THE OCCURRENCE OF TOXIGENIC MOULDS IN TRADITIONAL HOUSEHOLD *MOROGO* OF GIYANI

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For my parents

You have brains in your head. You have feet in your shoes.

You can steer yourself any direction you choose.

You're on your own. And you know what you know.

And you are the one who'll decide where to go.

And when things start to happen, don't worry. Don't stew.

Just go right along. You'll start happening too.

— Theodor Geisel / Dr Seuss

ABSTRACT

An estimated 57 % of the black Africans in South Africa live in rural areas. Traditional vegetables play an important role in providing nutrition for rural subsistence households. *Morogo* refers to traditional leafy vegetables that are well adapted to local growing conditions, produce high yields and can be cultivated cost-effectively. Some of these vegetables occur as weedy plants in cultivated lands. The dietary value and cultivation practices of traditional vegetables are largely embedded in indigenous knowledge systems of local communities and not well documented in scientific literature. The present study was conducted in a rural African community in the Mopani District of the Limpopo Province. Questionnaires were used to obtain and document information related to *morogo* types consumed, subsistence agricultural practices as well as traditional food preservation and processing methods. Since dietary safety of food produced for rural household subsistence has received little attention, the mycological safety of *morogo* was investigated. Standard techniques were employed to isolate potential toxigenic fungi from fresh and processed household *morogo*. Members of the fungal genera *Aspergillus* and *Penicillium* were present in low numbers. *Alternaria* was isolated in relatively high numbers mainly from internal leaf structures and *Fusarium* strains from leaf surfaces. *Fusarium* levels were found to be lower in samples of sun-dried, cooked and rinsed *morogo*. Molecular techniques were employed to confirm the identity of suspected fumonigenic *Fusarium* isolates and the presence of fumonisin-encoding genes. Fumonisin-producing *Fusarium* in the subsistence agro-environment implies a risk that *morogo* might be contaminated with fumonisin mycotoxins. Subsequent research should be aimed at investigating the source of *Fusarium* contamination in the subsistence agro-environment and identifying risk factors for toxin production in traditional *morogo*.

OPSOMMING

'n Geskatte 57 % van swart Afrikane in Suid Afrika woon in plattelandse gebiede. Tradisionele groente speel 'n belangrike rol in die voorsiening van voeding vir plattelandse onderhoud huishoudings. *Morogo* verwys na tradisionele blaar groentes wat goed aangepas is by plaaslike groei toestande, 'n hoë opbrengs lewer en koste-effektief verbou kan word. Sommige van hierdie groente kom as onkruid plante in landerye voor. Die dieetkundige waarde en verbouingspraktyke van tradisionele groentes is grootliks in inheemse kennis sisteme van plaaslike gemeenskappe ingebed en is skraps in wetenskaplike literatuur gedokumenteer. Hierdie studie is in 'n plattelandse Afrikaan gemeenskap in die Mopani Distrik van die Limpopo Provinsie uitgevoer. Vraelyste is gebruik om inligting te bekom en te dokumenteer wat betrekking het op *morogo* tipes wat verbruik word, onderhoudingslandbou praktyke sowel as tradisionele metodes van voedselbewaring en prosessering. Aangesien die dieetkundige veiligheid van voedsel wat vir huishouding onderhoud geproduseer word min aandag geniet, is die mikologiese veiligheid van *morogo* ondersoek. Standaard tegnieke is gebruik om potensieel toksigene fungi uit vars en geprosesseerde *morogo* te isoleer. Lede van die genera *Aspergillus* en *Penicillium* was in lae getalle teenwoordig. *Alternaria* is in relatief hoë getalle hoofsaaklik van interne blaarstrukture geïsoleer en *Fusarium* rasse vanaf blaaroppervlakke. Daar is bevind dat *Fusarium* vlakke laer is in songedroogde, gekookte en afgespoelde *morogo* monsters. Molekulêre tegnieke is gebruik om die morfologiese identiteit van vermoedelik fumonigeniese *Fusarium* isolate en die teenwoordigheid van fumonisien-koderende gene te bevestig. Die teenwoordigheid van fumonisien-produserende *Fusarium* in die onderhoudingslandbou omgewing impliseer 'n risiko dat *morogo* moontlik met fumonisien toksiene gekontamineer mag wees. Hieropvolgende navorsing behoort daarop gemik te wees om die bron van *Fusarium* kontaminasie in die onderhoudingslandbou omgewing te ondersoek en risiko faktore vir toksienproduksie in tradisionele *morogo* te identifseer.

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CHAPTER 1

INTRODUCTION

It is estimated that by 2010, the population in sub-Saharan Africa will increase to 910 million, and this figure could rise to 1,32 billion by 2025. Of these, 67 % will be rural and the majority will be poor (CCSU, 2005). At present, between forty and fifty percent of South Africa's population can be classified as living in poverty and this poverty is more pervasive in rural areas, particularly in the former homelands (FAO, 2005). Food insecurity is highest among the African population and rural households (Bonti-Ankomah, 2001). During the 1900s, thirty countries had over 20 % of their population undernourished and in eighteen of these, over 35 % of the population were chronically hungry. As of 2001, about 28 million needed emergency food and agricultural assistance (FAO, 2001). In 2002, a document by the New Partnership for Africa's Development (NEPAD) reported that some 97 % of the continent's food-insecure live in the countries of sub-Saharan Africa where over one-third of the population (34 %) is classified as undernourished. More recently, the development charity Oxfam (cited by Mason, 2006) found that the food crisis in Africa is continuing to worsen and on average, the number of African food emergencies per year has tripled since the mid 1980s.

Medaglini and Hoeveler (2003) speculate that endemic HIV / AIDS, malaria and tuberculosis are both cause and consequence of poverty, and probably account for more than half of all deaths in sub-Saharan countries. HIV / AIDS, in particular, is distinguished by the fact that infections are highest among adults between 20 and 40 years of age. This distinction has a marked impact on the income, expenditure pattern, food production and coping strategies of rural households (SADC FANR, 2003; SARPN, 2003). HIV / AIDS also breaks the chain of knowledge transfer and labour sharing between generations. As a result, survivors, including

both children and the elderly, often cannot manage the family farm due to lack of knowledge and experience (De Waal & Tumushabe, 2003).

The cumulative effects of these factors 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 (Mbaya, 2003; SADC FANR, 2003; SARPN, 2003; Wiggins, 2003). In view of the current food crisis and the prevailing HIV / AIDS situation, the Southern African Development Countries (SADC) Health Ministers deliberated on strategies to urgently and effectively address these. Establishment of a key intervention, with input from other sectors such as Agriculture, which would impact positively on the health and well-being of the general population including children, people living with HIV as well as other vulnerable populations, was recommended (SADC, 2003).

Agriculture may be regarded as the best vehicle to reduce rural poverty. The FAO (2004) reported that agricultural growth has a strong and positive impact on poverty, often significantly greater than that of other economic sectors. With regard to food security, Machethe (2004) observed from rural development studies that growth of the smallholder agricultural sector is the primary channel for achieving household food security. Another important finding was that households (in the rural sector) that are engaged in agricultural activities tend to be less poor and have better nutritional status than other households. Due to the generally acknowledged positive contribution of agriculture to poverty alleviation, one of the key priority action areas of NEPAD is to facilitate implementation of food security and agricultural development programmes (NEPAD, 2005).

Maize (*Zea mays*) dominates the production systems of smallholder farmers in the former homelands of South Africa (Machethe, 2004). This is probably because rural black communities in South Africa usually rely on maize as a major staple. However, de Waal & Tumushabe (2003) suggest that agricultural development focus on rapid adaptation of low-input but high-yielding food and cash crops, and high value food crops that are drought resistant. Indigenous plants are well adapted to local growing conditions, their requirements for soil fertility, water and plant protection are modest and subsistence farmers benefit from low production inputs (CGIAR, 2005).

However, these plants have seldom been subjected to scientific scrutiny either for their nutritional content or for their mycological quality. In light of the current nutrition status, SADC Health Ministers have urged their Member States to take stock of traditional foods and therapies with nutritional value, and promote their consumption. They recommend that research endeavours and programmes that seek to enhance the status of indigenous foods be supported. They also recommend the establishment of Centres of Research Excellence in the SADC region that would be employed to determine the safety, efficacy and quality of traditional herbs and foods and nutritional supplements (SADC, 2003). To this end, this study was cultivated to broaden our understanding of indigenous plants with a view to addressing the current high levels of food insecurity, in line with SADC and NEPAD philosophies.

Morogo is a vernacular term used to describe the leaves of various edible plant species indigenous to certain geographical regions. Rural communities within each of the nine provinces of South Africa have different types of plant species that are regarded as *morogo*. *Morogo* plants are cultivated for subsistence, while others may grow as weeds in cultivated lands or as members of the natural field flora (van Wyk & Gericke, 2000). At the onset of cultivation, these plants require low input and at harvest farmers are rewarded with a high

yield. *Morogo* may be used fresh or the leaves may be dried and stored, and used in times of food scarcity (pers. comm. Gugu Mothele; Mr R Mkhari, 2003). Accordingly, these plants may make an ideal contribution to agricultural development programmes with a view to reducing the threat of hunger that remains a peril for far too many people.

Fungal spores are ubiquitous in the environment and most plant species are subject to attack by a number of different types of fungal pathogens (Alexopoulos *et al.*, 1996). Surface colonisers refer to those fungi living on the exterior surfaces of the plant host, while internal colonisers live entirely within the host plant substrate and may have either a parasitic or symbiotic association with the host (Sinclair & Cerkauskas, 1996). Field fungi dominate in the field where moderate temperature and water activities prevail.

During storage, crops commonly undergo quality loss, rendering it increasingly susceptible to infection by storage fungi which, in addition to spoilage, deterioration and loss, may also produce mycotoxins (Ominski *et al.*, 1994). Indigenous food plants (like *morogo*) are not exempt from fungal infestation and are, as a result, at risk of possible mycotoxin contamination. 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 (Turner *et al.*, 1999). Home-grown foodstuffs are not subjected to quality control to ensure their dietary safety and consumers may therefore unknowingly be exposed to fungi and their associated mycotoxins. Chronic dietary exposure to mycotoxins is linked to the occurrence of various types of cancer, kidney toxicity and immune suppression (Bennet & Klich, 2003; Ferguson *et al.*, 2004).

The aim of the study was to quantify and identify mycotoxin-producing fungi from traditional staple foods consumed by rural subsistence communities in the Giyani region of the Limpopo Province. An objective of this study was to document which *morogo* types are commonly consumed by these indigenous communities. A second objective was to record idiosyncrasies of this population with respect to the *morogo* types. Questionnaires were employed to determine what plant types are used as *morogo* and which of these are the most commonly eaten. The questionnaires were also used to record customs regarding methods of preparation, preservation and storage of these indigenous plants. In addition, different agrarian strategies typical in these areas were recorded. Scientific names of commonly used *morogo* were identified by the South African National Biodiversity Institute (SANBI). A third objective was to isolate, identify and quantify potential mycotoxin-producing fungi associated with commonly eaten *morogo* types collected from households, schemes and a stall. *Morogo* leaves were assayed mycologically using methods such as, standard washing procedures, modified sterilisation regimes, single spore isolations, macroscopic and microscopic examinations. The final objective was to confirm the identity of *Fusarium* species and to determine whether *Fusarium* isolates from traditional *morogo* have the potential for fumonisin production. Positive amplification of *EF* gene fragment confirms *Fusarium* identity while isolates in which *FUM 1* gene fragment is positively amplified pose the risk for fumonisin production under favourable conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 THE FUNGI

Fungi are in general widespread and occur whenever moisture and organic material are available. These eukaryotic microorganisms act as decomposers, which is a role of enormous significance. They degrade complex organic materials in the environment to simple organic compounds and inorganic molecules. Carbon, nitrogen, phosphorus and other critical constituents of dead organisms are thereby released and made available for the metabolism of other living organisms (Prescott *et al.*, 2005). Some fungi are of economic importance since they are used in the processing and fermentation of foods as well as in the brewing industry, while others produce antibiotics and drugs of pharmaceutical value, agricultural fungicides, plant growth regulators, vitamins or enzymes (Carlile *et al.*, 2001).

Fungi can also play a destructive role causing immense economic losses. As saprophytes they cause damage to timber, fuel, various foods and manufactured goods. As parasites they are the major cause of plant diseases with over 5 000 fungal species attacking economically valuable crops, garden plants and many wild plants (Prescott *et al.*, 2005). Fungi also cause many diseases in domestic, farm and zoo animals. Examples include ringworm disease in various animals and birds, ear infection in dogs, superficial and mucosal candidosis in poultry, avian aspergillosis and mycotic abortions in cattle, horses, pigs and sheep (Carlile *et al.*, 2001). Humans also suffer from fungal infections ranging between superficial mycoses to opportunistic mycoses that may create life-threatening situations in the compromised host. Certain fungal diseases are increasing in incidence because of organ transplants, immunosuppressive drugs and the surge of the Acquired Immunodeficiency Syndrome (AIDS) virus (Latgé & Calderone, 2002).

2.1.1 Distribution of fungi

Fungi are primarily terrestrial organisms, although a few occur in freshwater or marine environments (Jay, 2000). In plants, they are associated with the leaves, stems, flowers, seeds and roots (Agrios, 1978). Fungi are also commonly isolated from the air, accumulated leaf litter, wood surfaces, animal feeds, dung, insects and other fungi (Baxter & van der Linde, 1999). Fungal genera that are nearly always present in soils include *Aspergillus*, *Botrytis*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus*, *Trichoderma* and *Trichothecium* (Jay, 2000; Carlile *et al.*, 2001). According to Hudson (1986), *Penicillium* and *Trichoderma*, in addition to being soil fungi, may also be regarded as litter fungi. Fungi that may be isolated from plant material include *Alternaria*, *Aureobasidium*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Helminthosporium* and *Rhizopus* (Frazier & Westhoff, 1988).

2.1.2 Field vs. storage fungi

Different fungi grow at different rates, determined by the temperature and water activity of the substrate they colonise (Ramakrishna *et al.*, 1996). Based on their individual responses to these two factors, fungi can be differentiated into two groups, namely field and storage fungi. In the field, where moderate temperatures and water activities persist, fungi are almost always and constantly associated with exposed freshly decaying green parts of plants (Hudson, 1986). Storage fungi, on the other hand, refer to those fungi that colonise crops (grain and other plant material) that have been harvested, processed and subsequently stored. Fungal spoilage in stored foods depends largely on the condition of the crop that is entering storage and maintenance of fungus-free conditions during storage (Usha *et al.*, 1993). Generally during storage, field fungi present on the stored material encounter low temperatures and water activities. Since field fungi cannot withstand these conditions they are succeeded by storage fungi that consider these conditions ideal.

Nonetheless, stored plant material may become damp or even water-logged if they are not kept dry (Carlile *et al.*, 2001), allowing the continued growth of various other microorganisms. Heat generated by the metabolism of these microorganisms present on or in the damp plant material cannot be readily dissipated, resulting in high temperatures and water activities. If these conditions persist, the growth rate of thermotolerant and thermophilic fungi may increase causing them to become dominant (Carlile *et al.*, 2001). In all eventuality, plants and plant products may be reduced in quality by diseases caused by fungi dominating either in the field (as is the case with most plant diseases) or during storage (mainly with grains) with the amount of losses ranging from slight loss to 100% loss (Agrios, 1978). With this in mind, and as an aid in selecting the most suitable storage conditions, it would be well to consider, and ultimately avoid, the temperature and water activity ranges ideal for fungal colonisation. Frazier and Westhoff (1988) have suggested that the two predominant genera of fungi present in stored products are probably *Aspergillus* and *Penicillium*. This was confirmed by Ramakrishna and colleagues (1996), while Jay (2000) stated that although the aspergilli are storage fungi, some species may be regarded as field fungi. Genera that are ordinarily considered field fungi include *Alternaria*, *Aureobasidium*, *Cladosporium* and *Fusarium* (Ominski *et al.*, 1994).

2.1.3 Parameters influencing fungal colonisation rate

Various factors play a hand in damaging valuable crops, leading to major economic losses. In order to limit crop loss it becomes necessary to determine what these factors are and what effect they have on the colonisation rate (refers to the time required for fungi to infect a particular field or an area of concern against how infected the field might become). Within a specific field and its surrounding areas, the colonisation rate may be evaluated according to

the (i) initial level of infection; (ii) frequency of spore production; (iii) types of spores common in the field and (iv) agent of spore dissemination.

Ecological parameters may also influence the colonisation rate. These parameters include (i) moisture, temperature and oxygen-carbon dioxide levels; (ii) mechanical and insect damage; (iii) competing microflora and (iv) the substrate itself.

Agrios, in 1978, reported that in an acre of heavily infected plants the number of spores produced is generally astronomical and as they are released there are enough spores to land and inoculate every conceivable surface in the field and the surrounding areas. The frequency of spore production differs, with some fungi producing spores more or less continuously while others produce spores in successive crops (Agrios, 1978). Spore dissemination in fungi may be through air, water, rain drops, rain splash, insects, mites, nematodes or man (Hudson, 1986), the distance of spore dispersal presumably varying with each agent. Carlile and co-authors (2001) described two spore types based on their method of dissemination. Dry spores (e.g. the conidia of *Aspergillus* and *Penicillium*) are those that have a hydrophobic surface and are difficult to wet. These are launched passively either by mechanical disturbance (wind) or electrostatic repulsion. Slime spores (e.g. the conidia of *Fusarium*) are produced with mucilage and moisture and form a slimy mass that is readily wettable. These spores are most often dispersed passively by water (i.e. dew, rain drops or rain splash).

The interactive effects of moisture, temperature and oxygen-carbon dioxide levels are important factors affecting colonisation rate (Ominski *et al.*, 1994; Jay, 2000). The presence of spores of fungi that favour prevailing conditions is an indication that their growth may be enhanced. Ominski *et al.* (1994) reported that mechanical and insect damage facilitates the penetration of inoculum into the interior of the host. They also reported that the presence of

other microorganisms, whether prokaryotic or eukaryotic, alters the growth of fungi. This is not only due to the competition for available nutrients but also because of the potential production of antimicrobial compounds by some of the microorganisms. The composition of substrates ensures that hosts differ in their ability to support fungal growth (section 2.2.4).

Complex interactions between each of these factors play a role in determining not only how speedily fungi may infect a particular field, but also to what extent the field may become infected.

2.1.4 Mycotoxins: Products of secondary metabolism

A large number of fungi produce toxic substances designated mycotoxins. Mycotoxins are low-molecular-weight natural products produced as secondary metabolites. Secondary metabolites are formed during the end of the exponential growth phase and have no apparent significance to the organism producing them, neither to growth nor metabolism (Jay, 2000). It is suspected that secondary metabolites are formed by manipulation and transformation of the large pools of metabolic precursors (such as amino acids, acetate and pyruvate) that have accumulated during primary metabolism. Mycotoxin production may be one way for the fungus to reduce the accumulated metabolic precursors that it no longer requires for primary metabolism. Other ways of reducing the products of primary metabolism include the synthesis of pigments, antibiotics, phytotoxins, animal toxins, plant hormones and pharmaceuticals (Moss, 2002). Mycotoxins fall into several chemically unrelated classes, are produced in a strain-specific way (D' Mello & Macdonald, 1997), and elicit some complicated and overlapping toxigenic responses in sensitive species. Responses include carcinogenicity, inhibition of protein synthesis, immunosuppression, dermal irritation and other dermal perturbations (Bennett & Klich, 2003). Turner and colleagues (1999) concluded from animal

model studies and human epidemiological data that mycotoxins possibly pose a considerable danger to human and animal health, depending on the concentration of the mycotoxins.

Common examples of these pharmacologically active metabolites include aflatoxins, citrinin, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone. Recently, the fumonisins have become increasingly significant to human health since they were implicated in the high occurrence of oesophageal cancer in China and South Africa (Rheeder *et al.*, 1992; Chu & Li, 1994). This group of mycotoxins and the fungi that produce them will receive more attention in section 2.3.

In 2001, Carlile and his fellow researchers suggested that up to 25% of the world's food supply may be contaminated with mycotoxins. Generally mycotoxins are associated with agricultural commodities going mouldy after harvest, but they can also be produced in the field before harvest (Moss, 2002). All the same, the distribution and severity of crop contamination tends to vary from year to year based on weather conditions, other environmental factors and also the type of commodity affected (Dutton, 1996). Crops with large quantities of mycotoxins may be destroyed but are likely to be diverted into animal feeds (Bennett & Klich, 2003), further exacerbating the mycotoxin problem. Mycotoxins usually enter the body by consumption of contaminated foods, inhalation of toxigenic spores, direct dermal contact or indirectly through the food chain as in milk from cows fed with contaminated feed (Kuiper-Goodman, 1994; Carlile *et al.*, 2001). Exposure to mycotoxins is more likely to occur in those parts of the world where poor methods of food handling and storage are common and where regulatory infrastructure and financial support required to protect exposed populations are not sufficiently enforced (Turner *et al.*, 1999).

2.2 THE LEAF AS A HOST SUBSTRATE

Fungal pathogens that live in the soil and infect roots face different problems from those infecting the above-ground parts (leaves, stems, flowers and fruit). Many root-infecting fungi produce spores that can remain viable for years and are highly tolerant of desiccation, heat and other environmental factors.

Fungi that infect above-ground parts release numerous spores that become air-borne or are spread by rain splash (Funnell & Schardl, 2001). The leaf surface serves as a landing site for fungal spores. In order for leaf infection to occur, appropriate growth, differentiation and interaction of fungal spore with leaf surface is essential (Funnel & Schardl, 2001). Fungal spores germinate and may colonise the surface of leaves or the intracellular spaces within leaves. Of these, some fungi are only capable of colonising a specific type of host substrate or geographical area thereby displaying host specificity or area specificity, respectively. In either case, the stage (age) of the leaf as host plays a role in determining the composition diversity of fungi on or in the leaf (Hudson, 1986). As the immature leaf gets older, enters senescence and later decomposition, the type of fungi that dominate and are isolated differs.

One can reason that isolation of a specific fungal genus may hint at either or all of three factors. Firstly, whether the leaf surface or the interior was infected. Secondly, the physiological state of the leaf at the time of fungal isolation (whether the leaf was immature or in a senescent or decomposing litter state). Thirdly, one could propose what the plant species, from which the fungus was isolated, might be. In turn, initial insight of these three factors may allow one to anticipate the fungal genera that could be isolated. Knowledge of these elements augments our understanding of the leaves and plants being analysed with the fungal genera that are recovered from these leaves / plants (i.e. plant-fungal interactions).

2.2.1 Surface colonisers

Surface colonisers refer to the many microscopic fungi growing actively on the surfaces of the living leaf (phylloplane). Fungal colonisation of the leaf surface begins as soon as the leaf emerges from the bud (Carlile *et al.*, 2001). As the leaf unfolds it is a relatively clean sheet that immediately provides a landing site for air-borne fungal spores.

The phylloplane serves as a differential spore trap; the efficiency depending on whether the leaves are horizontal or vertical, wet or dry, hairy or glabrous, glossy or matt, waxy or non-waxy and so on. Phylloplane inhabitants are not uniformly distributed over the leaf surface and are more prevalent on the upper surface of the leaf and along the veins (Hudson, 1986). This may be true since nutrients are transported along the veins of leaves making it the preferred site for fungal infection. As the living leaf matures, the number of phylloplane inhabitants increases (Hudson, 1986). This may be because there are only restricted amounts of nutrients available on immature leaves and this causes relatively poor development of the phylloplane inhabitants. Filamentous fungi growing on the phylloplane are mainly the mitosporic fungi and the Ascomycetes (Carlile *et al.*, 2001).

Fungi residing on leaf surfaces may be divided into three categories; (i) non-pathogenic epiphytes, (ii) pathogenic epiphytes and (iii) casual inhabitants. Non-pathogenic epiphytes are made up of phylloplane inhabitants and common primary saprophytes (Hudson, 1986). The phylloplane inhabitants are those fungi that are able to complete their life cycle or a significant part of it on the living leaf without damaging it. Common primary saprophytes are those fungi that are unable to grow to their full extent in the phylloplane until the onset of senescence. Examples of common primary saprophytes are species of *Cladosporium* and *Alternaria alternata* (Osono & Takeda, 1999). Forming the second category are the pathogenic epiphytes which are also divided further with the powdery mildews forming the

first group and virtually all remaining pathogens forming the second. Spores of pathogens that are unable to infect the leaves on which they have landed are also common (Hudson, 1986). These casual inhabitants make up the third category and may remain dormant whilst simultaneously contributing to the nutrient availability on the leaf surface.

2.2.2 Internal colonisers

In addition to fungi growing on leaf surfaces, many fungi are known to grow within the leaf and are aptly called internal colonisers or endophytes. These fungi live entirely within the leaf and grow between cells (Clay, 1990) with a tendency to cause infections in healthy tissues of plants without symptom expression (Saikkonen *et al.*, 1998). Despite being able to colonise a wide variety of hosts some endophytes show strong specificity toward certain host plants. On this basis, the foliar endophytes have been divided into two groups; (i) ubiquitous forms that can be isolated from a wide variety of host species in different ecological and geographical environments and (ii) unique forms that show a fair degree of host specificity and a higher degree of specialisation (Kriel *et al.*, 2000). In a study undertaken by Suryanarayanan and colleagues (2003) no host specificity was encountered. Despite this, they concur that unique forms of endophytic fungi could probably be host specific.

Internal colonisers are protected against sudden weather changes and other environmental factors (Kriel *et al.*, 2000) presumably because most of their life cycles occur within the leaf. Endophytic growth may initiate the onset of senescence (Carlile *et al.*, 2001), although the reverse also holds true (i.e. leaf senescence may trigger the growth and colonisation of endophytic fungi; Kriel *et al.*, 2000).

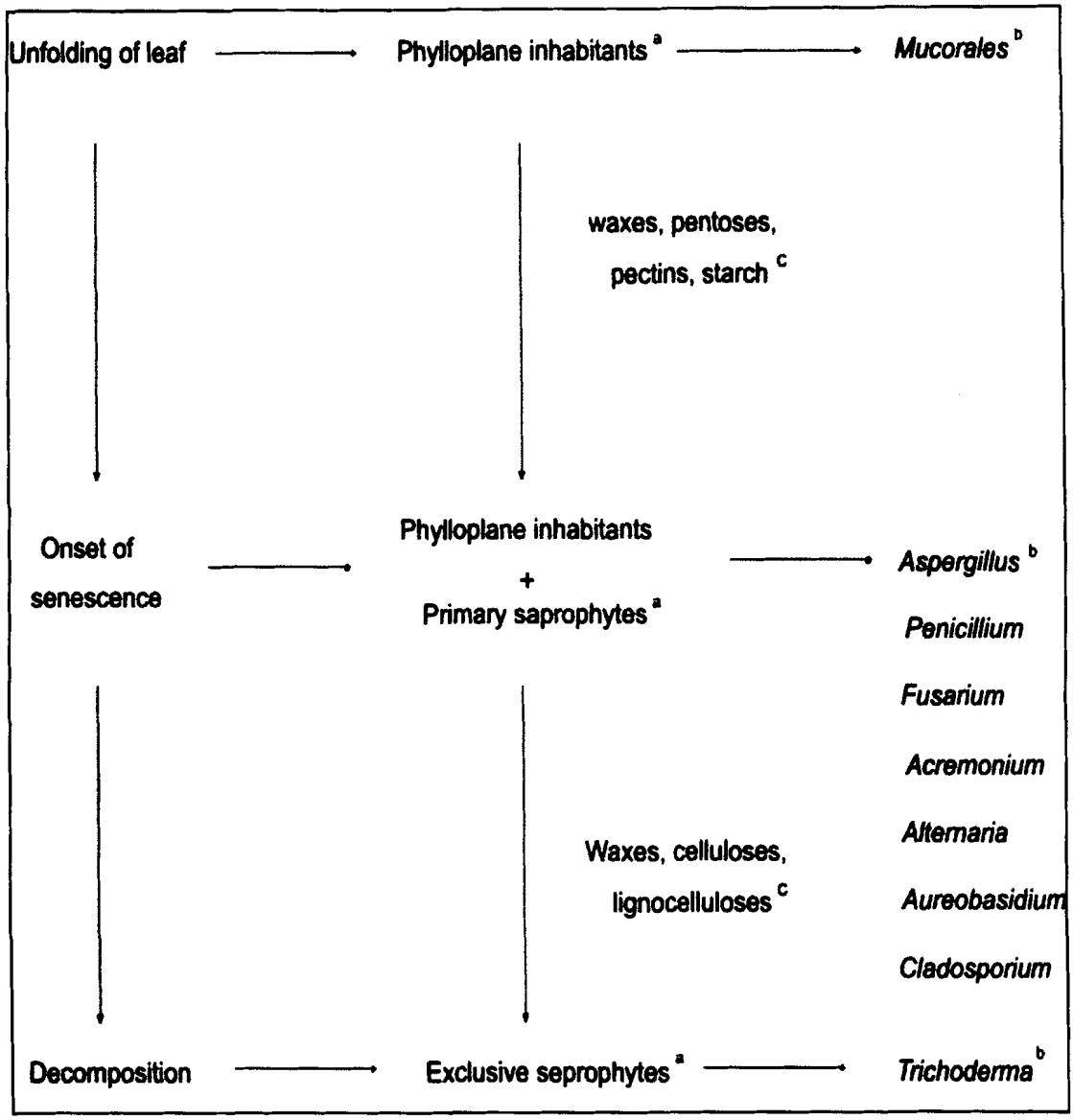

Figure 2.1: Schematic representation showing (a) fungal succession on the leaf substrate as the leaf unfolds, matures and enters the state of decomposition. Also shown are (b) examples of fungal genera common at each stage and (c) the carbon sources that may be utilised at each stage.

2.2.3 Fungal succession

The species composition and abundance of the phylloplane community changes as the leaf matures, senesces, dies and falls. Each stage brings a change in the phylloplane environment as the living processes of the leaf cells decline and there is an increase in substrates for saprophytes, as well as changes in the physical condition of the leaf (Carlile *et al.*, 2001). The primary colonisers that invade are the 'sugar' fungi. They are non-cellulolytic and rely upon readily available sugars, such as hexoses and pentoses, and other carbon sources simpler than cellulose (e.g. pectins and starch). These fungi normally have a high mycelial growth rate and a rapid capacity for spore germination. Mucorales is an order within the Zygomycetes and is a typical example of a primary coloniser (Hudson, 1986). Then, as the leaves die and fall, the initial primary surface colonisers are joined and gradually replaced by exclusively saprophytic fungi which decompose the waxes, cellulose and lignocellulose of the leaf (Kriel *et al.*, 2000). The genus *Trichoderma* is often found in these later stages of decomposition (Osono & Takeda, 1999). Figure 2.1 gives examples of fungi and the carbon sources utilised at each stage of fungal succession.

2.2.4 Substrate and host specificity

Both surface and internal colonisers may be confined to a particular host genus or a related group of plants thus showing signs of host specificity (Kriel *et al.*, 2000; Carlile *et al.*, 2001). In many cases host specificity is synonymous with substrate specificity, particularly in terms of leaf characteristic, position and age (Hudson, 1986). These factors, which aid the leaf in trapping or inhibiting fungal spores, have been discussed in section 2.2.1. In addition to these, a thickened cuticle layer and secretions from trichomes (leaf glands) may significantly inhibit fungal penetration while a variety of antibiotics that are stored in plant cells protect the plant against some fungal species (Funnell & Schardl, 2001). Each of these factors should be considered concomitantly when regarding substrate specificity and in turn host specificity.

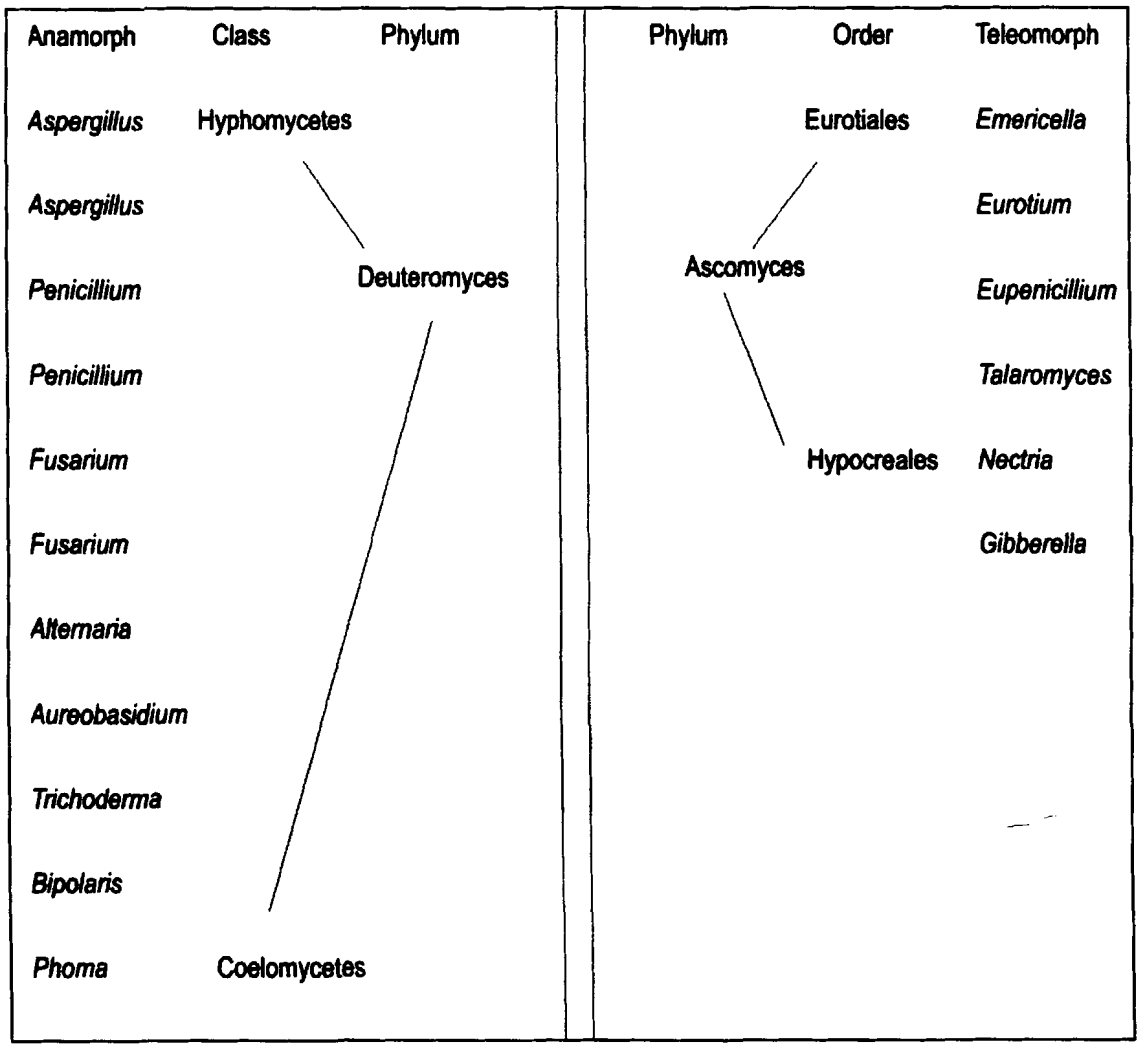

Figure 2.2: Comparison of taxonomy of Deuteromycetes and Ascomycetes that commonly colonise plants. Also shown is the teleomorph (sexual state) of the mitosporic fungi when known.

2.2.5 Common plant colonisers

Fungal genera that may commonly be isolated from the surface or from within include *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Bipolaris*, *Cladosporium*, *Fusarium*, *Penicillium*, *Phoma*, *Phomopsis*, *Rhizopus* and *Trichoderma* (Frazier & Westhoff, 1988). Most of these fungi belong to the Phylum Deuteromycota since their sexual states (teleomorphs) are not known. The group is also known as the mitosporic fungi because their

spores are produced following mitosis and not meiosis (Carlile *et al.*, 2001). Figure 2.2 gives a simplified scheme of the taxonomy of Deuteromycetes commonly isolated from plants. The sexual states of some of these genera are known and have thus been placed within the Phylum Ascomycota. Figure 2.2 also compares the classification of those fungi in which both the anamorph and teleomorph is known.

2.2.5.1 The genus *Acremonium*

The genus *Acremonium* was described in 1809 by Link ex Fries. *Acremonium* species are filamentous, cosmopolitan fungi that are commonly isolated from plant debris (indicating that they are saprophytes) and soil (Doctor Fungus, 2005). *Acremonium* species are endophytic and may infect tall fescue and perennial ryegrass, causing reduced productivity and neurological effects in cattle and sheep. Their mycotoxins (e.g. ergopeptine and lolitrem alkaloids) have been implicated as the causative agents. These fungi supply their host plant with defensive secondary compounds and the grasses provide essential nutrients for the fungus (D' Mello & Macdonald, 1997). Since they are cosmopolitan in nature *Acremonium* may be encountered as contaminants and their isolation should therefore be treated with caution.

2.2.5.2 The genus *Alternaria*

Alternaria was described by Nees ex Wallroth in 1816 (Doctor Fungus, 2005). The *Alternaria* are a cosmopolitan group of dermatiaceous fungi. They may be isolated from plant leaves and soil as well as from food and the indoor air environment (Delgado & Gómez-Cordovés, 1998). *Alternaria* species are capable of growth and reproduction in the phylloplane but they develop to a much greater extent in the initial stages of decomposition (Hudson, 1986). For this reason they may be considered as both phylloplane inhabitants and common primary saprophytes. *Alternaria* species are often involved in the spoilage of refrigerated products since they are capable of growing at low temperatures (Visconti & Sibilia, 1994). The conidia that are produced by *Alternaria* species are ovate or obclavate and have a melanin-like pigment (Blodgett *et al.*, 2000). Over seventy potentially toxic products have been isolated either from *Alternaria* species or directly from different foods (Visconti & Sibilia, 1994). The most important mycotoxins being alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), altertoxin I (ATX-I), tenuazonic acid (TA) and *Alternaria alternata* f.sp. *lycopersici* toxins (AAL toxins).

2.2.5.3 The genus *Aspergillus*

The aspergilli were described by Micheli ex Link in 1809 (Doctor Fungus, 2005). Some species within this genus belong to the Ascomycetes while the sexual state of others are not known and are therefore mitosporic. Members of the genus *Aspergillus* are filamentous and cosmopolitan and ubiquitous in nature having been recovered from many areas around the world (Pestka & Bondy, 1994). Although some species may be saprophytic and found in plant debris and soil, other *Aspergillus* species may be found in the indoor air environment (Jay, 2000). *Aspergillus* conidia are very hydrophobic and not readily wettable. On maturity however, they become easily separated and aerially dispersed (Hudson, 1986).

Aspergillus species are tolerant of low water activity, being able to grow on substrates of high osmotic potential and sporulate in an atmosphere of low relative humidity (Carlile *et al.*, 2001). Fungi of the genus *Aspergillus* usually appear abundantly since they grow well on rich nutrient media and their antibiotics may suppress the growth of other organisms. Generally, the aspergilli are regarded as storage fungi (Hudson, 1986) although some species may be field fungi (Jay, 2000). The genus includes over 185 species (Doctor Fungus, 2005) with various being capable of mycotoxin production. Well known mycotoxin-producers within the genera are *A. flavus* (aflatoxins and cyclopiazonic acid), *A. parasiticus* (aflatoxins), *A. ochraceus* (ochratoxins) and *A. terreus* (citrinin; Bennett & Klich, 2003). Aflatoxin was found to be the aetiological agent responsible for an outbreak of "Turkey X" disease when thousands of poultry died following consumption of contaminated groundnut meal. They are primarily hepatotoxic and hepatocarcinogenic but they also have numerous immunosuppressive effects (Pestka & Bondy, 1994).

2.2.5.4 The genus *Fusarium*

The *Fusarium* genus was described by Link ex Gray in 1821 (Doctor Fungus, 2005). Although most *Fusarium* species are regarded as mitosporic, the sexual states (teleomorph) of some species are known and these have subsequently been placed within the Ascomycete phylum. *Fusarium* species are field fungi (Hudson, 1986) and have been isolated from soil and the aerial and subterranean parts of many plant species (Nelson *et al.*, 1983). Members of the genus *Fusarium* are found worldwide in tropical and subtropical areas (Booth, 1971) as well as in colder climates (D' Mello & Macdonald, 1997) and some are capable of anaerobic growth (Carlile *et al.*, 2001). The fusaria have slime spores that are readily wettable (Carlile *et al.*, 2001) and as a result can be dispersed by dew, rain drops, rain splash or flowing water. Some *Fusarium* species cause major plant diseases such as maize ear infection and ear rot (Desjardins & Plattner, 2000).

Many species of *Fusarium* have been shown to produce a number of secondary metabolites, including a diverse range of mycotoxins, which may cause different physiological and pharmacological responses in plants, animals and humans (Nelson *et al.*, 1993). *F. verticillioides* and *F. proliferatum* are two species that are the most notable producers of the fumonisin group of mycotoxins. Other important toxigenic species include *F. sporotrichioides*, *F. culmorum*, *F. graminearum*, *F. poae* and *F. oxysporum*. These species synthesise a range of mycotoxins such as trichothecenes, zearalenone and moniliformin. The genus *Fusarium* and the fumonisin group of mycotoxins is dealt with in more detail in section 2.3.

2.2.5.5 The genus *Penicillium*

The penicilli were described in 1809 by Link (Schutte, 1992). Teleomorphs of some species are known, but the majority is considered mitosporic. The genus *Penicillium* is comparable to the genus *Aspergillus* in that they are both widespread and commonly isolated from decaying vegetation (thus saprophytic), soil and air (Prelusky *et al.*, 1994). The conidia of *Penicillium* (as of *Aspergillus*) are hydrophobic and difficult to wet but easily dispersed by air currents (Hudson, 1986). Many members of this genus are tolerant of low relative humidity making them important storage fungi and agents of biodeterioration (Carlile *et al.*, 2001). *Penicillium* species may be pathogenic to humans and are quite often encountered in immuno-compromised hosts (Doctor Fungus, 2005). In addition to their infectious potential, some penicilli also produce harmful mycotoxins such as ochratoxin (*P. verrucosum*) and patulin (*P. patulum* and *P. expansum*). Ochratoxins are potent nephrotoxins and teratogens and have been implicated as the causative agent responsible for human endemic nephropathy prevalent in areas of the Balkan countries (Prelusky *et al.*, 1994). They have therefore been classified as

(IARC) while patulin has been classified as "group 3" – no evidence in humans (Paterson *et al.*, 2004).

2.3 THE GENUS *FUSARIUM*

2.3.1 General characteristics

The fusaria are a widespread cosmopolitan group of fungi. A large number of species within the genus *Fusarium* are responsible for a broad range of plant diseases making them one of the most important plant pathogens in the world (Bennett & Klich, 2003). These pathogens are able to colonise the aerial and subterranean parts of many plant species (Nelson *et al.*, 1983). In maize (*Zea mays*), when *Fusarium* growth persists, diseases such as seedling, root, stalk and kernel rot as well as stunting and hypertrophy result (Booth, 1971; Torres *et al.*, 2003). *Fusarium* species have also been shown to be highly toxigenic to animals and humans. Some *Fusarium* species have also emerged as a major cause of invasive disease and mortality among neutropenic patients while others are known opportunistic nosocomial pathogens that cause invasive mycoses which is often fatal in humans and animals (Segal *et al.*, 1998; Hue *et al.*, 1999).

Many species of *Fusarium* have been shown to produce a number of secondary metabolites, including a diverse range of mycotoxins, which can cause different physiological and pharmacological responses in plants and animals (Nelson *et al.*, 1993). Examples of these mycotoxins include the fumonisins, fusarins, moniliformin, trichothecenes and zearalenone. The fumonisin group of mycotoxins in particular, is produced by a total of fifteen *Fusarium* species (Rheeder *et al.*, 2002), all within the Sections *Liseola, Dlaminia, Elegans* and *Arthrosporiella* (Table 2.1). The fumonisins are discussed further in section 2.3.3.

Because of their overall high levels of production, wide geographical distribution, frequent occurrence on food grains and association with known animal mycotoxicoses, *Fusarium verticillioides* and *F. proliferatum* are considered the principle fumonisin producing species (Ross *et al.*, 1991; Nelson *et al.*, 1993; Shephard *et al.*, 2000). *Fusarium napiforme* and *F. nygamai* may also be regarded as important species because of their association with the food grains millet and sorghum. On the other hand, *F. anthophilum* and *F. dlamini* may be considered of only minor importance since they are not associated with maize or any other major food grains and have only a limited geographic range (Nelson *et al.*, 1992).

Table 2.1: Fumonisin-producing *Fusarium* species with their associated fumonisin analogues (Rheeder *et al.*, 2002).

Section	Species within Section	Fumonisin analogues
Liseola	*F. verticillioides*	FA_{1-3}, FB_{1-5}, iso-FB_1, FAK_1, FBK_1, $FC_{1,4}$, FP_{1-3}, PH_{1a-b}
	F. proliferatum	FA_{1-3}, FB_{1-5}, FAK_1, FBK_1, FC_1, FP_{1-3}, PH_{1a-b}
	F. fujikuroi	FB_1
	F. sacchari	FB_1
	F. subglutinans	FB_1
	F. thapsinum	FB_{1-3}
	F. anthophilum	FB_{1-2}
	F. globosum	FB_{1-3}
Dlaminia	*F. nygamai*	FA_{1-3}, FB_{1-5}, FAK_1, FBK_1, FC_1, FP_1, PH_{1a-b}
	F. dlamini	FB_1
	F. napiforme	FB_1
	F. pseudonygamai	FB_{1-2}
	F. andiyazi	FB_1
Elegans	*F. oxysporum*	$FC_{1,3-4}$, *N*-acetyl-FC_1, iso-FC_1, *N*-acetyl-iso-FC_1, OH-FC_1, *N*-acetyl-OH-FC_1
Arthrosporiella	*F. polyphialidicum*	FB_1

2.3.2 *F. verticillioides* and *F. proliferatum*

Fusarium verticillioides (Sacc) Nirenberg [teleomorph: *Gibberella moniliformis* Wineland] is a soil-borne fungal pathogen that was described by Saccardo in 1881 and belongs in Section *Liseola* (Xu & Leslie, 1996). *F. verticillioides* is widespread, occurring from humid and subhumid temperate zones to subtropical and tropical zones. This *Fusarium* species causes many plant diseases such as seedling blight, scorch, foot rot, stunting and hypertrophy (Booth, 1971). *Fusarium proliferatum* (Matsushima) Nirenberg [teleomorph: *Gibberella intermedia*] is closely related to *F. verticillioides* and is also placed within the Section *Liseola*. Both species frequently contaminate foods, especially from the *Gramineae* family, intended for human and animal consumption. *F. verticillioides* and *F. proliferatum* have consistently been

isolated from maize in various countries worldwide including Argentina, Spain, the USA and China (Bacon *et al.*, 1992; Chu & Li, 1994; Cantalejo *et al.*, 1998; Torres *et al.*, 2003). Studies on maize samples from African countries such as South Africa, Zimbabwe, Kenya and Ghana, revealed similar results with the most predominant *Fusarium* species isolated being *F. verticillioides* (Marasas, 1988(a); Kedera *et al.*, 1999; Kpodo *et al.*, 2000; Gamanya & Sibanda, 2001). Both species also colonise rice, sorghum, wheat and barley (Marasas, 1996). *F. verticillioides* grows endophytically within the maize kernels and can infect either the vegetative or reproductive tissues (Bennett & Klich, 2003). Disease symptoms of infected maize vary from asymptomatic infection to severe rotting of all plant parts (Cavaglieri *et al.*, 2005). *F. verticillioides* and *F. proliferatum* have both been recovered from asymptomatic maize kernels in the field and during postharvest storage (Kedera *et al.*, 1999).

Development of *F. verticillioides* and *F. proliferatum* is reported to be differently affected by environmental conditions such as temperature and water activity. Samapundo and colleagues (2005) observed a general increase in the growth rate of both isolates as the temperature and water activity increased, the latter having a more notable effect on growth rates. They noted that at any temperature, the higher the water activity value the larger the growth of both *F. verticillioides* and *F. proliferatum* colonies.

Also, at any water activity, the higher the temperature the higher the growth rate for both strains. The group also found that at the same water activity, *F. verticillioides* grew at a faster rate compared to *F. proliferatum.* This was in contrast to another study that showed that although there was an increase in the growth rates of *F. verticillioides* and *F. proliferatum* as temperature and water activity increased, the overall growth rate of *F. verticillioides* at any water activity was slower than for *F. proliferatum* (Marin *et al.*, 1995). It should be noted that these studies were done in culture and that, when the maize is still on the field or in storage

facilities, other parameters probably also come into play and further affect the growth rates of *F. verticillioides* and *F. proliferatum* (Samapundo *et al.*, 2005). These other parameters may include, amongst others, the composition of the storage atmosphere, the effect of cycling temperature, the presence of competitors, cultivar of maize grain, cultural practices and the effect of anti-fungal agents.

2.3.3 The fumonisin group of mycotoxins

2.3.3.1 General and structural characteristics

Fumonisins were first isolated and chemically characterised in 1988 by researchers in the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the South African Medical Research Council (MRC; Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988). Mycotoxins of this group have subsequently been found to occur infrequently in various foodstuffs including sorghum, rice, asparagus, mung beans and beer (Creppy, 2002; Minorsky, 2002). However, the agricultural commodity most frequently contaminated by fumonisins is maize and its products.

Fumonisin production in maize occurs as a result of fungal invasion either in the field or after harvest during storage (Doko *et al.*, 1995). The environmental conditions of a specific area of cultivation may also play a role in the formation of fumonisins in maize. Nair (1998) reported that fumonisin contamination of maize is expected to be highest in countries with dry, warm climates (e.g. Egypt and Zimbabwe) and the lowest in countries that have cool, damp climates (e.g. Canada and New Zealand).

The worldwide natural occurrence of fumonisins has been well documented (Marasas, 1996). High levels of fumonisins in contaminated maize intended for human consumption or animal feed has been reported for the former Transkei region of South Africa (SA) and the Linxian

and Shangqiu Counties of the People's Republic of China (Rheeder *et al.*, 1992; Yoshizawa *et al.*, 1994).

In SA, mouldy as well as healthy maize (i.e. maize kernels with and without disease symptoms) from low and high oesophageal cancer areas were analysed for fumonisins. The mean fumonisin level in healthy maize from areas with a high rate of oesophageal cancer was three times higher compared to the fumonisin levels detected in healthy maize from areas with a low rate of oesophageal cancer. Even more significant were the results for the mouldy maize, which showed a mean fumonisin level that was twelve times higher in the area with a high rate of oesophageal cancer than in the low-rate area. In China, the incidence of fumonisin contamination of Shangqiu maize was 25 % while the incidence in Linxian maize was almost double at 48 %. Other regions where fumonisins have also been detected, albeit in lower concentrations, include areas within KwaZulu Natal (SA), Iran, Argentina, Northeast Mexico and Spain (Desjardins *et al.*, 1994; Sanchis *et al.*, 1994; Chulze *et al.*, 1996; Shephard *et al.*, 2000; Chelule *et al.*, 2001).

The basic fumonisin structure is shown in Figure 2.3. Structurally, the fumonisins have a linear 19- or 20- carbon backbone with hydroxyl, methyl and tricarboxylic acid moieties at various positions along the backbone (Shim & Woloshuk, 2001). However, 28 fumonisin analogues that are separated into four main groups have been described and are identified as the A, B, C and P series of fumonisin analogues (Rheeder *et al.*, 2002). The fumonisin B (FB) series is regarded as the main group while the others are considered to be minor and less well characterised (Jay, 2000).

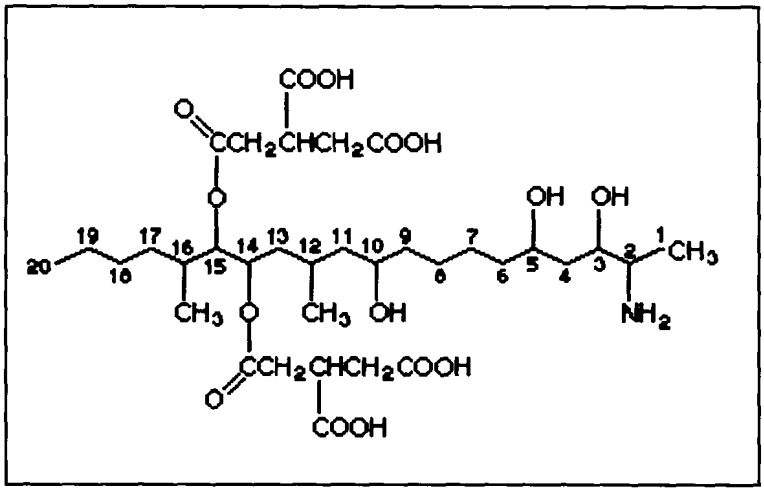

Figure 2.3: The basic structure of the fumonisin mycotoxin (Bennett & Klich, 2003).

Fumonisin B analogues, comprising toxicologically important FB_1, FB_2 and FB_3, are the most abundant naturally occurring fumonisins (Marasas, 1996). These analogues form a group of related polar metabolites that were originally isolated from *F. verticillioides* (Bezuidenhout *et al.*, 1988). The most prevalent amongst this group, FB_1, is also believed to be the most toxic (Minorsky, 2002). FB_1 is the diester of propane-1,2,3-tricarboxylic acid and a 2S-amino-12S,16R-dimethyl 1-3S,5R,10R,14S,15R-pentahydroxyicosane with both C-14 and C-15 hydroxy groups esterified with the terminal carboxy groups of the acids (Soriano *et al.*, 2005). FB_2 and FB_3 are homologs that lack one of the free hydroxyl groups on the backbone. FB_2 lacks the hydroxyl at C-10 while FB_3 lacks the hydroxyl at C-5 (Dutton, 1996). FB_4 was isolated even later with the difference also lying in the hydroxylation pattern. This homolog lacks the hydroxyl group at both C-5 and C-10 (Gelderblom *et al.*, 1992). Figure 2.4 shows the structural formula of FB_1, FB_2, FB_3 and FB_4.

Even though FB_1 is mainly restricted to maize, it has also been detected in sorghum and poultry feeds (Shetty & Bhat, 1997; Creppy, 2002). Results of analyses regarding the occurrence of FB_1 in maize samples from 18 countries worldwide established the presence of FB_1 in 93 % of the samples (Minorsky, 2002). Relatively high FB_1 levels were recorded in

Iran, China, the USA, India, Italy, Portugal, Zambia and Benin (Murphy *et al.*, 1993; Chu & Li, 1994; Doko *et al.*, 1995; Shetty & Bhat, 1997; Shephard *et al.*, 2000). FB_2 and FB_3 have been isolated from maize samples although at much lower concentrations than FB_1. In field exposures, the fungi apparently produce proportional amounts of each FB homolog (Murphy *et al.*, 1993). According to the authors, the correlation coefficient between FB_1 and FB_2 and between FB_2 and FB_3 was very close to one. In laboratory exposures, similar results were recorded by Marin *et al.* (1995). This group cultured FB_1, FB_2 and FB_3 on maize, rice or liquid media and reported that FB_1 typically accounts for 70 to 80 % of the total fumonisins produced, while FB_2 usually makes up 15 to 25 % with FB_3 making up 3 to 8 %. A study carried out by Seo and Lee (1999) showed small amounts of FB_4 occurring in mouldy maize samples.

FB_1 production by *F. verticillioides* is dependent on two extrinsic factors, namely water activity and temperature. Cahagnier *et al.* (1995) showed that as water activity was lowered, FB_1 production also decreased. The optimum temperature for FB_1 synthesis was found to be 25 °C (Alberts *et al.*, 1990). Continuous investigations done by a group of researchers on the combined effect of water activity and temperature showed firstly, an undoubted production of FB_1 and FB_2 and secondly, more FB_1 than FB_2 was likely to be produced regardless of water activity or temperature. It was also deduced that both water activity and temperature were statistically significant but that water activity was the more important factor affecting FB_1 production (Marin *et al.*, 1995; Marin *et al.*, 1999).

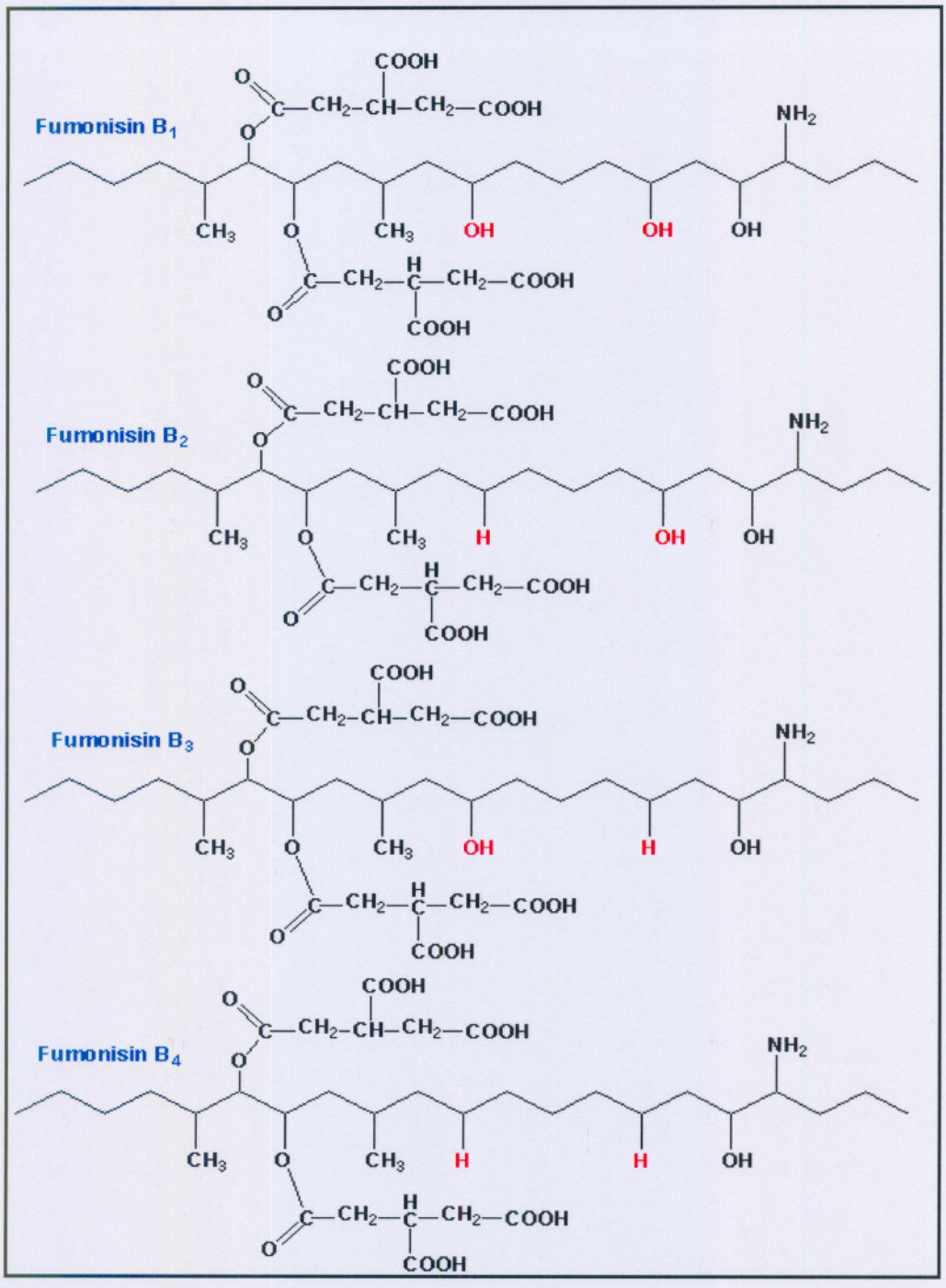

Figure 2.4: The chemical structures of FB_1, FB_2, FB_3 and FB_4 (Bennett & Klich, 2003).

Fumonisin stability during several food production and preparation processes has also been investigated. It was found that fumonisin levels decreased after being exposed to high temperature drying (i.e. temperatures that are reached during oven baking), alkaline cooking (nixtamilisation) and a process called wet milling (Bordson *et al.*, 1995; Bennett & Richard,

1996; Dombrink-Kurtzman *et al.*, 2000). Processes in which fumonisins remained stable include boiling (i.e. temperatures that occur during normal cooking processes), ammoniation and ethanol fermentation (Alberts *et al.*, 1990; Norred *et al.*, 1991; Bothast *et al.*, 1992).

2.3.3.2 Animal toxicity

Various toxicological investigations of *F. verticillioides* cultures or naturally contaminated maize revealed that fumonisins, in particular FB_1 and to a smaller extent FB_2, are the causal agents of several animal diseases. The species, age and health of the affected animal in conjunction with the level and duration of FB_1 exposure, determined the magnitude of effects associated with exposure (Stack & Carlson, 2003). Effects may be subtle, including reduced weight gain and minor behavioural abnormalities such as feed refusal, or may be more severe including reproductive dysfunction, organ failure and death. These effects point towards a real health risk to animals and an imminent threat to humans exposed to specific concentrations of naturally occurring fumonisins. Affected animals include horses, donkeys, rats, pigs, rabbits, calves and poultry.

In horses and other equine, FB_1 has been found to be the etiological toxic agent responsible for equine leukoencephalomalacia (ELEM; Marasas *et al.*, 1988(b)). Swine that were exposed to FB_1 developed pulmonary edema (PE) which proved to be lethal (Harrison *et al.* 1990). Similar results were observed in a more recent study with hepatic lesions consisting of apoptosis, necrosis and hepatocyte proliferation further being noticed, showing a specific affinity of FB_1 for the liver (Haschek *et al.*, 2001).

Dilkin *et al.* (2003) studied the effects of chronic oral exposure to FB_1 in weaned piglets and observed typical signs of porcine pulmonary edema (PPE) together with reduced feed consumption and body weight gain as well as pathological alterations. The FB_1-treated piglets

also exhibited detectable levels of FB_1 in the liver. Another study revealed an altered cardiovascular function in pigs exposed to FB_1 (Smith *et al.*, 2003). FB_1 has a cancer-promoting effect in rats as shown by Gelderblom and co-workers (1988). Further studies were undertaken by the same group as well as other researchers and results indicated that the liver and kidney were the principle target organs for FB_1 action in rats (Gelderblom *et al.*, 1991; Gelderblom *et al.*, 2001; Howard *et al.*, 2001; Theumer *et al.*, 2002). In a study involving fumonisin exposure to chicken eggs, a 100% mortality rate was observed and FB_1 was verified as being the most toxic of the fumonisins (Henry & Wyatt, 2001). Microscopic examinations carried out by Bucci *et al.* (1996) revealed small haemorrhages in the brains of those rabbits that died after exposure to FB_1. The animals also had marked degeneration (mainly apoptosis) of renal tubule epithelium and hepatocytes. Another study showed that in both male and female rabbit pups, the body, liver and kidney weights were lower than initial levels (LaBorde *et al.*, 1997). FB_1-treated calves were lethargic and had decreased appetite. Severe liver and bile duct injury and impaired hepatic function were observed and biochemical evidence revealed renal injury that chiefly involved the proximal convoluted tubule of exposed calves (Mathur *et al.*, 2001).

2.3.3.3 Human toxicity

In 1992, Thiel *et al.* showed that known naturally occurring levels of fumonisins posed a potential threat to human health and advised that realistic tolerance levels be set. In the same year, the group statistically associated fumonisins with the high incidence of oesophageal cancer in certain areas of the (former) Transkei region of the Eastern Cape Province, SA (Rheeder *et al.*, 1992). By 1993, considering the severity of the fumonisin threat, the IARC evaluated their carcinogenic risk to humans.

The fumonisins were evaluated as "Group 2B carcinogens", i.e. probably carcinogenic to humans (IARC, 1993). Studies were also undertaken in China and it was indicated that high levels of FB_1 might play an important role in carcinogenesis in humans in Cixian and Linxian counties (Chu & Li, 1994). Shephard *et al.* (2000) published the first report of fumonisin contamination of corn from Iran, in which samples from an area of high oesophageal cancer on the Caspian littoral were shown to contain high levels of fumonisins. This report further strengthens the epidemiological data that associates fumonisins with high incidences of oesophageal cancer. Despite this, oesophageal cancer has not been reproduced experimentally in animals with culture material of *F. verticillioides* nor pure FB_1 (Marasas, 1996). A causative relationship is therefore unproven and remains speculative.

2.3.3.4 Mechanism of action

It is expected that fumonisin toxicity is due to their structural similarity to the sphingoid base backbone of sphingolipids (Merrill *et al.*, 1996). This may explain the wide variety of health effects observed when fumonisin is ingested (e.g. LEM in equids, pulmonary edema in swine and the high rate of oesophageal cancer in humans). Sphingolipids can be found in all eukaryotic cells, where they are especially plentiful in the plasma and related cell membranes (Merrill *et al.*, 1997) as well as in nerve and brain tissues (Soriano *et al.*, 2005). They contain a ceramide backbone which anchors them in the outer leaflet of the lipid bilayer (van Echten-Deckert *et al.*, 1998).

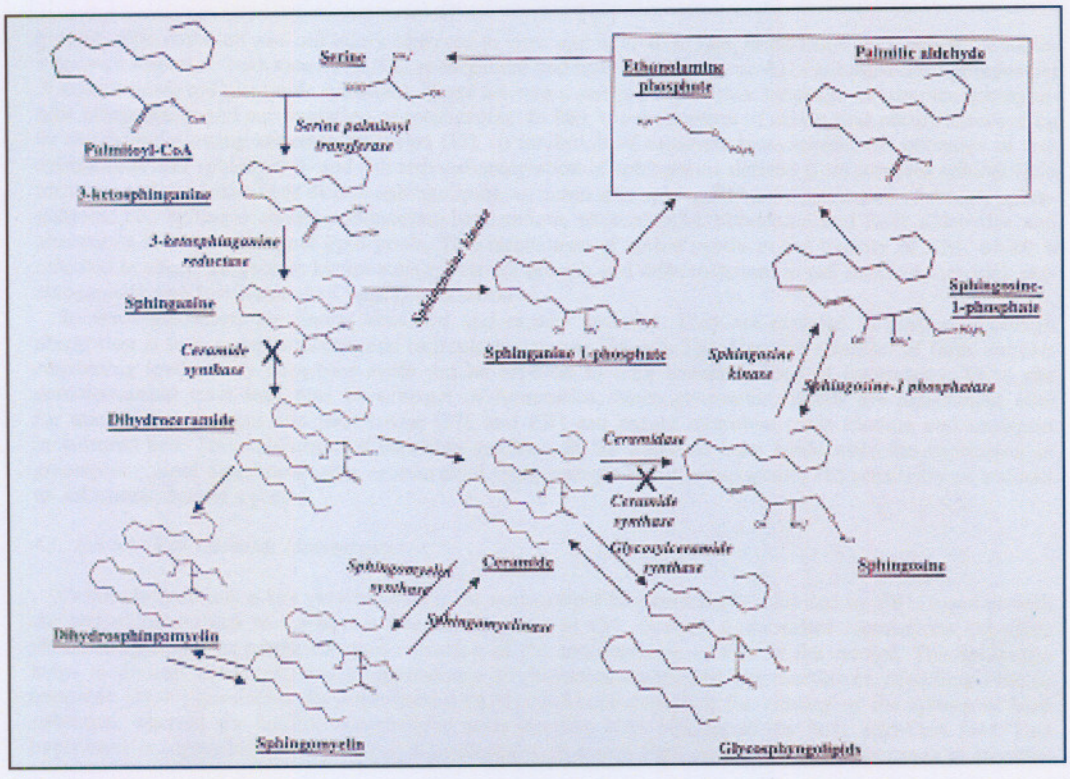

Figure 2.5: Cycle of *de novo* sphingolipid biosynthesis. The **X** symbol indicates the pathways that are inhibited by fumonisin B_1 (Soriano *et al.*, 2005).

De novo synthesis of sphingolipids begins with the condensation of serine and palmitoyl-CoA, catalysed by serine palmitoyl transferase, forming 3'-keto-sphinganine, which is reduced to form sphinganine. Sphinganine may either be phosphorylated to form sphinganine 1-phosphate, catalysed by sphinganine kinase, or *N*-acylated by ceramide synthase to form dihydroceramide. This product is then desaturated to ceramide, which may undergo either of 3 pathways. In the first step, ceramide may be transformed to sphingomyelin catalysed by sphingomyelin synthase. Ceramide may also be converted to glycolipids (glycosphingolipids) by the addition of oligosaccharides, catalysed by glycosylceramide synthase. The third step that ceramide may follow involves the catalytic action of ceramidase to form sphingosine. The resulting sphingosine is phosphorylated to yield sphingosine 1-phosphate, catalysed by sphingosine kinase.

Both sphingosine 1-phosphate and sphinganine 1-phosphate produce ethanolamine phosphate and a fatty aldehyde. These are converted into serine and palmitoyl CoA thereby closing the circle (Figure 2.5).

The intermediates formed during the synthesis of sphingolipids have various effects on cellular processes. Accumulated sphingoid bases (sphinganine and sphingosine) may either be growth inhibitory or growth stimulatory (Riley *et al.*, 2001) and are potent inhibitors of protein kinase C (Merrill *et al.*, 1996). They may also play a role in the regulation of cell growth, cell differentiation, cell morphology, apoptosis (programmed cell death) and endothelial cell permeability (Soriano *et al.*, 2005). At high concentrations, these sphingoid bases may be cytotoxic (Merrill *et al.*, 1996). Ceramide, another intermediate of sphingolipid biosynthesis, plays a role in the regulation and differentiation of cells, apoptosis and protein secretion, induction of cellular senescence and other processes (Soriano *et al.*, 2005). The end product, glycosphingolipid, forms cell- and species- specific profiles known to change characteristically during development, differentiation and transformation implying that they play a role in cell to cell interactions and in cell adhesion (van Echten-Deckert *et al.*, 1998).

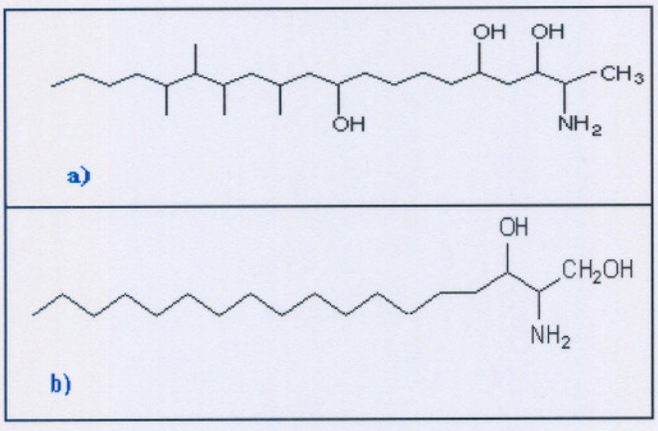

Figure 2.6: Comparison of (a) fumonisin B1 and (b) the sphingoid base sphingosine, structural analogues of each other (Bennett & Klich, 2003).

Fumonisin B_1 structurally resembles the sphingoid bases (Figure 2.6) and binds to the catalytic site of ceramide synthase effectively inhibiting the enzyme, which is responsible for catalysing the acylation of sphinganine and the reacylation of sphingosine. The biochemical consequences of fumonisin disruption of sphingolipid metabolism occurs in sequence beginning with (i) altered ceramide biosynthesis, (ii) increases in free sphinganine and sphingosine and their 1-phosphates and (iii) alterations in the cellular concentrations of specific glycosphingolipids (Riley *et al.*, 2001; Soriano *et al.*, 2005). Alteration of ceramide biosynthesis may explain a variety of effects in animals, including liver lesions, heart failure, inhibition of cell growth, apoptosis, pulmonary edema, carcinogenicity and genotoxicty (Soriano *et al.*, 2005). The genotoxic potential of FB_1 remains an enigma. Various authors have reported that FB_1 is not genotoxic (Merrill *et al.*, 1996; Creppy, 2002; Fotso *et al.*, 2002). However a study undertaken in 2002 by Galvano *et al* counters the common thought. Their results indicated that exposure to FB_1 at high concentrations and for prolonged times caused DNA damage of apoptotic type in human fibroblasts. In view of their study, they propose that the genotoxic potential of FB_1 has probably been underestimated and should be reconsidered. Increases in sphinganine and sphingosine can only occur once the capacity of sphinganine kinase to metabolise these sphingoid bases to their 1-phosphates has been exceeded. This is reflected in effects on cell growth and differentiation, on protein kinase activity, in apoptosis and the involvement of lipid peroxidation (Soriano *et al.*, 2005). In 2001, Riley's group reported that the loss of complete sphingolipids resulted in abnormal behaviour and altered morphology of fumonisin-treated cells. An earlier study showed that inhibition of complete sphingolipid formation by FB_1 accounted for the inhibition of neutrite outgrowth (Merrill *et al.*, 1996).

Taking these facts into account, fumonisin disruption of sphingolipid biosynthesis may be considered significant in the *in vitro* and *in vivo* fumonisin toxicity responsible for ELEM, PPE and possibly for human oesophageal cancer.

2.4 GENERAL METHODS USED IN THE ISOLATION OF FUNGI

Most natural substrates contain some degree of fungal spores and various methods have been devised to aid their study. Generally, mycological studies involve isolating the fungi from their host, growing the fungi in pure culture and identifying the fungi that have been isolated. In this way a list of species present on the host substrate may be compiled.

Isolation of fungi can be divided into the (i) direct method; in which visible spores and mycelia are transferred from the substrate to a suitable sterile nutrient culture medium, and (ii) indirect method; in which a sample of the substrate is used to inoculate the medium (Baxter & van der Linde, 1999; Carlile *et al.*, 2001). In the direct method, the spores or mycelia that are picked up usually belong to one specific fungus. On the other hand, when the indirect method is used, many colonies usually develop. These colonies are selected and transferred individually to a fresh sterile medium with the resultant pure culture being referred to as an isolate. When isolating fungi, it is standard to add antibiotics such as penicillin and streptomycin to the medium or to acidify the medium to about pH 4. The medium is then able to suppress bacterial growth while encouraging fungal growth.

In general, surface colonisers are absent among internal colonisers and vice versa. This could be attributed to the method of isolation (Kriel *et al.*, 2000). Leaf washings with subsequent dilution plating allows the isolation of phylloplane inhabitants and common saprophytes. This method of isolation also allows the isolation of spores that have become air-borne and consequently trapped onto leaves. The washing method however, tends to exclude internal

colonisers. Nevertheless, sterilising the leaf surface may isolate endophytes. This may be done by brief submersion in a mild sterilant such as a 3.5% sodium hypochlorite solution or a 1% hydrogen peroxide solution (Baxter & van der Linde, 1999). The sterilant washes away surface colonisers while internal colonisers are protected from it by the leaf within which they reside (Carlile *et al.*, 2001). The leaf is placed on a suitable medium that encourages the growth of internal colonisers.

Fungi that grow out of the leaf segments are periodically isolated. Each protocol (isolation of surface and interior colonisers) should be standardised so that efficient isolation of the maximum number of isolates is made possible. The protocol should also be validated to ensure proficient repeatability of the method.

Once a statistically representative number of fungi have been isolated from leaf surfaces and interior, the process of identification may begin. Because of their capacity for rapid change, species identification presents certain problems and fungi can therefore only be identified once a pure isolate has been obtained (Nelson *et al.*, 1983). In order to obtain a pure isolate it is imperative to start up colonies from a single uninucleate haploid cell that is genetically uniform. For the fusaria, it matters little whether these are single macroconidia or microconidia as both begin initially with a single nucleus (Booth, 1971). Characteristics employed to determine the class, order, family and genus of the pure fungal isolate include the shape, size, colour and manner of arrangement of conidia as well as conidiophores. This involves macroscopic and microscopic observations of both, the isolate itself and slides prepared from the isolate using a suitable mountant (pers. comm. Dr E van der Linde, 2003). The observed characteristics can be utilised to trace the fungus, through published analytical keys of the fungi, to the genus and species to which they belong (Agrios, 1978).

Traditionally, fungal identification was based primarily on morphology. However, fungal identification (particularly for the fusaria) based on morphological characters is time-consuming and requires unusual skills (Yergeau *et al.*, 2005). Ominski and colleagues (1994) reported on new identification techniques that may be used, including chemotaxonomy, electrophores, genetic and ultrastructure studies and immunological techniques. They concluded that when these techniques are employed concurrently with morphology-based taxonomy, they provide a more comprehensive identification of fungal species. Molecular techniques especially, will permit efficient and low-cost processing of large numbers of samples required in large-scale studies (Yergeau *et al.*, 2005).

The universal 18S gene region may be used initially to assess large fungal groups by examining the fungal community as a whole (Lowell & Klein, 2001). Further, it is generally necessary to isolate two genes that are known to be conserved in individual fungal genera. For the *Fusarium* genus, primers of Geiser *et al.* (2004) which amplifies a partial elongation factor 1 alpha (*EF*) genes is conserved. Furthermore, the gene responsible for synthesis of the polyketide backbone of fumonisin (*FUM 1*; Bluhm *et al.*, 2004) can be amplified to indicate that the polyketide synthase gene is present in certain *Fusarium* strains. These strains may be regarded as potential fumonisin-producing *Fusarium* strains. PCR and sequencing analysis of *18S* and *EF* genes, particularly the latter could thus be used to validate identification of *Fusarium* isolates (Leslie & Summerell, 2006).

To conclude, it was noted that fungi are ubiquitous in nature and their growth may be affected by various parameters. Under favourable conditions, fungi may produce mycotoxins which may impact negatively on plants, animals and humans. Mycological and / or molecular methods may be used to isolate fungi from the exterior and interior leaf surfaces and to identify the fungi and the toxins they are capable of producing. Prior to mycological or molecular analysis, it is necessary to identify a suitable area where feasible studies can be performed. For this reason, an in depth survey of the study area was carried out and is reported on in Chapter 3.

CHAPTER 3

STUDY AREA

3.1 INTRODUCTION

The mycological quality of foods is affected by different environmental conditions, especially temperature and water activity (Jay, 2000). Another factor that might contribute, either positively or negatively, to the mycological quality of foods is possible differences in agrarian practices. These conditions / factors may potentiate changes in the quantity and composition of expected fungal communities in the plant types and study area considered. It is of essence then, to contemplate climatic conditions (i.e. temperature and rainfall) as well as farming practices of the study area concerned.

Factors that determine the types, quantity and quality of foods consumed, are the socio-economic status and the ethnicity of the communities concerned. Different ethnic groups prefer different foods in spite of their geographical location (pers. comm. Mr PJ Lebea, 2003). Furthermore, people with a higher socio-economic standing consume larger quantities of foods and have a more diverse range of foods that are nutritionally more beneficial. For poorer households, not only is the portion of food consumed smaller but the types of foods eaten are also limited. In 2003, Bennett and Klich reported that when the food supply becomes limited (as in the case of poorer rural households), fungus-damaged, mycotoxin-containing foodstuffs are more likely to be consumed rather than discarded.

With this in mind, this Chapter aims to provide a detailed description of the non-mycological aspects that are likely to contribute to the mycological quality of foods staple to the study area of concern. Emphasis was also placed on ethnicity and socio-economic status of the different

communities within the study area. In addition, a general description of some plant types regarded as staple by the communities of the chosen region is given.

3.2 GEOGRAPHY, TOPOGRAPHY AND DEMOGRAPHY

The study was conducted in the Mopani District of the Limpopo Province in the summer season of 2002 / 2003. This sub-tropical region is situated approximately 215 meters above sea level and during the period of the study, an average annual minimum and maximum temperature of 20 °C and 34 °C, respectively and only 25 mm of rainfall was recorded (Appendix A). The Limpopo Province is the fourth most densely populated of the nine South African provinces (Anon, 2004). Consistent with the South African provincial demarcation, this most northern province of South Africa is divided into various districts, which are in turn allocated several municipalities that govern over a number of wards. Figure 3.1 shows the Limpopo Province and its six districts. The Limpopo Province extends over an area of 123 910 km^2 forming 10 % of South Africa's total land (Stats SA, 2003).

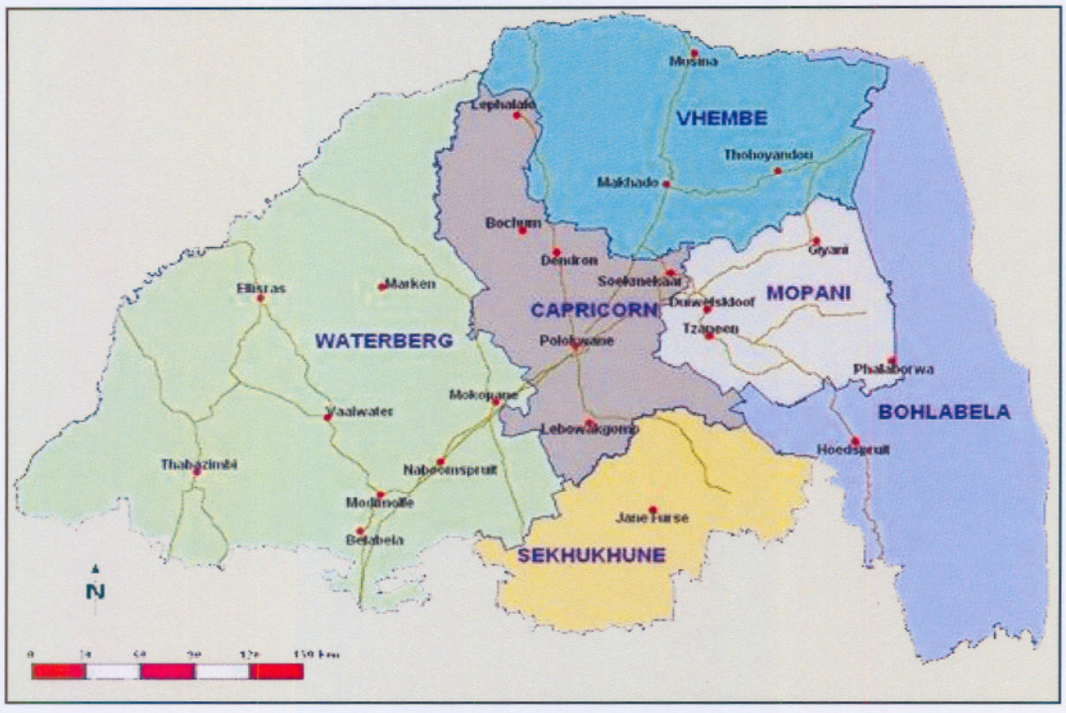

Figure 3.1: Map of the Limpopo Province showing its six districts.

This study was carried out among rural communities in one of the smallest districts of the Limpopo Province, namely the Mopani District, where 18 % of the province's population resides (Anon, 2004). Jurisdiction over the northern part of the Mopani District, where the population is predominantly Tsonga-speaking, is given to the Greater Giyani Municipality (GGM), while the north-western part is governed by the Greater Letaba Municipality (GLM) where the main language spoken is a Sepedi dialect, Khelobedu. The study areas identified within the GGM included the central business district (Giyani), one village (Sikhunyane) from ward 8 and one village (Nkomo A) from ward 18. Within the GLM, only one village (Shamfana), from ward 13, was selected as a study area. Figure 3.2 gives an overview of the study area chosen. The wards were chosen for their accessibility rather than their geography. The ethnicity of the community within the Mopani District was considered relevant since different ethnic groups have different food preferences. The plant types considered for this

study were those commonly used by both the Tsonga-speaking and Khelobedu-speaking locals.

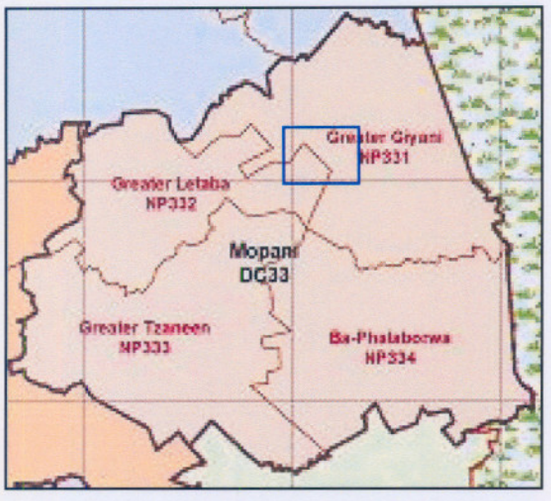

Figure 3.2: Map showing the four municipalities of the Mopani District. The square indicates the study area (Stats SA, 2003).

Although the number of villages and the total population within these three individual wards differ, there are common characteristics among them. These include the large percentage of the population that have no schooling experience, the high number of people that are unemployed or not economically active and the lack of basic amenities such as sanitation facilities, running water within each dwelling and refuse removal services (Stats SA, 2003; pers. comm. Mr N Nkuna, 2003). Statistics revealed that only a small percentage of the population within the 3 wards are employed and the majority of these are in the community / social / personal services industry to which the South African Police Service, Correctional Services, the South African National Defence Force and government employees belong to. On average, as much as 32 % of the employed hold only elementary positions and a further 30 % are employed as service workers, technicians or clerks and earn a monthly salary ranging between R1 and R3 200. In stark contrast, a mere 8 % are professionals while legislators and

senior officials make up a smaller 3 %. Ironically, these two groups earn a salary of not less than R6 400 rising to over R204 801 per month (Stats SA, 2003). From this, we can propose that 80 % of the population within the three wards chosen as study areas are unemployed and have no monthly income, only 19 % earn between R1 and R3 200 each month while less than 1 % earn a monthly salary that falls between the R6 400 and R204 801 bracket. The crux of this conclusion serves to highlight the critical importance of the farming community and farming practices as a means of continued survival for the majority of the population within these wards and possibly the Limpopo Province as a whole.

3.3 FARMING SECTOR

Cultivation of soil and rearing of livestock are the main agricultural activities practiced by the farming sector of the villages. Three groups of farmers that operate within the soil-cultivating farming community of these villages are distinguished, namely (i) the subsistence farming group, into which the majority of the farming community fall, (ii) the semi-commercial farming group that exists to a lesser extent and (iii) the small-scale commercial farming group which are found rather infrequently.

Factors of differentiation between the groups relate to their reasons for farming, level of formal education, accessibility to water, pesticides and fertilisers and the market each group targets. A summary of these differences is given in Table 3.1 while Figures 3.3 and 3.4 show fields that are typical of small-scale commercial farmers (SSCF), semi-commercial farmers (SCF) and subsistence farmers (SF).

The varying levels of formal education amongst the three farming groups play a determining role in each of the other factors of differentiation. Generally, SSCFs have tertiary qualifications (whether a degree, diploma or certificate). They therefore have a good

understanding of formal farming practices (i.e. those employed by conventional commercial farmers), in which advanced irrigation and pest control strategies are used (pers. comm. Mr R Mkhari, 2003). This leads to an increased crop yield of superior quality, which can be sold not only to households within villages, but can also be transported to and sold in different central business districts. The monthly income generated is sufficient to maintain equipment and other consumables and for the most part, SSCFs can continue with their farming activities uninterrupted (pers. comm. Mr P Molewa, 2003). The main difference between SSCFs and conventional commercial farmers is the restriction in the land area on which SSCFs cultivate their crops.

Table 3.1: Differences between subsistence farmers, semi-commercial farmers and small-scale commercial farmers.

	Subsistence farmers (SF)	Semi-commercial farmers (SCF)	Small-scale commercial farmers (SSCF)
Reason for farming	Nutritional gain	Cash gain	Cash gain
Level of formal education	None	Secondary schooling	Tertiary qualification
Water	Rely on rain	Borehole or generator pump	Borehole or generator pump
		Flood method of irrigation	Drip method of irrigation
Pest control measures	None	Inconsistent	Consistent
		Non-specific products	Various specific products
Fertilisers	None	Domestic (cow dung, kraal manure)	Consistent
			Various specific products
Target market	Own household	Own household	Own household
		Other households within village	Other households within own village and other villages
		Closest central business district	Various central business districts
			External markets (in other towns and cities)

Figure 3.3: Fields typical of **(a)** small-scale commercial farmers and **(b)** semi- commercial farmers. Compare with fields typical of subsistence farmers (Figure 3.4).

Figure 3.4: Fields typical of subsistence farmers. Compare with fields typical of small-scale commercial farmers and semi- commercial farmers (Figure 3.3 (a) and (b))

The majority of SCFs have some sort of secondary schooling. These farmers use mainly traditional farming methods but realize the significance of proper irrigation and pest control systems and practice these whenever possible (pers. comm. Mr P Baloyi, 2003). Their crop yield varies seasonally, according to the pest control systems in place for that period of time. The harvest is used within the family and the remaining is sold to other households and in the closest business district (pers. comm. Mr P Baloyi, Mr P Molewa, 2003). Because of the inconsistency of the crop yield, the income generated on a monthly basis is not fixed. As a consequence, maintenance of broken equipment and regular buying of pest control products is not guaranteed and farming activity and practices becomes inconsistent (pers. comm. Mr A Machete, 2003).

Subsistence farmers lack formal education and rely entirely on farming knowledge that was passed on by previous generations. This informal education is very basic and although effective, the holistic farming activity does not reach an optimum level. For this reason, crop

yield is sufficient to feed only the family within the household (pers. comm. Mr P Lebea, 2003; Mrs M Mabulana, 2003). Also, because SFs rely on rain to irrigate their crops, their farming activity is often disrupted or ceases completely during spells of drought thereby limiting the availability of food and nutritional variety.

Water availability is an important factor determining the types of crops cultivated by each farming group. Small-scale commercial farmers and SCFs have a constant water supply and are able to cultivate those crops with high water requirements such as spinach, cabbage and chillies (pers. comm. Mr R Mkhari, 2003). However, the method of irrigation used by SSCFs and SCFs vary. Small-scale commercial farmers prefer the drip system of irrigation to the flood or sprinkler systems while SCFs favour the flood system (Figure 3.5). The flood system is an inexpensive form of irrigation and for this reason is utilised by SCFs (pers. comm. Mr A Machete, 2003). The drip system waters crops from directly above and although more costly than the flood system, it is cheaper than the sprinkler system, easy to maintain, saves labour and conserves water, which makes it an attractive option for SSCFs (pers. comm. Mr R Mkhari, 2003).

Figure 3.5: Water irrigation method preferred by **(a)** SSCF, drip method, and **(b)** SCF, flood method.

Subsistence farmers who rely solely on rain to irrigate their ploughing fields are restricted to crops that require less water (i.e. plants that are more drought resistant). Examples of these

plant types include maize, pumpkin, watermelon and okra (pers. comm. Gugu Mothele, Mrs C Rasuku, 2003).

In the cases of SSCFs and SCFs the quantity and quality of crop yield is largely dependent on the type, as well as the constancy, of pesticide and fertiliser application. Small-scale commercial farmers use various fungicides and/or pesticides (e.g. Dithene, M45, Bravo and WondoZeb) simultaneously with different types of fertilisers (e.g. Potage, 101, Superphosphate, 2:3:4; pers. comm. Mr R Mkhari, 2003). This defined treatment of the crops and soil occurs on a regular basis and at harvest farmers are rewarded with a high crop yield of good quality. Semi commercial farmers do not have the same success rate as SSCFs, probably because of the following factors: (i) pesticides are only applied when the farmer is financially able to, and even then, the type of product bought is usually dependent on and limited to the budget available, (ii) the effective application of the product used for pest control. Most SCFs, although having adequate reading skills, do not interpret the instructions on the labels correctly and hence application of the product is rarely accurate and (iii) only domestic organic fertiliser (kraal manure and / or cow dung) is used, often in quantities not sufficient to fertilise the entire field (pers. comm. Mr. P Baloyi, Mr A Machete, Mr P Molewa, 2003). For these reasons the crop yield of SCFs is smaller and not of the same quality as the crops of SSCFs. Subsistence farmers make use of neither pesticides nor fertilisers and so the quantity and the quality of their crop yield is of inferior grade.

3.4 STAPLE FOODS OF RURAL LIMPOPO

The information in this section was obtained using the responses to questionnaires posed to each of the housewives within the study group (Appendix B).

The types of foods eaten by a specific community may vary according to the geographical area of the community, the ethnicity of that community and the plant species indigenous to that area. Food types that are customarily eaten in the Mopani District include various cereals, fruits and berries, vegetables, seeds, nuts, roots, tubers and bulbs. Most of these food types are eaten only when available, which is usually during the specific season that they ripen. Some families begin the day with tea and bread while some higher income earners have the opportunity to supplement their staple with meat. Children may occasionally eat other delicacies such as locusts, termites and mopani worms and more recently they consume sweets, crisps, ice creams and baked fish. Maize (*Zea mays*) is the most commonly eaten cereal, and together with a number of leafy vegetables (*morogo*), it forms the staple diet of both the Tsonga-speaking and the Khelobedu-speaking people of the Limpopo Province. *Morogo* is a vernacular term used to describe the leaves of various edible plant species indigenous to certain geographical regions. Other vernacular names used to describe the leafy green vegetables include *miroho* (Venda) and *imfino* (Xhosa). Rural communities within each of the nine provinces of South Africa may then effectively have different types of plant species that are regarded as *morogo*. Because maize serves as the primary nutritional source for the majority of the rural population, it is the chief crop cultivated in these regions. Different types of *morogo* form a secondary source of nutrition and are therefore also cultivated on a large scale. Traditionally, the leafy green *morogo* is eaten as an accompaniment to maize porridge. Children may consume approximately 100 g of *morogo* while adults consume about 200 g. The type of *morogo* eaten each day varies according to the choice of the housewife. Maize porridge and *morogo* is usually cooked in the morning and may be eaten at any time of the day. In most households, this staple is eaten two to three times a day.

Most subsistence farmers intercrop different types of *morogo* with maize (Figure 3.6). In some cases though, a section of the field may be sectioned off and used solely for *morogo* cultivation. On average, cultivated *morogo* may be harvested after three weeks. Only the young leaves are picked thus ensuring sustained growth of new leaves. Typically, the *morogo* leaves are picked only when required and are not washed unless covered in excessive soil or mud, caused by rain splash. The customary method of cooking *morogo* is by placing the leaves in a pot of boiling water and allowing to simmer for ten to fifteen minutes while stirring occasionally. For different *morogo* types salt, bicarbonate of soda, oil, tomatoes or ground peanuts may be added. The bicarbonate of soda is used to soften *morogo* leaves in dry months. Certain types of *morogo* leaves are used as a spice, in which case a few leaves are added to the main *morogo* dish.

Figure 3.6: Typical field of a subsistence farmer showing intercropping of maize and different types of *morogo*.

Leaves of some *morogo* types may be dried and stored for use in winter when fresh *morogo* becomes scarce (Figure 3.7). The fresh leaves of different *morogo* types are boiled separately in water until cooked halfway. The water is discarded and clumps of half-cooked *morogo* are placed on metal, clay or plastic surfaces and left in the sun to dry. The drying process usually takes a few days after which the dried *morogo* may be stored in plastic packets for short-term use or in plastic buckets for medium-term storage. For long-term storage (± 2 years) empty mealie meal sacks are used. Air passes freely through the pores of the sack keeping the *morogo* dry for longer without allowing a build-up of moisture, which encourages fungal growth. The plastic bags and buckets and mealie meal sacks are kept in the cooking hut of each household unit.

Figure 3.7: *Morogo* that has been sun-dried and stored from the previous harvest van Wyk & Gericke, 2000).

Nutritional analyses of the most popular *morogo* have shown that they are rich in minerals (iron, calcium and magnesium), amino acids (especially thiamin, riboflavin and nicotinic acid) and vitamins A and C (van Wyk & Gericke, 2000). However, the use of bicarbonate of soda ($NaHCO_3$) has been shown to have a detrimental effect on the vitamin C content contained in *morogo* (Nesamvuni *et al.*, 2001). Scientific data regarding the mycological quality and health-related risks of *morogo* are frugal, at best. As a consequence, this study focused on the main types of leafy vegetables considered the staple of rural communities in this region. Plant types commonly used by Tsonga- and Khelobedu-speaking people in the Mopani District as *morogo* include *dinawa*, *ditaka*, *lerotho*, *ligushe,* okra and *theepe*. The vernacular, common and botanical names were determined from different sources and are listed in Table 3.2.

Table 3.2: Vernacular (Khelobedu, Tsonga and Venda), common and scientific names of commonly used *morogo* in the Mopani District of the Limpopo Province of South Africa.

Khelobedu[a]	Tsonga[a]	Venda[a,b]	Common name[c]	Scientific name[c]
dinawa	*tinyawa*	*munawa*	cowpea, black-eyed pea	*Vigna unguiculata*
ditaka	*xiphaswa*	—[d]	pumpkin	*Cucurbita* sp.
lerotho	*bangala*	*murudi, mutohotoho*	african cabbage	*Cleome* spp.
ligushe	*guxe (xilogo, muciki-njovo)*	*delele lupfumo*	—	*Corchorus* spp.
madanda	—	—	okra, lady finger	*Abelmoschus* sp.
theepe	*thyeke*	*thebe*	common pigweed	*Amaranthus hybridus*

[a] pers. comm. Mr PJ Lebea, 2003
[b] Nesamvuni *et al.*, 2001
[c] van Wyk & Gericke, 2000
[d] vernacular, common or scientific name not determined

3.4.1 *Dinawa* (*Vigna unguiculata*)

Dinawa is an annual herb that is cultivated in many parts of the world for its seeds, pods and / or leaves (Duke, 1983). In the Mopani District, the pods containing fresh seeds may be boiled and the seeds eaten. *Dinawa* seeds form a nutritious component in the human diet and are high in carbohydrates and proteins and have a low fat content (Davis *et al.*, 1991). More recently, *dinawa* seeds were reported to have high concentrations of minerals (potassium, magnesium and phosphorus) as well as essential and non-essential amino acids (Iqbal *et al.*, 2006). The purple flowers are medium in size and are not eaten. The leaves are green and flexible becoming maroon and waxy with age (Figure 3.8). Leaves may be eaten fresh but dried leaves are preferred, usually cooked with oil, salt and tomatoes. A study undertaken in Nigeria showed that the total nutrient intake of a mixture of maize and *dinawa* leaves seemed substantial enough to meet the nutrition requirement of children (Obatolu, 2003). In the Mopani District of the Limpopo Province, *dinawa* is eaten throughout the year, whether fresh or dried. It may therefore be regarded as a valuable supplement to the staple maize meal eaten daily.

Figure 3.8: Waxy leaves, purple flowers and bean pod typical of *dinawa* type *morogo*.

3.4.2 *Ditaka* (*Cucurbita* sp.)

Ditaka is a creeping plant that grows annually and may be compact or semi-shrubby (Saade & Hernández, 1994; Figure 3.9). The leaves are large and hairy and together with the large, yellow flowers are eaten when fresh. If crop is in excess then leaves and flowers may be dried and stored. Before the leaves are boiled they are usually deveined. *Ditaka* is usually cooked with salt, tomatoes and sometimes ground peanuts. The plant may be cultivated during the dry season although bicarbonate of soda has to be added to the leaves when cooking. The pod or fruit may also be boiled and eaten when ripe and is reported to have high carotene content while the seeds may be high in oil and protein (Saade & Hernández, 1994). *Ditaka* is often confused with *Lagenaria siceraria* (calabash) because the fruits are somewhat similar (van Wyk & Gericke, 2000). *Ditaka* is distinguished from calabash by their smaller, more coarsely hairy leaves, yellow flowers and small, smooth seeds. Flowers of calabash plant are white while seeds are angular and winged.

Figure 3.9: Creeping growth, large yellow flowers, young pod and hairy leaf characteristic of the *ditaka* plant.

3.4.3 *Lerotho* (*Cleome* spp.)

Two types of *lerotho* are common. They are both annual herbs with white and purple flowers that are not eaten. Young leaves are eaten fresh and may be dried after cooking. As with *ligushe*, dried *lerotho* is not preferred in terms of taste. Nutritionally, *C. gynandra* has high levels of folate (417.6 μg) and calcium (206.0 mg; Nesamvuni *et al.*, 2001). *C. gynandra* is also particularly high in magnesium and iron and has relatively high levels of nicotinic acid (van Wyk & Gericke, 2000).

Figure 3.10: The three types of *ligushe* plants, each having similar serrated leaf margins and small yellow flowers.

3.4.4 *Ligushe* (*Corchorus* spp.)

Three types of *ligushe* are eaten by the locals of the Mopani District. All three types are not cultivated and grow naturally among maize fields. They are annual herbs with hairlike teeth at

the base of the leaf blades (van Wyk & Gericke, 2000). Their leaf margins are serrated and flowers of all three types are small and yellow in colour (Figure 3.10). The three types of *ligushe* may be combined when picking from fields and are not separated when cooking. The flowers are not eaten and the leaves are mainly eaten fresh. *Ligushe* is typically cooked in boiling water with bicarbonate of soda, salt and tomato. The leaves are mucilaginous and require continuous stirring after being removed from direct heat in order to reduce the slimy texture. *Ligushe* leaves may be dried raw but since it is plentiful throughout the year and the taste of dried *ligushe* is not preferred, drying is not common. The nutrient content of one type of *ligushe* (*C. tridens*) was determined (Nesamvuni *et al.*, 2001). While varying levels of energy, protein, fibre, beta-carotene, folate, vitamin C, calcium and zinc were detected in leaves of *C. tridens*, iron levels were highest (11.50 mg per 100 g). Vitamin C levels may have been higher than the amount detected (2.5 mg) but because bicarbonate of soda was added during cooking, the levels may have diminished.

3.4.5 Okra (*Abelmoschus* sp.)

The leaves and fruits of the okra plant are eaten mainly by the Tsonga people while Khelobedu-speaking people customarily use the leaves as a spice. In this case, a few okra leaves may be added to the main *morogo* dish for a distinct flavour. While the leaves are not mucilaginous, the young fruits have a slimy texture when cooked.

3.4.6 *Theepe* (*Amaranthus hybridus*)

Theepe is an erect annual herb with stalk and leaf colour varying between green and purple. These plants may grow naturally or may be cultivated within maize fields. Fresh leaves are eaten when young and may be dried raw, but again dried *theepe* is not preferred. *Theepe* can grow up to 1.5m tall, produce a large amount of biomass in a short time and complete up to six generations per year. This species is a nutritious leafy vegetable that can be cultivated

throughout the world's semiarid regions (Blodgett & Swart, 2002). Trials done at the Vegetable and Ornamental Plant Institute (VOPI) of the Agricultural Research Council (ARC) have shown that cultivation of *theepe* in rural areas can contribute significantly to a reduction in the incidence of malnutrition among children (van Wyk & Gericke, 2000). Mycological studies indicated varying levels of toxigenic fungi (*Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*) from the interior of seeds as well as leaves (Bresler *et al.*, 1995; Blodgett *et al.*, 2000). Nutritional studies done on *theepe* show varying levels of protein, calcium and iron (van Wyk & Gericke, 2000; Nesamvuni *et al.*, 2001).

CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION

A list of plant types utilised as traditional *morogo* (whether fresh or dried) in the villages surrounding Giyani was determined by verbal communication with the housewives of various households. Questionnaires were also employed to record customs regarding cultivation, preparation, preservation and storage of each *morogo* type. Information concerning consumption frequency was also noted. Appendix B shows the templates of the questionnaires employed to obtain information from households, schemes and town-stalls.

Typical farming groups (section 3.3) within village A (VA) and village B (VB) were identified as collection sites for fresh samples. From VA, fresh samples were collected from a household (subsistence farm, SF), and two schemes (a semi-commercial farm, SCF, and a small-scale commercial farm, SSCF). In village B (VB), subsistence farming is not commonly practised. For this reason fresh samples were only collected from two schemes (both SCFs). At the collection fields of each of these households and schemes, an X pattern with each arm extending 30 m was used to mark off five sampling points. Fresh plant material (leaves, stems, roots, flowers) of the two types of *ligushe* (*Corchorus* sp., cf. *trilocularis* L. and *Corchorus* sp. aff. a*splenifolius* Burch) and *ditaka* (cf. *Cucurbita* or *Lagenaria*) were collected from the sampling points of each collection field in the early hours of the morning to delay wilting. Leaves from individual plants were kept intact and placed in separate, sterile, labeled plastic bags.

No farming activity occurs in Giyani town (GT) and *morogo* is usually bought from schemes within villages and sold at stalls in the market area to people in the town. Fresh leaves of

ligushe and *ditaka* were bought from an identified stall in GT. A total of 250 leaves was randomly chosen from the bought *morogo* and was regarded as samples.

Approximately 1 kg dried material of *dinawa* (*Vigna unguiculata* ssp. *unguiculata*) was obtained from one household in VA since no other household had any dried *morogo* available. The dried sample was placed in a sterile plastic bag. Cooked samples were collected from five households in VA and five from village C (VC). About 200 g fresh leaves of *ligushe* and *ditaka* together with 200 g of dried material of *dinawa* was distributed to the households. Housewives were asked to cook these and 50 g of the cooked dishes were weighed out and sampled. All samples were kept at cool temperatures while being transported and stored at 4 ^{0}C until analysis took place. It should be noted that samples obtained from VA, VB and VC were representative of *morogo* that is consumed by villagers while those bought from GT, despite being initially bought from areas similar to VA and VB, represents *morogo* that is typically consumed by townsfolk. Mycological analyses were carried out in duplicate on subsamples collected separately from the same collection fields and stored apart.

4.2 BOTANICAL SPECIES IDENTIFICATION

Leaves, stems and roots (where possible) of each type of *morogo* were collected for plant species identification. Flowers were collected only from the pumpkin plant, as these were the only plants that had already flowered. Bean pods were collected from the bean plant. All the plant material collected were placed between sheets of newspaper and stacked into a plant press to dry. In addition, characteristics of the plant types were recorded. The following were included; place of growth, height of plant, growth pattern, type of root system, colour of leaves and flowers and any other unique feature.

Once the plant material was completely dry, they were glued onto an A3 size cardboard sheet. The dried plant material and the recorded information were submitted to the South African National Biodiversity Institute (SANBI) in Pretoria for plant species identification. Results were recorded in Table 5.1. The common and vernacular names (Khelobedu and Tsonga) of all the plant species were determined and also recorded in Table 5.1.

4.3 MYCOLOGICAL ANALYSIS

Mycological analysis included washing regimes, to isolate external colonisers from leaf surfaces, sterilisation regimes, for the isolation of internal colonisers from leaf interior and purification of isolates for purpose of genus and species identification.

4.3.1 The washing regime

A standard mycological washing method was employed to isolate surface colonisers from the host substrate (Medina-Martinez & Martinez, 2000). Although this method is time consuming, it allows the isolation of a variety of fungi. From the two types of *ligushe*, twenty leaves were chosen for further mycological analysis while from *ditaka* only five leaves were chosen as subsample. In both cases the number of leaves chosen was due to the size of individual leaves. Each subsample was added to 99 mL sterile 1 % peptone water containing 0.01 % Tween 80. This mixture was shake-incubated at 200 (revolutions per minute (rpm) for 10 minutes at 25 ^{0}C. Serial dilutions were then prepared using the same diluent.

Aliquots (0.1 mL) of the dilutions were used to prepare spread plates on the following media, each supplemented with bacterial inhibiting substances; (i) potato dextrose agar (PDA; Merck, SA) containing 5 % sodium chloride (Merck, SA) and 40 mg L^{-1} oxytetracycline (Separations, SA), (ii) 1.5 % malt extract agar (MEA; Merck, SA) containing 150 mg L^{-1} tetracycline (Separations, SA) and (iii) rose bengal chloramphenicol agar (RBCA; Merck,

SA). The plates were then sealed and incubated at 25 ^{0}C (± 1 ^{0}C) for a minimum of 7 days. Following incubation, colonies were purified by single spore isolation for subsequent identification.

Three sets of controls were included for each of the duplicate analyses. The first set of controls involved the basic washing method, but *in lieu* of the leaf subsample, 1 mL of sterile distilled water was used. A second 1 mL aliquot of the distilled water and 1 mL of the peptone water that was used as diluent in the leaf analyses were spread separately and individually on petri plates of the three media types. These served as the second set of controls. The third set of control plates were also prepared by randomly selecting and incubating uninoculated (i.e. sterile) plates of each media type. Control plates were subjected to the same incubation conditions as the plates generated from each of the duplicate analyses.

4.3.2 The sterilisation regime

Because no previous mycological work has been done on *ligushe* and *ditaka* plants, it was necessary to standardise a protocol in order to isolate internal fungal colonisers from the leaves of these plant types. Leaves collected at each sample site were therefore combined and treated as the end sample. The surface sterilisation regime of Blodgett *et al.* (2000) was used as a starting point for our analysis. Since these treatments seemed too stringent for the plant types (i.e. no fungal isolates were obtained), four modified sterilisation regimes (MSRs) were developed based on the studies of Osono & Takeda (1999) and Suryanarayanan *et al.* (2003).

4.3.2.1 Original sterilisation regime

Blodgett *et al.* (2000) circumscribed a series of 1 minute in 96 % ethanol, 5 minutes in a 3,5 % NaOCl solution (m/v), and 30 seconds in 96 % ethanol. On completion of surface sterilisation, the leaves were allowed to dry. Approximately 2 mm^2 pieces were then cut from

the leaves at random points and a total of 100 such pieces were plated on corn-meal agar (CMA; Oxoid, SA) containing tetracycline (150 mg L^{-1}). All plates were sealed and incubated at 25 °C (± 1 °C) for a minimum of 7 days.

Only one set of controls was applied for the surface sterilisation regime and this involved choosing sterile uninoculated plates randomly. These plates were sealed and incubated with the plates containing sample leaf pieces.

4.3.2.2 Modified sterilisation regime

Each of the MSRs commenced with 30 s in 96 % ethanol and terminated with 20 s in 96 % ethanol. The critical variable that was altered was the middle step of each sterilisation regime wherein the period of leaf exposure to the sterilising agent (3.5 % NaOCl) was increased from 20, 30, 45 to 60 seconds, for each of the regimes respectively.

The modified regime in which leaf exposure to NaOCl was only for 20 seconds served as the least intense sterilisation regime. Modified sterilisation regime 4 was regarded as the most intense sterilisation regime since the time of leaf exposure to NaOCl was the longest (60 seconds). For each sterilisation regime one hundred leaf pieces were plated. A comparison of the surface sterilisation regimes used in this study and those used by other authors, on which the modified sterilisation regimes employed in this study was based, is given in Table 4.1.

Table 4.1: Comparison of surface sterilisation regimes employed by other authors and the modified sterilisation regimes (MSRs) employed in this study.

	Times of exposure (s)		
	96 % Ethanol	Sterilising agent	96 % Ethanol
Blodgett *et al.*, 2000	60	300[a]	30
Osono and Takeda., 1999	60[b]	60[c]	60[b]
Suryanarayanan *et al.*, 2003	5[b]	90[d]	10[e]
MSR[f] 1	30	20	20
MSR[f] 2	30	30	20
MSR[f] 3	30	45	20
MSR[f] 4	30	60	20

[a] 3.5 % sodium hypochlorite
[b] 70 % ethanol
[c] 15 % hydrogen peroxide
[d] 4 % sodium hypochlorite
[e] sterile water
[f] modified sterilisation regime

4.3.3 Analysis of dried and cooked samples

A subsample of dried material was ground using a Waring blender which was cleaned and drenched with 70 % ethanol prior to analysis. One gram of the ground product was sprinkled onto each of ten sterile PDA plates. The plates were incubated at 25 °C for a minimum of 7 days. A combination of surface and internal fungal colonisers were expected to be recovered.

Cooked samples were collected in sterile plastic bottles and kept at cool temperatures during transportation. On return to the laboratory, fungal colonies were already present on the cooked samples. These colonies were transferred aseptically to separate sterile PDA plates and incubated at 25 °C.

4.3.4 Purification

In order for fungal colonisers, obtained from the washing and sterilisation regimes, to be further characterised, the dilution plate technique (i.e. a single spore isolation technique) was used to obtain pure colonies of each isolate. An initial spore suspension was made in 10 mL

sterile water. This suspension was poured onto a single petri plate containing potato dextrose agar (PDA; Merck, SA). The mixture was distributed onto the agar by moving the plate gently in a circular motion. After approximately 30 seconds the excess suspension was poured off. The plates were incubated in a slanted position for 16 – 24 hours at room temperature, after which any excess liquid was poured off. Using a dissection microscope, single germinated spores were removed and placed onto a sterile PDA plate. The plates were incubated at 25 ^{0}C for 7 days or until growth was sufficient to commence with identification procedures.

4.3.5 Genus identification

Once colonies were purified on PDA, they were ready for identification to genus level. This was carried out microscopically using a squash mount and lactophenol blue as a mountant. A small piece of approximately 1 mm^2 in size was picked up from the edge of the chosen isolate using a sterile needle and placed into the mountant. A cover slip was then placed on top of the fungal material and using a soft plastic cap the entire piece with the fungus was squashed. The squash mount was then viewed under a calibrated phase contrast microscope and various characteristics were noted. These included sporulating and nonsporulating structures as well as presence or absence of spores. If spores were present the shapes were recorded. In this manner, the genera of all the isolates were determined.

4.3.6 Species identification

Species identification was performed on *Alternaria, Aspergillus, Fusarium* and *Penicillium* isolates. For this specialised technique, both macroscopic and microscopic investigations were undertaken. Macroscopic investigations involved growing fungal isolates on different media types according to the genus of the isolate. *Alternaria* isolates were grown on potato carrot agar (PCA) and after a 7-day incubation period at 25 °C, the colour of the colony was recorded. Each *Fusarium* isolate was grown on a half-plate, one half containing PDA, the

other, carnation leaf agar (CLA). The carnation leaves were exposed to 26.3kGy of gamma irradiation at 25 °C from a ^{60}Co source by Isotron, SA. This was sufficient to eliminate all fungi, bacteria and yeasts on or within the carnation leaves. Colonies growing on PDA were used for macroscopic observations like texture, and colour of the top- and under-surface of the colony. The CLA was used to enhance sporodochia development. Slides were prepared and examinations were made based on characteristics of macroconidia, microconidia, phialides and chlamydospores. Colonies of genus *Aspergillus* were grown on four media types namely; Czapek Solution Agar (CZ; incubated at 25 °C), Czapek Yeast Extract Agar (CYA; 1 plate incubated at 25 °C, another at 37 °C), MEA (incubated at 25 °C) and Czapek Yeast Extract Agar with 20 % Sucrose (CY20S; incubated at 25 °C). Growth and other characteristics growing on all four media types were noted while slides were prepared using only CYA colonies. Various microscopic features were observed and measured, including the shape, colour, length and breadth of conidial heads, conidiophores, vesicles, sterigmata and conidia. Assessment of the teleomorph status of each *Aspergillus* isolates was also carried out. Species identification of the penicilli was similar to that of *Aspergillus* in that different media were employed to compare growtn characteristics and other macroscopic features. For *Penicillium*, CYA (incubated at 5 °C, 25 °C and 37 °C), MEA (incubated at 25 °C and 25 % Glycerol Nitrate Agar (G25N; incubated at 25 °C) were used. Microscopically, the shape and colour of the stipe, penicilli, rami, metulae, phialide and conidium were noted in addition to having their dimensions measured. Again, each isolate was examined for signs of possible sexual states.

Microscopic analysis for all genera involved preparation of squash mounts for each isolate. However, the mountant used to determine the species of isolates varied depending on the genus of the isolate. For *Alternaria* isolates, a basic lactophenol solution, with no stain added, was used as mountant. For the fusaria, fuchsin was added to the basic lactophenol solution

and this served as the staining agent. In the cases of *Aspergillus* and *Penicillium* isolates, pieces of the isolates were primarily placed in 70 % ethanol and teased apart. Once the ethanol dried out, lactofuchsin was applied to stain the material.

For each of the macroscopic and microscopic analyses, observations and measurements of various features were recorded on individual isolate identification forms (see Appendix C). The information recorded in these forms was used in ascertaining the species names of isolates according to the keys of Simmons (1996), Nelson *et al.*, (1983), Pitt (1979) and Klitch and Pitt (1988).

4.3.7 Statistical analysis

The frequency of sample contamination in the three areas of study was compared statistically using non-parametric analyses. The p-value gives the probability that the obtained value (or more extreme) could be obtained under the assumption that the null hypothesis (e.g. no difference between the population means) is true (Ellis & Steyn, 2003). A p-value smaller than 0.05 (5 % level of significance) is generally considered as sufficient evidence that the result is statistically significant. However, in cases of scarceness of data, a significance level of 10 % may be considered sufficient evidence of statistical significance by statisticians (pers. comm. Dr SM Ellis, 2006). When only two variables are being compared, the Mann-Whitney test can be used to determine the p-value. When more than two variables are being compared, the Kruskal-Wallis test may be used. Once the p-value has been determined, 2-tailed multiple comparison tests can be performed to identify where the exact difference lies. In this study, the Kruskal-Wallis test and 2-tailed multiple comparison test was performed where appropriate.

4.4 MOLECULAR ANALYSIS

4.4.1 Sample Preparation

Purified isolates were used for DNA isolation and subsequent amplification. Approximately 2 mm^2 of hyphal biomass of isolates and reference cultures (*Fusarium verticillioides* MRC 4319, 4317, 826) were inoculated, in duplicate, into Yeast, Peptone, Dextrose (YPD) broth. These were shake-incubated at 200 rpm for 48 hours at room temperature. After the incubation period, the samples were centrifuged at 4000 rpm (Harriet 18/80, Vacutec, RSA) for 5 minutes, the supernatant discarded and the hyphal biomass washed with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) 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 a maximum of 2 hours in preparation for the subsequent lyophilisation process. DNA was extracted from the lyophilised sample.

4.4.2 DNA Extraction Procedure

A cetyltrimethyl ammonium bromide-polyvinyl pyrrolidone (CTAB-PVP) DNA extraction procedure was used to extract DNA from 50 mg of freeze-dried fungal material (Leslie & Summerell, 2006). The weighed sample was transferred to 2 mL microfuge tubes. Double strength CTAB isolation buffer (2 % CTAB, Tris-HCl, pH 8.0, 1.2 M NaCl), heated to 65 °C and to which 0.2 % (v/v) β- mercaptoethanol was added immediately prior to use. A 5 % (v/v) (PVP) solution and 1 mg/ml final proteinase K 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 13 400 rpm 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 13 400 rpm for a further 5 minutes after which the aqueous phase was transferred to a clean microfuge tube. The DNA was allowed to precipitate for a minimum of 1 hour at -80 °C in NaCl (5 M) and 2 volumes of ice cold ethanol (99.5 %). In order to pellet the precipitated DNA, the tubes were centrifuged at 13 400 rpm 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 50 µL of TE buffer for 1 hour at 65 °C. All DNA samples were stored at 4 °C until required.

4.4.3 DNA Amplification Procedure

DNA was amplified by polymerase chain reaction (PCR) using an OmnE (Hybaid, 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 4.2) and template DNA (100 ng). 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).

Table 4.2: The primers employed in this study.

Primer	Sequence	Gene Target	Product Size (bp)
FCC-1 F FCC-1 R	5'-CGGTCCGACGACAAATGACTGG-3' 5'-CGACACAATGTCGCTTCTGG-3'	*Fusarium* Cyclin C-1 (FCC-1)[a]	1500
FUM-1 F	5'-GTCGAGTTGTTGACCACTGCG-3'	Polyketide Synthase (PKS) (FUM-1)[b]	800
FUM-1 R	5'-CGTATCGTCAGCATGATAGC-3'		
EF-1	5'-ATGGGTAAGGAGGACAAGAC-3'	Translation elongation factor 1-α (*EF*)[c]	700
EF-2	5'-GGAAGTACCAGTGATCATGTT-3'		
SSU 0017F[d] SSU 1196R[e]	5'- AGT AGT CAT ATG CTT GTC-3' 5'- TCT GGA CCT GGT GAG TTT CC -3'	18S Small Sub-unit (*18S*)	1200

[a] Shim & Woloshuk, 2001;
[b] Bluhm *et al.*,2004;
[c] Geiser *et al.*, 2004;
[d] Kolwalchuk *et al.*, 1997;
[e] Borneman & Hartin, 2000.

PCR conditions began with an initial denaturation step of 5 minutes at 95 °C. This step was followed by 29 cycles of denaturing (95 °C for 30 seconds), primer annealing (55 °C for 30 seconds) and primer extension (72 °C for 60 seconds). Amplification was terminated by a final extension period of 72 °C for 5 minutes. Agarose gel electrophoresis was employed to confirm DNA amplification.

4.4.4 Confirmation of DNA Amplification

Electrophoresis was conducted on 5 μL of PCR product through agarose gel (1 % or 2 % w/v; Roche, Germany) containing 1 μg mL^{-1} ethidium bromide (BioRad, UK). Each gel was also loaded with a DNA molecular weight standard (Molecular Weight Marker XIV; Roche Diagnostics, Germany or Fermentas Life Sciences USA, 100 bp GeneRuler) to which the intensities of the template DNA bands could be compared. Electrophoresis was performed for 105 minutes at 80 V using 1 x TAE buffer (pH 8.3). Gel images were captured using a Gene

Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software.

4.4.5 Sequence analysis

Amplified DNA fragments were sequenced by Inqaba Biotech, South Africa. BLASTN searches (http://www.ncbi.nlm.nih.gov/BLAST for *FUM 1* and *18S* gene fragments or http://fusarium.cbio.psu.edu for *EF*) were used to confirm the identity of the amplified sequences.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. INTRODUCTION

Interviews with the housewives of various households revealed that the leaves of different plant types are regarded as *morogo*. *Morogo* commonly used in the Mopani District of the Limpopo Province includes *ligushe*, *ditaka*, *dinawa*, *lerotho*, *theepe* and okra. These plant types are discussed individually in Chapter 3 with regards to traditional customs of cultivation, preparation, preservation and storage. Each of the three farming sectors as described in section 4.1 was considered.

Results from botanical species identification, the washing and sterilisation regimes of fresh *morogo* leaves, dried and cooked sample analysis as well as molecular analysis are described in the following paragraphs.

5.2. BOTANICAL SPECIES IDENTIFICATION

Morphological characteristics were used by South African National Biodiversity Institute (SANBI) to determine the genus and species of each plant type. Characteristics used for identification by SANBI, and scientific species naming of the four plant types are recorded in Table 5.1. Samples 1 and 2 were placed within the same genus with sample 2 not being previously described and according to the SANBI report it might be a new subspecies. Morphological characteristics of samples 1 and 2 in terms of leaves, flowers and roots were very similar.

Table 5.1: Physical characteristics and scientific names of the four samples as identified by SANBI. Also included are the vernacular and common names of the samples.

Sample number	Physical characteristics	Scientific name	Vernacular names		Common name
			Khelobedu	Tsonga	
1 (Figure 3.10)	Grows upward with a height of 25-50cm. Has a tap root system. Leaves are dark green and very thin. Leave edges are serrated. Flowers are yellow and very small. Are never cultivated. Instead, grows wild between maize in cultivated fields.	*Corchorus* sp., cf. *trilocularis* L.	*Ligushe*	*Guxe* (*xilogo*)	–
2 (Figure 3.10)	Grows horizontally along the ground. Height ±5cm. Has a tap root system. Leaves are dark green and narrower than sample 1. Flowers are yellow and very small. Never cultivated. Grows wild between maize. Very similar to sample 1.	*Corchorus* sp. aff. a*splenifolius* Burch (possible new subspecies)	*Ligushe*	*Guxe* (*muciki-njovo*)	–
3 (Figure 3.9)	Grows along the ground with a height of 10-15cm. Fibrous root system. Leaves are lobed, thick and hairy. Flowers are yellow, large and develop into a pod (pumpkin). Stems also thick and hairy. Are cultivated between maize or in demarcated area at edge of maize field.	cf. *Cucurbita* or *Lagenaria*	*Ditaka*	*Xiphaswa*	Pumpkin plant
4 (Figure 3.8)	Grows along the ground. Has a height of ±30cm. Tap root system. Leaves are dark green, flexible and do not break easily. Older leaves change colour to maroon, become waxier. Flowers are purple and large. Develop into long pods (beans). Are planted only between maize in fields.	*Vigna unguiculata* ssp. *unguiculata*	*Dinawa*	*Tinyawa*	Cowpea plant

Besides having the same name in Khelobedu (*ligushe*) and Tsonga (*guxe*), samples 1 and 2 have a similar taste and local villagers do not differentiate between these *morogo* types when collecting them from fields or cooking them (i.e. samples 1 and 2 may be combined when picking and cooking; pers. comm. Mrs A. Lebea, 2003). Under this premise, samples 1 and 2, respectively identified by SANBI as *Corchorus* sp., cf. *trilocularis* L. and *Corchorus* sp. aff. *asplenifolius* Burch were combined and treated as one sample for mycological analysis.

Sample 3 belonged to either the genus *Cucurbita* (pumpkin) or *Lagenaria* (calabash). Some light was shed on the matter by local villagers, each of whom described sample 3 as being the pumpkin plant with yellow flowers as opposed to the calabash plant with white flowers (pers. comm. Gugu Mothele, Mr P Baloyi, Mr P Lebea, Mr R Mkhari, 2003). Given that the pumpkin plant is a member of the genus *Cucurbita*, it is more likely that sample 3 belonged to genus *Cucurbita* rather than *Lagenaria*. Nonetheless, without the pod, the scientific name remains unconfirmed.

The botanical name of sample 4 was identified as *Vigna unguiculata* ssp. *unguiculata*. Vernacular names of the *morogo* types namely *ligushe* for samples 1 and 2, *ditaka* for sample 3 and *dinawa* for sample 4 are given in Table 5.1 and used throughout this report.

5.3. MYCOLOGICAL ANALYSIS

Mycological analysis was only carried out on *ligushe*, *ditaka* and *dinawa* since these appear to be the most commonly eaten *morogo*.

5.3.1. The washing regime

The quantity and population composition of surface colonisers contaminating *ligushe* (*Corchorus* sp., cf. *trilocularis* L. and *Corchorus* sp. aff. a*splenifolius* Burch) and *ditaka*

(*Cucurbita*), from schemes in Village A (VA) or Village B (VB), a household in VA and a stall in Giyani Town (GT), was recorded in Table D.1 (Appendix D). The table shows the average number of fungal isolates obtained from duplicate assays carried out on three different selective media (section 4.3.1). Table D.1 also indicates that a number of fungal genera, namely *Acremonium*, *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* were present. *Fusarium* isolates were identified further to species level. Also included in Table D.1 is "other" isolates made up of (i) other identified isolates which included fungi belonging to the genera *Aureobasidium, Phoma,* and *Trichoderma* that were obtained in relatively low numbers, (ii) unidentified isolates including nonsporulating mycelial fungi and those isolates that were not of interest to the study and (iii) contaminants, into which bacterial and yeast isolates were placed. No fungi were isolated from controls indicating that no contamination occurred in the diluent (1% peptone containing 0.01% Tween 80), the distilled water that was used in place of the leaf sample, or on the agar plates. The washings were therefore deemed successful.

Results in Table D.1, Appendix D showed that 162 colonies were isolated in total. Of these, the same numbers (24) were isolated from *ligushe* and *ditaka* collected from VA while similar numbers were recorded from GT (*ligushe* 9; *ditaka* 10). In VB however, the number of isolates recovered from *ligushe* (46) were approximately two times the number isolated from *ditaka* (25).

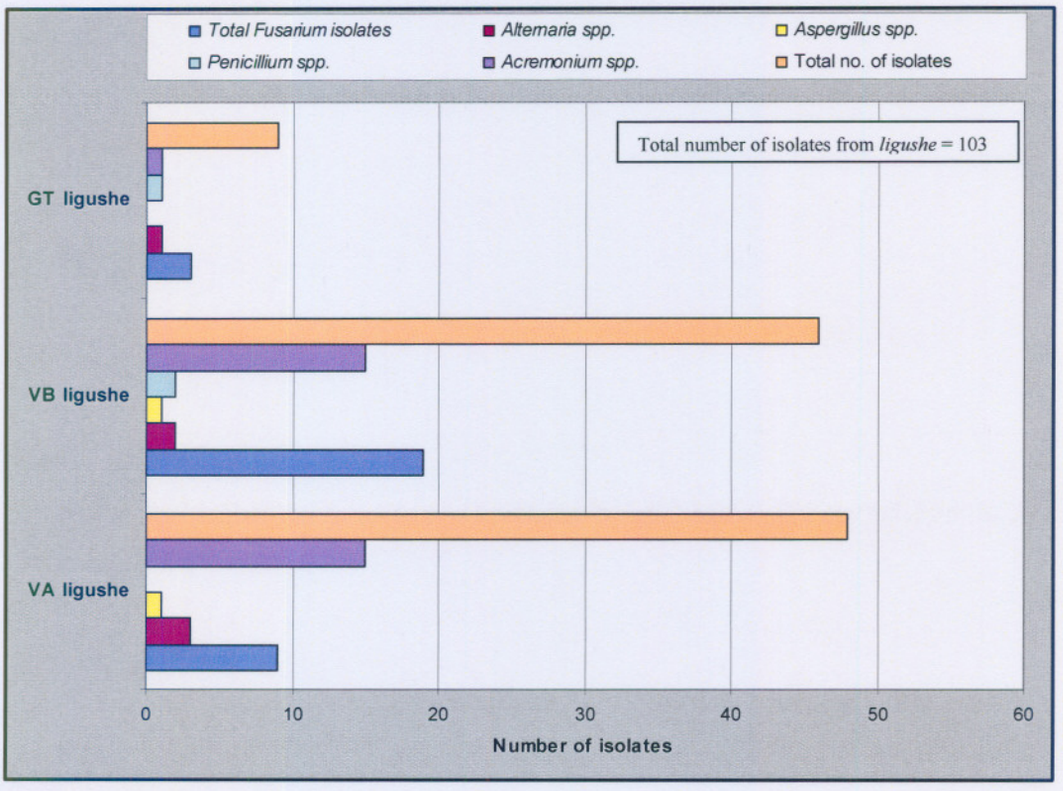

Figure 5.1: Graph comparing the relative levels of toxigenic fungal genera on surfaces of *ligushe* type *morogo* obtained from the different sampling sites.

The total number of isolates recovered from *ligushe* in VA (schemes and households combined) and in VB was in the same range (48 and 46, respectively) while the number obtained from GT (9) was approximately five times lower (Figure 5.1). The number of isolates retrieved from *ditaka* leaves of VA (24) and VB (25) were also notably higher compared to the number from GT (10; Figure 5.2). Overall, a similar trend was observed between *ligushe* and *ditaka* leaves in that isolate levels obtained from VA were similar to those obtained from VB while levels from GT were much lower.

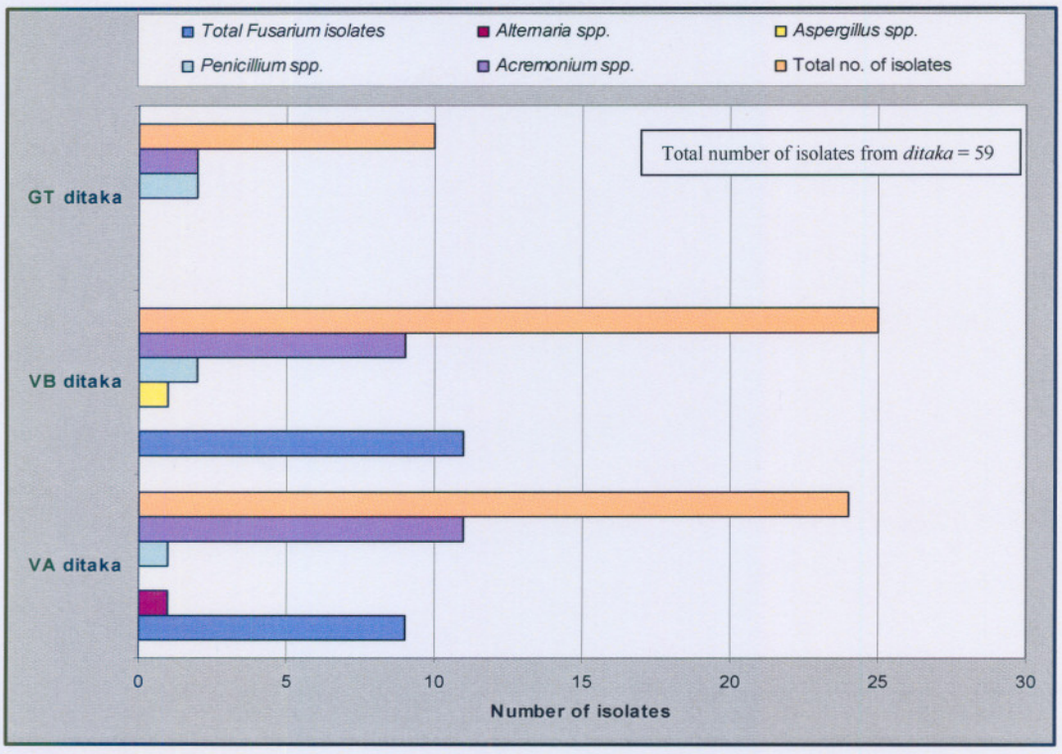

Figure 5.2: Graph comparing the relative levels of toxigenic fungal genera on surfaces of *ditaka* type *morogo* obtained from the different sampling sites.

When the total number of isolates obtained from the three sampling sites were combined and the plant types compared, results indicated that almost double the number of isolates were obtained from *ligushe* (103; Figure 5.1) compared to *ditaka* (59; Figure 5.2) leaves. An explanation for this observation may be provided by either of two reasons or a combination of both. Firstly, surfaces of *ligushe* leaves may be different to surfaces of *ditaka* leaves, with *ligushe* leaves probably providing a more favourable landing site for fungal spores. Secondly, *ditaka* plants, because they are cultivated, are watered frequently which may lead to frequent dispersal of fungal spores present on leaf surfaces, leaving little chance for spores to colonise the leaf. *Ligushe*, on the other hand, grows wild and is not watered (pers. comm. Gugu Mothele, 2003). Fungal spores may therefore not be easily dispersed, unless by rain, and so a higher rate of leaf infection and colonisation might result.

The distribution of the different fungal genera among isolates obtained from both plant types and each sampling area are also shown in Figures 5.1 and 5.2. Varying numbers of isolates from approximately five genera were isolated from *ligushe* and *ditaka* and from VA, VB and GT. The five genera were isolated from both plant types but not necessarily from all three sample areas. The genera isolated were mainly field fungi belonging to the mitosporic group of fungi and included *Acremonium*, *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*. The presence of these fungal genera might indicate that sample leaves, despite seeming fresh on collection, were at the onset of senescence since the isolated fungi tend to be common primary saprophytes (Carlile *et al.*, 2001). This view is supported by the fact that a few *Trichoderma* spores were also isolated. *Trichoderma* spp. has been reported to follow common primary saprophytes in later stages of decomposition (Osono & Takeda, 1999). The isolation of the various genera from asymptomatic leaves could be an indication of a non-pathogenic association between the fungi and *ligushe* and *ditaka* leaves.

Fusarium and *Acremonium* were the predominant genera among isolates from *ligushe* and were isolated from all three sample areas (Figure 5.1). Isolates belonging to genus *Alternaria* were obtained from *ligushe* from all three sample areas. *Penicillium* and *Aspergillus* strains were isolated from *ligushe* but from only two of the three sampling areas. A comparable trend in genus distribution was observed for isolates from *ditaka* leaves collected from VA and VB with either *Acremonium* or *Fusarium* isolate numbers dominating. *Alternaria*, *Aspergillus* and *Penicillium* strains were isolated in lower numbers (Figure 5.2).

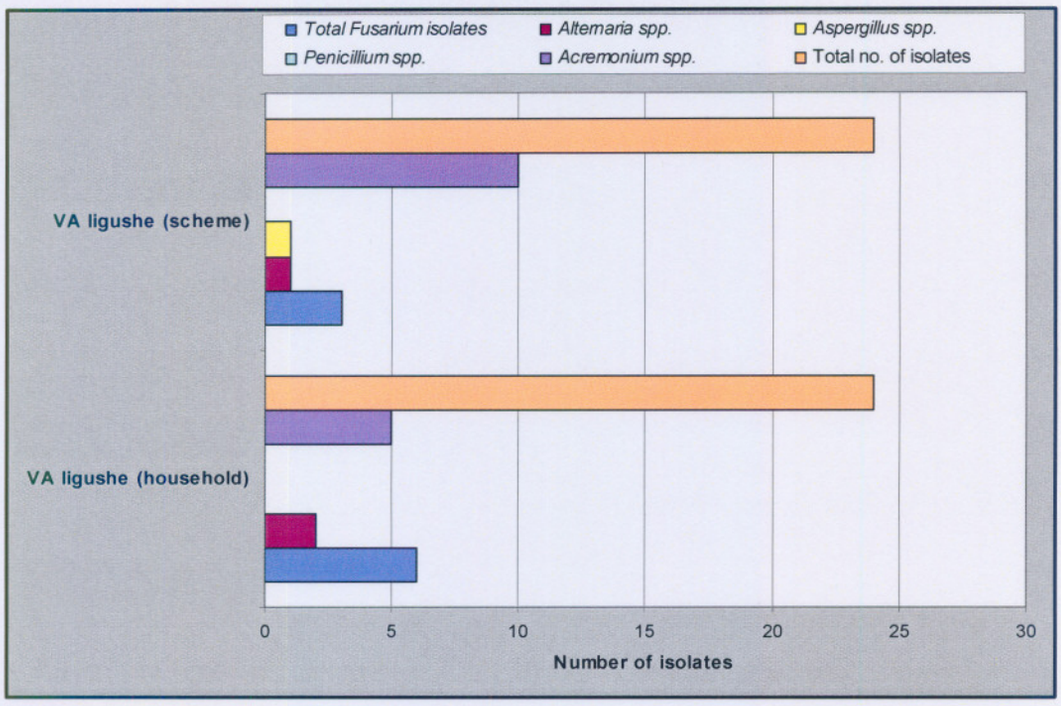

Figure 5.3: Graph illustrating the relative levels of toxigenic fungal genera on surfaces of *ligushe* type *morogo* obtained from a household compared to a scheme within Village A.

Ligushe from VA was collected from a household as well as a scheme. It was interesting to note the variation in the numbers of *Acremonium* and *Fusarium* isolates obtained from these two collection areas (Figure 5.3). The number of *Acremonium* isolates (10) retrieved from *ligushe* of the scheme was twice the number (5) of that isolated from the household. The reverse was true for *Fusarium* isolates with numbers isolated from the household *ligushe* (6) being double that obtained from the scheme (3). These results might be due to differences with regard to traditional farming practises (section 3.3).

Fungi, in general, are ubiquitous in nature and are commonly isolated from plant material, decaying vegetation, wood, soil, air, water and food (Jay, 2000; Prescott *et al.*, 2005). Samples analysed in this study were collected and handled in the same manner as is customary to communities within rural areas of the Limpopo Province. Therefore, leaves of

both plant types remained unwashed prior to mycological analysis and hence may have had soil particles present on their surfaces. It is possible that any anticipated fungi were either external leaf colonisers or common soil contaminants. Taking this into account and considering that the genera isolated in this study (*Acremonium, Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*) are cosmopolitan in nature and are common in plants, plant debris and soil (Nelson *et al.*, 1983; Pestka & Bondy, 1994; Prelusky *et al.*, 1994; Delgado & Gómez-Cordovés, 1998), their presence on the surface of *ligushe* and *ditaka* leaves was to be expected. Whether these mitosporic fungi originated from soil or were indigenously associated with leaf surfaces, individuals will, in all probability, be inclined to ingest the spores of these fungi.

Comparing each of the sample areas with respect to the combined number of isolates from the two plant types, there was no notable difference in the number of isolates obtained from VA (72) and VB (71; Figure 5.4). However, only about 25% of the number of isolates obtained from VA or VB was isolated from GT (19). Statistical analysis (10% level of significance) showed a significant difference ($p = 0.0559$) between the total number of isolates obtained and the three sample sites. Multiple comparisons further indicated that the difference was between VB and GT ($p = 0.0619$).

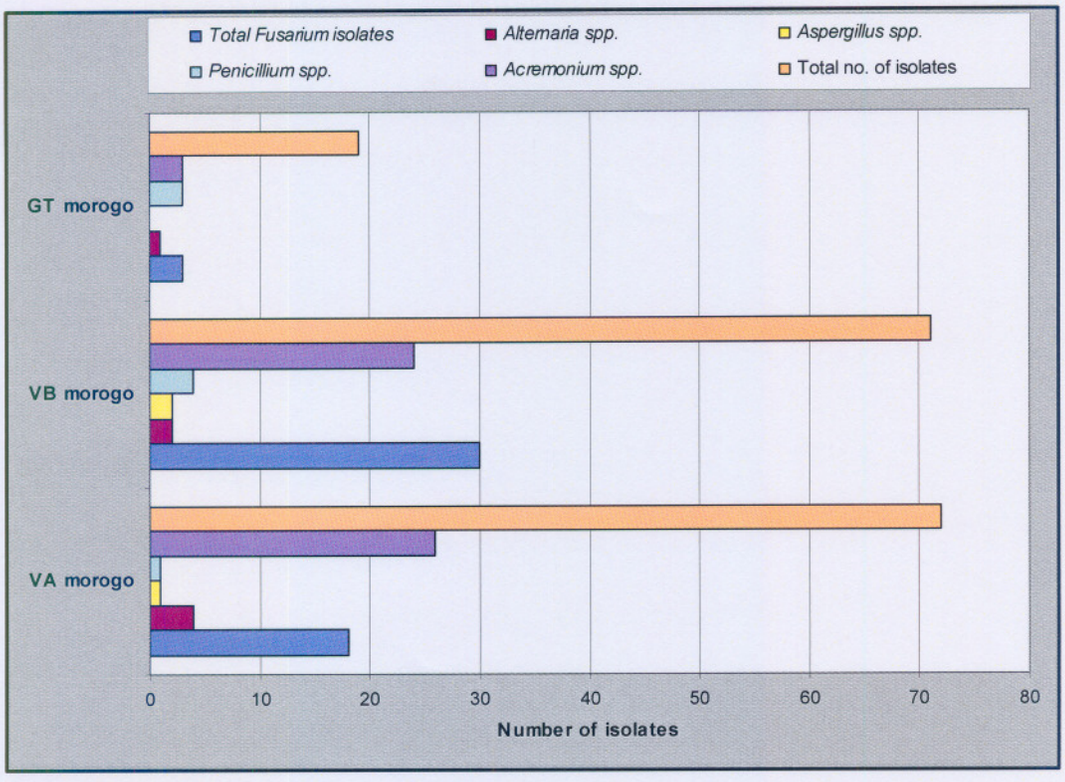

Figure 5.4: Graph comparing the occurrence of the different toxigenic fungal genera on surfaces of *morogo* (*ligushe* and *ditaka* combined) obtained from Village A, Village B and Giyani Town.

The similarity and / or dissimilarity between the levels of fungal isolation obtained from VA, VB and GT may be explained by the dispersal effect that water may have on the quantities of fungal spores present on leaf surfaces. It should be remembered that samples from VA and VB were collected directly from fields either belonging to a household or a scheme while samples from GT were bought from a stall in the market area. *Morogo* grown in fields are sold unwashed to residents in the surrounding villages who typically cook the *morogo* without prior washing. One should also bear in mind that *morogo* sold in GT are primarily bought from scheme owners. This *morogo* is usually transported to and sold in the central business district to individuals, the majority of whom reside in the town area. The *morogo* sold from stalls in GT are frequently sprinkled with water to maintain the freshness and crispness of the

leaves (pers. comm. Mrs A Mayimele, 2003). *Morogo* obtained from GT was originally bought from areas similar to VA and VB. The lower number of isolates obtained from GT compared to those isolated from VA and VB might be due to the constant sprinkling of water washing the spores off the leaves. The washing-off effect of fungal spores was not unexpected since flowing water has long been reported an agent of spore dispersal (Hudson, 1986). Since *morogo* from VA and VB are consumed predominantly by villagers and *morogo* from GT mainly by townsfolk, it can be deduced from results that people residing in village areas may be exposed to a higher level of fungal spores than people residing in towns. Overall, the washing of *morogo* leaves with water seems to be an effective practice that could be employed to decrease the load of surface colonisers associated with *morogo* leaves.

With regards to the types of fungi obtained, it was observed that *Acremonium*, *Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium* were isolated from the surfaces of *morogo* collected at the three sample sites. While high numbers of *Acremonium* isolates were retrieved from VA (26) and VB (24), only 3 were obtained from GT. A similar trend was observed for *Fusarium* isolates, namely; 18 from VA, 30 from VB and 3 from GT. Relative to the total number of isolates obtained from VA (72), VB (71) and GT (19), the numbers of *Fusarium* and *Acremonium* isolated from *morogo* of VA and VB were approximately double the number isolated from GT *morogo*. The number of *Penicillium* isolates recovered varied between VA (1), VB (4) and GT (3). However, when regarding these values relative to the total number of isolates obtained from each area, only one eighth the number of *Penicillium* isolates obtained from GT was obtained from VA and about one third was obtained from VB. An overall increase in the number of *Fusarium* and *Acremonium* isolates and an overall decrease in *Penicillium* numbers was evident when comparing *morogo* collected from VA and VB to *morogo* from GT.

Acremonium and *Fusarium* are field fungi that have commonly been isolated from soil and various plant parts (Nelson *et al.*, 1983; D' Mello & Macdonald, 1997). Both these genera have slime spores that are readily wettable and are therefore easily dispersed by water (Carlile *et al.*, 2001). Bearing in mind that *Acremonium* and *Fusarium* dominated in VA and VB, it seems likely that the number of their readily wettable spores on leaf surfaces were reduced successfully by the constant sprinkling of water on *morogo* leaves being sold in GT. The dominance of *Penicillium* on *morogo* from GT may be explained by either of two scenarios. Firstly, spores of genus *Penicillium* are hydrophobic (Hudson, 1986) and therefore not as easily dispersed by water. It may be expected that the constant sprinkling of water on GT *morogo* leaves could have reduced the number of *Acremonium* and *Fusarium* spores, but had only a marginal effect on the number of *Penicillium* spores. Furthermore, once the spores of *Acremonium* and *Fusarium* were washed off, the leaves became more accessible to colonisation by *Penicillium* spores already present on leaf surfaces. Another factor that might have contributed to the high number of *Penicillium* isolates from *morogo* of GT is the fact that some members of the penicilli are common air contaminants (Prelusky *et al.*, 1994). Some of the *Penicillium* isolates from GT *morogo* were probably air contaminants.

Of the total number of *Fusarium* isolates recovered from the two plant types ten different species were identified (Figures 5.5 and 5.6). These included *F.* cf. *camptoceras*, *F. chlamydosporum*, *F. equiseti*, *F. lateritium*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, *F. solani*, *F. subglutinans* and *F. verticillioides*. *Fusarium* isolates that could not conclusively be identified to species level were grouped as *Fusarium* spp. For each of the species recovered from *ligushe* and *ditaka*, the Kruskal-Wallis test was applied to determine statistical significance, of which none was found. A range of *Fusarium* species were reportedly isolated from various plant types in temperate to subtemperate and subtropical to tropical areas

(Booth, 1971). The isolation of various *Fusarium* species from different plant types in the Limpopo region, which is generally a tropical region, was thus not unusual.

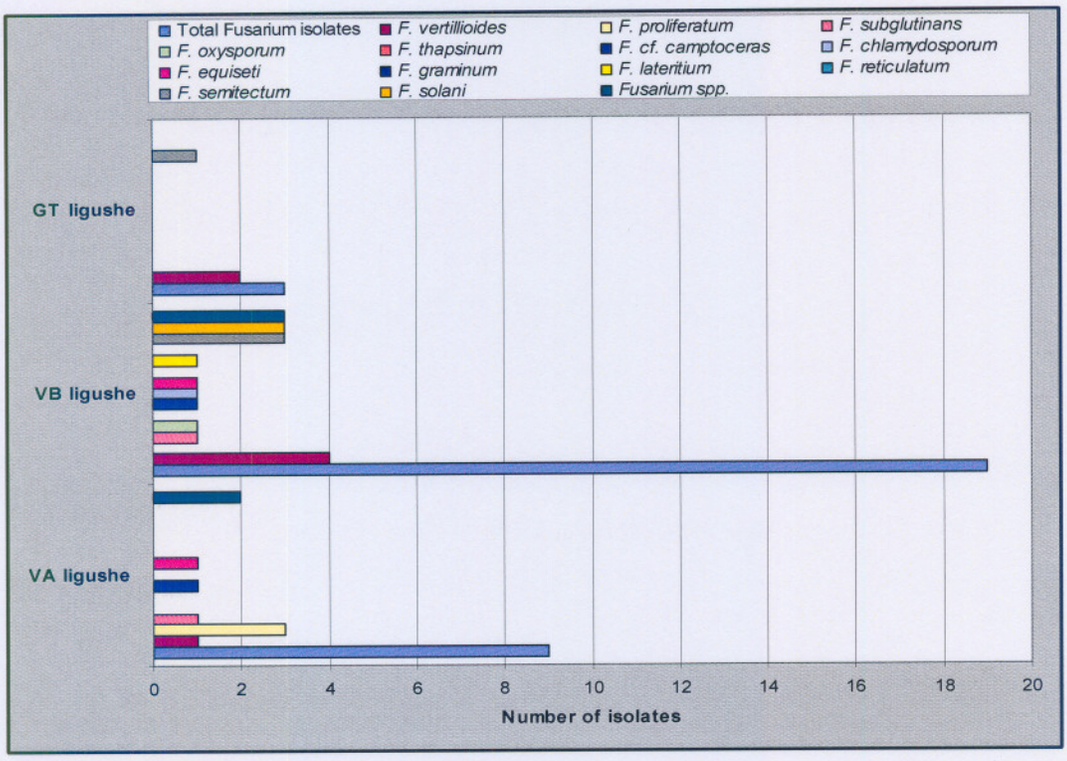

Figure 5.5: Graph comparing *ligushe* type *morogo* from different sampling sites with respect to species distribution among the *Fusarium* isolates associated with leaf surfaces.

While all ten species were recovered from *ligushe* leaves, only *F. equiseti*, *F. oxysporum*, *F. proliferatum*, *F. solani* and *F. verticillioides* were obtained from *ditaka* leaves. The relative numbers of the different *Fusarium* species obtained from *ligushe* and *ditaka* leaves varied. *F. verticillioides* was the dominating species isolated from *ligushe* leaves (7; Figure 5.5). From *ditaka* leaves, while *F. verticillioides* was recovered in similar levels (6) as *ligushe* leaves, *F. proliferatum* occurred as the dominating species (9; Figure 5.6). The remaining species obtained from both plant types were isolated in lower levels.

In a recently published study, Fandohan and co-workers (2005) reported that 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 was *F. verticillioides* and *F. proliferatum*. Closer to home, Rheeder *et al.* (2002) reported the frequent occurrence of *F. verticillioides* in non-mouldy home-grown maize of rural communities in the (former) Transkei region.

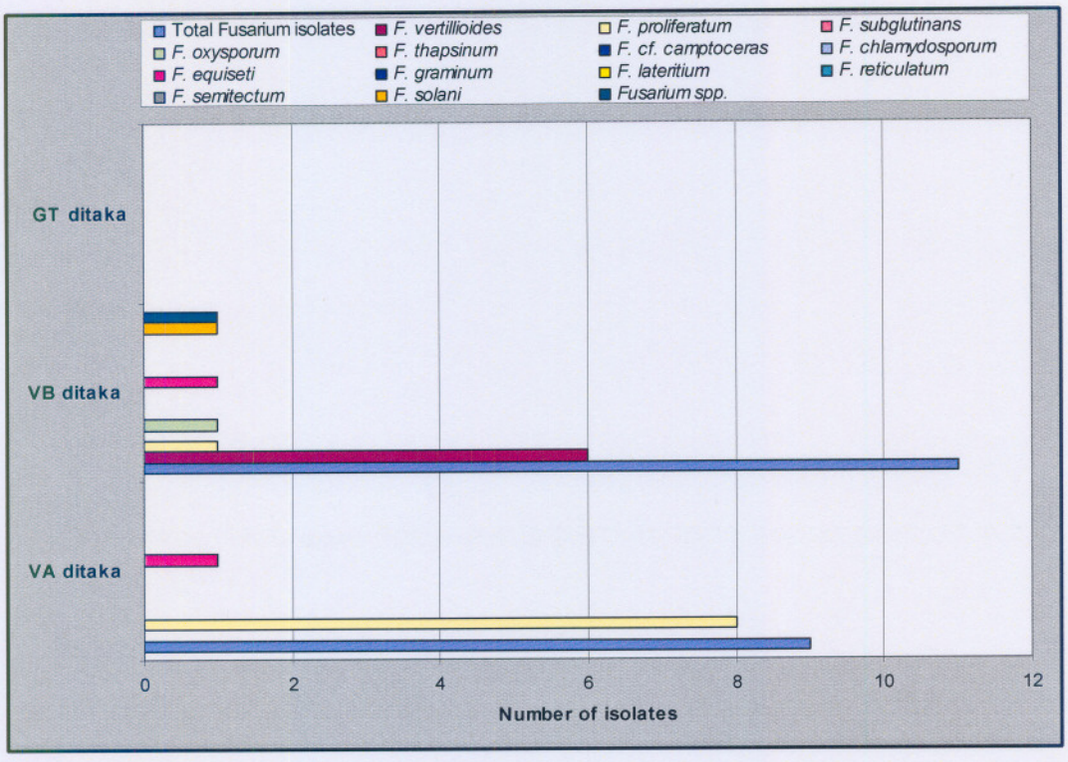

Figure 5.6: Graph comparing *ditaka* type *morogo* from different sampling sites with respect to species distribution among the *Fusarium* isolates associated with leaf surfaces.

Environmental conditions resulting from traditional framing practices are believed to have influenced the levels of *F. proliferatum* and *F. verticillioides*, since development of these two *Fusarium* species was reported to be differently affected by temperature and water activity. Marin and colleagues (1995) reported that as temperature and water activity increased, the overall growth rate of *F. proliferatum* was higher compared to that of *F. verticillioides*. Results illustrated in Figures 5.5 and 5.6 probably relate to differences in water availability to *ligushe* and *ditaka* plants and to the fungi associated with these plant types. Differences in

water availability may possibly be a consequence of the different traditional farming practices common in the study area. *Ligushe* grows as a weedy plant and relies on rain water to flourish. *Ditaka* plants are cultivated and watered frequently and are not solely reliant on rain water as a means of irrigation (pers. comm. Gugu Mothele, Mr R Mkhari, 2003). Rain during the 2002 – 2003 season was sporadic (Appendix A) implying that less water was available to *ligushe* plants and fungi associated with *ligushe* compared to *ditaka* plants, and any fungi related to *ligushe*.

Figure 5.7 compares the *Fusarium* species distribution in *morogo* (*ligushe* and *ditaka* combined) from the respective sample sites. Results indicate that the highest number of *Fusarium* occurred in *morogo* of VB (30), followed by VA (18) then GT (3). The Kruskal-Wallis test, at a 10 % level of significance, showed a statistical significant difference ($p = 0.0840$) between the total number of *Fusarium* strains isolated from VB and those obtained from GT. The difference is probably not due to climatic conditions (temperature and rainfall) since sample areas are relatively close to each other. Weather data received from the South African Weather Service (SAWS; Appendix A) was an average measurement of temperature and rainfall for the sample areas.

Harvesting practices within the different sample areas could also have influenced the occurrence of species differently. GT differs from VA and VB as explained in Chapter 3. In brief, GT can be described as a peri-urban town as opposed to VA and VB which are rural communities where subsistence farming (SF) is practiced mainly. Also in both VA and VB are a number of semi-commercial farms (SCF) and small-scale commercial farms (SSCF). From VA, samples were collected from each of the farming groups (i.e. SF, SCF and SSCF) while from VB samples were only collected from SCFs. Agriculture practiced by these farming groups differed in terms of accessibility to water, pest control measures employed,

fertiliser application and soil type (Table 5.2) which might to some extent explain results illustrated in Figure 5.7.

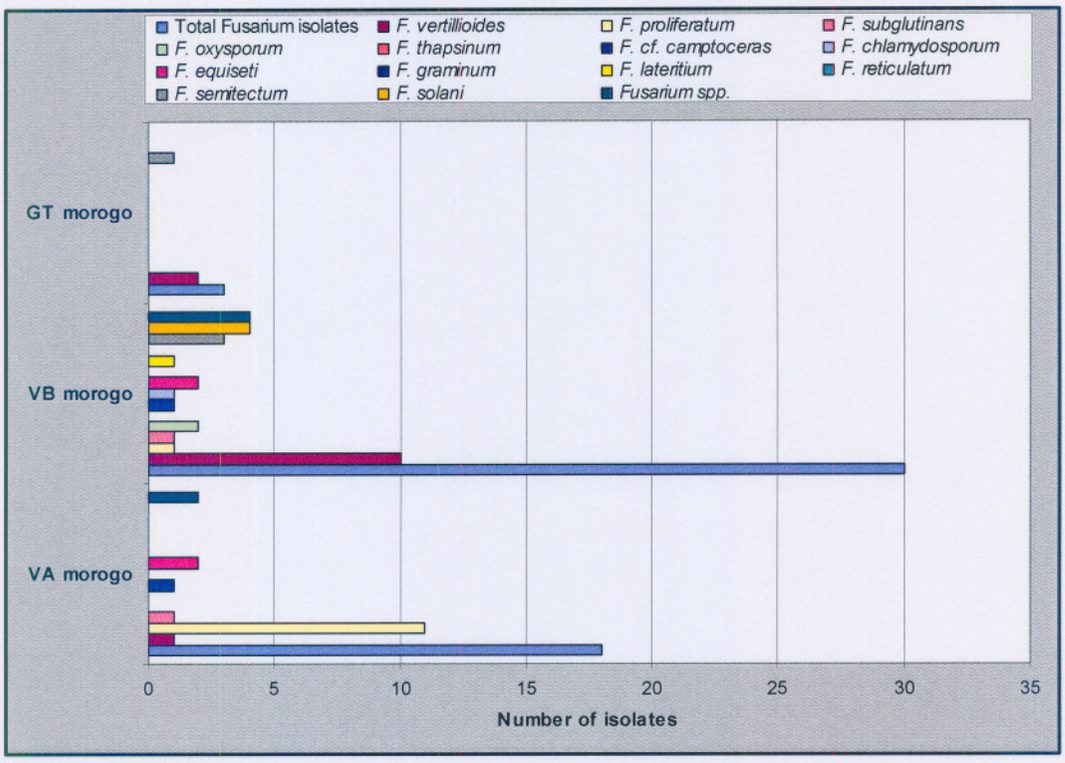

Figure 5.7: Graph illustrating the overall species distribution among *Fusarium* strains retrieved from surfaces of *morogo* (*ligushe* and *ditaka* combined) collected at the different sampling sites.

A major difference detected between VA and VB is the consistent application of commercial pesticides and fertilisers on some samples collected from VA. The numbers of *Fusarium* isolates obtained from VB (30) was higher than those isolated from VA (18). It appears that a combination of pesticides and fertilisers might impede the growth of fungi belonging to the *Fusarium* genus. Another difference observed is the soil types found in VA and VB. Sample areas within VB had black, loose sandy soil types. Within VA, soils in sample areas included those with black, loose sand in addition to red clay areas and white, rocky, salty areas (pers. comm. Mr PJ Lebea, 2003; Table 5.2). The inference that red clay or white, salty, rocky soils inhibit *Fusarium* while black, loose sandy soils are conducive for *Fusarium* growth may be

suggested. Studies regarding the effect of pesticides and fertilizers on different fungi, in addition to soil study analyses, need to be undertaken to confirm or dispel the distribution of *Fusarium* species on different *morogo* types.

Table 5.2: Differentiating factors of the farming sectors (SF, SCF and SSCF) within the sample areas (VA, VB and GT).

Sample area	Farming sector	Pest control measure[a]	Fertiliser[a]	Water[a]	Soil type[b]
VA	SF	none	none	rain[c]	red, clay
	SCF	none	domestic, inconsistent	river, flood	white, rocky, salty
	SSCF	commercial, consistent	commercial, consistent	river, drip	black, loose sand
VB	SCF	none	domestic, inconsistent	river, flood	black, loose sand
GT	none	-	-	-	-

[a] determined using questionnaires
[b] pers. comm. Mr PJ Lebea
[c] limited rain was recorded for the 2002-2003 season

A number of potential fumonisin-producing *Fusarium* strains were identified among the total fungal isolates obtained from both *ligushe* and *ditaka* (Figure 5.8). They included *F. oxysporum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides*. The remaining *Fusarium* species were grouped as non-fumonisin producing strains. A higher number of fumonisin producers were retrieved from *ditaka* (16 of 20) compared to *ligushe* (13 of 31). From *ditaka* leaves, *F. proliferatum* was the dominant potentially fumonisin-producing species recovered (9). *F. verticillioides* was detected in moderate levels (6). Only one *F. oxysporum* was isolated while no *F. subglutinans* isolates were obtained. From *ligushe* leaves, potential fumonisin-producers comprised *F. verticillioides* (7), *F. proliferatum* (3), *F. subglutinans* (2) and *F. oxysporum* (1).

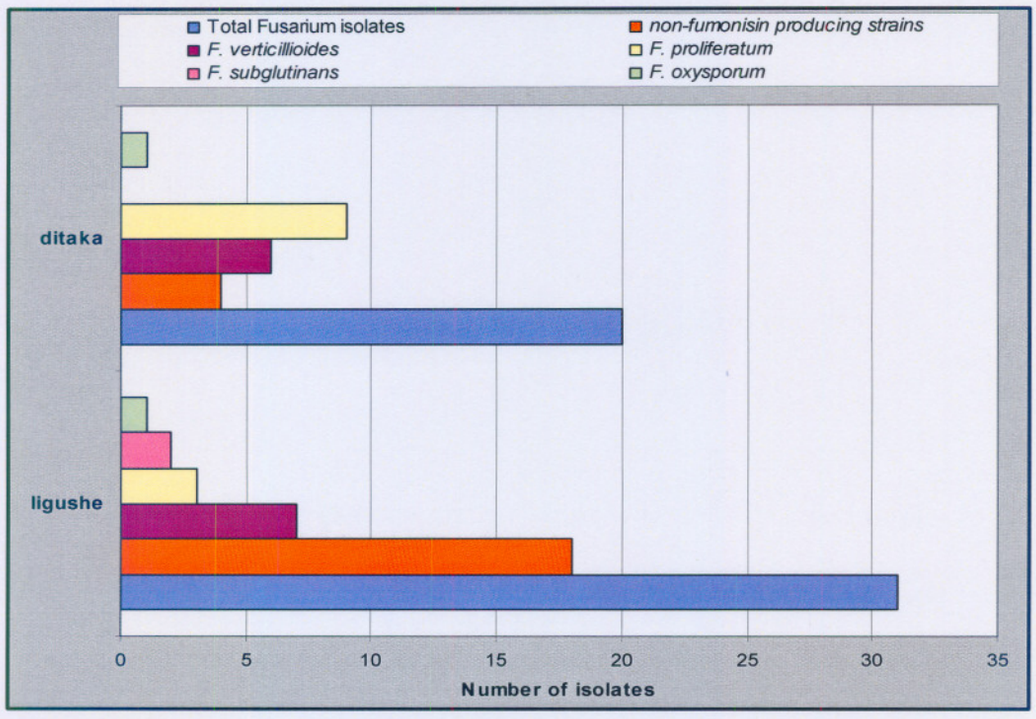

Figure 5.8: Graph comparing the relative numbers of fumonisin-producing *Fusarium* species with non-fumonisin species, isolated from leaf surfaces of *ligushe* and *ditaka* type *morogo*.

Comparison between sample areas showed a variation among potential fumonisin-producing isolates and non-producing *Fusarium* isolates (Figure 5.9). From VA, a proportionally higher number of possible fumonisin producers were recovered (13 of 18) compared to non-fumonisin producers (5 of 18). Of the thirteen potential fumonisin-producing species, *F. proliferatum* occurred in the highest number (11). Only one isolate of *F. subglutinans* and *F. verticillioides* and no *F. oxysporum* isolates were recovered. In VB, almost half (14) the total number of *Fusarium* species (30) obtained were potentially fumonisin producing, with *F. verticillioides* being the dominant fumonisin-producing species (Figure 5.9). Other fumonisin-producing species obtained were *F. oxysporum* (2), *F. proliferatum* (1) and *F. subglutinans* (1). Of the three *Fusarium* isolates recovered from GT, two were potential fumonisin-producers. Both of these isolates were identified as *F. verticillioides*.

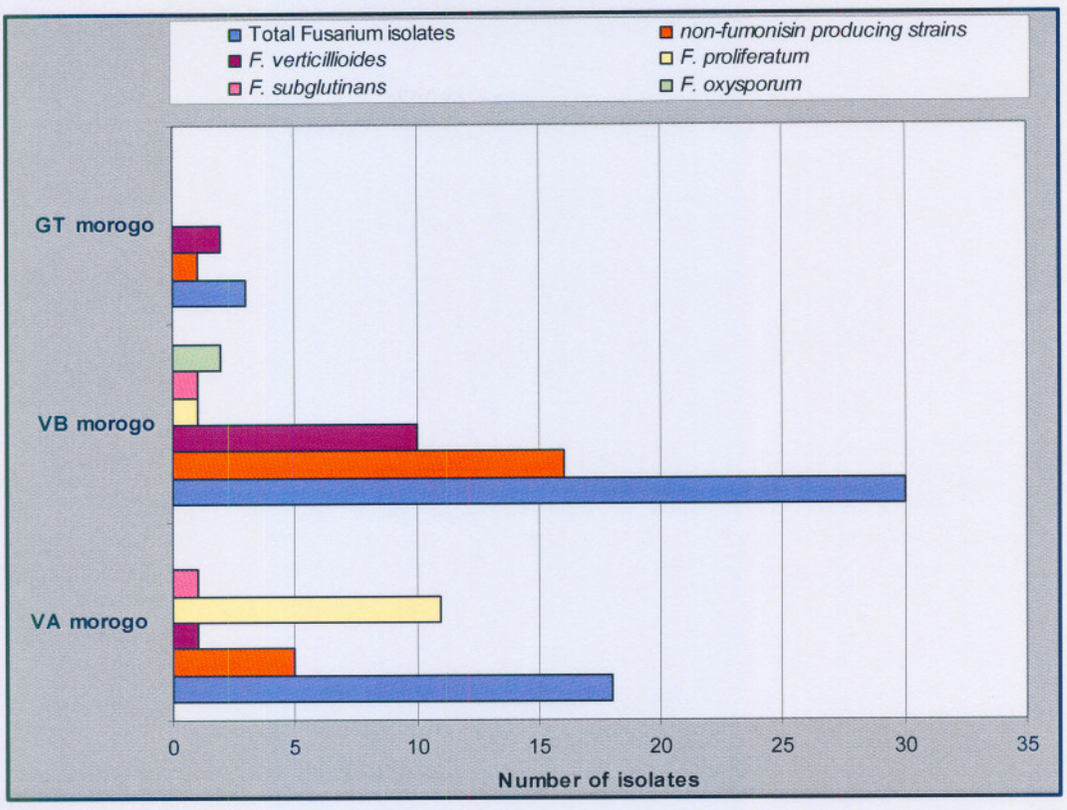

Figure 5.9: Graph comparing the relative numbers of fumonisin-producing *Fusarium* species with non-fumonisin species isolated from VA, VB and GT.

Figure 5.10 illustrates the relative numbers of potential fumonisin-producing species compared to non-fumonisin producers isolated from all *morogo* (*ligushe* and *ditaka*) samples analysed. Over half the total number of *Fusarium* species (51) obtained as surface colonisers on *ligushe* and *ditaka* plants from VA, VB and GT were possible fumonisin producers. *F. proliferatum* and *F. verticillioides* were the dominating fumonisin-producing species (12 and 13, respectively) while two each of *F. oxysporum* and *F. subglutinans* were found.

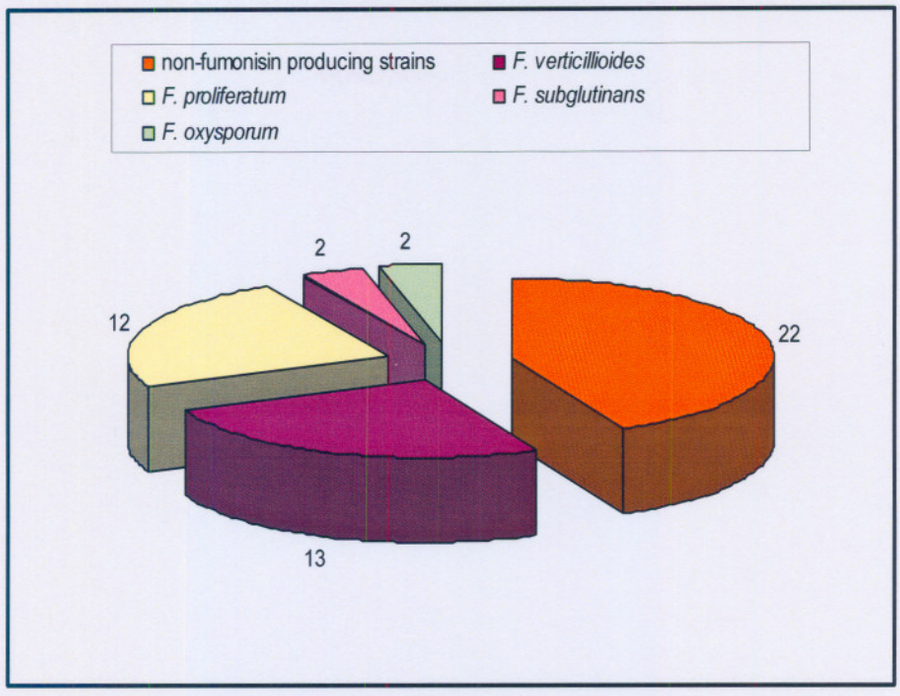

Figure 5.10: Graph comparing the relative percentages of fumonisin producing species and non-producers among the total number of *Fusarium* strains isolated from surfaces of *morogo* (*ligushe* and *ditaka* combined) collected from VA, VB and GT.

The fumonisin group of mycotoxins have been evaluated as "Group 2B carcinogens" due to their carcinogenic risk to humans (IARC, 1993). They have been associated with the high incidence of oesophageal cancer observed in parts of South Africa, China and Iran (Rheeder *et al.*, 1992; Chu & Li, 1994; Shephard *et al.*, 2000). Fumonisin production has recently been reported in other *morogo* types cultivated in similar rural settings (van der Walt *et al.*, 2006). If the *Fusarium* species described here are indeed fumonisin-producing strains, and conditions for fumonisin production are favourable, the result in Figure 5.10 bodes ill for the communities within all three sample areas.

5.3.2 The sterilisation regime

Four modified sterilisation regimes (MSRs) were employed to retrieve internal colonisers from fresh leaves of *ligushe* and *ditaka* type *morogo* from VA, VB and GT. The total number

of isolates as well as the composition diversity of the isolated colonies from each of the four MSRs was recorded in Table D.2, Appendix D. Fungal isolates mainly belonged to the genera *Alternaria*, *Aspergillus*, *Aureobasidium*, *Penicillium* and *Phoma*. "Other" isolates refer to (i) other identified isolates belonging to the *Bipolaris* and *Cladosporium* genera, (ii) unidentified isolates including nonsporulating mycelial fungi and (iii) contaminants such as bacteria and yeasts. No growth was visible on the uninoculated plates used as controls.

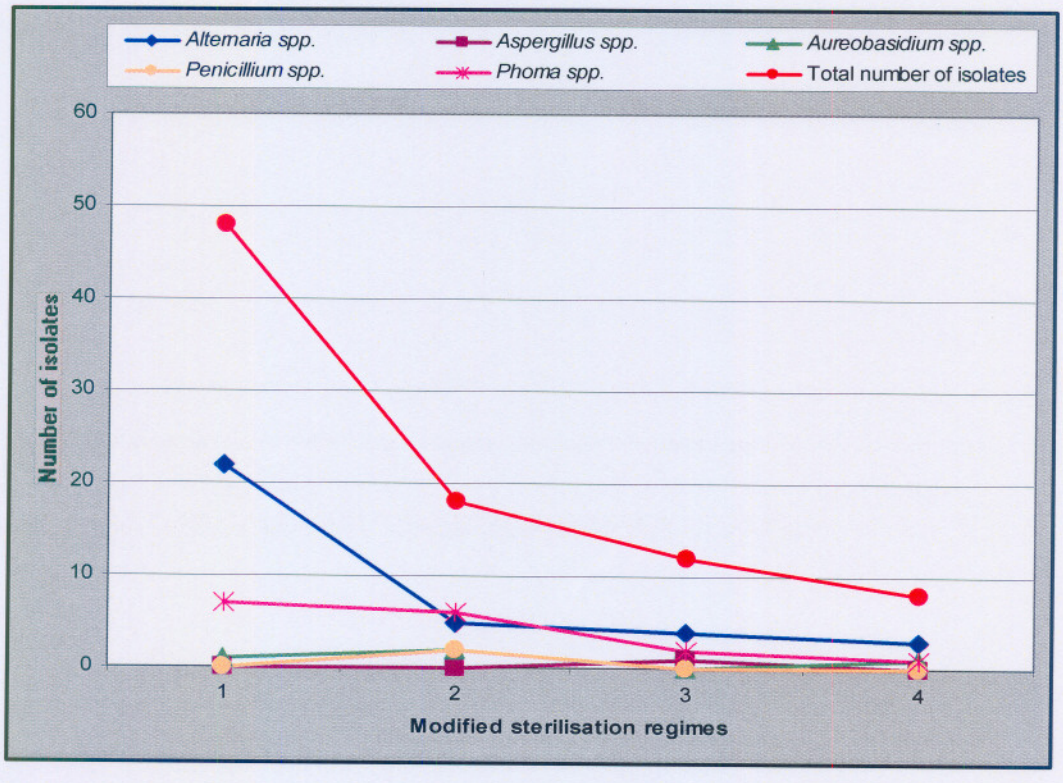

Figure 5.11: Graph illustrating the relative numbers of various fungal genera obtained from the interior of fresh *ligushe* leaves at each sterilisation attempt.

Figure 5.11 illustrates that for *ligushe* leaves, an inversely proportional relationship exists between time of leaf exposure to sterilising agent (NaOCl) and total number of isolates obtained. In other words, the regime with the shortest time of exposure (MSR1, in which the time of leaf exposure to sterilising agent was only 20s) had the least effect on reducing isolate

numbers while the regime with the longest exposure time (MSR4, in which the leaves were exposed to the sterilising agent for 60s) had a better effect at reducing isolate numbers with only eight isolates being retrieved. The most common genus isolated from the interior of *ligushe* leaves was *Alternaria*. Comparison between the increase in leaf sterilisation times with the decrease in the total number of isolates and retrieved *Alternaria* spp., gave an positive correlation ($r = 0.99$; $p = 0.067$). Another genus obtained in relatively high levels (compared to other genera) was *Phoma*, with isolation numbers decreasing with increasing times of leaf sterilisation. *Aureobasidium* isolates, which may grow as endophytes (Carlile *et al.*, 2001), were retrieved from three of the four sterilisation regimes albeit in low numbers. *Aspergillus* and *Penicillium* strains were also isolated in low numbers while no *Fusarium* or *Acremonium* isolates were obtained. These results seem to be congruent with results reported for *A. hybridus* leaves (another type of *morogo*) in which *Alternaria* species were most frequently isolated followed by *Epicoccum nigrum* and *Phoma* spp. (Blodgett *et al.*, 2000).

One of the four sterilisation regimes should be used as the standardised protocol for future quantification of internal colonisers from fresh *ligushe* leaves. The regime that should be regarded as standard ought to be the MSR in which mostly internal colonisers were recovered and not necessarily the regime from which the highest number of isolates were recovered. Since MSR1 gave the highest number of isolates as well as the most fungi regarded as endophytes (*Alternaria* spp., *Phoma* spp. and *Aureobasidium* spp.), it was considered the most appropriate sterilisation regime for the isolation of fungal colonisers from the interior of *ligushe* leaves. It is therefore recommended that hereafter, MSR1 be regarded as the standard when isolating internal colonisers from fresh *ligushe* leaves.

Results for *ditaka* leaves showed that isolates were recovered in low numbers in the case of all four MSRs (Table D.2, Appendix D). The highest number of isolates (10) was obtained

following MSR1 while approximately half this number was isolated following MSR2 (5), MSR3 (4) and MSR4 (5). Figure 5.12 shows that from each MSR, fungi regarded as internal colonisers (*Alternaria*, *Aureobasidium* and *Phoma*) as well as surface colonisers (*Aspergillus* and *Penicillium*) were isolated. Also, the numbers of isolates obtained within each genus showed no specific trend or relationship (i.e. decrease or increase) with an increase in time of leaf exposure to sterilising agent.

As a consequence, it was not possible to conclude which MSR was the most successful sterilisation method. We suggest increasing the number of isolation attempts at each exposure time (i.e. >100 attempts at 20s, 30s, 45s and 60s each) to improve the results and to determine the most appropriate regime to use as a standard protocol for future isolation of internal fungal colonisers from fresh *ditaka* leaves.

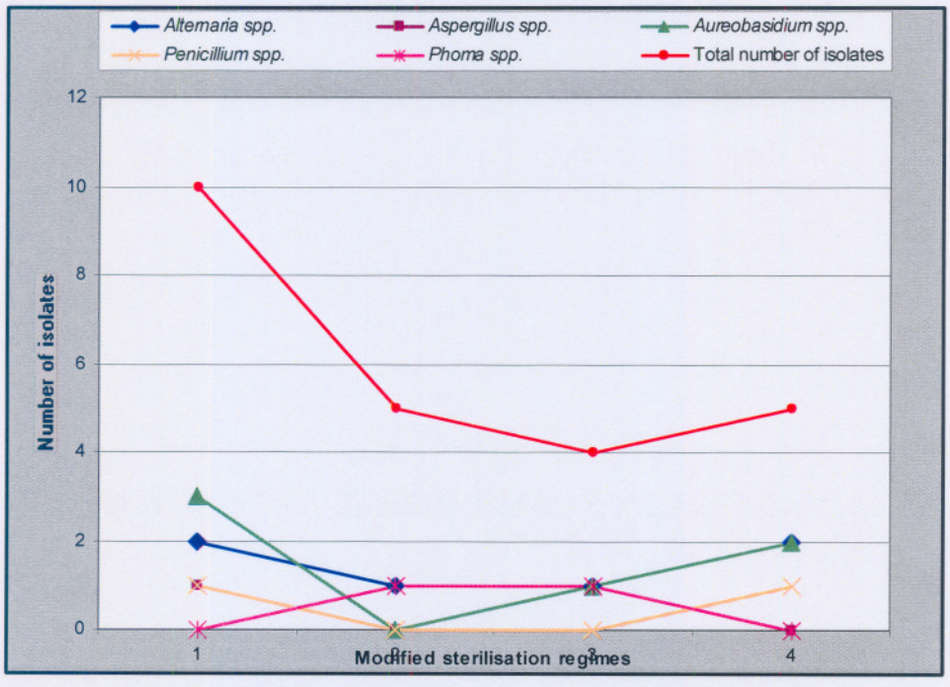

Figure 5.12: Graph illustrating the relative numbers of various fungal genera obtained from the interior of fresh *ditaka* leaves at each sterilisation attempt.

5.3.3 Analysis of cooked and dried samples

Table D.3, Appendix D gives the total number of isolates obtained from dried and cooked *dinawa* and cooked *ligushe* and *ditaka*. Dried samples of *ligushe* and *ditaka* leaves were not sampled due to their unavailability, either because of lack of rain (section 3.4.2) or because dried *morogo* of these plant types are not preferred (section 3.4.4). Table D.3 (Appendix D) also indicates the relative numbers of isolates representative of various genera (*Fusarium*, *Alternaria*, *Aspergillus*, *Penicillium*, *Phoma* and *Aureobasidium*). Species distribution of *Fusarium* isolates is also shown. "Other" isolates were made up of (i) unidentified isolates including nonsporulating mycelial fungi and (ii) contaminants comprising bacteria and yeast isolates.

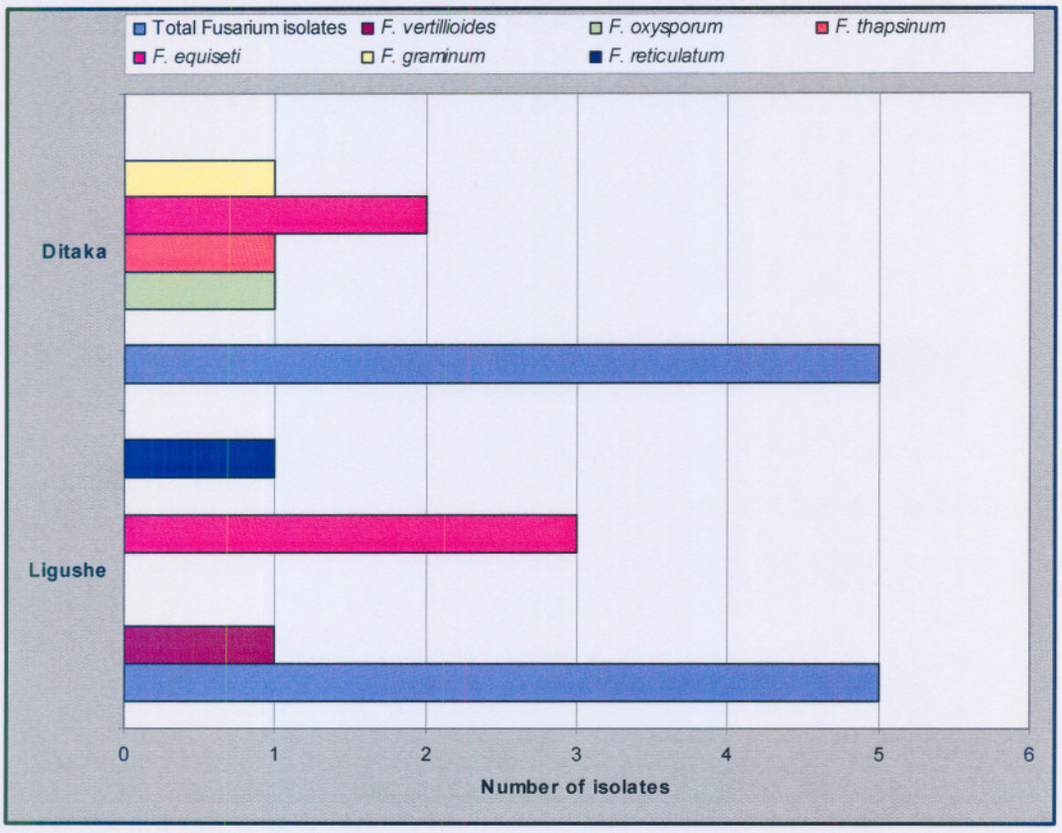

Figure 5.13: Graph comparing *Fusarium* species distribution among isolates obtained from cooked *ligushe* and *ditaka* type *morogo*.

From cooked *ligushe* samples, a total of ten isolates were recovered, half of which were *Fusarium* isolates. From cooked *ditaka* samples, seven isolates were retrieved, five being *Fusarium* isolates. Figure 5.13 compares the *Fusarium* species isolated from cooked *ligushe* and *ditaka* leaves. Among the *ligushe* isolates, one was *F. verticillioides*, a potential fumonisin-producing isolate. *F. equiseti* (3) and *F. reticulatum* (1) were also recovered. While these two species are not known for producing the fumonisin mycotoxin, they have been reported to produce other mycotoxins (Nelson *et al.*, 1993). Among fusaria retrieved from *ditaka*, two potential fumonisin-producers were detected, namely *F. oxysporum* and *F. thapsinum*. It seems that by ingesting *ligushe* and *ditaka* leaves that has been cooked in the traditional manner of people in the Mopani District of the Limpopo Province, there might be a

risk for mycotoxin exposure, in view of various mycotoxin-producing fungal genera apparently associated with these leaves.

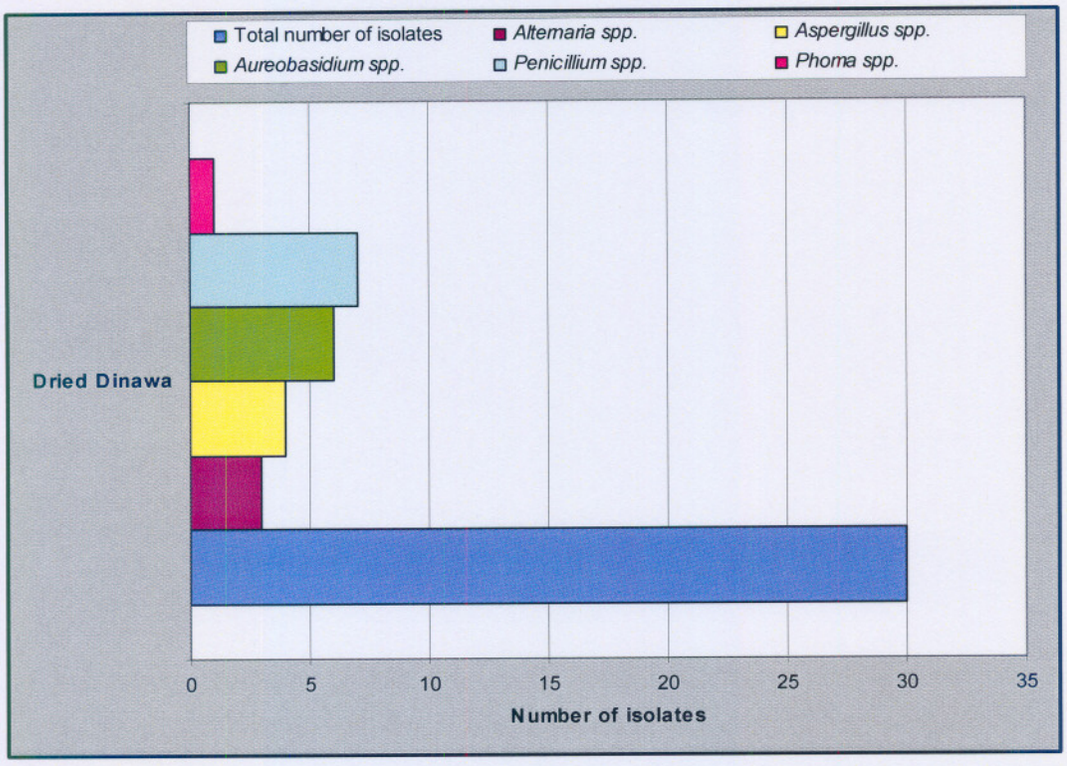

Figure 5.14: Graph illustrating the relative numbers of various fungal genera isolated from dried *dinawa*.

A total of thirty isolates were obtained from dried *dinawa*. Figure 5.14 depicts species distribution data of fungi isolated from dried *dinawa* samples and shows the presence of surface (*Aspergillus* and *Penicillium*) as well as internal (*Alternaria*, *Phoma* and *Aureobasidium*) colonisers. The occurrence of both surface and internal colonisers was expected since the method of analysis (section 4.3.3) was not specific for surface colonisers or for internal colonisers. Approximately equal numbers of surface (*Aspergillus* 4 + *Penicillium* 7 = 11 isolates) and internal (*Alternaria* 3 + *Phoma* 1 + *Aureobasidium* 6 = 10 isolates) colonisers were obtained.

It was noted that while some storage fungi (*Aspergillus* and *Penicillium*) were retrieved, no field fungi (e.g. *Fusarium*) were isolated from dried *dinawa*. Field and storage fungi colonise substrates differently, based on their individual responses to temperature and water activity conditions within the environment (section 2.1.2). In brief, field fungi dominate in the field where higher temperatures and water activities prevail. Storage fungi succeed field fungi during storage conditions when temperature and water activity levels decrease. The dried *dinawa* analysed in this study was dried during the 2001 - 2002 season and stored in a mealie meal sack in a storage hut. Drying of *morogo* would continue while stored in mealie meal sacks (section 3.4). It is therefore possible that during the storage period, the water activity level of dried *dinawa* decreased and that storage fungi subsequently succeeded field fungi that may have been associated with the dried *dinawa* before storage.

Whereas results from uncooked dried *dinawa* show relatively high numbers of isolates (30), results from cooked dried *dinawa* show that no isolates were recovered (Table D.3, Appendix D). It is supposed that the number of fungi associated with dried *dinawa* were reduced as a result of the cooking process. Comparison of uncooked and cooked *morogo* show that while *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Fusarium*, *Penicillium* and *Phoma* were retrieved from uncooked *ligushe* and *ditaka* (Tables D.1 and D.2, Appendix D), only *Fusarium* isolates were recovered from cooked *ligushe* and *ditaka* (Table D.3, Appendix D).

It is proposed that, similar to dried *dinawa*, the cooking of *ligushe* and *ditaka* leaves probably reduced the number of *Acremonium*, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Penicillium* and *Phoma* isolates. Even though these genera may not be present, it cannot be assumed that the cooked samples are clear of their toxins. Mycotoxin detection in processed foods has been reported (Alberts *et al.*, 1990; Bennett & Klich, 2003).

5.4 MOLECULAR ANALYSIS

In Figure 5.15, a negative image of the successfully amplified fragments is depicted. The template DNA was a reference *Fusarium verticillioides* strain (MRC 4319) from the Medical Research Council's PROMEC collection. This strain is well characterised and is known to produce relatively high levels of fumonisins in culture.

All the targeted gene fragments could be amplified using the PCR protocol described in section 4.4.3. These fragments were all of the expected size. The fragments were sequenced (Inqaba Biotech, SA) and analysed by BLASTN searches in the relevant databases to verify the identity of the fragments.

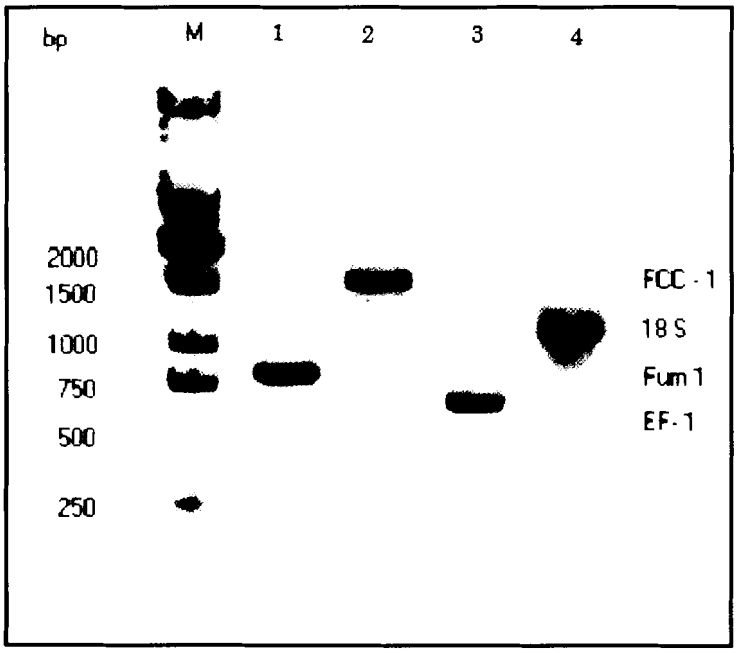

Figure 5.15: A negative image of an ethidium bromide stained gel showing successful amplification of the various gene fragments. The DNA was from *Fusarium verticillioides* MRC strain 4319.

The EF primers were used for the amplification of elongation factor 1 alpha (*EF*) gene fragment. These primers are conserved for members of the genus *Fusarium* (Geiser *et al.*, 2004). Amplification of any potential fusaria with these primers could thus be regarded as a result indicating that the isolate belongs in this genus. Sequencing of this amplicon can be used for identification of the isolate to species level, using the *Fusarium* databank (http://fusarium.cbio.psu.edu; Geiser *et al.*, 2004).

Furthermore, amplification of the 800 bp *FUM 1* gene fragment indicates that the polyketide synthase gene is present in the isolate. Polyketide synthase is responsible for the biosynthesis of polyketide backbone of fumonisin (Bluhm *et al.*, 2004). The potential of fusarial isolates to produce fumonisins can be verified using these primers.

In Table 5.3 is a list of morphologically identified isolates and their associated PPRI numbers. The elongation factor and *FUM* gene fragments were amplified for all of these isolates and the sequences are given in Appendix F. Since the *EF* primers used in this study are conserved for *Fusarium* (Geiser *et al.*, 2004), positive amplification results of the *EF* gene confirms genus identification of these *Fusarium* isolates. Amplification of *FUM 1* gene implies that these *Fusarium* isolates from *morogo* from the Mopani District are potentially fumonisin mycotoxin producers.

Due to some inconsistencies and condradictions between detailed sequence and morphological data, the former was not further explored. Statistical analyses of BLASTN searches are thus not provided. Follow-on studies are in progress to address these issues.

Table 5.3: List of isolated *Fusarium* species and the gene fragments amplified. *F. verticillioides* MRC 4319 was the reference strain.

PPRI Number	Species name	*FUM 1*	*EF*
7363	*F. verticillioides*	✓	✓
7365	*F. subglutinans*	✓	✓
7370	*F. verticillioides*	✓	✓
7371	*F. verticillioides*	✓	✓
7372	*F. verticillioides*	✓	✓
*MRC 4319	*F. verticillioides*	✓	✓

The *Fusarium* cyclin C1 gene controls secondary metabolism in this genus. However, the potential of the *FCC 1* and the *FUM 1* genes to answer *Fusarium* phylogenetic and other evolutionary relationships has not been tested. This could be due to the amount of sequences that are available at present.

A 1200 bp *18S* fragment was included in the analysis and was used as a control. The primer sequences are based on general fungal small subunit ribosomal (18S) genes (Borneman & Hartin, 2000). These primers were thus included to particularly test negative results and eliminate false negatives that could have been due to reaction failure.

In a subsequent study, Bezuidenhout *et al.* (2006) showed that *EF*, *FUM 1* and *18S* primers can be used in a multiplex PCR for the simultaneous detection and identification of fumonisin positive *Fusarium* species, in subsistence as well as commercial crops.

The results from the preceeding sections showed certain trends. Relatively high levels of mycotoxin-producing fungi were retrieved from surfaces of *ligushe* and *ditaka* leaves while

only moderate levels were isolated from surfaces and interior of dried *dinawa*. Cooking of these *morogo* types (*ligushe*, *ditaka* and dried *dinawa*) seems to lower the levels of fungi naturally associated with them. Molecular results indicated that the risk for fumonisin production is present in *Fusarium* isolates from the Mopani District of the Limpopo Province.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

To address the poverty crisis in rural South African communities as well as undernourished food-insecure regions of other developing countries, agricultural development is encouraged. Emphasis is placed on crops that are of low-application but are high-yielding. Consumption of indigenous foods that are sustainable, safe, nutritious and cost-effective should be promoted.

The present study was conducted in the Mopani District of South Africa's Limpopo Province with the aim to quantify and identify mycotoxin-producing fungi from traditional staple foods consumed by these rural communties. Four well-formulated objectives were set for this study. The trends and conclusions of each of these are briefly listed and dicussed in the following sections:

(i) Documentation of commonly consumed *morogo* types

In the Mopani District of South Africa's Limpopo Province, the farming community and farming practices play a critical role in food availability and should therefore be promoted. The most commonly consumed indigenous plants are *ligushe* (*Corchorus* sp., cf. *trilocularis* L. and *Corchorus* sp. aff. a*splenifolius* Burch), *ditaka* (*Cucurbita* sp.) and *dinawa* (*Vigna unguiculata* ssp. *Unguiculata*).

(ii) Recording idiosyncrasies of this community with respect to *morogo* types

The role that these indigenous plant types play in the lifestyles of rural communities has been determined and broadens our limited knowledge of traditional foods. These *morogo* types are eaten everyday as a side dish. The dish may be prepared from fresh or dried *morogo* types. Typically, dried *morogo* is used during lean months.

Traditional *morogo* plants from the Mopani District were found to contain relatively high levels of folate (van der Walt *et al.*, 2005). Further studies are being conducted to determine the nutritional content of some of these indigenous plants. Recently results were presented at an international conference which demonstrated that these plants may contain important fatty acids, high levels of minerals and substances with anti-oxidant properties (Ibrahim *et al.*, 2006). More studies of this nature are required.

(iii) Isolation, identification and quantification of potential mycotoxin-producing fungi associated with *morogo* types

Mycological characterisation indicated a non-pathogenic association between *morogo* leaves and a number of surface and interior fungal colonisers, many of them potentially mycotoxin producing. The fungi were suspected of either being air and / or soil contaminants or having originated from maize plants around which some *morogo* plants are intercropped. Further studies are necessary to determine the origin of these fungal spores so that preventive measures can be put in place. Food preparation and preservation processes (i.e. cooking and drying) seem to lower the levels of fungi associated with external and internal surfaces of *morogo* plants. In addition, the number of fungal spores on leaf surfaces was reduced when water was sprinkled on them. Villagers can therefore be advised to wash *morogo* leaves with water prior to preparation or preservation.

Traditional farming strategies, environmental conditions and some soil types seemed to have a negative effect on fungal growth, particularly *Fusarium* species. Further studies are required to corroborate this notion. If confirmed, traditional agricultural practices can contribute positively to delivering crops that are safe and more nutritious. These farming techniques may then be implemented in other areas where poverty and food insecurity remains rife.

(iv) Molecular confirmation of *Fusarium* sp. and determining the potential to produce fumonisin.

Molecular analysis revealed that the fumonisin-producing gene was present in *Fusarium* isolates, indicating that these mycotoxins could potentially be produced during post-harvest environmental stresses. In studies parallel to this, varying levels of the fumonisin B (FB) group of mycotoxins were detected in similar or other fresh or dried *morogo* types (van der Walt *et al.*, 2006; Alli, 2007; Mogakabe, 2007). Laboratory scale studies also demonstrated that certain MRC strains of *F. verticillioides* produce similar levels of fumonisins in *morogo* extract medium as in corn meal medium (Poswa, 2006).

Follow-on studies to determine FB levels in traditional *morogo* from the Mopani District are integral to determining their safety. Currently though, the exposure risk implied by tentative molecular findings in this study has important consequences for poor rural communities that are dependent on these indigenous food crops.

In a molecular study subsequent to this, Bezuidenhout *et al.* (2006) showed that primers used for the molecular analysis in this study, were used successfully in a multiplex PCR for the simultaneous detection and identification of fumonisin positive *Fusarium* species.

This study sets the stage to enhance the status of indigenous foods. Since the mycological quality of these traditional plants have been determined, their nutritional value can be ascertained to substantiate our knowledge of these food plants and to provide the panacea of food insecurity, which is linked interchangeably with the poverty crisis and the HIV / AIDS pandemic, in South Africa and other developing countries. Result from this and follow- on studies have been presented in article format or as poster- or oral- presentations at national and international conferences. Some of these are listed in Appendix E.

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APPENDIX A

A.1. Daily maximum temperatures for the years 2002 – 2003 as measured by the South African Weather Service (SAWS)

---- indicates that data is not yet available or was not requested

*** indicates that data is missing or not yet available in the current month

= indicates that the average for the month is unreliable due to missing daily values

Daily Maximum Temperature (C) Data for station [0682141A2] - LETABA **2002**. Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/25 08:33)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01	***	29.3	35.5	***	26.3	19.0	24.3	***	***	36.3	***	32.5
02	***	30.3	36.0	***	29.3	21.0	23.5	***	***	24.1	***	37.0
03	***	32.0	35.0	***	32.0	21.2	23.5	***	***	33.1	***	34.5
04	***	34.5	***	***	29.0	23.2	22.7	***	***	23.4	***	29.9
05	***	35.5	36.0	***	28.3	24.2	25.0	***	***	30.0	***	32.0
06	***	38.0	35.2	***	32.2	25.1	26.3	29.0	***	36.2	***	28.0
07	***	26.0	36.0	***	27.1	26.2	27.9	***	***	29.0	***	30.0
08	***	27.0	27.2	***	23.8	22.9	24.1	25.8	***	20.6	***	32.8
09	***	30.5	31.8	***	25.3	28.4	29.0	***	***	23.5	***	33.5
10	***	35.0	32.5	***	27.1	***	27.2	***	***	28.9	***	31.5
11	***	41.0	33.0	***	26.3	24.9	22.4	***	***	31.5	29.0	32.0
12	***	38.0	31.4	***	28.2	24.8	26.8	29.5	***	30.5	28.2	33.5
13	***	33.0	30.1	***	29.4	23.7	23.0	***	***	31.0	34.5	36.0
14	32.5	37.5	34.0	***	29.6	28.4	28.1	31.1	***	34.5	30.5	34.4
15	33.0	38.0	32.5	***	24.6	22.8	29.2	28.5	***	32.3	27.2	38.8
16	35.0	35.5	35.0	***	26.0	25.1	30.7	31.5	***	35.0	34.0	36.7
17	39.0	26.5	34.0	***	29.8	24.7	22.1	***	***	38.0	31.1	35.0
18	39.0	27.0	34.5	***	26.0	25.1	***	***	***	39.4	31.5	29.3
19	30.0	31.5	38.0	***	24.2	24.2	22.0	28.0	***	35.7	36.0	31.7
20	35.0	33.0	37.0	***	26.0	15.5	***	24.5	***	25.0	37.3	39.8
21	37.0	35.0	36.0	***	27.1	18.7	***	28.5	***	30.0	29.0	35.5
22	35.4	34.0	38.5	***	30.0	23.2	***	***	***	35.9	33.9	35.3
23	38.0	35.0	36.0	***	33.1	27.5	***	***	32.0	37.2	37.0	38.0
24	33.5	33.0	34.5	***	28.9	27.5	***	***	38.0	41.5	24.0	38.6
25	31.0	35.5	32.5	***	28.7	28.4	***	***	37.3	29.2	27.8	36.0
26	31.0	28.5	34.5	***	26.6	23.9	32.5	***	35.6	32.3	28.0	30.7
27	31.5	33.0	36.0	***	24.0	24.1	***	***	29.1	37.3	30.5	34.0
28	34.5	34.0	41.0	***	26.0	26.4	***	***	35.2	***	35.7	38.3
29	34.0	***	30.0	32.5	28.0	23.7	***	***	33.9	***	36.0	33.5
30	36.0	***	31.0	27.5	27.2	19.3	***	***	***	***	25.0	35.2
31	32.5	***	32.5	***	25.2	***	***	***	***	***	***	36.5
Avg	34.3=	33.1	34.2=	30.0=	27.6	23.9=	25.8=	28.5=	34.4=	31.9=	31.3=	34.2

Daily Maximum Temperature (C) Data for station [0682141A2] - LETABA **2003** Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/23 10:16)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01	34.2	37.5	35.1	30.9	32.5	26.9	25.1	***	30.1	35.0	31.0	33.5
02	36.6	41.1	33.0	32.7	26.0	28.1	28.9	***	30.2	38.3	23.1	32.0
03	38.5	38.7	32.5	36.0	28.0	29.2	22.7	***	27.1	35.0	28.8	38.0
04	37.5	39.6	30.0	33.6	36.0	25.0	25.7	28.1	28.4	33.1	31.5	30.4
05	29.5	40.6	27.7	31.8	25.5	22.3	25.1	23.0	27.7	40.0	37.7	35.4
06	35.0	34.4	31.4	30.7	24.5	20.0	20.2	22.1	19.8	39.0	22.7	37.5
07	39.3	34.0	31.2	31.5	24.1	27.7	22.0	22.3	21.4	27.0	31.5	36.4
08	38.5	35.2	31.7	32.8	28.4	24.7	22.3	24.6	29.7	23.1	34.3	42.3
09	33.1	34.5	32.0	34.2	28.0	26.8	23.1	24.9	33.5	30.0	37.0	43.0
10	21.3	30.4	32.6	30.8	28.5	20.2	24.7	29.5	29.1	36.5	28.0	29.2
11	28.6	34.6	31.0	28.4	27.0	23.3	26.5	31.6	23.5	29.5	37.0	35.9
12	32.7	36.3	31.8	31.6	29.5	25.1	21.3	30.7	29.5	26.5	31.0	43.7
13	***	36.0	33.4	31.2	29.0	27.9	24.0	28.4	22.9	28.0	26.0	36.9
14	40.6	36.0	34.7	27.2	25.5	27.1	24.6	28.9	17.1	35.0	32.0	44.0
15	37.5	38.5	***	29.7	25.8	27.3	23.6	26.0	23.0	25.5	37.6	30.0
16	41.6	34.5	37.0	29.4	25.5	25.7	26.0	26.0	24.0	35.0	29.5	32.5
17	34.4	37.2	33.5	29.7	27.0	27.4	27.0	31.3	27.0	24.5	33.2	39.5
18	38.3	34.5	35.5	30.4	26.5	25.0	28.3	28.9	31.0	14.0	34.2	32.0
19	40.4	35.0	35.6	29.4	22.0	25.6	28.3	33.0	37.5	15.2	28.6	35.5
20	41.5	38.0	32.6	29.8	26.0	23.5	24.9	24.8	37.5	15.2	33.1	36.5
21	35.4	26.0	33.6	31.8	27.4	23.2	23.5	21.6	26.9	19.0	38.0	41.2
22	37.6	32.0	38.1	31.6	28.2	23.6	26.0	22.5	16.1	28.0	37.1	39.5
23	39.9	34.0	38.7	33.5	27.2	24.9	26.0	26.5	24.8	32.5	35.6	39.5
24	33.5	35.0	34.5	33.5	31.5	24.5	26.8	28.7	30.5	33.1	32.0	37.0
25	35.6	34.7	35.5	34.0	34.3	22.5	27.8	30.0	31.1	35.5	36.0	39.9
26	37.8	31.5	31.7	26.6	34.2	20.0	26.9	31.8	32.6	39.4	36.2	39.4
27	31.5	32.6	31.2	27.0	27.2	22.0	30.7	24.9	33.0	22.0	37.5	22.5
28	34.3	35.4	31.9	29.4	25.7	21.4	32.1	29.4	33.3	24.0	33.0	27.7
29	36.0	***	31.4	29.4	27.0	21.1	26.6	31.9	28.1	28.0	27.1	31.7
30	40.6	***	32.2	31.5	26.6	21.5	25.4	24.6	28.0	32.0	30.1	35.5
31	35.0	***	33.2	***	27.2	***	27.6	29.5	***	0.0	***	37.5
Avg	35.9	35.3	33.1	31.0	27.8	24.4	25.6	27.3	27.8	28.4	32.3	36.0

A.2. Daily minimum temperatures for the years 2002 – 2003 as measured by (SAWS)

---- indicates that data is not yet available or was not requested

*** indicates that data is missing or not yet available in the current month

= indicates that the average for the month is unreliable due to missing daily values

Daily Minimum Temperature (C) Data for station [0682141A2] - LETABA **2002** Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/25 08:33)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01	***	24.0	21.0	20.5	19.9	13.7	14.1	***	***	18.0	***	17.3
02	***	22.8	24.0	***	13.0	10.6	4.4	***	***	17.0	***	21.0
03	***	21.2	23.6	***	14.5	8.9	0.2	***	***	18.3	***	20.7
04	***	20.0	21.8	***	18.6	3.1	4.5	***	***	17.5	***	22.0
05	***	20.0	***	***	16.5	4.2	2.5	***	***	18.5	***	20.8
06	***	21.0	23.0	***	13.0	12.6	3.2	***	***	15.0	***	22.0
07	***	22.0	21.8	***	7.2	4.7	5.0	4.6	***	15.3	***	20.6
08	***	17.0	20.8	***	3.4	13.1	2.5	***	***	***	***	19.5
09	***	17.0	18.7	***	3.4	12.7	4.3	12.7	***	***	***	25.6
10	***	15.5	18.4	***	4.1	12.9	6.0	***	***	***	***	***
11	***	18.0	19.4	***	9.2	***	4.5	***	***	***	***	***
12	***	25.0	19.7	***	6.3	12.3	2.9	***	***	***	***	***
13	***	23.0	20.2	***	6.7	12.2	11.2	7.2	***	***	***	***
14	22.0	20.0	20.1	***	7.3	13.6	9.1	***	***	***	***	***
15	22.0	21.0	20.5	***	14.2	4.3	9.5	9.5	***	***	***	***
16	20.0	23.5	21.0	***	13.8	9.2	4.5	18.2	***	***	***	***
17	22.0	22.0	16.3	***	8.7	4.3	11.4	19.0	***	***	***	27.5
18	22.5	20.5	17.0	***	9.8	6.3	13.5	***	***	***	***	24.4
19	24.0	19.0	15.4	***	12.0	7.5	***	***	***	***	18.8	22.0
20	22.5	18.0	19.4	***	13.9	11.6	5.5	16.2	***	***	19.3	22.3
21	21.5	19.0	21.0	***	11.2	12.0	***	16.0	***	***	23.3	22.0
22	23.0	21.0	18.7	***	10.0	11.5	***	13.5	***	***	16.3	20.6
23	17.5	20.0	24.0	***	13.1	11.8	***	***	***	***	16.6	22.0
24	22.0	21.5	22.0	***	9.8	6.6	***	***	14.2	***	19.2	23.8
25	21.4	20.0	18.0	***	7.4	6.6	***	***	13.0	***	18.0	22.5
26	21.5	22.0	18.0	***	13.8	15.5	***	***	15.5	***	14.0	22.5
27	20.5	22.0	18.0	***	19.0	5.5	1.5	***	20.0	***	15.5	23.7
28	20.0	21.0	16.2	***	10.0	6.8	***	***	18.5	***	14.5	23.5
29	21.5	***	21.0	***	8.9	9.5	***	***	17.3	***	19.0	25.7
30	22.5	***	21.0	11.5	10.4	12.2	***	***	16.3	***	18.5	24.2
31	22.0	***	14.2	***	14.3	***	***	***	***	***	***	21.1
Avg	21.6=	20.6	19.8=	16.0=	11.1	9.5=	6.0=	13.0=	16.4=	17.1=	17.8=	22.4=

Daily Minimum Temperature (C) Data for station [0682141A2] - LETABA **2003** Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/23 10:16)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01	21.0	25.5	23.4	22.7	14.4	10.6	12.1	***	9.4	19.5	18.0	18.1
02	23.0	25.0	24.3	22.6	21.1	7.6	8.4	***	6.9	16.0	20.0	***
03	23.0	26.0	24.5	19.3	15.5	8.0	14.5	***	9.8	20.0	18.5	17.0
04	25.0	23.0	24.2	19.3	15.0	13.8	7.2	***	12.0	21.0	19.0	23.0
05	25.0	28.6	24.0	20.7	22.9	17.7	10.4	11.4	15.6	16.0	19.5	22.0
06	***	22.5	23.0	21.7	16.0	16.0	5.5	16.5	12.5	17.5	21.0	21.5
07	***	24.5	23.4	17.9	13.7	13.0	***	10.0	8.5	***	19.3	21.0
08	***	24.7	21.6	16.1	9.5	15.0	***	10.7	8.0	18.5	18.0	23.0
09	***	25.6	19.9	17.1	11.0	15.3	***	***	8.9	18.7	19.5	27.0
10	***	22.1	20.0	24.6	11.4	14.8	***	***	13.0	16.6	20.0	21.5
11	***	20.6	18.7	21.5	16.5	6.5	***	9.9	14.2	22.5	12.5	23.0
12	***	19.6	18.0	21.1	13.0	6.1	***	13.9	11.0	18.0	23.0	25.8
13	***	24.6	21.3	21.9	7.0	7.4	***	14.1	16.0	15.3	18.5	21.8
14	***	24.0	19.0	18.0	20.0	8.6	***	12.3	9.5	19.9	15.0	24.1
15	27.3	26.6	20.0	17.5	18.5	7.8	***	6.9	***	18.0	18.5	22.5
16	25.0	25.4	***	20.3	13.5	12.1	***	8.2	14.5	16.5	21.0	22.0
17	22.0	25.9	25.1	14.9	10.5	10.5	***	7.9	12.0	20.5	19.0	21.0
18	24.0	24.5	24.0	19.5	10.0	14.6	***	9.1	13.5	13.4	17.9	24.0
19	24.6	26.4	20.5	16.0	15.5	17.0	***	9.6	14.3	12.0	22.0	22.5
20	26.6	27.2	22.5	14.3	10.0	13.7	***	15.3	18.0	12.0	19.2	23.0
21	25.8	22.5	22.5	15.4	13.9	10.6	***	12.5	21.0	13.5	25.5	25.0
22	25.0	23.0	20.1	10.5	16.5	10.3	***	4.3	15.5	11.2	27.9	22.0
23	26.1	20.5	23.0	14.0	17.5	15.5	***	4.5	15.4	16.5	24.0	26.0
24	25.9	22.0	24.1	17.6	11.6	10.9	***	10.9	15.0	19.0	25.5	26.0
25	24.5	24.0	23.9	17.4	9.4	10.5	***	12.6	15.5	23.0	23.5	23.5
26	24.5	23.5	21.2	21.5	13.5	15.0	***	8.5	17.5	25.0	22.8	14.5
27	22.7	22.4	20.2	20.5	14.5	13.0	***	7.3	16.5	19.0	26.5	16.5
28	20.5	20.5	18.1	15.1	14.3	15.8	***	5.2	13.1	19.0	24.0	19.0
29	23.0	***	16.5	15.0	10.8	16.0	7.5	8.6	20.5	19.0	22.0	20.0
30	22.7	***	20.1	12.6	9.8	12.3	15.0	17.4	19.0	18.0	18.0	20.0
31	25.2	***	20.3	***	12.9	***	6.6	10.8	***	18.0	***	22.0
Avg	24.2	24.0	21.6	18.2	13.9	12.2	9.7	10.3	13.7	17.8	20.6	21.9

A.3. Daily rainfall for the years 2002 – 2003 as measured by SAWS

Only rainfall >= 0.1 mm is reflected on this report

---- indicates that data is not yet available

(**blank**) indicates that no rain fell on that day

*** indicates that data is missing or not yet available in the current month

= indicates that the total for the month is unreliable due to missing daily values

"**A**" or "**B**" indicates that no rainfall reading was taken on the day, but that any rainfall that did occur is included in the accumulation total at the end of the period

"**C**" next to a value indicates that the rainfall was accumulated over a number of days

Daily Rain (mm) Data for station [0682141A2] - LETABA **2002** Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/25 08:33)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01						7.0						
02												
03												
04												
05												
06												
07		6.0				5.2				5.5		
08										3.4		
09												8.6
10												
11												
12												
13			2.0									17.2
14								1.3				
15												2.7
16												
17		1.6										
18		2.3										
19												
20						6.3						
21												
22												
23			0.9									
24	11.0	2.6										
25	1.7											
26	4.0											
27		2.0							2.6			
28												
29		***										
30	12.0	***										
31		***		***		***			***		***	
Tot	28.7	14.5	2.9	0.0	0.0	18.5	0.0	1.3	2.6	8.9	0.0	28.5

Daily Rain (mm) Data for station [0682141A2] - LETABA **2003** Measured at 08:00
Lat:-23.8500 Lon:31.5800 Height:215 m (Extracted 2005/02/23 10:16)

Day	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
01								***				
02								***				
03			13.0					***				
04			17.0									
05		68.4	41.0		4.1				2.6		3.0	
06			0.5									
07												
08												
09	17.7					0.5						
10	0.6			1.0								
11				1.1								
12											1.3	1.3
13									9.0			
14												
15		15.0										
16												
17				1.0						2.5		
18										10.5		
19										19.0		
20										9.0		3.0
21									0.3			
22												0.0 E
23												0.0 E
24											13.0	13.0
25	8.4											4.0
26						3.0				2.5		83.0
27						0.5						1.2
28						1.5						0.0 E
29		***										0.0 E
30		***										0.0 E
31		***		***		***			***		***	0.0 E
Tot	26.7	83.4	71.5	3.1	4.1	5.5	0.0	0.0=	11.9	43.5	17.3	105.5

APPENDIX B

B.1. Template of questionnaire employed for subsistence farmers and households.

Household number:

Season:

Name:

Occupation of father:

Occupation of mother:

Number of household members in specific age group:

0-10	11-20	21-30
31-40	41-50	50-

---

1. What is the staple diet of the household?
2. What is the consumption frequency of the staple diet? once a day
3. What other foods do you eat?
4. What is the consumption frequency of these foods? daily
   Further comment:
5. What is the consumption frequency of *morogo*? once a day
   Further comment:
6. What is the average *morogo* eaten by members of the household per meal?
   Children Teenagers Adults
7. Do children eat foods that are not cooked at home?
8. If yes, where do they get these foods?

---

9. Where do you usually get the *morogo* that you eat from?
   Ploughing ☐ Schemes ☐ Relatives ☐ Other ☐
10. Do you plough? Yes ☐ No ☐
    Further comment:
11. If yes, where do you plough? At home ☐ Fields ☐
12. Do you rely only on rain for water? Yes ☐ No ☐
13. Did you plough this season (2002-2003)? Yes ☐ No ☐
14. How did the lack of rains during this season affect ploughing and plant growth?

15. How do you cultivate your *morogo*?

- How many plant types do you use as *morogo*?
- How do you plant your *morogo*?
- How long does it take for the *morogo* to grow until it is ready for picking?
- Do you pick young and old leaves for cooking?
- Which plants do you use as fresh / dry *morogo*?

- How do you cook the fresh / dry *morogo*?

- Do you combine different *morogo*s when you cook or dry them?

- Do you wash the *morogo* before cooking it?

- What other ingredients do you add when you cook the *morogo*?

- How do you dry the *morogo*?
- Where and how do you store your dried *morogo*? How consistent is this storage method?
- How long do you keep your dried *morogo*?

16. Do you use seeds for planting? Yes ☐ No ☐

17. Where do you get the seeds from?

Previous years crop ☐ Relatives ☐

Bought ●

Further comment:

18. Do you find any mouldy leaves? Yes ☐ No ☐

19. What do you do with these leaves?

Throw them away ☐ Use them with other leaves ☐

Leave them on the plant ☐

20. If you throw them away, where do you discard of them?

B.2. Template of questionnaire employed for semi-commercial farmers and small-scale farmers.

Scheme number:

Name and surname:

Current season:

---

1. What types of *morogo* do you plant?
2. Do you use seeds? Yes ☐ No ☐
3. Where do you get the seeds from?
   Previous years crop ☐ Relatives ☐
   Bought ☐
   Further comment:
4. How many times a year do you plough?
5. Do you rely only on rain for water? Yes ☐ No ☐
   Further comment:
6. If not, what method of watering do you employ?
   Flooding ☐ Sprinkler ☐ Other ☐
7. How often do you water the plants?
   Summer Winter
   Further comment:
8. How do you sell the *morogo*?
   Washed ☐ Unwashed ☐
   Roots intact ☐ Leaves only ☐
9. Who do you supply the *morogo* to?
   Villagers ☐ Giyani market ☐ Other ☐
   Further comment:
10. Have you noticed any of the plants going mouldy or exhibiting brown spots?
    Yes ☐ No ☐
11. If yes, how often do you notice this?
12. Does it affect your harvest? Yes ☐ No ☐
13. What have you done / are going to do about this problem?
14. Would you like us to arrange talks / consultations with an agricultural institute?
    Yes ☐ No ☐

B.3. Template of questionnaire employed for the town area.

Stall number:

Name and surname:

Current season:

---

1. Where do you get the *morogo* that you are selling from?

   Ploughing ☐ Schemes ☐ Other ☐

2. If you plough yourself, do you use seeds?

   Yes ☐ No ☐

3. Where do you get the seeds from?
4. How many times a year do you plough?
5. Do you rely only on rain for water?

   Yes ☐ No ☐

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

   Flooding ☐ Sprinkler ☐ Other ☐

7. How often do you water the plants?

   Summer Winter

   Autumn Spring

8. What types of *morogo* do you sell?
9. How do you sell the *morogo*?

   Washed ☐ Unwashed ☐

   Roots intact ☐ Leaves only ☐

10. Do you sell young and / or old leaves?

    Yes ☐ No ☐

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

    Villagers ☐ Giyani townspeople ☐ Other ☐

12. What time do you start selling?
13. When / what time do you get your stock to sell for the day?the morning of the same day
14. How long does it take you to sell your stock? half a day
15. If your stock is not finished by the end of the day, what do you do with it?

    Sell it the next day ☐ Throw it away ☐

    Use it yourself ☐ Give it to relatives/other people ☐ Other ☐

16. Have you noticed any of the leaves going mouldy or exhibiting brown spots?

    Yes ☐ No ☐

17. If yes, when do you notice this? around 1 o' clock
18. If yes, how often do you notice this? every day
19. What have you done / are going to do about this problem?

_____

_____

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

Yes ☐ No ☐

APPENDIX C

C.1: Template used for the identification of *Alternaria* isolates.

Sample no._____ ***Alternaria*** _____

Date: _____

Macroscopic observations

Colony colour: olivaceous, grey, black

Microscopic observations

Conidiophores: length _____ µm, breadth _____ µm //

morphology: single, small groups, simple branched //

scars: present, absent // number _____ // apex: smooth, verrucose //

Conidia: length _____ µm, breadth _____ µm //

colour: pale, golden brown, brown straw //

chains: solitary (no chain), short, long and branched //

form: straight, curved, ovoid, ellipsoidal, conical, obclavate, obpyriform, cylindrical, subcylindrical, tapering to a beak, variable, extremely variable //

ornamentation: smooth, smooth-minutely verrucose, smooth with minutely verrucose base, verrucose, distinctly verrucose, tuberculate, thick-walled //

septa: smooth, constricted // close together, far apart //

transverse: present, absent // quantity _____ //

longitudinal: present, absent // quantity _____ //

oblique: present, absent // quantity _____ //

beak: short, up to but not more than one third the conidial length, up to half the conidial length, shorter than half the conidial length, long and septate //

conical, cylindrical, narrow, tapering, tapering with a swollen tip //

scar: present, absent // quantity _____ //

C.2: Template used for the identification of *Aspergillus* isolates.

Sample no._____ ***Aspergillus*** _____

Date: _____

Macroscopic observations

CYA: diameter _____mm	// low, medium, deep		//
sparse, medium, dense	// plane, sulcate, convolute		//
mycelium colour: _____	// conidiogenesis colour: _____		//
velutinous, granular, floccose	// reverse colour: _____		//
margins: subsurface (yes / no)	// low, deep	// entire, irregular	//
exudate:	// sol. pigment:		//
MEA: diameter _____mm	// low, medium, deep		//
sparse, medium, dense	// plane, sulcate, convolute		//
mycelium colour: _____	// conidiogenesis colour: _____		//
velutinous, granular, floccose	// reverse colour: _____		//
margins: subsurface (yes / no)	// low, deep	// entire, irregular	//
exudate:	// sol. pigment:		//
CY20S: diameter _____mm	// low, medium, deep		//
sparse, medium, dense	// plane, sulcate, convolute		//
mycelium colour: _____	// conidiogenesis colour: _____		//
velutinous, granular, floccose	// reverse colour: _____		//
margins: subsurface (yes / no)	// low, deep	// entire, irregular	//
exudate:	// sol. pigment:		//
CZ: diameter _____mm	// low, medium, deep		//
sparse, medium, dense	// plane, sulcate, convolute		//
mycelium colour: _____	// conidiogenesis colour: _____		//
velutinous, granular, floccose	// reverse colour: _____		//
margins: subsurface (yes / no)	// low, deep	// entire, irregular	//
exudate:	// sol. pigment:		//
CYA37: diameter _____mm	// low, medium, deep		//
sparse, medium, dense	// plane, sulcate, convolute		//
mycelium colour:	// conidiogenesis colour: _____		//
velutinous, granular, floccose	// reverse colour: _____		//
margins: subsurface (yes / no)	// low, deep	// entire, irregular	//
exudate:	// sol. pigment:		//

Microscopic observations

Conidial heads: globose, radiate, columnar, compactly columnar, broadly/loosely columnar, splitting columnar, clavate // colour: _____ //

Conidiophores: length _____ µm, breadth _____ µm // short, long // sinuous, recurved // smooth, rugose // constricted // colourless, pigmented //

Vesicles: length _____ µm, breadth _____ µm // globose, subglobose, ellipsoidal, pyriform, clavate // fertility: entire, three-quarter, half //

Sterigmata: uniseriate, biseriate // smooth, coarse // hyaline, pigmented // metulae / primaries: length _____ µm, breadth _____ µm // phialides / secondaries: length _____ µm, breadth _____ µm //

Conidia: length _____ µm, breadth _____ µm // globose, subglobose, ellipsoidal, fusiform, pyriform, ovoid, cylindrical // colourless, yellow, brown // smooth, roughened, rugulose, echinulate //

Hűlle cells:

Ascocarp: diameter _____ µm // no. of weeks before formation _____ // CYA, MEA, CY20S, CZ, CYA37 // rare, abundant // colour: _____ //

Ascospores: length _____ µm, breadth _____ µm // Spheroidal, subspheroidal, ellipsoidal // smooth, rugose, spinose //

Dimensions

Conidiophores		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Vesicles		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Conidia		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Phialides		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Metulae		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

C.3: Template used for the identification of *Fusarium* isolates.

Sample no._____ ***Fusarium*** _____

Day: 7 / 14 **Date:** _____

Macroscopic observations

Colour: surface colour: white, cream, yellow, orange, peach, pink, purple, salmon, carmine red, tan, brown

//

undersurface colour: _____ //

texture: slimy, powdery, woolly, filamentous //

Sporodochia: present, absent // *colour*: _____ //

Sclerotia: present, absent // *colour*: _____ //

Microscopic observations

Sporodochial Macroconidia: abundant, sparse, absent // size: short, medium, long //

septate: (yes / no) // number of septa: _____ //

shape: sickle, slightly sickle, straight, stout, cylindrical, spindle, distinctly curved //

basal cell: distinct, foot-shaped, notched, rounded, papillated //

apical cell: attenuated, blunt, rounded, elongated, tapered //

Aerial Macroconidia: abundant, sparse, absent //

size: short, medium, long //

septate: (yes / no) // number of septa: _____ //

shape: sickle, slightly sickle, straight, stout, cylindrical, spindle, distinctly curved //

basal cell: distinct, foot-shaped, notched, rounded, papillate //

apical cell: attenuated, blunt, rounded, elongated, tapered //

Microconidia: abundant, sparse, absent // size: short, medium, long //

septate: (yes / no) // number of septa: _____ //

shape: oval, kidney, club, comma, ellipsoidal, lemon, pear //

formed in: chains, singly, false heads //

Phialides: Monophialides: unbranched, branched //

Polyphialides: unbranched, branched //

Chlamydospores: present, absent //

Apical: singly, paired,chains, clumps // smooth, rough //

Hyphal: singly, paired,chains, clumps // smooth, rough //

C.4: Template used for the identification of *Penicillium* isolates.

Sample no._____ ***Penicillium*** _____

Date: _____

Macroscopic observations

CYA: diameter _____mm	// low, medium, deep	//
sparse, medium, dense	// plane, sulcate, radial, umbonate, convolute	//
velutinous, granular, floccose	// reverse colour:	//
funiculose, fasciculate, coremiform, synnematous		//
synnemata formed in: dark, light	// 7, 14, 21 days // height: 3, 5, 10, + mm	//
mycelium colour:	// conidiogenesis colour:	//
margins: subsurface (yes / no)	// low, deep // entire, irregular	//
exudate:	// sol. pigment:	//
MEA: diameter _____mm	// low, medium, deep	//
sparse, medium, dense	// plane, sulcate, radial, umbonate, convolute	//
velutinous, granular, floccose	// reverse colour:	//
funiculose, fasciculate, coremiform, synnematous		//
synnemata formed in: dark, light	// 7, 14, 21 days // height: 3, 5, 10, + mm	//
mycelium colour:	// conidiogenesis colour:	//
margins: subsurface (yes / no)	// low, deep // entire, irregular	//
exudate:	// sol. pigment:	//
G25N: diameter _____mm	// low, medium, deep	//
sparse, medium, dense	// plane, sulcate, radial, umbonate, convolute	//
velutinous, granular, floccose	// reverse colour:	//
funiculose, fasciculate, coremiform, synnematous		//
synnemata formed in: dark, light	// 7, 14, 21 days // height: 3, 5, 10, + mm	//
mycelium colour:	// conidiogenesis colour:	//
margins: subsurface (yes / no)	// low, deep // entire, irregular	//
exudate:	// sol. pigment:	//
CYA5: diameter _____mm	// low, medium, deep	//
sparse, medium, dense	// plane, sulcate, radial, umbonate, convolute	//
velutinous, granular, floccose	// reverse colour:	//
funiculose, fasciculate, coremiform, synnematous		//
synnemata formed in: dark, light	// 7, 14, 21 days // height: 3, 5, 10, + mm	//
mycelium colour:	// conidiogenesis colour:	//
margins: subsurface (yes / no)	// low, deep // entire, irregular	//
exudate:	// sol. pigment:	//
CYA37: diameter _____mm	// low, medium, deep	//
sparse, medium, dense	// plane, sulcate, radial, umbonate, convolute	//
velutinous, granular, floccose	// reverse colour:	//
funiculose, fasciculate, coremiform, synnematous		//
synnemata formed in: dark, light	// 7, 14, 21 days // height: 3, 5, 10, + mm	//
mycelium colour:	// conidiogenesis colour:	//
margins: subsurface (yes / no)	// low, deep // entire, irregular	//
exudate:	// sol. pigment:	//

Microscopic observations

Stipes: length _____ µm, breadth _____ µm //
smooth, rugose, tuberculate // non-septate, septate //

Penicilli: mono-, bi-, ter-, quarter-verticillate // number of apices _____ //

Rami: quantity: _____ // length _____ µm, breadth _____ µm //
divergent, appressed // spath, vesic // smooth, rugose //

Metulae: quantity: _____ // length _____ µm, breadth _____ µm //
divergent, appressed // spath, vesic // smooth, rugose //

Phialides: quantity: _____ // length _____ µm, breadth _____ µm //
ampulliform, acerose, cylindroidal //
collula: short, average, long // truncate, tapered //

Conidia: length _____ µm, breadth _____ µm //
smooth, rugose, verrucose, spinose, striate //
chains, disordered columns //
spheroid, subspheroid, ellipsoid, cylindroid, fusiform, pyriform, apiculate //

Ascocarp: diameter _____ µm // no. of weeks before formation _____ //
CYA, MEA // rare, abundant // colour _____ //
gymno initials: swollen // hypha: cylindrical // cell: helical, coiled //

Asci: length _____ µm, breadth _____ µm // singly, chains //

Ascospores: length _____ µm, breadth _____ µm //
colour: _____ //
flanged 2,4 // smooth, rugose, spinose, striate //
spheroidal, subspheroidal, ellipsoidal //

Dimensions

Stipes		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Rami		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Metulae		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Phialides		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

Conidia		Length	X	Breadth	X
	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
Averages					

APPENDIX D

Table D.1: Average number of fungal isolates obtained as surface colonizers from *ligushe* and *ditaka* collected from households and schemes within two villages and from a stall in the town.

	Village A				Village B				Giyani Town		
	Schemes		Households		Schemes		Households		Stall		
	Ligushe	*Ditaka*	*Ligushe*	*Ditaka*[a]	*Ligushe*	*Ditaka*	*Ligushe*[a]	*Ditaka*[a]	*Ligushe*	*Ditaka*	Total
F. vertillioides	0	0	1	-	4	6	-	-	2	0	13
F. proliferatum	0	8	3	-	0	1	-	-	0	0	12
F. subglutinans	1	0	0	-	1	0	-	-	0	0	2
F. oxysporum	0	0	0	-	1	1	-	-	0	0	2
F. cf. *camptoceras*	0	0	1	-	1	0	-	-	0	0	2
F. chlamydosporum	0	0	0	-	1	0	-	-	0	0	1
F. equiseti	1	1	0	-	1	1	-	-	0	0	4
F. lateritium	0	0	0	-	1	0	-	-	0	0	1
F. semitectum	0	0	0	-	3	0	-	-	1	0	4
F. solani	0	0	0	-	3	1	-	-	0	0	4
Fusarium spp.	1	0	1	-	3	1	-	-	0	0	6
Total *Fusarium* isolates	**3**	**9**	**6**	**-**	**19**	**11**	**-**	**-**	**3**	**0**	**61**
Acremonium spp.	10	11	5	-	15	9	-	-	1	2	53
Alternaria spp.	1	1	2	-	2	0	-	-	1	0	7
Aspergillus spp.	1	0	0	-	1	1	-	-	0	0	3
Penicillium spp.	0	1	0	-	2	2	-	-	1	2	8
Other[b]	9	2	11	-	7	2	-	-	3	6	40
Total number of isolates	**24**	**24**	**24**	**-**	**46**	**25**	**-**	**-**	**9**	**10**	**162**

[a] did not sample

[b] refers to other identified and unidentified isolates and contaminants (section5.3.1)

Table D.2: Relative number of fungal isolates obtained as interior colonisers from fresh leaves of *ligushe* and *ditaka* plants collected from VA, VB and GT.

	Ligushe				*Ditaka*			
	MSR[a] 1	**MSR[a] 2**	**MSR[a] 3**	**MSR[a] 4**	**MSR[a] 1**	**MSR[a] 2**	**MSR[a] 3**	**MSR[a] 4**
Time of exposure to sterilant (s)	**20**	**30**	**45**	**60**	**20**	**30**	**45**	**60**
Acremonium spp.	0	0	0	0	0	0	0	0
Alternaria spp.	22	5	4	3	2	1	1	2
Aspergillus spp.	0	0	1	0	1	0	1	0
Aureobasidium spp.	1	2	0	1	3	0	1	2
Fusarium spp.	0	0	0	0	0	0	0	0
Penicillium spp.	0	2	0	0	1	0	0	1
Phoma spp.	7	6	2	1	0	1	1	0
Other[b]	18	3	5	3	3	3	0	0
Total number of isolates	**48**	**18**	**12**	**8**	**10**	**5**	**4**	**5**

[a] refers to modified sterilisation regime

[b] refers to other identified and unidentified isolates and contaminants (section 5.3.2)

Table D.3: Total number of fungal isolates obtained from dried and cooked samples of *dinawa, ligushe* and *ditaka* type *morogo.*

	Dried samples			Cooked samples		
	Dinawa	***Ligushe***[a]	***Ditaka***[a]	***Dinawa***	***Ligushe***	***Ditaka***
F. equiseti	0	-	-	0	3	2
F. graminum	0	-	-	0	0	1
F. oxysporum	0	-	-	0	0	1
F. reticulatum	0	-	-	0	1	0
F. thapsinum	0	-	-	0	0	1
F. vertillioides	0	-	-	0	1	0
Total *Fusarium* isolates	**0**	**-**	**-**	**0**	**5**	**5**
Acremonium spp.	0	-	-	0	0	0
Alternaria spp.	3	-	-	0	0	0
Aspergillus spp.	4	-	-	0	0	0
Aureobasidium spp.	6	-	-	0	0	0
Penicillium spp.	7	-	-	0	0	0
Phoma spp.	1	-	-	0	0	0
Other[b]	9	-	-	0	5	2
Total number of isolates	**30**	**-**	**-**	**0**	**10**	**7**

[a] did not sample
[b] refers to unidentified isolates and contaminants (section 5.3.3)

APPENDIX E

OUTPUTS AS PART OF THE *MOROGO* RESEARCH PROGRAM (MRP)

CONFERENCES – PARTICIPANTS

- *International Round Table: Health, Food and Environmental – exploring IKS and its interfaces with other knowledge systems. 28 November – 1 December 2005. Cairo, Egypt.*

(i) Traditional African vegetables can combat hunger, disease and malnutrition in rural sub-Saharan Africa. HJ Vorster, WS Jansen van Rensburg, **SD Jivan**, AS Raedani, JAL Denner, P Modjadji and AM van der Walt. Poster presentation.

- *Pan African Environmental Mutagen Society Fifth International Meeting (PAEMS 2005). 24 – 26 November 2005. Fez, Morocco.*

(i) Molecular characterisation of potential fumonisin-producing *Fusarium verticillioides* isolated from traditional leafy vegetables (*morogo*) of southern Africa. CC Bezuidenhout, M Prinsloo, BG Dreyer, TP Taje, **SD Jivan** and AM van der Walt. Poster presentation.

- *Cape to Cairo Conference (satellite conference of the "18th International Congress of Nutrition". September 2005. North-West University, Potchefstroom.*

(i) Potential fumonisin B group-producing fusaria in traditional African vegetables. AM van der Walt, **SD Jivan**, E van der Linde and CC Bezuidenhout. (Oral presentation).

(ii) Molecular studies of potential fumonisin-producing *Fusarium verticillioides* isolated from traditional leafy vegetables (*morogo*). CC Bezuidenhout, **SD Jivan**, M Prinsloo, G Dreyer, TP Taje and AM van der Walt. (Poster presentation).

- *Biodiversity: Science and Governance. 24 – 28 January 2005. Paris, France.*

(i) Initiative for the Development of Indigenous Food-plants of Africa (IDIFA) – exploring uses, risks and benefits of traditional African crops. AM van der Walt, CC Bezuidenhout, **SD Jivan** and B Bouwman. (Poster presentation).

(ii) Diversity of potential mycotoxin-producing fungal species associated with traditional *morogo*. CC Bezuidenhout, AM van der Walt and **SD Jivan**. Poster presentation.

- *Indigenous Knowledge Systems (IKS) Colloquium. 29 February – 2 March 2004. University of the Free State, Bloemfontein.*

(i) Indigenous African food-plants: Vehicles for disease or sources of protection? AM van der Walt, **SD Jivan**, KSA Mossanda and CC Bezuidenhout.

PUBLICATIONS – SCIENTIFIC

(i) AM van der Walt, E van der Linde, M Alberts, P Modjadji, **SD Jivan** and CC Bezuidenhout. March 2006. Fumonisin-producing *Fusarium* strains and fumonisins in traditional vegetables (*morogo*). *South African Journal of Science*. Vol 102(3/4).

(ii) AM van der Walt, KSA Mossanda, **SD Jivan** and WJ Swart. June 2005. Indigenous African food plants: Vehicles of disease or sources of protection. *INDILINGA – African Journal of Indigenous Knowledge Systems*. Vol 4(1).

APPENDIX F