.41 ± 0.06	0.14 ± 0.04	3.70	62.5	0.0005
Thepe	0.26 ± 0.05	0.04 ± 0.02	4.42	46.2	0.0001

Table 2. Relative *Fusarium* species distribution (log numbers) at localities near and far from maize, respectively.

Fusarium species	Near maize (mean ± s.e.)	Away from maize (mean ± s.e.)	*t*	d.f.	*P*
F. verticillioides	0.85 ± 0.9	0.3 ± 0.1	4.18	29.5	0.0001
F. proliferatum	0.78 ± 0.09	0.24 ± 0.07	4.57	28.7	<0.0001
F. solani	0.69 ± 0.1	0.15 ± 0.07	4.33	27.6	0.0002
F. oxysporum	0.52 ± 0.1	0.14 ± 0.06	3.15	25.1	0.0021
F. subglutinans	0.4 ± 0.11	0.09 ± 0.05	2.60	20.7	0.0085
F. poae	0.25 ± 0.08	0.05 ± 0.03	2.21	19.8	0.0194
F. semitectum	0.22 ± 0.07	0.04 ± 0.04	2.28	22.5	0.0162
F. chlamydosporum	0.15 ± 0.05	0	2.88	15.0	0.0057
F. equiseti	0.11 ± 0.04	0	2.53	15.0	0.0115

Discussion

With the exception of *F. poae*, *Fusarium* species isolated from the environment of peri-urban food gardens have been reported to be capable of causing opportunistic infections in immune-suppressed individuals.[21] Moreover, all species targeted for detection in the present survey produce mycotoxins[22,23] that could compromise immune functioning.[3,24,25] Results illustrated in Fig. 1 suggest these mycotic and mycotoxigenic *Fusarium* species are common members of autochthonous microbial populations in the environment of peri-urban food gardens. *Fusarium* species were retrieved in significantly higher numbers from localities in the proximity of home-grown maize than away from maize (Table 1). Though true of all nine *Fusarium* species, the observation was most pronounced for *F. verticillioides*, *F. proliferatum*, *F. subglutinans* and *F. oxysporum* (Table 2). Commonly associated with commercial maize ecosystems,[13,26,27] the first three species are maize plant pathogens that cause seedling disease, root and crown rot, stalk rot and ear rot.[28] Apart from the study of Sreenivasa and co-workers,[29] in which *F. solani* was detected in freshly harvested maize, the presence of this species in maize ecosystems is not often reported.

The isolation of *F. solani* in significant numbers ($P = 0.0002$) is of particular interest in view of the high incidence of HIV infection in South Africa.[20] Mid-year estimates indicated that 10.9% of the South African population was HIV-positive in 2006.[20] Pujol *et al.*[21] describe *F. solani* as the most dangerous filamentous fungus for immunocompromised patients after *Aspergillus fumigatus*, whereas Boutati and Anaissie[30] report it as the species predominantly isolated from fatal cases of disseminated fusarioses in patients with haematologic malignancies. *Fusarium poae*, usually associated with destructive diseases of wheat,[31,32] was also re-

Table 3. *Fusarium* species distribution (log numbers) in air, soil, lerotho and thepe, respectively.

Component	Species–distance interaction*	*F. verticillioides*	*F. proliferatum*	*F. solani*	*F. oxysporum*	*F. subglutinans*	*F. poae*	*F. semitectum*	*F. equiseti*	*F. chlamydosporum*
Air	0.028	$^{d}0.83^{a}$	$^{d}0.66^{a}$	$^{d}0.61^{a,b}$	$^{d}0.43^{a,b,c}$	$^{d}0.4^{a,b,c}$	$^{d}0.16^{b,c}$	$^{e}0^{c}$	$^{d}0.13^{c}$	$^{d}0.08^{c}$
Soil	0.051	$^{d,e}0.71^{a}$	$^{d}0.68^{a}$	$^{d}0.45^{a,b}$	$^{d}0.45^{a,b}$	$^{d}0.28^{a,b}$	$^{d}0.11^{b}$	$^{d,e}0.09^{b}$	$^{e}0^{b}$	$^{d}0.06^{b}$
Lerotho	0.772	$^{d,e}0.5^{a}$	$^{d}0.42^{a,b}$	$^{d}0.38^{a,b}$	$^{d,e}0.41^{a,b}$	$^{d}0.12^{a,b}$	$^{d}0.23^{a,b}$	$^{d}0.33^{a,b}$	$^{e}0^{b}$	$^{d}0.06^{a,b}$
Thepe	0.809	$^{e}0.26^{a}$	$^{d}0.27^{a}$	$^{d}0.23^{a}$	$^{e}0.04^{a}$	$^{d}0.17^{a}$	$^{d}0.1^{a}$	$^{d,e}0.1^{a}$	$^{d,e}0.08^{a}$	$^{d}0.1^{a}$
Source-distance interaction**		0.699	0.285	0.490	0.030	0.616	0.998	0.402	0.020	0.958

*Interaction between *Fusarium* species and distance from maize (*P*-values).
**Interaction between source and distance from maize (*P*-values).
Superscripts a, b and c in the same row: entries with the same symbol do not differ significantly at the 5% level.
Superscripts d and e in the same column: entries with the same symbol do not differ not significantly at the 5% level.

Table 4. Relative *Fusarium* species distribution in maize cobs (silk and kernels).

Species–maize cob interaction	*F. proliferatum*	*F. verticillioides*	*F. solani*	*F. oxysporum*	*F. subglutinans*	*F. poae*	*F. semitectum*	*F. equiseti*	*F. chlamydosporum*
0.395	0.79^{a*}	$0.75^{a,b}$	$0.38^{a,b,c}$	$0.36^{a,b,c}$	$0.32^{b,c}$	$0.29^{b,c}$	0.23^{c}	0.04^{c}	0.04^{c}

*Entries with the same superscript symbol do not differ significantly at the 5% level.

trieved in significant numbers ($P = 0.02$) from localities near maize. *F. equiseti* and *F. chlamydosporum* were not isolated from sites away from maize, but in relatively small numbers near these plants. Relative to *F. verticillioides*, which was isolated as the predominant species, *F. equiseti* and *F. chlamydosporum* represented a small percentage of *Fusarium* isolated from commercial maize in Ghana.[26]

Figure 1 and Tables 1 and 2 indicate that maize may play a role in maintaining *Fusarium* species in the environment of peri-urban home gardens. Table 3 reveals significant interaction of maize-associated *F. verticillioides* and *F. proliferatum* with air ($P = 0.03$) and soil ($P = 0.05$). Cotton and Munkvold[28] found *F. verticillioides* and *F. proliferatum* multiplied rapidly during the growing season on maize leaf surfaces as well as in rainwater trapped in leaf sheaths, and subsequently survived for up to two years in soil and maize plant residue on the soil surface. Mean log numbers depicted in Table 4 show that *F. verticillioides* (0.75) and *F. proliferatum* (0.79) isolated from maize cobs were primarily associated with maize kernels and silk, which might indicate a pathogenic relationship. *Fusarium* establishes infection when spores in the environment land on the silk, germinate and enter the ear after pollination, according to Cardwell *et al.*[33] Nesci *et al.*[27] attributed the occurrence of *F. verticillioides* and *F. proliferatum* in soil of pre-harvest maize ecosystems to the survival of these species in plant debris on the soil surface. Rossi *et al.*[34] found that, under humid field conditions, fusarial spores in crop debris germinate and continue to produce macroconidia. These spores become airborne by splash dispersal during rain showers or irrigation and are disseminated over substantial distances by air currents.[35,36]

It thus seems likely that in home gardens, *F. verticillioides*, *F. proliferatum* and possibly *F. subglutinans* and *F. oxysporum*, occurring in association with home-grown maize, are maintained in maize residues and debris on the soil surface. Dispersed from plant debris, and depending on spore and leaf surface characteristics, airborne spores are trapped in differing degree by *morogo* plants growing in association with maize. This scenario might explain why, in the same environment, *F. verticillioides* was retrieved in higher numbers from thepe than from lerotho, while *F. oxysporum*, *F. poae* and *F. semitectum* were isolated in greater numbers from lerotho than from thepe (Table 3).

The multiplex PCR method described by Bezuidenhout *et al.*[17,37] confirmed the presence of the *FUM 1* gene in *F. oxysporum*, *F. proliferatum*, *F. solani* and *F. subglutinans* isolated from *morogo* plants. Furthermore, HPLC analysis detected 44.8 ng g^{-1} fumonisin B_1 in thepe and 58.7 ng g^{-1} in lerotho.[38] Similar findings were reported from maize-based subsistence agriculture in the Limpopo province.[39]

Fusarium species in home gardens have public-health implications. Urban activities are expected to enhance dissemination of fusarial spores, while human population numbers in urban settings put more individuals at risk of opportunistic fusarial infections. Numerous case studies identified *F. verticillioides*, *F. proliferatum*, *F. solani* and *F. oxysporum*[30,40–43] as causative agents of disseminated fusariosis in immunocompromised individuals, in most instances with fatal consequences.[8,44] The prevalence of *Fusarium* infections of the skin, the upper respiratory tract and eyes suggests that these organs serve as portals of entry, eventually leading to multiple organ infection.[30,45] In the present survey these pathogens were isolated in considerable numbers from air, indicating an unavoidable risk for residents of inhalation, skin contact, or eye exposure to fusarial spores. *Fusarium* spores in the environment enhance the risk of HIV-positive individuals contracting secondary fusarial infections which, according to Dignani and Anaissie[8] and Pujol *et al.*,[21] are most difficult to treat. Common features of opportunistic *Fusarium* infections include the presence of fusaria in the blood stream, the high frequency of skin lesions and a high mortality rate in patients with suppressed immunity as a result of pathogens resistance to drugs.[39,46]

All the *Fusarium* species targeted for isolation in the present study are producers of potent toxins including beauvericin,[22] fumonisins[23] and trichothecenes.[47] Dietary mycotoxins may produce a range of biological effects, depending on which of the following properties they possess: antinutritional,[48] oxidative,[5] pro-mutagenic,[6] pro-inflammatory,[49] tumorgenic,[50] genotoxic[51] and/or immune-suppressive.[3,25,52]

Concluding remarks

Food gardens play a vital role in providing poor families with nutrition.[9,10] The present study suggests, however, that home-grown maize in food gardens may play a cardinal role in maintaining harmful *Fusarium* species in the peri-urban environment. The findings reported here invite further attention, in view of the public-health implications. A database on possible sources and mechanisms of dissemination of toxigenic and mycotic *Fusarium* in peri-urban food production settings

should serve as a basis for future strategies to ensure safe food-production practices among vulnerable populations.

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Appendix

Sample collection. Samples were collected from food gardens of selected households at four localities in the township of Phokeng, near Rustenburg, North West province, during the maize-growing season in Febru-

ary and April 2006 as well as in February 2007. Two naturally growing *morogo* types commonly consumed in the study area, namely thepe (*Amaranthus hybridus*) and lerotho (*Cleome gynandra*), were sampled at four localities where *morogo* vegetables were growing with maize on the same plot of land, and four sites where these plants grew some distance from maize. At each locality, 10 leaves were collected from two separate plants each of thepe and lerotho. Five leaves of a maize plant as well as a maize cob were also sampled. At each locality samples were taken of the top soil layer at three random positions around each of the plants. Three agar plates containing pentachloronitrobenzene (PCNB) medium selective for *Fusarium* were exposed at three random positions around each plant for three minutes to trap *Fusarium* spores from the air. Air plates were immediately closed and secured with parafilm. Thepe, lerotho, soil and maize samples were transferred separately to 'ziplock' plastic bags. Samples were transported to the laboratory on ice and immediately processed for mycological analysis upon arrival.

Culture media for isolation and morphological identification of Fusarium. The culture medium, selective for the isolation of *Fusarium*, contained Peptone PCNB (Terraclor, Sigma, South Africa) to which were added the following antibiotics for inhibition of bacterial growth: benzylpenicillin (Fresenius, South Africa), pendistrep (Virbac Animal Health, South Africa) and chloramphenicol pure (Pharmachemie, South Africa). The following growth media were used for the purification and preparation of single spore cultures of *Fusarium* isolates: Water Agar plates (WA; Agar Bacteriological, Biolab, Merck, South Africa) and Carnation Leaf Agar (CLA; consisting of a Water Agar plate with a piece of carnation leaf γ-sterilized by Isostar, South Africa, placed on the surface). To identify species, single-spore cultures were subsequently transferred to the following culture media: to Potato Dextrose Agar (PDA; Biolab, Merck, South Africa) to observe colony morphology; to CLA plates and Synthetic Nutrient Agar (SNA; Sigma, South Africa) to examine microscopic structure. Culture media were prepared as described in ref. 15.

Isolation of Fusarium. We used the washing procedure described by Medina-Martinez and Martinez[16] to isolate *Fusarium* that had colonized the external surfaces of *morogo* leaves, maize leaves and kernels. Each leaf and kernel was separately added to 99 ml sterile 1% peptone water (Biolab, Merck) containing 0.01% Tween 80 and shaken for 10 min on a rotary shaker at room temperature to remove spores from leaf surfaces. Diluents containing the rinsed-off *Fusarium* spores were subsequently diluted (10^{-3} to 10^{-5}) and 0.1-ml aliquots of each dilution were used for spread plating onto PCNB agar. Internal colonizing *Fusarium* was isolated by sterilizing leaf and kernel surfaces in 1% hydrogen peroxide for 1 min followed by aseptical rinses in distilled water three consecutive times.[17] Five approximately 1-cm^2 squares cut from each *morogo* leaf, and 10 pieces from each maize leaf, were transferred separately to the surface of a separate PCNB agar plate, employing aseptic procedures. After surface sterilization according to a similar procedure, ten maize kernels from each maize cob were each placed on a separate PCNB agar plate. The three soil samples from each site were thoroughly mixed in the laboratory into a composite soil sample, of which 1 g was carefully weighed and transferred to 9 ml sterile distilled water (10^{-1} dilution). A dilution series of 10^{-2} to 10^{-5} was subsequently prepared aseptically and 0.1-ml aliquots of each dilution used for surface plating onto PCNB agar. All plates (including parafilmed air plates) were subsequently incubated at 25°C for a minimum of 7 days.

Purification of Fusarium *colonies.* After incubation, plates were examined under a stereo-microscope. Colonies suspected of being *Fusarium* were selected, and then purified, on the basis of characteristics and procedures described in ref. 15. A 1-cm^2 piece of PCNB agar containing the selected colony was aseptically cut from the PCNB plate and transferred to a CLA plate. After incubation for 7–10 days at 25°C, during which time plates were exposed to a 12:12-hour light/dark cycle, single-spore cultures were prepared by flooding each plate with 9 ml sterilized distilled water, and pouring the mixture aseptically over the surface of a WA plate. The WA plates were carefully rotated and the excess water was drained off before being incubated at 25°C in an inclined position. After 16–24 hours of incubation, the WA plates were examined under a stereo-microscope and a piece of agar with a single germinating spore was aseptically transferred to the surface of each of a PDA, CLA and SNA plate. Inoculated plates were incubated at 25°C for 7–14 days.

Morphological identification of Fusarium *isolates.* After incubation, single-spore cultures of *Fusarium* isolates growing on the PDA, SNA and CLA were identified from morphology and other features according to illustrated laboratory manuals.[15,18] Macroscopic observations of single-spore colonies growing on PDA plates included colony morphology, colony colour, as well as the presence and position of the sporodochia. Using lactophenol for slide preparation, microscopic observations and identifications were based on such characteristics as the position, size, shape, and arrangement of microconidia, macroconidia and chlamydospores of single spore cultures on CLA plates.[15,18]

Statistical methods. Fusarium isolates from the various sources were subject to two-way and three-way analyses of variance (ANOVA), using Statistica 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-transformed (i.e. by taking the logarithm to base 10 of the count + 1) to establish normality of data. 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 these factors could be concluded whenever there were no significant interactions and the *P*-values were smaller than 0.05. Where appropriate, Tukey post hoc comparisons were performed between mean values of the different components and species. In cases of significant interaction effects with distance from maize, Student's *t*-tests (assuming unequal variances) were applied to compare means of distance with means for each species or for each source.[19]

Multiplex PCR-Based Detection of Potential Fumonisin-Producing *Fusarium* in Traditional African Vegetables

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ABSTRACT: Culture-independent methods employed in fungal genetic studies using *in vitro* amplification (PCR) and analysis of specific genes or gene fragments have proved to be useful for detection, identification, and molecular taxonomy of various plant pathogens including *Fusarium* spp. This approach may be faster than culture-dependent methods, and could especially be of value for the rapid detection of slow-growing toxin-producing species in food samples. The present study was aimed at the development and evaluation of multiplex PCR-based methods for the detection and identification of potential fumonisin-producing *Fusarium* spp. in traditional *morogo*—leafy vegetables supplementing the maize-based staple diet of rural communities in South Africa. In these rural subsistence settings, some *morogo* plants grow as weeds in maize fields where they might become contaminated with potential fumonisin-producing *Fusarium* strains before being collected for consumption. Substances released by senescent vegetables could induce toxin production during storage. Using fumonisin-positive MRC *Fusarium verticillioides* strains as reference cultures, the following primer sets for the detection of specific gene fragments in fumonisin-positive *Fusarium* spp. were evaluated: (i) the conserved transcription elongation factor gene (EF-1), (ii) the *FUM1* gene encoding polyketide synthase for fumonisin B_1 production, and (iii) 18S rRNA gene. Preliminary results indicated that, these DNA fragments were amplified from MRC reference strains as well as *Fusarium* spp. isolated from *morogo*. The annealing temperature for the multiplex PCR was 55°C and each reaction contained 25 pmol of each of the primer sets *EF* and *FUM1* and 12.5 pmol of the 18S primer set. The detection limit of the individual primers was up to 1 ng and for the multiplex up to 10 ng. This demonstrates the potential of this method for the detection of potential fumonisin-positive strains.
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Keywords: *Fusarium* sp.; multiplex PCR; *morogo*; fumonisin; elongation factor

INTRODUCTION

Leafy wild vegetables (*Marogo*) have been part of the diet of African people for many generations (Khumbane, 1997). These are unrelated plants and are known by various names in different parts of the African continent. The plants are commonly used to supplement staple diets of maize. Besides the high nutritional value of these plants, they also possess substances with health protecting properties (van Wyk and Gericke, 2000; Van der Walt et al., unpublished results). However, recent mycological analysis of *morogo* from rural areas in the Limpopo Province of South Africa indicated that a considerable proportion of fungal isolates belonged to the notorious genus *Fusarium* (Van der Walt et al., unpublished results). Several of the *Fusarium* sp. isolates were *F. verticillioides*, *F. proliferatum*, *F. subglutinans*

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(Van der Walt et al., unpublished results). These are generally pathogens of grains and cereals but have also been implicated as opportunistic pathogens that cause dermal, blood, respiratory, and other ailments, particularly in immuno-compromised individuals (Hue et al., 1999; Dornbusch et al., 2004; O'Donnell et al., 2004). A further concern was that these species also produce fumonisins [mycotoxins that were demonstrated to cause birth defects, alimentary canal irritations, as well as a number of other diseases in humans and animals (Rheeder et al., 2002)]. Furthermore, the fumonisin group of toxins has, among others, mutagenic and thus carcinogenic properties (Galvano et al., 2002). In the study of Van der Walt et al. (unpublished results), what was particularly of concern was that a large number of the mentioned Fusariums isolated, were from cooked, fresh, and stored *morogo* samples. It is thus important that the levels of these fungi are determined in foods in general, but in particular in the foods of rural African communities that may have large numbers of immuno-compromised individuals (babies, HIV positive individuals, elderly, etc.) among them.

The identification of *Fusarium* sp. in food crops is problematic. This is due to a number of factors including diversity among the *Fusarium* sp., problems with clear morphological characteristics that separates the species, variation and mutation of isolates in culture (Bluhm et al., 2002). Present species differentiation is based on morphological characteristics such as shape and size of macroconidia, presence or absence of macroconidia, and colony morphology (Bluhm et al., 2002). These limitations have had serious implications on the taxonomy of toxigenic and pathogenic species (Geiser et al., 2004). Extensive training and expertise is required to isolate and identify these species. The process is laborious and time-consuming. Rapid and reliable methods to identify toxigenic *Fusarium* sp. in food sources would be one of the first steps toward the protection of humans and animals from the effects of the toxins.

Polymerase chain reaction (PCR) assays were developed to identify these species in samples (Grimm and Geisen, 1998). Conserved segments of structural and functional genes have been characterized and used in PCR assays to identify toxigenic *Fusarium* sp. (Grimm and Geisen, 1998; Hue et al., 1999; Bluhm et al., 2002). These include internal transcribed (ITS) regions (between ribosomal genes). Several candidate primers, based on the ITS, were developed for the detection and identification of *Fusarium* sp. in blood, environmental, and food samples (Grimm and Geisen, 1998; Hue et al., 1999; Bluhm et al., 2002). Conserved segments of genes that are directly involved in the biosynthesis of toxins are also useful for PCR assays. Bluhm et al. (2002, 2004) developed a multiplex PCR assay for the simultaneous detection of fumonisin (using polyketide synthase, *FUM1* gene) and trichothecene producing *Fusarium* sp. in cornmeal. A recent phylogenetic study of the polyketide synthase gene was conducted by Kroken et al. (2004). They demonstrated that within the subphylum Pezizomycotina, of the phylum Ascomycota to which the *Fusarium* sp. belong, polyketide synthase can be coded for by between 7 and 25 different genes (Kroken et al., 2004). This could have implications for reliability of the use of the *FUM1* gene. The database for this particular gene is, however, limited and thus requires further investigation.

Other candidate genes should also be surveyed for the development of PCR-based assays. Particularly useful should be the translation elongation factor 1-α (TEF) gene (Geiser et al., 2004). Conserved primers were developed and a database (FUSARIUM-ID v. 1.0) is available on a local BLAST server. Thus, from multiplex PCRs, the fragment for TEF could be isolated, reanalyzed by sequencing to determine the identity of the species present in the sample. Genes regulating the biosynthetic pathway (*FCC1* and *PAC1*) had been genetically characterized and sequenced (Shim and Woloshuk, 2001; Flaherty et al., 2003). These sequences could be used to construct primers to increase the sensitivity of such a PCR-based detection of potential fumonisin producing *Fusarium* sp. The reliability of using these particular genes for identification is, however, untested.

The aim of this study was to develop a PCR-based diagnostic test for simultaneous detection and identification of a potential fumonisin producing *Fusarium* sp. The objectives were to (i) test existing primer combinations (Elongation factor primers EF) for detection and identification of *Fusarium* sp., (ii) test existing primers (*FUM1*) for determining the potential of isolates to produce fumonisin B1, and (iii) combine the various individual PCR steps into a single diagnostic PCR assay (multiplex-PCR).

MATERIALS AND METHODS

Sample Preparation

Fusarium verticilliodes reference strain (MRC 4319) was obtained from PROMEC, Medical Research Council, South Africa. The *Fusarium* spp. isolates (Table I) were obtained as described by Van der Walt (unpublished results) and identified by the South African Agricultural Research Council's Plant Protection Research Institute (PPRI), National Collection of Fungi.

Single spore purified isolates and reference cultures were used for DNA isolation and subsequent amplification. Approximately 2 mm^2 of each isolate was inoculated, in duplicate, into yeast, peptone, dextrose (YPD) broth. Each sample was shake-incubated at 200 rpm for 48 h at room temperature. After the incubation period, 45 mL samples were collected and centrifuged at 4000 rpm for 5 min. The supernatant was then carefully discarded. TE buffer (15 mL) was added to remove as much of the growth medium as possible. The sample was centrifuged for a second time at 4000 rpm for 5 min and the supernatant discarded.

TABLE I. A list of the *Fusarium* sp. isolates used in this study

PPRI Number	*Fusarium* sp.	PPRI Number	*Fusarium* sp.
7363	*Fusarium verticillioides*	7370	*Fusarium verticillioides*
7365	*Fusarium subglutinans*	7372	*Fusarium verticillioides*
7367	*Fusarium verticillioides*		

The portion that remained was frozen at −65°C for 2 h in preparation for the subsequent freeze drying process. The dried material obtained was regarded as the sample from which extraction of DNA was performed.

DNA Extraction Procedure

A CTAB-PVP DNA extraction procedure was used to extract DNA from 50 mg of freeze-dried fungal material. The weighed sample was transferred to 2 mL microfuge tubes. Double strength CTAB isolation buffer, heated to 65°C and to which 0.2% (v/v) β-mercaptoethanol was added immediately prior to use, together with a 5% PVP solution and proteinase K (20 mg/mL) was added to each sample tube. All tubes were incubated in a 65°C water bath for 30 min with gentle inversion of tubes every 3 min. An equal volume of TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1) was added to each tube and extraction of the aqueous phase was allowed to transpire at room temperature for 10 min. Sample tubes were then centrifuged at 13 400 rpm in a bench-top centrifuge (MiniSpin Eppendorf, Germany) for 5 min at room temperature. A measured volume of the aqueous phase was transferred to a new microfuge tube and was re-extracted with an equal volume of TE-buffered chloroform: isoamyl alcohol (24:1) for 10 min at room temperature. The tubes were centrifuged at 13 400 rpm for a further 5 min after which the aqueous phase was transferred to a clean microfuge tube. The DNA was allowed to precipitate at −80°C for a minimum of 1 h in NaCl (5 M) and 99.5% ice cold ethanol. To pelletize the precipitated DNA, the tubes were centrifuged at 13,400 rpm for 5 min at 4°C. After pouring off the supernatant, the NaCl was removed by washing the pellet with ice cold 70% ethanol. Following a brief spin, the supernatant was poured off and the pellet dried under vacuum. The DNA was reconstituted by suspending it in 50 μL of TE buffer for 1 h at 65°C. All DNA samples were stored at 4°C until required.

DNA Amplification

DNA was amplified by polymerase chain reaction (PCR) using an ICycler (BioRad, UK) thermal cycler. The total volume for each reaction was 25 μL and constituted single strength PCR master mix, Supertherm *Taq* polymerase (1 U; JM Holdings, UK), bovine serum albumin (BSA; 50 ng), DNase/RNase-free distilled water, primer mix (Table II), and template DNA. The PCR master mix employed was double concentrated and contained 2.5 U *Taq* polymerase in 20 mM Tris-HCl, 100 mM KCl, 3.0 mM $MgCl_2$, Brij 35, 0.01 % (v/v), dNTP mix (dATP, dCTP, dGTP, dTTP each 0.4 mM) with a final pH of 8.3 (at 20°C; PCR Master; Roche, Germany).

The following protocol was followed for all PCRs (Table II: single primers as well as multiplex): initial denaturation for 5 min at 95°C, followed by 29 cycles of denaturing (95°C for 30 s), annealing (55°C for 30 s), and extension (72°C for 60 s). Amplification was terminated after a final extension period of 72°C for 5 min. This protocol was optimized by determining the common annealing temperature at which each of the primers (Table II) amplified the target genes without any nonspecific fragment amplification. Furthermore, annealing temperatures between 50 and 62°C were tested and for the multiplex PCR, determining primer concentrations in the reaction was critical. Various

TABLE II. Primers used in this study. The expected product sizes are ideal for multiplex diagnostic PCR assay. SSU 0017F contained the GC clamp CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC (Muyzer et al., 1996)

Ref	Primer Name	Sequence	Gene Target	Product Size (bp)
1	FCC-1 F	5′-CGGTCCGACGACAAATGACTGG-3′	Fusarium Cyclin C-1	1500
	FCC-1 R	5′-CGACACAATGTCGCTTCTGG-3′		
2	SSU 0017F	5′-AGT AGT CAT ATG CTT GTC-3′	18S Small Sub-unit	570
	SSU 0583R	5′-TCT GGA CCT GGT GAG TTT CC-3′		
3	FUM-1 F	5′-GTCGAGTTGTTGACCACTGCG-3′	Polyketide Synthase (PKS)	800
	FUM-1 R	5′-CGTATCGTCAGCATGATAGC-3′		
4	EF-1	5′-ATGGGTAAGGAGGACAAGAC-3′	Translation elongation factor 1-α	700
	EF-2	5′-GGAAGTACCAGTGATCATGTT-3′		

1 = Shim & Woloshuk (2001); 2 = Kowalchuk et al. (1997); 3 = Bluhm et al. (2002); 4 = Geiser et al. (2004).

primer concentrations of each of the primer sets were tested for successful amplification of fragment of the expected size (results of the various optimization experiments not shown). It was also essential to add an additional 1 U of *Taq* to each reaction.

Confirmation of DNA Amplification

Electrophoresis was conducted on 5 μL of PCR product through agarose gels (2% w/v; Roche, Germany) containing 1.0 μg/mL ethidium bromide (BioRad, UK). Each gel was also loaded with a DNA molecular weight standard (100 bp Molecular Weight Marker; BioRad, UK) to which the sizes and intensities of the template DNA bands could be compared. Electrophoresis was performed for 105 min at 80 V using 1× TAE buffer. Gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software.

Sequence Analysis

Amplified DNA fragments were sequenced by Inqaba Biotec, South Africa. Blastn searches (http://www.ncbi.nlm.nih.gov/BLAST for FUM1 or http://fusarium.cbio.psu.edu for TEF) were used to confirm the identity of the amplified sequences.

RESULTS

Four agarose (1 % w/v) gels are depicted in Figure 1 and show the sizes of the four gene fragments targeted in this study. The DNA was from *F. verticillioides* MRC 4319 and the sizes of the fragments were as expected (Table II). Gels B, C and D also demonstrate that up to 1 ng of Fusarium DNA could be detected by the individual *EF*, *FUM1*, and *FCC1* primers. Bluhm et al. (2002) defined detection limits as: "the minimal amount of DNA template that yielded a clearly visible product on agarose gels containing ethidium bromide." The detection limit in our study was less sensitive than a previous study (Bluhm et al., 2002) that showed PCR detection limits between 100 pg and 1 ng of *Fusarium* DNA. This lower detection limit in our study could have been due to the lower $MgCl_2$ concentrations that we used in our PCRs. Blumh et al. (2002) used 2.5 mM, whereas we used 1.5 mM in each of our reactions. The goal of our study was to optimize the multiplex PCR instead of only optimizing PCR detection of individual genes.

From Figure 1, it is also evident that agarose concentrations greater than 1% (w/v) and electrophoresis times longer than 105 min should be used in the multiplex PCR.

The primers were combined and various annealing temperatures as well as primer concentrations were also tested. From the results, it was evident that annealing temperature of 55°C worked the best. Another aspect that had to be considered was optimizing the PCR for the 18S rRNA primers. The 18S primers were selected, since they could amplify a spectrum of fungal species and was useful in fungal dynamics studies. These primers would thus act as positive controls i.e., they should amplify the 550 bp fragment of any fumonisin negative as well as nonFusarium species. When we attempted to use the 18S rRNA gene primers, we consistently obtained three fragments (1500, 1200, and the expected 550 bp). However, when a GC clamp, typically used in PCR denaturing gradient gel electrophoresis (DGGE) was added to the 5′ end of the SSU 0017F primer, only one fragment was obtained (Fig. 1, gel A).

Another aspect to determine was the optimum concentration for the various primers. We found that it was also important to combine the *FUM* and EF primers at equal

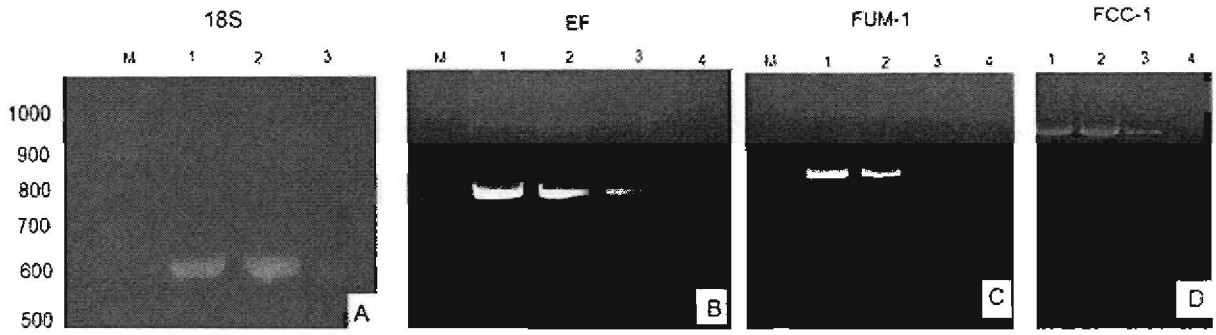

Fig. 1. PCR reactions showing the size of the various fragments. Serial dilutions of genomic DNA is demonstrated for B, C and D. In the latter three cases electrophoresis was for 30 minutes at 80 V. For A the electrophoresis was for 105 minutes. The letters A, B, C and D on the gel indicates the 18S rRNA gene fragment, elongation factor (EF), polyketide synthase gene (*FUM1*) and fusarium cyclin C-1 (*FCC1*) respectively. Numbers 1 to 4 in gels B, C and D indicate 1000, 100, 10 and 1 ng of target DNA. The numbers 1, 2 and 3 in A indicate DNA from different individuals.

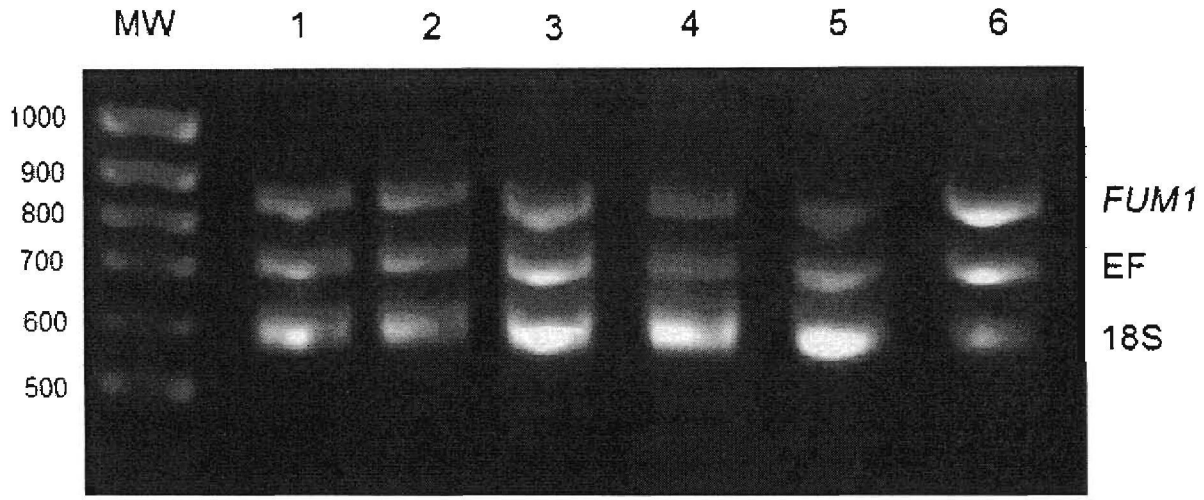

Fig. 2. A 2% (w/v) agarose gel depicting the successful multiplex PCRs in which *FUM1*, EF and 18S primers were used. The DNA concentration 100 ng per individual reaction and presented *F. verticillioides* MRC 4319 and the identified isolates listed in Table I.

concentrations and then to adjust the 18S primers (containing the GC clamp) to a 1:2 ratio with respect to the *FUM1* and EF primer combination. The concentration of the latter two primers were thus 25 pmol per set and the concentration of the 18S primer was 12.5 pmol. The *FUM1* optimized primer concentration in the multiplex PCR was consistent with findings of Bluhm et al. (2002). No information was available for the 18S and EF primers. Unfortunately, the *FCC1* primers did not amplify in the multiplex and was thus left out for further analysis. The composite agarose gel in Figure 2 shows several *Fusarium* DNA samples at 100 ng that were amplified by multiplex PCR. These include the *F. verticillioides* MRC 4319 strain as well as all the identified isolates listed in Table I.

It is evident from Figure 2 that these primers could be utilized for detection of fumonisin positive *Fusarium* sp. when 100 ng of target DNA is present in the PCR mixture. Detection limits of this multiplex primer combination was evaluated and the results depicted in Figure 3. At 1 and 0.1 ng of target DNA, the 18S fragments were weakly amplified. When the amount of target DNA was above 1 ng, then all three PCR products were observed.

Although the detection limit of the primer set for detection the fumonisin biosynthesis gene (*FUM1*) was lower than previously described (Bluhm et al., 2002), this approach may have certain advantages over conventional methods. It is relatively fast and combining them with quantitative PCR and/or DNA fingerprinting methods could be powerful. Quantitative PCR methods would allow for determining levels of contamination by *Fusarium* spp. and DNA fingerprinting methods for determining the species diversity of this genus in the sample.

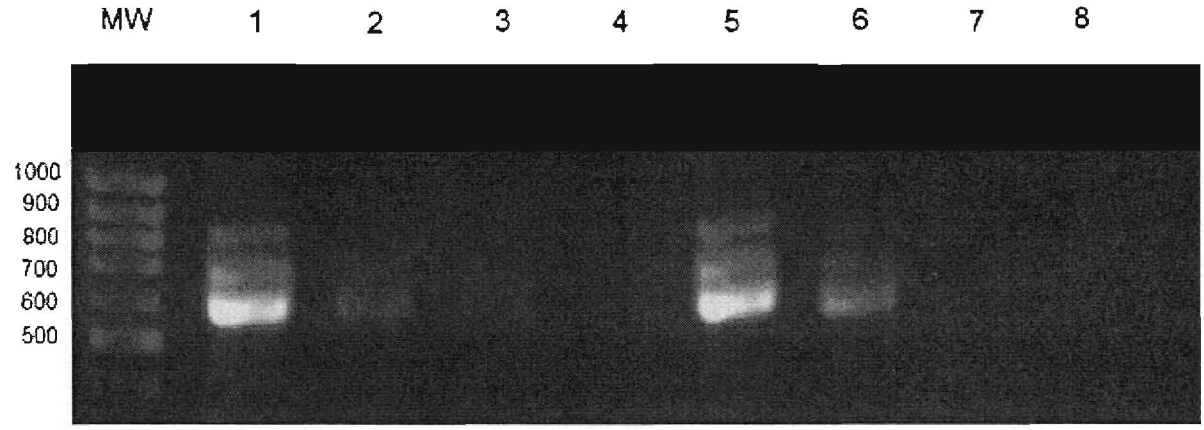

Fig. 3. A 2% (w/v) agarose gel depicting the successful multiplex PCRs in which *FUM1*, EF and 18S primers were used. The DNA was from *F. verticillioides* (Lanes 1 to 4) and *F. subglutinans* (Lanes 5 to 8). DNA concentrations in the various reactions were 100, 10, 1 and 0.1 ng in each of the Lanes 1 to 4 and 5 to 8 respectively.

DISCUSSION

The selected primers amplified the fragments that were targeted. Fragment identities were confirmed by sequence analysis. When the primers were combined in multiplex PCRs, three fragments of appropriate sizes were obtained. This demonstrates the potential of using these fragments in a multiplex PCR that aims at detection of fumonisin positive *Fusaroium* spp.

In diagnostic multiplex PCR processes, it is essential that the target sequences are well selected to ensure good sensitivity but also that PCR products yielded by the various primers are of distinct sizes. The primers used in this study targeted three types of sequences: (i) one that is specific for detection of a gene involved in fumonisin biosynthesis (*FUM1*), (ii) a sequence of the elongation factor (EF) of *Fusarium* spp., and (iii) a sequence of conserved fungus 18S regions. These were all of distinct sizes and products of the multiplex PCRs could be resolved using 2% (w/v) agarose gels.

To eliminate the limitation of potential false negatives that could be associated with general PCR failure, we included the 18S primer set as an internal control. PCR failure could be associated with PCR-inhibiting compounds that are coextracted with the DNA (Fortin et al., 2003). An internal control such as the one in this study is thus essential in multiplex PCRs.

Although the primer sets yielded the expected PCR fragments in multiplex PCRs, further careful optimization of this process is essential. Such optimization steps could improve the detection limits of the primers sets. Besides determining the optimum primers concentrations in the PCR mix, it will also be important to determine the detection limit in the presence of related and unrelated fungal genera, as well as host plant, DNA.

Assays that could detect toxigenic *Fusarium* spp. *in situ* were previously described by Bluhm et al. (2002, 2004). In these cases, ITS (internally transcribed spacers region) primers were used as internal controls. The ITS primers were specific for *Fusarium* spp. In our study, the elongation factor is used as a control for *Fusarium* spp. This gene has the additional advantage that when detection of *Fusarium* spp. directly from plant material is used, the opportunity exist to cut the fragments from the agarose gels, reanalyze them by PCR-DGGE and then followed by sequencing one could rapidly determine which species are present. Results from the approach as described in the preceding sentence would also be informative about the diversity of the *Fusarium* species present in such plant crops. Yergeau et al. (2005) used EF primers in PCR-DGGE application to assess the diversity of *Fusarium* spp. in asparagus crops. They showed the usefulness of these primers in such an application but moreover, demonstrated the added advantage of using the elongation factor primers i.e., that a well established database is available for identification of *Fusarium* sp. obtained by such a study.

This multiplex PCR method can thus be combined with the PCR-DGGE technique to determine the dynamics of fungal species, particularly in stored food crops. The 18S rRNA fragment primer combination used in this study could be evaluated for this purpose when DNA from food crops are analyzed. Subsequent to multiplex PCR and agarose electrophoresis, the band representing the 18S fragments could be excised, reamplified by the same primer set, and subjected to DGGE. The DNA profiles could then be analyzed and individual bands excised and sequenced. The sequence data could be analyzed by GeneBank or ribosomal data bases.

Moreover, the system could also be optimized for quantitative multiplex PCR. This could increase the sensitivity of the method. Further advantage of the latter technique is that it would further reduce the analysis time and give a quantitative measurement of the amount of target DNA fragments present in the sample. Schnerr et al. (2001) used several approaches ranging from hotstart PCR, inclusion of pyrophosphatase, and Uracil DNA glycosylase to increase the quantitative PCR detection limits, specificity of the reaction, and reduce the risk of PCR carryover. These approaches could thus be useful to explore.

A need exist for the accurate and sensitive detection of pathogenic fungi from environmental, crops, and clinical samples due to the increase in fungal infections (Dornbusch et al., 2004; Diaz and Fell, 2005). New opportunistic pathogenic fungal infections are also increasing and may emerge as new species within genera (Diaz and Fell, 2005). This scenario worsens in the case of immuno-compromised individuals. Rural African communities may consist of a large proportion of such individuals. Some food sources that were mycologically analyzed (Van der Walt et al., unpublished results) contain potential opportunistic pathogens such as *F. verticillioides* and *F. proliferatum* (Dornbusch et al., 2004; O'Donnell et al., 2004). These species are also known fumonisin producers. It is thus important that methods are evaluated and optimized for rapid and accurate detection of these and other mycotoxigenic fungi in the food sources of these communities. Such information could be used to recommend improved storage methods and thus decreasing the risk of exposure to the fungal species and their toxins.

In summary, this study provides evidence that primers *FUM1*, EF, and 18S described could be useful tools for rapid detection and identifying mycotoxigenic fungi in food samples. The primers could also be useful to study the dynamics of the fungal colonizers in food crops and additionally could be useful to construct fungal rDNA libraries for DGGE analysis. Information from such studies will additionally provide information to discuss the scope of the functional roles. Studies are underway to further optimize the multiplex PCR and to test the detection limits in the presence of competing DNA.

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