

**DISTRIBUTION AND QUANTIFICATION OF
FUSARIUM VERTICILLIOIDES IN SOUTH
AFRICAN MAIZE AND ITS EFFECT ON GRAIN
QUALITY AND TOXICITY**

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ABSTRACT

Maize is the most important cereal crop produced in southern Africa. Worldwide, *Fusarium verticillioides* and *F. proliferatum* are the most commonly reported fumonisin-producing fungi to infect maize. Both are important producers of fumonisins that are associated with animal mycotoxicoses. *F. verticillioides*-infected maize has also been associated with human oesophageal cancer in South Africa, northern Italy and Iran. Increased incidence of liver cancer has also been reported from certain endemic areas of the People's Republic of China due to ingestion of *F. verticillioides*-infected maize. The carcinogenic risk that fumonisins pose to humans was evaluated by the World Health Organisation's - International Agency for Research on Cancer (WHO-IARC). They were classified as Group 2B carcinogens, which means that they are probably carcinogenic to humans. This potential threat highlights the necessity for screening human and animal foodstuffs for fumonisins. A primary concern in evaluating potential health risks associated with mycotoxin-contaminated foodstuffs is the reliability of fumonisin detection methods. A number of detection methods have been developed, but results are not consistent when compared. Substantial mycotoxin research has been carried out but high variation in mycotoxin results and species identification confounded statistical analysis of data. The aim of this study was to identify and address sources of variation in the quantification of species identification and fumonisin quantification. Chapter 1 provides a general overview of the importance of maize as a primary crop for subsistence, resource-poor and commercial farmers and the potential threat fumonisins pose to the safety of humans and animals. The objective of chapter 2 was to determine whether fumonisin levels in milled maize samples increase or decrease over time prior to testing for fumonisins. Grain samples from Sannieshof, Ventersdorp and Lichtenburg was evaluated for fumonisin levels every two months for a year. High variation in fumonisin levels quantified with the same samples was observed over time, indicating sources of variation which needs to be studied. Chapter 3 addressed sources of variation associated with sampling and improving sampling procedures to reduce this variation. Maize kernel samples were selected from five localities with high fumonisin levels. These samples were used for investigating the following four sources of variation, namely 1) subsample size (increasing from 25 g to 1000 g, 2) establish variation of fumonisins within a single maize sample, 3) number of

replications (5 to 25) using prescribed 25 g subsamples and modified 250 g subsamples and 4) variation between laboratories and techniques for fumonisin detection. In chapter 4 the incidence of *F. verticillioides*, *F. subglutinans*, *F. proliferatum* and fumonisins in maize from warmer production areas of South Africa was evaluated to determine 1) the incidence and geographic spread of *Fusarium* pathogens from maize grain silos in South Africa, 2) to study the relationship between isolation frequency, fumonisin incidence and ergosterol concentration and 3) to establish the relationship between fumonisin levels and weather parameters. Chapter 5 aimed to 1) determine *Fusarium* spp. variation in maize samples, 2) compare the accuracy of *Fusarium* spp. identification on rose bengal-glycerine-urea (RbGU) as selective and identification medium with split plates containing standard Carnation Leaf (CLA) & Potato Dextrose Agars (PDA) as identification media and 3) detect and quantify potential bias among enumerators (inter-enumerator reliability) in direct microscopic identifications of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. Reducing variation in quantification of fumonisins and improving *Fusarium* spp. identification on maize kernels, would increase confidence and accuracy in this technology. This will improve the value of research on this topic. Improved accuracy in detection and quantification of fumonisins will also contribute directly to the establishment of realistic and safe tolerance levels for this toxin in basic and processed foodstuffs. This in turn is important in ensuring food safety for humans and animals.

OPSOMMING

Mielies is die belangrikste graangewas wat deur kommersiële en bestaansboere in suidelike Afrika geproduseer word. *Fusarium verticillioides* en *F. proliferatum* is fungusse wat mielies wêreldwyd die meeste infekteer. Die fungusse is belangrike fumonisienproduseerders en word geassosieer met gevalle van mikotoksikose by diere. Mielies wat met *F. verticillioides* besmet is, word ook statisties geassosieer met slukdermkanker by mense. Sulke gevalle is aangeteken in Suid Afrika, noordelike Italië en Iran. 'n Verhoogde voorkoms van lewerkanker is ook aangeteken in sekere endemiese gebiede in die Republiek van China nadat *F. verticillioides*-besmette mielies vir voedsel gebruik is. Die Wêreld Gesondheid Organisasie se Internasionale Agentskap vir Navorsing op Kanker (WGO-IANK) het fumonisiene geklassifiseer as Groep 2B-karsinogene. Dit beteken dat fumonisiene waarskynlik karsinogenies is vir mense. Dit is dus uiters belangrik om voedsel vir mens en dier noukeurig te evalueer vir die voorkoms van fumonisiene. 'n Belangrike komponent in die kwantifisering van die gesondheidsrisiko wat geassosieer word met mikotoksien-gekontamineerde voedsel, is die betroubaarheid van die verskillende mikotoksientoetse wat beskikbaar is. 'n Verskeidenheid mikotoksientoetse is tans beskikbaar, maar resultate varieer wanneer dit met mekaar vergelyk word. Tot op hede is daar 'n aansienlike hoeveelheid mikotoksien navorsing gedoen, maar die hoë variasie in mikotoksientoetse, sowel as fungusspesie-identifikasies belemmer statistiese ontleding van data. Die doel van die studie was om bronne van variasie in die kwantifisering van fungusspesie-identifikasie en fumonisienkwantifikasie te identifiseer en om dit aan te spreek. Hoofstuk 1 gee 'n algemene oorsig oor die belangrikheid van mielieproduksie as voedselbron, sowel as die gevaar wat fumonisiene inhou vir die veiligheid van mens en dier. Die doel van hoofstuk 2 was om vas te stel of fumonisienvlakke in gemaalde meelmonsters toeneem of afneem oor tyd voordat dit getoets word vir fumonisiene. Graan vanaf Sannieshof, Ventersdorp en Lichtenburg was elke twee maande vir 'n jaar lank getoets vir fumonisiene. Baie variasie in fumonisienvlakke het oor die tydperk plaasgevind en die bronne van variasie moet nog vasgestel word. Hoofstuk 3 spreek die variasie wat geassosieer word met monsterneming aan deur bronne van variasie te identifiseer en metodes van monsterneming te verbeter in 'n poging om variasie te verminder. Monsters van vyf lokaliteite met hoë fumonisienvlakke is geselekteer en aangewend om

vier bronne van variasie te bestudeer, naamlik 1) submonstergrootte (vermeerder van 25 g na 1000 g), 2) variasie van fumonisien vlakke binne 'n enkele mieliemonster, 3) aantal herhalings benodig (5 tot 25) deur gebruik te maak van voorgestelde 25 g submonsters en aangepaste 250 g submonsters en 4) variasie tussen laboratoriums en tegnieke vir fumonies kwantifisering/bepaling. In hoofstuk 4 word die voorkoms van *F. verticillioides*, *F. subglutinans* en *F. proliferatum* in mielies van verskillende warm mielieproduksie gebiede geëvalueer om te bepaal 1) wat die voorkoms en geografiese verspreiding van *Fusarium*-patogene van mieliegraan in silos in Suid Afrika is, 2) om vas te stel of daar 'n ooreenkoms is tussen fungusplaattellings, fumonisienproduksie en ergosterolkwantifisering is en 3) om die verhouding tussen fumonisienvlakke en weerparameters vas te stel. In hoofstuk 5 word daar gepoog om 1) *Fusarium*-spesie-variasie in mieliemonsters te bepaal, 2) om die akkuraatheid van *Fusarium*-spesie-identifikasies op roos bengal-gliserien-ureum (RbGU) as 'n isolasie- en identifikasie-medium vir mieliegraan-*Fusariums* te vergelyk met plate wat beide Angelier Blaar Agar (ABA) sowel as Aartappel Dekstrose Agar (ADA) bevat en 3) om bevooroordeelheid tussen opsommers (tussen-opsommerbetroubaarheid) vas te stel deur mikroskopiese identifikasie van *F. verticillioides*, *F. subglutinans* en *F. proliferatum*. Deur al die bogenoemde probleme te bestudeer om variasie in die kwantifikasie van fumonisiene te verminder, en *Fusarium*-spesie-identifikasies te verbeter, kan navorsing met vertroue en akkuraatheid uitgevoer word. Verhoogde akkuraatheid in die opsporing en kwantifisering van fumonisiene sal regstreeks bydra tot die daarstelling van realistiese en veilige verdraagsaamheidsvlakke van die toksien in basiese sowel as verwerkte kos. Dit is uiters belangrik om voedselveiligheid vir mens en dier te verseker.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Maize production in South Africa

Maize (*Zea mays* L.) is grown worldwide and is an important component of the diet of millions of people due to relatively high yields, ease of cultivation, adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Fandohan *et al.*, 2003). Maize is the most important cereal crop produced by resource-poor farmers in southern Africa and an increase in population pressure has resulted in an intensification of land use (Ofori & Kyei-Baffour, 2006). Total estimated maize production by commercial farmers in South Africa in 2004 was 4.3 million tonnes according to the Food and Agriculture Organization (FAO) (Anonymous, 2002). The final production estimate for maize in South Africa for the 2003/2004 season by the National Crops Estimates Committee was 9 482 000 tons (Anonymous, 2004e).

The area planted to maize by the developing sector in South Africa for 2003/04 was estimated at 360 810 ha (228 070 tonnes white and 57 180 tonnes yellow maize), which amounts to ± 0.8 tonnes/ha (Anonymous, 2004a). No data could be found for the subsistence farming community. According to Ofori & Kyei-Baffour (2006) several African countries have focused attention on increasing maize production, but efforts have been ineffective due to heavy pre- and post-harvest losses caused by diseases, weeds and pests. Factors that may contribute to the poor yield within the developing sector can be insufficient rainfall, a lack of inputs such as fertilizers, improved seeds, irrigation and labour (Anonymous, 2004e). Lepidopterous stemborers that infest maize cause significant yield losses in east and southern Africa (Van den Berg, 2005), which increase fungal infections and toxin production (Munkvold & Desjardins, 1997).

Maize grown by the developing sector contributes to approximately 4% of South Africa's national production (Anonymous, 2004a). The developing agricultural sector which relies on maize as a staple food is highly vulnerable to yield losses, poor grain quality

resulting in nutrient loss, increased fungal infections and possible fumonisin toxicosis when maize products are consumed.

Bankole & Adebajo (2003) reported that bacteria constitute the greatest hazard to human food safety, followed by mycotoxins, while the latter poses the greatest threat to livestock feeds. Fandohan *et al.* (2003) consider insects to be the primary cause of grain loss followed by fungi. Fungi could cause 50-80% of the damage to farmers' maize during storage, should conditions be favourable for their development (Fandohan *et al.*, 2003). Major fungal genera encountered on maize in tropical and subtropical regions are *Fusarium*, *Aspergillus*, and *Penicillium* (Turner *et al.*, 1999, Orsi *et al.*, 2000) where it is common for *Aspergillus* and *Penicillium* spp. to co-infect with *Fusarium* spp. (Bush *et al.*, 2003).

1.2 Importance of *Fusarium* species on maize

The genus *Fusarium* includes economically important plant pathogens that cause billions of dollars of losses worldwide each year (Jurgenson *et al.*, 2002). *F. verticillioides* and *F. proliferatum* are the most commonly reported fumonisin-producing fungi infecting maize worldwide (Shepard *et al.*, 1996, Cotten & Munkvold, 1998) and produce over 100 secondary metabolites that adversely affect human and animal health (Bankole & Adebajo, 2003). *Fusarium* species can cause seedling blight, root and crown rot, stalk rot and ear rot on maize (King & Scott, 1981; Glenn, 2005). *F. verticillioides* (synonym: *F. moniliforme*) is considered a major parasite of the Gramineae, particularly in tropical and subtropical regions, resulting in severe economic losses (Kpodo *et al.*, 2000). *F. verticillioides* also occurs on rice and sugarcane and Bacon *et al.* (1996) calculated that more than 11 000 plant species may serve as a host for this fungus. *F. moniliforme sensu* Snyder & Hansen encompass strains in species other than *F. verticillioides*. The name *F. verticillioides* should be used only for strains that have the *G. moniliformis* teleomorph. Because of the abovementioned nomenclature confusion, and lack of understanding that there were more than one species in older species definition it is difficult to determine the true causal agent in many cases, and therefore pathogenic associations need to be re-evaluated to confirm

that they are caused by *F. verticillioides* and not by another member of the *G. fujikuroi* complex that used to be included in *F. moniliforme*.

Rheeder *et al.* (2002) reported *F. verticilloides* and *F. proliferatum* to be the most important fumonisin producers due to high levels of fumonisin production, worldwide distribution, frequent occurrence on maize and the association with animal mycotoxicoses. The presence of high levels of fumonisins in maize seeds might have deleterious effects on seedling emergence (Doehlert *et al.*, 1994). Rheeder *et al.* (2002) reported up to 17.9 mg/g fumonisin from isolates of *F. verticillioides* from South Africa and 31.0 mg/g from isolates of *F. proliferatum* from Spain. *F. subglutinans* also frequently infects maize worldwide, but is known to produce low fumonisin levels (Rheeder *et al.*, 2002).

According to Marasas (2001) fumonisins are produced by several *Fusarium* species:

- F. verticillioides* (Sacc.) Nirenberg (synonym: *F. moniliforme* Sheldon)
- F. proliferatum* (Matsushina) Nirenberg
- F. nygamai* Burgess & Trimboli
- F. anthophilum* (Braun) Wollenweber
- F. dlamini* Marasas, Nelson & Toussoun
- F. napiforme* Marasas, Nelson & Rabie
- F. thapsinum* Klittich, Leslie, Nelson & Marasas
- F. globosum* Rheeder, Marasas & Nelson
- F. subglutinans* (Wollenw. et Reinking) Nelson

Economic effects of *F. verticillioides* impact on all sectors involved in the production and consumption of maize products. Maize producers are negatively affected by limited yields, restricted markets and price discounts. Maize traders are affected by restricted storage options, costs of testing grain lots and loss of markets. Processors incur higher

costs due to product losses, monitoring costs and restricted end-markets. Consumers pay higher product prices due to increased monitoring at all levels of handling and, in extreme cases may have to cope with health problems due to consumption of contaminated products. Society as a whole ends up paying a higher cost due to increased regulations, necessary research, lower export costs and higher import costs (Anonymous, 1999). It is, however subsistence farmers that pay the highest price due to a lack of knowledge of toxin-producing *Fusarium spp.* and are more prone to exposure to significant levels of fumonisins because maize production is primarily for own consumption (Desjardins, 2006).

1.3 Taxonomy

F. verticillioides, *F. proliferatum* and *F. subglutinans* are grouped in *Fusarium* section *Liseola*, based on morphological characteristics (Nelson *et al.*, 1983). The anamorph species *F. verticillioides* corresponds to mating population A (Kuhlman, 1982; Leslie, 1995) and F (Munkvold & Desjardins, 1997), *F. proliferatum* corresponds to mating population C or D (Desjardins, 2006), and *F. subglutinans* corresponds to mating population B or E (Leslie, 1992; Cotten & Munkvold, 1998). Mating type A population contain many prolific fumonisin-producing strains, while F population strains produce little or no fumonisin.

Based on the structure in or on which conidiogenous hyphae are borne, *Fusarium spp.* are classified under the Hyphomycetidae subclass of the Deuteromycetes (Agrios, 1997). *F. verticillioides* and *F. proliferatum* have small, hyaline microconidia that are abundant and primarily single-celled, oval to club shaped and have a flattened base (Glenn, 2005). Microconidia of *F. verticillioides* and *F. proliferatum* are abundantly produced in long, catenate chains (Figure 1) developing on phialides (Nirenberg, 1990; Glenn, 2005). The length of chains increase as KCl concentrations in water agar increase making these chain-forming species difficult to identify on the basis of chain length alone (Fisher *et al.*, 1983). Spore chains developing on polyphialides separates *F. proliferatum* from *F. verticillioides*, which produce monopialides (Nelson *et al.*, 1983).

The main difference between *F. subglutinans* and *F. verticillioides* is the absence of microconidial chains and the presence of polyphialides in *F. subglutinans*. Conidia of *F. subglutinans* are abundant, oval and usually single-celled, but may be one- to three-septate. Microconidia are produced only in false heads (Figure 2).

Macroconidia in *F. verticillioides* are present but are according to Nelson *et al.* (1983) sometimes rare. Macroconidia vary from slightly sickle-shaped to almost straight, with the dorsal and ventral surfaces almost parallel and they have thin, delicate walls. Basal cells are foot-shaped, chlamyospores are absent (Nirenberg & O'Donnell, 1998) and the perfect state is known as *Gibberella fujikuroi* (Sawada) Wollenw. Macroconidia are abundant in *F. proliferatum* and are slightly sickle-shaped to almost straight, with the dorsal and ventral surfaces parallel for most of the length of the macroconidium. Basal cells are foot-shaped and chlamyospores are absent. The perfect stage for *F. proliferatum* is *G. intermedia* (Leslie & Summerell, 2006). Macroconidia of *F. subglutinans* are abundant, slightly sickle-shaped to almost straight with the dorsal and ventral surfaces almost parallel and with thin delicate walls. The basal cell is foot-shaped and chlamyospores are absent (Nelson *et al.*, 1983).



Figure 1 Microconidia of *F. verticillioides* and *F. proliferatum* are abundantly produced in long catenate chains.

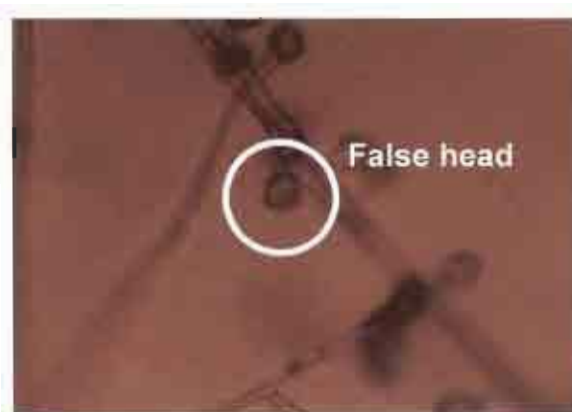


Figure 2 Microconidia of *F. subglutinans* produced in false heads.

1.4 Symptomless infection

F. verticillioides is one of the most common fungi that occurs without producing symptoms in seeds of maize and teosinte (Desjardins *et al.*, 2005). The endophytic nature of *F. verticillioides* is typical for a number of species within the genus *Fusarium* (Bacon *et al.*, 2001). Endophytic fungi are classified by Bacon *et al.* (2001) as "intercellular infections that are at least transiently symptomless but are functionally relevant to the association as a viable, growing, and biochemically important component". Endophytic fungi actively colonise host tissues and establish long-term associations with the host, without disease symptoms being observed for extended periods of time (Jardine & Leslie, 1999). Detection and control of endophytic infections in maize are difficult because kernels appear to be sound (Figure 3). Symptomless infection of kernels are often frequent, but fumonisin levels could be very low (Bush *et al.*, 2003). Presence of fumonisins in visually sound maize intended for human consumption supports the hypothesis by Bacon *et al.* (2001) that low concentrations of fumonisins are synthesised by symptomless, endophytic fungi. Endophytic hyphae of *F. verticillioides* are neither latent or dormant but are important in seed and plant infection (Bacon *et al.*, 2001). Endophytic hyphae act as a reservoir from which infection of each generation of plants take place and serves as a source of renewed toxin synthesis *in planta* (Bacon *et al.*, 2001).



Figure 3 Maize ear appearing healthy (left) and one with typical symptoms of *Fusarium* infection (right) (Source: Fandohan *et al.*, 2003).

1.5 Disease cycle

1.5.1 Inoculum

F. verticillioides can be transmitted to uninfected plants by inoculum from field stubble (Munkvold & Desjardins, 1997) or airborne conidia (micro- and macro conidia) abundant in maize fields during a growing season. The relative importance of silk infection, insect-assisted infection and systemic infection is likely influenced by the availability of inoculum for each type of infection (Munkvold *et al.*, 1996).

1.5.2 Infection

F. verticillioides has a saprophytic as well as parasitic stage and infects maize at all stages of plant development, either via the silk channel, infected seed, or wounds (Reid *et al.*, 1999).

1.5.2.1 Silk and kernel infection

The most commonly reported method of kernel infection is through airborne or water-splashed conidia that land on the silks (Oren *et al.*, 2003). The exact conditions that favour silk infection are not known, but infection is enhanced by maintaining moisture on the silks (Munkvold & Desjardins, 1997). Once there, the spores germinate and infect the silks, especially as they turn green-brown and brown (Vincelli & Parker, 2002). The fungus then grows down the silk channel and among the developing kernels. According to Vincelli & Parker (2002) green silks are relatively resistant to infection and colonization, whereas green-brown and brown silks can be colonized by the fungus.

1.5.2.2 Seed infection

Another proposed infection pathway by Oren *et al.* (2003) is systemically through the seed. Systemic infection can start from fungal conidia or mycelia that are either carried inside the seeds or on the seed surface. The fungus develops inside the young plant and moves from the roots to the stalk and finally to the cob and kernels (Munkvold & Desjardins 1997). Mature maize kernels may also be infected after sowing, by soilborne inoculum penetrating fissures in the pericarp, or at germination where the pericarp is torn by the emerging seedling (Galperin *et al.*, 2003).

1.5.2.3 Infection through wounds

Feeding activities of lepidopterous insects may spread *F. verticillioides* spores to silks, kernels and stems and the feeding channels are then colonized by the fungus (Vincelli & Parker, 2002). Birds causing physical injury to stalks and ears are also suspected to promote infections by *Fusarium* spp. (Papst *et al.*, 2005).

1.5.3 Colonization (growth and reproduction)

F. verticillioides often persists as a symptomless endophyte, systemically colonising all plant tissues, including kernels (Bacon *et al.*, 2001, Galperin *et al.*, 2003). Oren *et al.* (2003) showed that systemic growth of *F. verticillioides* occurs in maize seedlings within 10 days, but relatively little fungal biomass develops at this stage. Bacon *et al.* (2001) suggests that conditions that favour symptomless infections result in fungal growth that is restricted to root and mesocotyl tissues.

Transmission of *F. verticillioides* from maize seed to kernels of the same plant can be divided into four steps: 1) transmission from seed to seedling, 2) ramification within the stalk, 3) ramification into the ear and 4) spread within the ear (Munkvold & Desjardins, 1997).

1.5.4 Inoculum dispersal

Small, hyaline mostly single celled microconidia are abundantly produced and are well adapted for wind, rain and vectoral dispersal (Glenn, 2005). Insects can act as wounding agents, spreading the microconidia from origin of inoculum to plants. Wounding of insect may provide an opportunity for the fungus to establish infection sites on the host plant (Fandohan *et al.*, 2003).

1.5.5 Survival

F. verticillioides overwinters on stalks partially buried in the soil, only to re-infect plants in the growing season. Infected stalks are known to be major overwintering sites (Payne, 1999) and can be a long-term source of *F. verticillioides* inoculum for infection of maize plants (Cotten & Munkvold, 1998). *F. verticillioides* does not produce chlamydospores, but can produce thickened hyphae that apparently prolong its survival (Desjardins & Munkvold, 1997).

1.6 Fumonisin

Mycotoxins are toxic secondary metabolites (Miller, 2001) of fungal origin that when ingested, inhaled or absorbed through the skin could cause reduced performance, sickness or death in humans and animals (Bankole & Adebajo, 2003). Mycotoxicoses are diseases caused by the ingestion of foods or feeds that are toxic due to high levels of mycotoxins (Nelson *et al.*, 1993). Many mycotoxigenic *Fusarium* species are aggressive pathogens of agricultural plants and can cause mycotoxin contamination of cereal grains or other plant-based foods (Desjardins, 2006). Desjardins (2006) reported that the three major classes of *Fusarium* mycotoxins are the fumonisins, trichothecenes and zearalenones, with a few less important mycotoxins such as beauvericin, fusaproliferin, fusarins and moniliformin. According to Desjardins (2006), fumonisin production has only been identified in *Fusarium* species but closely related compounds are produced by *Alternaria* spp. The ability to produce fumonisins appears to be absent from the *F. solani* species complex as well as from all the other trichothecene-producing species (Rheeder *et al.*, 2002, Leslie *et al.*, 2004).

Fumonisin B¹, B² and B³ were first isolated in 1988 by South African researchers (Bezuidenhout *et al.*, 1988) from cultures of *F. verticillioides* strain MRC 826 (Marasas, 2001). These three B-series fumonisins account for the majority of fumonisins that occur in grain samples naturally contaminated with *F. verticillioides*, *F. proliferatum* and

other potential fumonisin-producing *Fusarium* species.

1.6.1 Occurrence and distribution of fumonisins

Fumonisin B¹ accounts for 70 to 80% of total fumonisins produced, while B² usually makes up 15 to 25 and B³, 3 to 8% (Dilkin *et al.*, 2002; Rheeder *et al.*, 2002). The distribution of fumonisins is global and their presence has been confirmed in at least twenty five countries (Mazzani *et al.*, 2001). Damaged, *Fusarium*-rotted kernels typically contain higher fumonisin levels than intact, healthy grain (Vincelli & Parker, 2002).

According to Nelson *et al.* (1993) some of the factors involved in field outbreaks of mycotoxicoses caused by plant pathogenic fungi are:

- 1) the infection of a susceptible host plant by a mycotoxin-producing fungus,
- 2) environmental and other factors favourable for the development of the disease,
- 3) genetic capability of the pathogen to produce a metabolite or metabolites that are harmful to animals or humans,
- 4) environmental and other conditions favourable for the production and accumulation of sufficient quantities of toxic metabolites in the diseased plant to cause toxicosis to the consumer
- 5) and the consumption of sufficient quantities of toxin-containing plant material by a susceptible consumer.

1.6.2 Factors affecting fumonisin production

Fungal growth as well as mycotoxin production result from the complex interaction of several factors. An understanding of the factors involved is essential for understanding

the overall process and to predict and prevent mycotoxin development (Velluti *et al.*, 2000).

1.6.2.1 Plant senescence

Miller (2001) and Bacon *et al.* (2001) suggested that fumonisin is produced in senescent maize tissue or during saprophytic fungal growth on damaged or dead tissue (Bacon *et al.*, 2001). Fumonisin *per se* would not be particularly stable or biologically active within actively growing maize tissue (Miller, 2001).

1.6.2.2 Physiological stress factors

Physiological stresses such as drastic variations in rainfall and relative humidity prior to harvesting are likely to create favourable conditions for fumonisin production (Visconti, 1996). Drought, rather than temperature stress increases levels of fumonisins (Miller, 2001). Low soil moisture content, high day maximum temperatures, high night minimum temperatures and nutrient-deficient soils are only some of the interacting factors that stress maize plants and cause fungal growth of *F. verticillioides* and toxin production (Abbas *et al.*, 2006). Marin *et al.* (1995) studied the effects of different temperatures and pH on *F. verticillioides* and *F. proliferatum* growth and fumonisin production and concluded that both species increased as moisture and temperature increased. *F. verticillioides* grew better at pH 7.0 and 30°C, whereas *F. proliferatum* grew better at pH 5.5 and 25°C.

1.6.2.3 Insect damage

Drought stress results in greater insect herbivory and a strong relationship between insect damage and *Fusarium* ear rot has been reported (Ajanga & Hillocks, 2000; Miller, 2001). *F. verticillioides* growth in a maize plant causes the release of volatiles that

attract lepidopterous and coleopteran pests, thereby encouraging infestation of plants by these pests (Cardwell *et al.*, 2000; Schulthess *et al.*, 2002). Insect feeding on ears renders grain more susceptible to *F. verticillioides* and *A. flavus*, resulting in possibly higher fumonisin and aflatoxin levels in field and storage grain (Hell *et al.*, 2000).

1.6.2.4 Infection by other pathogens

Maize ears infected by pathogens such as *F. graminearum* may be predisposed to *F. verticillioides* infection and fumonisin accumulation (Miller, 2001).

1.6.3 The chemical structure of fumonisins

The active component in fractionated maize cultures of *F. verticillioides* strain MRC 826 was designated fumonisin B¹ (Figure 4a) and was shown to be a di-ester of propane-1,2,3-tricarboxylic acid and a 2-amino-12,16-dimethyl, 3,5,10,14,15-pentahydroxyicosane with both C-14 and C-15 hydroxy groups esterified with the terminal carboxy group of the acids (Nelson *et al.*, 1993; Barna-Vetró, 2000). Fumonisins B² and B³ (Figure 4b & 4c) are homologues that lack one of the three hydroxyl groups on the backbone. B² lacks the hydroxyl at C-10 while fumonisin B³ lacks the hydroxyl at C-5 (Nelson *et al.*, 1993).

1.6.4 Animal mycotoxicology

According to Thiel *et al.* (1991) fumonisins B¹ and B² occur naturally in maize and feeds that are associated with field outbreaks of mycotoxicoses in animals. Although fumonisins have a relatively simple chemical structure, their inhibition of sphingolipid metabolism can have diverse and complex effects in animal systems (Desjardins, 2006). Fumonisins cause leukoencephalomalacia (LEM) in horses (Kellerman *et al.*, 1990; Ross *et al.*, 1990), a brain lesion that can be fatal to horses after only a few days

of consumption of contaminated feed. Fumonisin also causes pulmonary oedema in swine (Harrison *et al.*, 1990) and is hepatotoxic and carcinogenic to rats (Gelderblom *et al.*, 1988).

1.6.5 Human mycotoxicology

F. verticillioides-infected maize has been statistically associated with human oesophageal cancer in South Africa (Marasas *et al.*, 1981; Marasas, 1982; Marasas, 1988; Rheeder *et al.*, 1992), northern Italy (Franseschi *et al.*, 1990) and Iran (Shephard *et al.*, 2000). Chu & Li (1994) and Li *et al.* (2001) reported an increased incidence of primary liver cancer in people that ingest maize infected by *F. verticillioides* in certain endemic areas of The People's Republic of China. Recent studies by Stack (1998) and Placinta *et al.* (1999) have shown a strong correlation between consumption of fumonisin-contaminated tortillas and neural-tube defects in humans. The potential carcinogenic risk of fumonisins to humans was evaluated and classified by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) (Anonymous, 1993) as Group 2B carcinogens which means they are probably carcinogenic to humans. Alberts *et al.* (1990) reported that fumonisin B¹ is not destroyed by cooking and could therefore easily enter the human food chain. This emphasises the importance of screening human and animal foodstuffs for the presence of fumonisins.

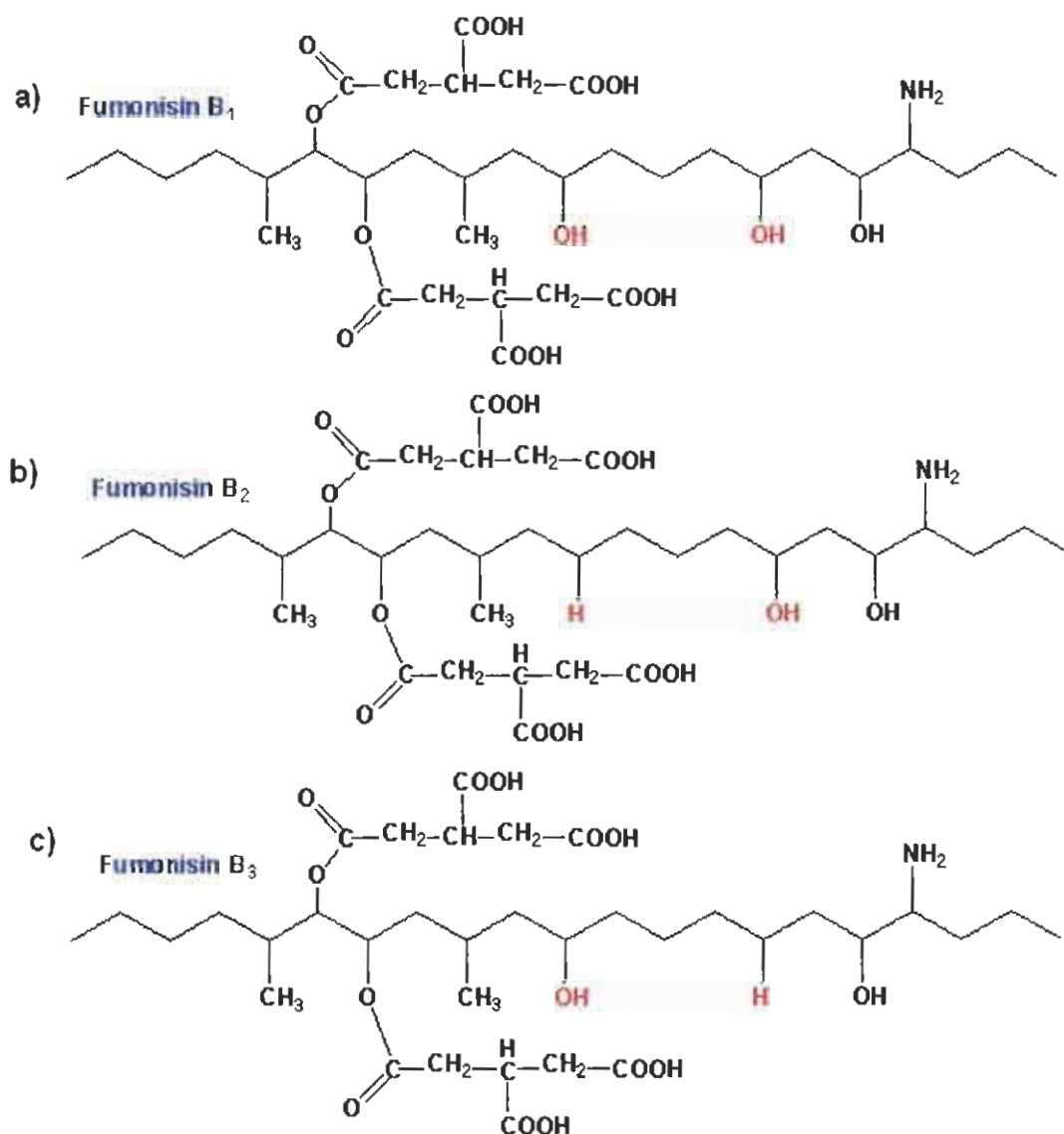


Figure 4 Chemical structure of fumonisins B¹, B² and B³ (Source: Barna-Vetró, 2000).

1.6.6 Analytical detection methods

Several analytical detection methods have been developed for determining fumonisin levels in maize and maize-based foods and feeds. These methods include capillary gas chromatography, thin-layer chromatography (TLC), direct competitive enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis with mass spectrometry and high-performance liquid chromatography (HPLC) (Dilkin *et al.*, 2001). The officially approved method of testing mycotoxins according to the Association of Official Analytical Chemists (AOAC) was initially the TLC technique but, recently HPLC has replaced TLC (Sydenham *et al.*, 1996) because of greater sensitivity. ELISA are quicker and cheaper than HPLC techniques, with accuracy levels being acceptable to the grain processing and feed industries (Dilkin *et al.*, 2001). HPLC is used as reference method to gauge the accuracy of the other tests and is used primarily in laboratories where greater accuracy is required (Anonymous, 1999).

1.6.6.1 Multitoxin and rapid detection methods

Well established methods based on chromatographic principles are still valid, but recently a transition to multitoxin and rapid methods have been observed (Krska, 2006). Analytical detection methods are usually optimized for one target mycotoxin or at best a group of closely related mycotoxins. The use of multitoxin detection methods such as liquid chromatography (LC) with tandem mass spectrometry (LC-MS/MS) enables the simultaneous determination of up to 40 different mycotoxins.

The development of rapid tests are very important for mycotoxin detection, as mycotoxins are present in different kinds of food and feed, and their presence should be controlled at various stages, such as pre- and post-harvest, pre- and post-processing (De Saeger *et al.*, 2006). A number of rapid methods based on immunochemical techniques, mostly do not require any clean-up or analyte enrichment. ELISA has become one of the most useful tools for the rapid monitoring of single target

mycotoxins, especially for the screening of raw materials (Krska, 2006). Dipstick tests (Krska, 2006) as well as flow-through devices (De Seager *et al.*, 2006) and affinity columns have been developed (Powers, 2006) for rapid, multitoxin detection.

1.6.6.2 Molecular detection methods

Detection tools for *Fusarium* spp. and mycotoxins are constantly evolving. Electrical chip technology, molecular biology and advanced immunology can be integrated for fast, cheap and robust detection of mycotoxins as well as the corresponding fungi (Klerks *et al.*, 2006).

1.7 Fumonisin exposure

During 1992 a high incidence of oesophageal cancer was reported in rural populations of the Butterworth and Kentani districts of Transkei (Rheeder *et al.*, 1992), while only 175-200 km away at Bizana and Lusikisiki oesophageal cancer was relatively low. *F. verticillioides* incidence was reported to be greater in home-grown maize collected from high oesophageal cancer- incidence areas compared to low-incidence areas (Rheeder *et al.*, 1992). Maize meal porridge is the staple diet of people in these rural areas and adults consume beer fermented deliberately from visibly rotten and asymptomatic maize kernels (Miller, 2001). Males were found to consume more beer than females, which explains the higher incidence of oesophageal cancer in the male population (Isaacson, 2005). The Cancer Association of South Africa (CANSA) published statistics showing the South African black male population to have an age-standardized incidence rate (ASR) per 100 000 (world standard) of 16.22 and a lifetime risk rate (LR) of developing oesophageal cancer of 1 in 51 people, compared to black women with an ASR rate of 7.33 and a LR rate of 1 in 33 (Anonymous, 1997). Statistics for oesophageal cancer incidence in Asians, white and coloured populations (male and female) were not included because of the low incidences. From this information it is clear that black males were twice more likely to develop oesophageal cancer than black females.

In 2005 Isaacson reported an epidemic "of comparatively recent origin" of squamous cell carcinoma of the oesophagus in black South Africans. Until the 1950's carcinoma of the oesophagus was comparatively rare in the black population. In Johannesburg no cases of oesophageal cancer were diagnosed in black patients from 1912 to 1927 (Isaacson, 1982). Higginson & Oettle (1960) reported 53 cases from 1953 to 1955. During 1960 the histopathology laboratory at Baragwanath Hospital diagnosed 87 cases. From 1966 to 1975 Isaacson (1982) reported 1331 cases with a male: female ratio of 5:1. The current incidence at Baragwanath hospital is more than one case per day (Isaacson, 2005). Isaacson (2005) and Leslie *et al.* (2005) describe the change of staple diet from sorghum and pearl millet to maize during the mid 20th century to be the primary cause of this epidemic.

1.8 Legislation and legal limits

In many parts of the world the continuously increasing human population places higher demands on food supply. The need to eat outweighs considerations such as food safety and health (Bankole & Adebajo, 2003). As food supply becomes limited the mycotoxin hazard increases since more fungus-damaged, potentially mycotoxin-containing foodstuffs are consumed rather than being discarded. Malnutrition enhances susceptibility to lower concentrations of food-borne mycotoxins (Nelson *et al.*, 1993). As previously indicated, the area planted to maize by the local developing sector for 2003/04 was estimated at 360 810 ha (Anonymous, 2004). Thiel *et al.* (1992) estimate that the daily intake of fumonisins via maize-based food in South Africa is 14 mg/kg - 440 mg/kg body weight per day for a population in a high-risk area and 5 mg/kg - 59 mg/kg body weight per day in a low-risk area. Research has shown that maize consumption in developing areas is as high as 4.6 mg/kg per person per day. In contrast the consumption in developed countries is not higher than 0.7 mg/kg per person per day.

During 2004 Shepard (2004) conducted a worldwide maize consumption survey and the rural populations of the Transkei in South Africa were amongst the highest maize

consumers. Due to high consumption and high fumonisin contamination of maize, these communities were exposed to fumonisin levels well in excess of the Tolerable Daily Intake (TDI) of 2 mg/kg body weight/day that was set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). These JECFA guidelines are designed to protect consumers in developed countries where maize consumption is low, but much lower tolerance levels than 2 mg/kg would be required to protect subsistence farming communities that consume maize as a staple diet in developing countries (Shepard, 2004).

It is necessary that safety of food and feed for human and animal consumption be regulated (Bankole & Adebajo, 2003). Internationally agreed legislation on fumonisins is essential to protect public health, avoid trade barriers and competition distortions (Soriano & Dragacci, 2004a). Legal limits is not the only solution to the global fumonisin problem, but should be applied together with control of fungal infections and reduction of fumonisin production in maize grain and products from field-to-fork. Rural communities should also be educated and informed about the health hazard of fumonisins.

1.9 Maximum limit

In certain countries mycotoxin regulations are implemented using a defined maximum limit and a sampling plan to detect and divert mycotoxin-contaminated products from food and feed markets. The dilemma in any standardization process is to decide how low to set the maximum limit. Lowering the maximum limit will reduce contamination in the food and feed markets but increase the amount of product rejected in the testing programme (Whitaker, 2003). Any standardized maximum limit should be low enough to protect the health of the consumer, but not so low as to limit the much needed supply of food. Differences in maximum limits and sampling plans make marketing of commodities on world markets difficult for exporters and importers.

To date there has been no standardized international introduction of fumonisin limits,

but 77 nations have mycotoxin regulations primarily for aflatoxins in both food and feed (Whitaker, 2003). The world community, working through FAO/WHO has acknowledged the need to standardize mycotoxin sampling plans and maximum limits to facilitate international trade and improve consumer protection (Whitaker, 2003). The following need to be considered: method of selecting the sample, sample size, subsample size, degree of sample grind (particle size reduction), type of analytical method including the cost of the method, number of analytical measurements, maximum allowable mycotoxin limits (Whitaker, 2003) as well as trained analysts. According to Soriano & Dragacci (2004a) the European Commission has recommended but not legislated maximum levels for combinations of fumonisins B¹ and B², which range from 2 mg/kg for unprocessed maize to 1 mg/kg for infant food. Guidelines for total fumonisins allowed (B¹+B²+B³) in maize and products in food and animal feeds by the United States Food and Drug Administration (FDA) were drafted based on data from countries such as the United States of America, Canada and Western Europe where human consumption of maize products are modest (Soriano & Dragacci, 2004b).

Although legislative measures may reduce the already low risk associated with fumonisins in many affluent societies where maize is a small component of the diet, research is needed to reduce the exposure of rural communities that rely on home-grown maize as their main dietary staple and are hence at most risk from fumonisins (Shepard *et al.*, 1996).

Establishment of guidelines in South Africa for total fumonisins allowed (B¹+B²+B³) in maize and maize products in food and animal feed is necessary. Shepard (2004) suggests that the Provisional Maximum Tolerable Daily Intake (PMTDI) should be based on detailed knowledge of maize consumption in various populations, taking maize consumption of the local population into account.

1.10 Dry milling and fumonisins

Dry milling of whole maize grain results in production of fractions called bran, flaking

grits, grits, meal and flour. Fumonisin is concentrated in the germ and hull of whole maize kernels, therefore the dry milling process results in fractions with different concentrations of fumonisins. For example, dry milled fractions (except for the bran fraction) obtained from degermed maize kernels contain lower levels of fumonisins than dry milled fractions obtained from non-degermed or partially-degermed maize (Anonymous, 2004b). Dry milling results in fumonisin-containing fractions in descending order of highest to lowest fumonisin levels being; bran, flour, meal, grits, and flaking grits (Anonymous, 2004b). The following guidelines (Table 1) have been issued by the FDA (Anonymous, 2004b) for total fumonisins allowed ($B^1+B^2+B^3$) in foodstuffs:

Table 1 Guidelines set by the FDA (USA) for total fumonisins allowed ($B^1+B^2+B^3$) in foodstuffs (Anonymous, 2004b).

Degermed dry milled maize product	2 ppm
Whole/partly degermed, dry-milled maize product	4 ppm
Dry-milled maize bran	4 ppm
Cleaned maize intended for popcorn	3 ppm
Cleaned maize for mass production	4 ppm

The following guidelines (Table 2) for total fumonisins allowed ($B^1+B^2+B^3$) in maize and maize products in food and animal feeds has also been issued by the FDA (Anonymous, 2004c):

Table 2 Guidelines set by the FDA (USA) for total fumonisins allowed ($B^1+B^2+B^3$) in maize and maize products in food and animal feeds (Anonymous, 2004c).

Humans	2 ppm
Equids and rabbits	5 ppm; no more than 20 % of diet
Swine	20 ppm; no more than 50 % of diet
All other livestock species and pet animals	10 ppm; no more than 50 % of diet

Switzerland is the only country that has set a regulatory limit for fumonisins in maize-based foods (Visconti *et al.*, 1999). Current human exposure estimates (Shephard, 2004) or risk assessments, proposed for South Africa regarding mycotoxin legislation are effective to regulate grain produced by commercial farmers, and protect consumers of these products. Subsistence farmers that consume home-grown maize as a staple diet are classified by Visconti *et al.* (1999) as high risk, and have no regulation systems in place. Even if legislation was set in place it would not be considered particularly when drought and famine conditions occur and subsistence farmers are forced to eat their produce whether it is contaminated or not. Fungal contamination and mycotoxins therefore remain a serious threat for subsistence and small farmers.

Asymptomatic infections are easily overlooked by subsistence farmers and mycotoxins may pose a serious health risk to consumers. Farmers who plant second-generation seed saved from the previous crop may aggravate infections as the fungus survives as a pathogen and saprophyte, thus providing an inoculum source for the new crop. Raising awareness levels among these communities would be the only way of attempting to alleviate the undesirable consequences of consuming fumonisin-contaminated maize.

1.11 Grain sampling problems

Mycotoxin analysis requires representative and practical sampling methods. Sampling procedure and preparation prior to analysis are the least considered steps in solving analytical problems despite the fact that they may result in large sources of error (Garfield, 1989). Correct sampling procedures are important as mycotoxins are generally heterogeneously distributed in contaminated goods (Brera & Miraglia, 1996; Stroka *et al.*, 2004). A small percentage of the kernels within a sample may be contaminated but one contaminated kernel may have extremely high mycotoxin-levels (Whitaker & Wiser, 1969), which may indicate false positive results as the infected kernel was not representative of the total sample. The greatest effect of variation associated with the analysis process is in the sampling procedure (Stroka *et al.*, 2004).

An incorrect sampling plan affects all further quantification activities, up to the final result, due to higher coefficients of variation (Brera & Miraglia, 1996). Studies by Garfield (1989) and Vincelli & Parker (2002) show that 90% of variability in test results comes from sampling variation. Park & Pohland (1989) identified three factors that increase an accurate and precise estimate of the true mycotoxin concentration of a given component in a sample viz: 1) sampling, 2) sample preparation and 3) analysis. The preparation of the final test portion must maintain the representativeness of the original sample. The ideal would be to sample equal portions at random points throughout the entire sample, with a sufficient number of points sampled (Park & Pohland, 1989). According to Whitaker (2003) increasing sample size, sub-sample size and number of analytical measurements (replicates) will reduce variation associated with mycotoxin test results and thus reduce the buyer's and seller's risk. Despite existence of sophisticated analytical instrumentation used to quantify fumonisin levels in foods, accurate exposure assessment is problematic, making study design and representative sampling of utmost importance in epidemiological studies (Turner *et al.*, 1999). The USA FDA (Anonymous, 2005) have recommended sample sizes for fumonisin quantification in maize (Table 3).

Table 3 Recommended sample sizes set by the FDA for fumonisin quantification in maize (Anonymous, 2005).

Product	Package type	Number of sample units*	Unit size (minimum)	Total sample size (minimum)
Maize-shelled, meal flour or grits	Consumer or bulk	10	450 g	4.5 kg

*To be collected from as many random sites in the sample as possible.

The problem with this recommendation is that the entire sample size of a grainload, silo or container is not taken into consideration. The fumonisin level in a bulk sample is generally estimated by measuring fumonisins in a small portion of the sample (Whitaker, 2004). It is then assumed that the bulk sample concentration is the same than the small portion concentration and decisions about the bulk sample are based upon the sample value. If a total sample size of 4.5 kg is taken from a 45 000 kg bulk sample, the quantity that would be inspected will be increased by a factor of 10 000 at the sampling step. The 4.5 kg sample is then milled and only 25 g (ELISA technique) is removed for quantification of fumonisins. The quantity of the product inspected is further reduced by a factor of 180 000. Finally only 5 g of the 25 g milled product is represented in the solvent mixture. In this example only 5 g out of the original 45 000 000 g is used to estimate the fumonisin concentration in the bulk sample. Obviously the larger the bulk sample, the greater the chances of not having a final sample that is representative of the bulk sample when only 4.5 kg is taken. The total sample size should increase with the bulk sample size to reduce error (Whitaker, 2003).

1.12 CONCLUSIONS

Fungal infection and concomitant mycotoxin contamination of maize grain constitutes a threat to the food safety and security of millions of people in Africa who are dependent

on this crop as a major food source. *Fusarium* spp. are the most important mycotoxin-producing fungi that infect maize worldwide. *F. verticillioides* and *F. proliferatum* are isolated most frequently from maize kernels, including those that appear healthy (Visconti *et al.*, 1999). These species cause *Fusarium* kernel rot of maize, which is one of the most important ear diseases in warm maize growing areas and is primarily associated with warm, dry weather and/or insect damage (Visconti *et al.*, 1999). Factors such as geographical region, season, environmental conditions, insect infestation, pre- and post- harvest handling and interaction with other fungi affect the production of fumonisins (Fandohan *et al.*, 2003). Moisture level and temperature are important factors regulating the growth of *F. verticillioides* and *F. proliferatum* and the production of fumonisins. Information on the minimum, optimum and maximum temperature for fumonisin production is uncertain. According to Alberts *et al.* (1990) the best temperature range for fumonisin production is 20-28°C. Leslie & Summerell (2006) reported that production of fumonisins is improved at $\geq 15^{\circ}\text{C}$.

Globally *F. verticillioides* is the dominant species isolated from grain associated with field outbreaks of leukoencephalomalacia of equines and pulmonary oedema of swine (Marasas, 2001). Fumonisins were found to be carcinogenic to rodents and consumption of grain contaminated with fumonisins has been epidemiologically associated with human diseases, particularly oesophageal cancer. Cancer of the oesophagus occurs worldwide, but incidence rates are significantly higher in certain geographical areas and ethnic groups in Africa, Asia and Latin America (Desjardins, 2006). Subsistence farmers who consume home grown maize have the highest maize intakes and also consume maize with the highest levels of fumonisin contamination. These people are at the highest risk for mycotoxin exposure and are least protected or educated in this regard.

This study aimed at evaluating the natural incidence of *F. verticillioides*, *F. subglutinans* and *F. proliferatum* at different maize production localities in the warmer areas of South Africa, by collecting 50 kg maize grain samples from various silos. Fungal plate isolations and identifications based on visual morphological differences were carried out. Because fungal plate isolations are time-consuming and not applicable to meal

samples (Schwadorf & Müller, 1989), ergosterol was quantified to determine a possible relationship between fungal biomass and toxin production. Minimum temperature, maximum temperature and rainfall from weather stations closest to each silo location, were collected from November 2003 to July 2004. Data were analysed to determine any possible relationship between fumonisin production and the abovementioned weather parameters.

Accurate measurement of fumonisins is essential for establishing the safety of maize or its products for consumption. Vincelli (2002) found that 90% of differences in measured toxin levels result from sample variation. Several sensitive and accurate analytical methods are currently available for toxin-detection, but their results may differ. Variation in detection levels was increased by small grain samples as well as inaccurate evaluation techniques, which may produce false positive and/or negative results. Currently the ELISA-Veratox protocol is used by the Agricultural Research Council - Grain Crops Institute because it is quicker and cheaper than HPLC, making it easier for wide application in grain processing and feed industries. HPLC takes longer but is much more sensitive (Sydenham *et al.*, 1996) and is used as reference method to gauge the accuracy of other tests.

To date substantial mycotoxin research using the ELISA-Veratox protocol has been carried out, but high variation in toxin tests and species identification confound statistical analysis of data. Studies were therefore conducted to determine sources of variation in order to improve sampling methods and reduce variation. This was done by studying three sources of variation: subsample size, number of replications and variation between laboratories. This study also aimed to determine if fumonisin levels will increase or decrease in milled maize samples over time. If the variation of the overall fumonisin test procedure can be reduced, the lot mycotoxin concentration can be estimated with improved confidence and accuracy (Whitaker *et al.*, 2003).

REFERENCES

- Abbas, H.K., Cartwright, R.D., Xie, W. & Shier, W.T. 2006. Aflatoxin and fumonisin contamination of corn (maize, *Zea mays*) hybrids in Arkansas. *Crop Protection* **25**: 1-9.
- Agrios, G.N. 1997. *Plant pathology*. Fourth Edition. Academic Press. 635pp.
- Ajanga, S. & Hillocks, R.J. 2000. Maize cob rot in Kenya and its association with stalk borer damage. *Crop Protection* **19**: 297-300.
- Alberts, J.F., Gelderblom, W.C.A., Thiel, P.G., Marasas, W.F.O., Van Scalkwyk, D.J. & Behrend, Y. 1990. Effects of temperature and incubation period on production of fumonisin B¹ by *Fusarium moniliforme*. *Applied and Environmental Microbiology* **56**: 1729-1733.
- Anonymous. 1993. WHO-IARC. Toxins derived from *Fusarium moniliforme*: Fumonisin B¹ and B² and fusarin. C. Lyon (F): IARC
- Anonymous. 1997. Cancer association of South Africa - Cancer statistics. [Available on Internet:] www.givengain.com/unique/cansa/documents/cancer_%20statistics_1997.pdf [Date of access: 28 September 2006].
- Anonymous. 1999. USDA Grain fungal diseases and mycotoxin reference. [Available on Internet:] www.usda.gov/gipsa/pubs/mycobook.pdf [Date of access: 4 June 2004].
- Anonymous. 2002. FAOSTAT Database, Food and Agriculture Organisation, Roma, Italy. [Available on Internet:] <http://apps.fao.org/lim500/nph-wrap.pl>. [Date of access: 11 February 2005].
- Anonymous. 2004a. Trends in the agricultural sector. Printed and published by the Department of Agriculture, Pretoria. 66pp.

Anonymous. 2004b. The Food and Drug Administration food and cosmetic compliance program: Mycotoxins in imported food. [Available on Internet:] <http://www.cfsan.fda.gov/~comm/cp07002.html> [Date of access: 27 February 2006].

Anonymous. 2004c. The Food and Drug Administration regulatory guidance for toxins and contaminants. [Available on Internet:] <http://www.ngfa.org/toxinsPDF-1.pdf> [Date of access: 11 February 2005].

Anonymous. 2004e. Famine early systems warning network. South Africa food security brief. [Available on Internet:] <http://www.fews.net/centers/innerSections.aspx?f=r3&m=1001213&pageID=monthliesDoc> [Date of access: 16 October 2006].

Anonymous. 2005. The Food and Drug Administration Investigations Operations Manual: Mycotoxin sample sizes. [Available on Internet:] http://www.fda.gov/ora/inspect_ref/iom/ChapterText/sschedule6.html [Date of access: 28 February 2006].

Bacon, C.W., Porter, J.K., Norred, W.P. & Leslie, J.F. 1996. Production of fusaric acid by *Fusarium* species. *Applied Environmental Microbiology* **62**: 4139-4143.

Bacon, C.W., Yates, I.E., Hinton, D.M. & Meredith, F. 2001. Biological control of *Fusarium moniliforme* in maize. *Environmental Health Perspectives* **109**: 325-332.

Bankole, S.A. & Adebajo, A. 2003. Mycotoxins in food in West Africa: current situation and possibilities of controlling it. *African Journal of Biotechnology* **2**: 254-263.

Barna-Vetró, L. 2000. Development of a sensitive ELISA for the determination of fumonisin B1 in cereals. *Journal of Agricultural Food Chemistry* **48**: 2821-2825.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.F.O., Spiteller, G. & Vleggaar, R. 1988. Structure elucidation of the fumonisins,

mycotoxins from *Fusarium moniliforme*. *Journal of the Chemistry Society for Chemical Communications*: **4**: 743-745.

Brera, C. & Miraglia, M. 1996. Quality assurance in mycotoxin analysis. *Microchemical Journal* **54**: 465-471.

Bush, B.J., Carson, M.L., Cubeta, M.A., Hagler, W.M. & Payne, G.A. 2003. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. *Phytopathology* **94**: 88-93.

Cardwell, K.F., Kling, J.G., Maziya-Dixon, B. & Bosque-Pérez, N.A. 2000. Interactions between *Fusarium verticillioides*, *Aspergillus flavus*, and insect infestation in four maize genotypes in lowland Africa. *Phytopathology* **90**: 276-284.

Cotten, T.K. & Munkvold, G.P. 1998. Survival of *Fusarium moniliforme*, *F. proliferatum*, and *F. subglutinans* in maize stalk residue. *Phytopathology* **88**: 550-555.

Chu, F.S. & Li, G.Y. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Applied and Environmental Microbiology* **60**: 847-852.

De Saeger, S, Yu, I., Goryacheva, B., & Van Peteghem, C. 2006. Flow-through device for rapid, simultaneous detection of multiple mycotoxins. Proceedings of the Myco-Globe International Conference, Monopoli, Bari, Italy, 26-29 September.

Desjardins, A.E., Plattner, R.D., Stressman, R.J., McCormick, S.P. & Millard, M. 2005. Identification and heritability of fumonisin insensitivity in *Zea mays*. *Phytochemistry* **66**: 2474-2480.

Desjardins, A.E. 2006. *Fusarium* mycotoxins. Chemistry, genetics and biology. The American Phytopathological Society. APS Press. 260pp.

Dilkin, P., Mallmann, C.A., de Almeida, C.A.A. & Corrêa, B. 2001. Robotic animated clean-up for detection of fumonisins B¹ and B² in corn and corn-based feed by high performance liquid chromatography. *Journal of Chromatography* **925**: 151-157.

Dilkin, P., Mallmann, C.A., de Almeida, C.A.A., Stefanon, E.B., Fontana, F.Z. & Milbradt, E.L. 2002. Production of fumonisins by strains of *Fusarium moniliforme* according to temperature, moisture and growth period. *Brazilian Journal of Microbiology* **33**: 111-118.

Doehlert, D.C., Knutson, C.A. & Vesonder, R.F. 1994. Phytotoxic effects of fumonisin B¹ on maize seedling growth. *Mycopathologia* **127**: 117-121.

Fandohan, P., Hell, K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology* **2**: 570-579.

Fisher, N.L., Marasas, W.F.O. & Toussoun, T.A. 1983. Taxonomic importance of microconidial chains in *Fusarium* section *Liseola* and effects of water potential on their formation. *Mycologia* **75**: 693-698.

Franseschi, S., Bidoli, E., Baron, A. E. & La Vecchia, C. 1990. Maize and risk of cancers of the oral cavity, pharynx and esophagus in Northeastern Italy. *Journal of National Cancer Institute* **82**: 1407-1411.

Galperin, M., Graf, S. & Kenigsbuch, D. 2003. Seed treatment prevents vertical transmission of *Fusarium moniliforme*, making a significant contribution to disease control. *Phytoparasitica* **31**: 344-352.

Garfield, F.M. 1989. Sampling in the analytical scheme. *Association of Official Analytical Chemists* **72**: 405-411.

Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, M.J., Vleggaar, R. & Kriek, N.P.J. 1988. Fumonisin - novel mycotoxins with cancer promoting activity produced by *Fusarium moniliforme*. *Applied Environmental Microbiology* **54**: 1806-1811.

Glenn, A.E. 2005. Natural variation of ascospore and conidial germination by *Fusarium verticillioides* and other *Fusarium* species. *Mycological Research* **110**: 211-219.

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E. & Cole, J.R. 1990. Pulmonary edema and hydrothorax in swine produced by fumonisin B¹, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation* **2**: 217-221.

Hell, K., Cardwell, K.F., Sétamou, M. & Schulthess, F. 2000. Influence of insect infestation on aflatoxin contamination of stored maize in four agroecological regions in Benin. *African Entomology* **8**: 169-177.

Higginson, J. & Oettle, A.G. 1960. Cancer incidence in the bantu and Cape coloured races of South Africa. Report of a cancer survey in the Transvaal. *Journal of the National Cancer Institute* **24**: 589-671.

Isaacson, C. 1982. Pathology of a black African population. Current topics in pathology. Berlin: Springer Verlag. pp 88-91.

Isaacson, C. 2005. The change of the staple diet of black South Africans from sorghum to maize (corn) is the cause of the epidemic squamous carcinoma of the oesophagus. *Medical Hypotheses* **64**: 658-660.

Jardine, D.J. & Leslie, J.F. 1999. Aggressiveness to mature maize plants of *Fusarium* strains differing in ability to produce fumonisin. *Plant Disease* **83**: 690-693.

Jurgenson, J.E., Zeller, K.A. & Leslie, J.F. 2002. Expanded genetic map of *Gibberella moniliformis* (*Fusarium verticillioides*). *Applied and Environmental Microbiology* **68**: 1972-1979.

Kellerman, T.S., Marasas, W.F.O., Thiel, P.G., Gelderblom, W.C.A., Cawood, M. & Coetzer, J.A.W. 1990. Leukoencephalomalacia in two horses induced by oral dosing of fumonisins B¹. *Onderstepoort Journal of Veterinary Research* **57**: 269-275.

King, S.B. & Scott, G.E. 1981. Genotypic differences in maize to kernel infection by *Fusarium moniliforme*. *Phytopathology* **71**: 1245-1247.

Krska, R. 2006. Emerging rapid methods for the determination of mycotoxins. Proceedings of the Myco-Globe International Conference, Monopoli, Bari, Italy, 26-29 September.

Klerks, M., Beekwilder, J., van Houwelingen, A., van Gent-Peltzer, M., de Vries, I. & Waalwijk, C. 2006. EU-BIOSENSE, electrical bio-sensor arrays for the analyses of mycotoxins and toxigenic fungi. Proceedings of the European Fusarium Seminar, Wageningen, the Netherlands, 19-22 September.

Kpodo, K., Thrane, U. & Hald, B. 2000. *Fusaria* and fumonisins in maize from Ghana and their co-occurrence with aflatoxins. *International Journal of Food Microbiology* **61**: 147-157.

Kuhlman, E.G. 1982. Varieties of *Gibberella fujikuroi* with anamorphs in *Fusarium* section *Liseola*. *Mycologia* **74**: 759-768.

Laurent, D., Pellegrin, F., Kohler, F., Costa, R., Thevenon, J., Lambert, C. & Huerre, M. 1989. La fumonisine B¹ dans la pathogenie de la leucoencephalomalacie equine. *Microbiologie Aliments Nutrition* **7**: 285-291.

Leslie, J.F., Plattner, R.D., Desjardins, A.E. & Klittich, C.J.R. 1992. Fumonisin B¹ production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section *Liseola*). *Phytopathology* **82**: 341-345.

Leslie, J.F. 1995. *Gibberella fujikuroi*: Available populations and variable traits. *Canadian Journal of Botany* **73**: 282-291.

Leslie, J.F., Zeller, K.A., Logrieco, A., Mulè, G., Moretti, A. & Ritieni, A. 2004. Species diversity of and toxin production by *Gibberella fujikuroi* species complex strains isolated from native prairie grasses in Kansas. *Applied Environmental Microbiology* **70**: 2254-2262.

Leslie, J.F., Zeller, K.A., Lampbrecht, S.C., Rheeder, J.P. & Marasas, W.F.O. 2005. Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from Sorghum and Millet. *Phytopathology* **95**: 275-283.

Leslie, J.F. & Summerell, B.A. 2006. *The fusarium laboratory manual*. Blackwell Publishing. 386pp.

Li, F.Q., Yoshizawa, T., Kawamura, O., Luo, X.Y. & Li, Y.W. 2001. Aflatoxins and fumonisins in corn from the high incidence area for human hepatocellular carcinoma in Guangxi, China. *Journal of Agriculture and Food Chemistry* **49**: 4122-4126.

Marasas, W.F.O., Wehner, F.C., Van Rensburg, S.J. & Van Schalkwyk, D.J. 1981. Mycoflora of corn produced in human oesophageal cancer areas in Transkei, Southern Africa. *Phytopathology*: **71**: 792-796.

Marasas, W.F.O. 1982. Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. In: Pfeiffer, C.J. (Ed.). *Cancer of the Osophagus*. CRC Press Inc, Boca Raton, Florida. p29-40.

Marasas, W.F.O., Jaskiewicz, K., Venter, F.S. & Van Schalkwyk, D.J. 1988. *Fusarium moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *South African Medical Journal* **74**: 110-114.

Marasas, W.F.O. 2001. Discovery and occurrence of fumonisins: a historical perspective. *Environmental Health Perspectives* **2**: 239-243.

Marin, S., Sanchis, V. & Magan, N. 1995. Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Canadian Journal of Microbiology* **41**: 1063-1070.

Mazzani, C., Borges, O., Luzón, C., Barrientos, V. & Quijada, P. 2001. Occurrence of *Fusarium moniliforme* and fumonisins in kernels of maize hybrids in Venezuela. *Brazilian Journal of Microbiology* **32**: 345-349.

Miller, J.D. 2001. Factors that affect the occurrence of fumonisin. *Environmental Health Perspectives* **109**: 321-324.

Munkvold, G.P., McGee, D.C. & Carlton, W.M. 1996. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. *Phytopathology* **87**: 209-217.

Munkvold, G.P. & Desjardins, A.E. 1997. Fumonisins in maize. Can we reduce their occurrence? *Plant Disease* **81**: 556-564.

Nelson, P.E., Tousson, T.A. & Marasas, W.F.O. 1983. *Fusarium species* : An illustrated manual for identification. The Pennsylvania State University Press, University Park and London. 193pp.

Nelson, P.E., Desjardins, A.E. & Plattner, R.D. 1993. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annual Review of Phytopathology* **31**: 233-252.

Nirenberg, H.I. 1990. Recent advances in the taxonomy of *Fusarium*. *Studies in Mycology* **32**: 91-101.

Nirenberg, H.I. & O'Donnell, K. 1998. New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex. *Mycologia* **90**: 434-458.

Ofori, E. & Kyei-Baffour, N. 2006. Agrometeorology and maize production. [Available on Internet:] <http://www.wmo.ch/web/wcp/agm/RevGAMP/chap13C-draft.pdf> [Date of access: 16 October 2006].

Oren, L., Ezrati, S., Cohen, D., & Sharon, A. 2003. Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Applied and Environmental Microbiology* **69**: 1695-1701.

Orsi, R.B., Corrêa, B., Possi, C.R., Schammass, E.A., Nogueira, J.R., Dias, S.M.C. & Malozzi, M.A.B. 2000. Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *Journal of Stored Products Research* **36**: 75-87.

Papst, C., Utz, H.F., Melchinger, A.E., Eder, J., Magg, T., Klein, D. & Bohn, M. 2005. Mycotoxins produced by *Fusarium* spp. in isogenic Bt vs. non-Bt maize hybrids under european corn borer pressure. *Agronomy Journal* **97**: 219-224.

Park, D.L. & Pohland, A.E. 1989. Sampling and sample preparation for detection and quantification of natural toxicants in food and feed. *Association of Official Analytical Chemists* **72**: 399-403.

Payne, G.A. 1999. Ear and kernels rots. In: White, D.G. (Ed.), *Compendium of corn diseases*. Third Edition. The American Phytopathological Society. 78pp.

Placinta, C.M., D'Mello, J.P.F. & Macdonald, A.M.C. 1999. A review of worldwide contamination of cereal grains in animal feed with *Fusarium* mycotoxins. *Animal Feed Science Technology* **78**: 21-37.

Powers, S. 2006. Development and application of multi-mycotoxin affinity columns. Vicam, Watertown. Proceedings of the Myco-Globe International Conference, Monopoli, Bari, Italy, 26-29 September.

Reid, L.M., Nicol, R.W., Ouellet, T., Savard, M., Miller, J.D., Young, J.C., Stewart, D.W. & Schaafsma, A.W. 1999. Interaction of *Fusarium graminearum* and *F. moniliforme* in maize ears: disease progress, fungal biomass, and mycotoxin accumulation. *Phytopathology* **89**: 1028-1034.

Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Syndeham, E.W., Shepard, G.S. & Van Schalkwyk, D.J. 1992. *Fusarium moniliforme* and fumonisins in corn in relation to human esophageal cancer in Transkei. *Phytopathology* **82**: 353-357.

Rheeder, J.P., Marasas, W.F.O. & Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology* **68**: 2101-2105.

Ross, P.F., Nelson, P.E., Richard, J.L., Osweiler, G.D., Rice, L.G., Plattner, R.D. & Wilson, T.M. 1990. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Applied Environmental Microbiology* **56**: 3225-3226.

Schulthess, F., Cardwell, K.F. & Gounou, S. 2002. The effect of endophytic *Fusarium verticillioides* on infestation of two maize varieties by Lepidopterous stemborers and Coleopteran grain feeders. *Phytopathology* **92**: 120-128.

Schwadorf, K. & Müller, H.M. 1989. Determination of ergosterol in cereals, mixed feed components, and mixed feeds by liquid chromatography. *Association of Official Analytical Chemists* **72**: 457-462.

Shepard, G.S., Thiel, P.G., Stockenstrom, S. & Syndeham, E.W. 1996. Worldwide survey of fumonisin contamination and corn-based products. *Association of Official Analytical Chemists* **79**: 671-686.

Shepard, G.S., Marasas, W.F.O., Leggott, N.L., Yazdanpanah, H., Rahimian, H., & Safavi, N. 2000. Natural occurrence of fumonisins in corn from Iran. *Journal of Agriculture and Food Chemistry* **48**: 1860-1864.

Shephard, G.S. 2004. Mycotoxins worldwide: Current issues in Africa. In Barug, D., Van Egmond, H.P., López Garcíá, R., Van Osenbruggen, W.A. & Visconti, A. (Eds) *Meeting the mycotoxin menace*. Wageningen Academic Publishers, the Netherlands. pp. 81-88.

Soriano, J.M. & Dragacci, S. 2004a. Intake, decontamination and legislation of fumonisins in foods. *Food Research International* **37**: 367-374.

Soriano, J.M. & Dragacci, S. 2004b. Occurrence of fumonisins in food. *Food Research International* **37**: 985-1000.

Stack, M.E. 1998. Analysis of fumonisin B¹ and its hydrolysis product in tortillas. *Journal of Analytical Chemistry* **81**: 737-740.

Stroka, J., Spanjer, M., Buechler, S., Barel, S., Kos, G. & Anklam, E. 2004. Novel sampling methods for the analysis of mycotoxins and the combination with spectroscopic methods for the rapid evaluation of deoxynivalenol contamination. *Toxicology Letters* **153**: 99-107.

Sydenham, E.W., Shepard, G.S., Thiel, P.G., Stockenström, S., Snijman, P.W. & Van Schalkwyk, D.J. 1996. Liquid chromatographic determination of fumonisins B1, B2 and B3 in corn: UAPAC/AOAC interlaboratory collaborative study. *Association of Official Analytical Chemists* **79**: 688-696.

Thiel, P.G., Marasas, W.F.O., Sydenham, E.W., Shepard, G.S., Gelderblom, W.C.A. & Nieuwenhuis, J.J. 1991. Survey of fumonisin production by *Fusarium* species. *Applied and Environmental Microbiology* **57**: 1089-1093.

Thiel, P.G., Marasas, W.F., Sydenham, E.W., Shepard, G.S. & Gelderblom, W.C. 1992. The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **117**: 3-9.

Turner, P.C., Nikiema, P. & Wild, C.P. 1999. Fumonisin contamination of food: progress in development of biomarkers to better assess human health risks. *Mutation Research* **443**: 81-93.

Van den Berg, J. 2005. "Push-pull" strategies for management of stemborers in South Africa. [Available on Internet:] http://southafrica.push-pull.net/ppsa_sa.html [Date of access: 8 September 2006].

Velluti, A., Marin, S., Bettucci, L., Ramos, A.J. & Sanchis, V. 2000. The effect of fungal competition on colonization of maize grain by *Fusarium moniliforme*, *F. proliferatum* and *F. graminearum* and on fumonisin B¹ and zearelanone formation. *International Journal of Food Microbiology* **59**: 59-66.

Vincelli, P. & Parker, G. 2002. Fumonisin, vomitoxin, and other mycotoxins in corn produced by *Fusarium* fungi. [Available on Internet:] <http://www.ca.uky.edu>. [Date of access: 11 February 2005].

Visconti, A. 1996. Fumonisin in maize genotypes grown in various geographic areas. In: Jackson, L.S., de Vries, J.W. & Bullerman, L.B. (Eds.). Fumonisin in food. Plenum Press, New York. p193-204.

Visconti, A., Marasas, W.F.O., Miller, J.D. & Riley, R. 1999. Fumonisin. Third joint FAO/WHO/UNEP international conference on mycotoxins. Tunisia. [Available on Internet:] <http://venetoagricoltura.regione.veneto.it/archive/00000048/01/myco5a.pdf> [Date of access: 17 October 2006].

Whitaker, T.B. & Wiser, E.H. 1969. Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. *Journal of American Oil Chemists' Society* **46**: 377-379.

Whitaker, T.B. 2003. Standardization of mycotoxin sampling procedures: an urgent necessity. *Food Control* **14**: 233-237.

Whitaker, T.B. & Johansson, A.S. 2005. Sampling uncertainties for the detection of chemical agents in complex food matrices. *Journal of Food Protection* **68**: 1306-1313.

CHAPTER 2

THE ROLE OF MAIZE MEAL STORAGE ON FUMONISIN LEVELS

2.1 INTRODUCTION

Maize (*Zea mays* L.) plays an important role in the diet of people and livestock worldwide. *Fusarium verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg are two of the most commonly isolated fungi infecting maize kernels (Fandohan *et al.*, 2003). Both have the ability to produce fumonisins, which adversely affect humans and animals. The potential carcinogenic risk of fumonisins to humans was evaluated and classified by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) (Anonymous, 1993) as Group 2B carcinogens which means they are probably carcinogenic to humans. Alberts *et al.* (1990) reported that FB¹ is not destroyed by cooking and could therefore easily enter the human food chain. This necessitates the importance of screening human and animal foodstuffs for the presence of fumonisins.

A major concern in quantifying health risk associated with mycotoxin contaminated foodstuffs is the time frame within receiving a maize meal sample in the laboratory and testing for fumonisin levels. A significant amount of literature is available on the role of maize grain storage on fumonisin levels, but little information is available on the role of maize meal storage on fumonisin levels.

Where analytical tests for fumonisin contamination cannot be carried out within 12 to 14 hours after receiving a grain sample, the sample should be dried to below 16% moisture level before storage (Vincelli & Parker, 2002). When a grain sample with a high moisture content is kept for an extended period before being tested, the fungi already present in the grain sample can continue to develop and may produce mycotoxins in the sample (Vincelli & Parker, 2002). Consequently incorrect sample storage procedures could also be an important source of variation in the analysis of fumonisins (Vincelli & Parker, 2002).

Laboratories often receive milled maize samples for fumonisin quantification, which are stored prior to testing. The purpose of this study was to determine fumonisin levels, in milled maize samples, over time in storage prior to being tested.

2.2 MATERIALS AND METHODS

Based on fumonisin concentrations determined for grain samples from 23 localities, grain from three localities (Sannieshof, Ventersdorp and Lichtenburg) were selected to study effects of meal storage in the laboratory on fumonisins. Grain (750 g) from each locality was drawn using the European Mycotoxin Awareness Network (EMAN) criteria (Anonymous, 2005). Each sample was separately milled and stored in a brown paper bag in an air-conditioned laboratory at an approximate temperature of 25°C. At the end of every second month, six replications of 25 g aliquots of maize meal from each of the three localities were drawn for fumonisin testing using the ELISA-Veratox protocol (Anonymous, 2006) and the mean fumonisin value was calculated. Data were analysed using multifactor ANOVA and means were separated using Fisher's LSD (Statgraphics Version 5.0).

2.3 RESULTS

A significant interaction recorded between storage time and locality from which samples were obtained ($P \geq 0.01$). Grain samples from Lichtenburg showed an increase in fumonisin levels from 1.9 to 3.8 ppm from March to July with a subsequent decrease to 2.6 ppm from July to November (Table 2.1). Similar results were obtained for the Sannieshof sample with an initial increase in fumonisin levels from 0.7 ppm to 2.5 ppm from May to July, and a decrease to 1.3 ppm in November. Ventersdorp grain showed little fluctuation with fumonisin levels ranged from 1.3 - 1.8 ppm over time.

2.4 DISCUSSION

Fumonisin concentrations may increase during planting, harvesting and during the storage process (Orsi *et al.*, 2000; Ngoko *et al.*, 2001; Fandohan *et al.*, 2006) leading

to variation in fumonisin data (Scott *et al.*, 1999). In this study fumonisins increased initially, but decreased over time in two of the samples. This is in line with the findings of Gelderblom *et al.* (2002, PROMEC, Medical Research Council, Tygerberg, South Africa, personal communication) who reported that maize meal samples stored at 4°C in air-tight tin containers with a screw cap over 13-20 years, lost approximately 30% of total FB¹. Fandohan *et al.* (2006) reported a significant decrease in *Fusarium* incidence in maize cobs stored from 0 - 8 months in bamboo granaries, platforms, mud silos or a grain storage house. A general decrease in fumonisin levels was observed in all storage systems over their storage study period, but was only significant in the bamboo granary. The fumonisin levels of maize stored on platforms initially decreased (0 - 4 months) but later increased slightly. In contrast to these findings, Ngoko *et al.* (2001) reported from Cameroon that the incidence of *Fusarium* spp. isolated from maize kernels and fumonisin concentrations in infected maize kernels increased after a four month storage period. Maize was harvested manually, dried, shelled and stored using traditional methods. HPLC fumonisins analyses conducted after two months of storage yielded a mean FB¹ concentration of 3.5 ppm and after four months of storage a mean concentration of 10.4 ppm.

The Ventersdorp sample that showed little variation in fumonisin levels, could not be explained and needs further study.

The significant locality x storage time interaction could be due to a number of factors known to influence fumonisin levels such as moisture levels, insect infestation, temperature variation, fungal incidence as well as milled versus whole maize kernels (Fandohan *et al.*, 2003).

Future research is needed to establish the effect that moisture, insect infestation, temperature and fungal incidence will have on stored grain and milled maize. Previous work has been done on storage of grain in different storage systems, the effect of storage of milled maize and fumonisin levels over time still needs to be researched. Fluctuations in fumonisin levels can also be contributed to the fact that small sub-samples of 25 g were used, creating variation in fumonisin levels. Therefore greater

sub-samples sizes will be applied in future research to reduce variation of fumonisin levels and more accurately determine increases and or decreases in fumonisin levels over time.

REFERENCES

- Alberts, J.F., Gelderblom, W.C.A., Thiel, P.G., Marasas, W.F.O., Van Scalkwyk, D.J. & Behrend, Y. 1990. Effects of temperature and incubation period on production of fumonisin B₁ by *Fusarium moniliforme*. *Applied and Environmental Microbiology* **56**: 1729-1733.
- Anonymous. 1993. WHO-IARC. Toxins derived from *Fusarium moniliforme*: Fumonisin B¹ and B² and fusarin. C. Lyon (F): IARC
- Anonymous, 2005. European Mycotoxin Awareness Network. Fact sheets on sampling and legislation. [Available on Internet:] http://193.132.193.215/eman2/fsheet6_3.asp [Date of access: 19 October 2006].
- Anonymous, 2006. Veratox Quantitative fumonisin test instructional pamphlet. Neogen Corporation, 620 Leshar Place, Lansing, MI 48912.
- Fandohan , P., Hell,K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in africa. *African Journal of Biotechnology* **2**: 57-579.
- Fandohan, P., Gnonlonfin, B., Hell, K., Marasas, W.F.O & Wingfield, M.J. 2006. Impact of indigenous storage systems and insect infestation on the contamination of maize with fumonisins. *African Journal of Biotechnology* **5**: 546-552.
- Ngoko, Z., Marasas, W.F.O., Rheeder, J.P., Shepard, G.S., Wingfield, M.J. & Cardwell, K.F. 2001. Fungal infection and mycotoxin contamination of maize in the humid forest and the western highlands of Cameroon. *Phytoparasitica* **29**: 352-360.
- Orsi, R.B., Corrêa, B., Possi, C.R., Schammas, E.A., Nogueira, J.R., Dias, S.M.C. & Malozzi, M.A.B. 2000. Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *Journal of Stored Products Research* **36**: 75-87.

Scott, P.M., Lawrence, G.A., Lombaert, G.A. 1999. Studies on extration of fumonisins from rice, corn-based foods and beans. *Mycotoxin Research* **15**: 50-60.

Vincelli, P. & Parker, G. 2002. Fumonisin, Vomitoxin, and other Mycotoxins in corn produced by *Fusarium* fungi. [Available on Internet:] <http://www.ca.uky.edu>. [Date of access: 11 February 2005].

Table 2.1 Effect of storage over a 12-month period on fumonisins (ppm) in milled maize grain from three localities.

Time (months)	Locality			Mean
	Sannieshof	Ventersdorp	Lichtenburg	
January	0	1.3	1.9	1.1a
March	0.7	1.4	1.6	1.2a
May	0.7	1.9	2.7	1.8b
July	2.4	1.4	3.9	2.6c
September	2.2	1.9	3.6	2.6c
November	1.1	1.8	2.6	1.8b
Mean	1.2c	1.6b	2.7a	1.8
LSD ($P \geq 0.05$) (Interaction table = 0.05)				

CHAPTER 3

REPRESENTATIVENESS OF GRAIN SAMPLES AND METHODOLOGY FOR FUMONISIN EVALUATIONS

3.1 INTRODUCTION

Maize (*Zea mays* L.) is an important food source for humans and livestock worldwide and *Fusarium* is one of the major fungal genera associated with maize (Fandohan *et al.*, 2003). This genus comprises several toxigenic species including *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg, which are the most prolific producers of fumonisins. Therefore the screening of resultant maize foodstuffs for fumonisins is important to ensure food safety. This can be achieved by identifying potentially dangerous grain kernels or grain products and removing them from the production system. To date substantial mycotoxin research has been carried out at the Agricultural Research Council - Grain Crops Institute (ARC-GCI), but high variation in toxin test results and species identification confounded statistical analysis of data (Dr. B.C. Flett, personal communication). The grain sampling technique used is critical in ensuring accurate estimates of mycotoxin contamination. Mycotoxin production is not homogenous and therefore poor grain sampling procedures result in highly infected grains being missed (Whitaker & Wiser, 1969). Alternately, coincidental sampling of contaminated grain in what otherwise is a relatively healthy grain sample can result in grain being discarded unnecessarily with considerable economic implications. Studies have shown that up to 90% of variation in test results comes from sampling variability (Vincelli, 2002). The sampling step, preparation step and analytical step contribute to the total variation associated with any test procedure (Vincelli, 2002).

A major concern in quantifying health risks associated with mycotoxin contaminated foodstuffs is the reliability of fumonisin detection methods. A variety of detection methods have been proposed (Dilkin *et al.*, 2001), but results vary when compared to one another. The problem is compounded by variation in results from a single grain sample using a single evaluation method, which gives rise to false positives and/or negatives.

Methods of fumonisin detection include capillary gas chromatography, thin layer chromatography (TLC), Neogen Veratox, which uses direct competitive enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis with mass spectrometry and high-performance liquid chromatography (HPLC) (Dilkin *et al.*, 2001). Subsequent to 1990 the officially approved method of testing by the Association of Official Analytical Chemists (AOAC) for mycotoxins was TLC which was recently replaced by the HPLC technique (Sydenham *et al.*, 1996). ELISA is quicker than HPLC, with accuracy levels that are acceptable to the grain processing and feed industries. It allows for screening of large numbers of samples, which would be difficult with conventional chromatographic methods such as HPLC (Shelby *et al.*, 1994). HPLC is used as a reference method to gauge the accuracy of various other tests and in laboratories where greater accuracy is required (Anonymous, 1999).

The aim of this study was to address variation associated with sampling by identifying sources of variation and improving sampling methods. Reduced variation in the overall fumonisin test procedure could ensure that the mycotoxin concentration of the primary sample is estimated with more confidence and classified more accurately (Whitaker *et al.*, 1998).

3.2 MATERIALS AND METHODS

In April 2004, nineteen maize samples of 50 kg each were collected from silos (Figure 3.1) from sixteen localities in the dry western area (western Free State and North-West provinces) and seven maize samples of 50 kg each were collected from silos from six localities in the wetter eastern maize production area (eastern Free State, Gauteng, Mpumalanga and Kwa-Zulu Natal) (Du Toit *et al.*, 1996).

Silo samples ranged from grade 1 to 3 (Table 3.1), based on the general maize grading system used locally. Silo samples included yellow and white maize depending on availability at the time of collection (Table 3.2). Each silo sample was obtained by randomly sampling small portions from a moving conveyer belt at each maize silo (Whitaker, 2003). Each sample was placed in a polyethylene bag and stored at 5°C for

a month before conducting fumonisin evaluations.

Prior to further investigation fumonisin levels were determined at the ARC - GCI, using the ELISA - Veratox protocol (Anonymous, 2006). Five replications per silo were used and each test was repeated three times to provide 15 fumonisin readings. Data determined was used to select grain sub-samples from five localities with fumonisin levels varying from 0 - 7.5 ppm for use in further studies. Selected localities included Hartebeesfontein (1.7 ppm), Derby (7.5 ppm), Sannieshof (2.4 ppm), Ventersdorp (2.3 ppm) and Bronkhorstspuit (0 ppm). These maize sub-samples were used to study 1) the effect of sub-sample size, 2) variation within a single maize sub-sample, 3) the effect of number of replications, using prescribed 25 g sub-samples and modified 250 g sub-samples and 4) variation between analytic laboratories.

3.2.1 Increasing sub-sample size:

Sub-samples of grain from the selected five localities were drawn from the 50 kg silo grain sample following the criteria of the European Mycotoxin Awareness Network (EMAN) fact sheet on sampling and legislation (Anonymous, 2005). These sub-samples were replicated five times for each locality. Sub-samples of increasing increments of 25 g to 1000 g for each of the 26 maize silo-samples were taken. Each sub-sample was individually milled in a Cyclotech mill to approximately the particle size of fine instant coffee. The mill was thoroughly cleaned with high pressure air between each sub-sample to prevent cross contamination of mycotoxins in samples. A final 5 g aliquot was independently drawn from each of the milled sub-samples for fumonisin extraction and quantification using the ELISA-Veratox protocol (Anonymous, 2006).

Regression analysis ($Y=aX^b$) (Statgraphics Version 5.0) was used to quantify the relationship between maize sub-sample sizes and detected fumonisin concentrations.

3.2.2 Variation within a single maize sub-sample

Grain collected from Leeudoringstad was selected for this study because of the low

fumonisin levels determined in previous data. Maize grain from Vredefort, which was naturally highly contaminated by fumonisins, was separated by hand into diseased grain and healthy looking grain. Diseased grain tested with ELISA for fumonisins had a mean of 5.3 ppm and was used to spike the low fumonisin sub-samples for fumonisin analysis. Fumonisin-contaminated grains were added at rates of 100%, 75%, 50% and 25% by volume to 5 kg Leeudoringstad grain batches and thoroughly mixed. Variation of fumonisin quantifications within each percentage spiked maize sample was carried out. Twenty-one replications of 25 g samples for each of the 5 kg spiked sub-samples were then separately milled as previously discussed. A 5 g aliquot from each 25 g milled sub-sample was analysed as described by the ELISA-Veratox protocol to determine fumonisin concentrations (Anonymous, 2006).

The entire procedure was repeated using a 250 g sub-sample from which the 5 g aliquots were drawn for fumonisin testing. Fumonisin data for the 25 g and 250 g samples were analysed by calculating the coefficient of variation (CV) for each spiked sample (100%, 75%, 50% and 25%). CV's were plotted against percentage spiked samples.

3.2.3 Increasing replications

Grain samples from the initial five maize silo localities identified above were used. Five sub-samples of 25 g each were drawn according to EMAN criteria (Anonymous, 2005) by taking random samples from each of the 50 kg polyethylene bags. Sub-sampling was repeated five times to obtain a total of 25 replicates. Each sub-sample was milled as described above and a final 5 g aliquot was taken for ELISA fumonisin quantification.

This study was repeated with fumonisin-contaminated grain which was added at rates of 100%, 75%, 50% and 25% volume to 5 kg Leeudoringstad grain batches as described above (see 3.2.2). Twenty-five sub-samples of 25 g each were drawn, separately milled, and a final 5 g aliquot was taken for fumonisin determination. This procedure was repeated by drawing 25 replicates of 250 g each from which a final 5 g aliquot was taken for fumonisin quantification.

Fumonisin data of the 25 g and from the 250 g sub-samples were analysed by calculating the standard deviation and coefficient of variation (CV) for each spiked sub-sample (100%, 75%, 50% and 25%). CV's were plotted against the percentage spiked samples.

3.2.4 Variation between laboratories

Two replications of 500 g of each of the five selected samples were sent to the Medical Research Council - PROMEC (MRC) in Cape Town and the Société Generale de Surveillance Laboratory (SGS) in Pretoria. MRC and SGS analysed the samples using the HPLC technique (Shepard, 2000). Two replicates of fumonisin data obtained using the ELISA-Veratox protocol (Anonymous, 2006) at the ARC-GCI, were correlated with MRC and SGS data to quantify the relationship between the two techniques and repeatability between laboratories.

3.3 RESULTS

3.3.1 Increasing subsample sizes

A non-linear regression ($Y=aX^b$) with a $R^2=0.64$ and $P=0.05$ explained the relationship between fumonisin concentration and sample size (Figure 3.2). It indicated an initial rapid increase in fumonisins which was reduced when the sub-sample size got greater than 250 g.

Similarly, regression analysis ($Y=aX^b$) with a $R^2=0.81$ and $P=0.001$ between sample size and number of positive hits confirmed the above result (Figure 3.2). Fumonisin evaluations of the 25 g sub-samples indicated that 23 out of the 25 sub-samples tested negative for fumonisins. Results from the 250 g sub-samples indicated that 23 out of the 25 sub-samples tested, were positive for fumonisins, ranging from 1 - 17 ppm.

3.3.2 Variation within a single maize sample

High variation in CV for the 25 g sub-samples existed with an insignificant fit ($R^2=61.8$,

$P=0.136$) and an intercept of 218.33 and slope of -1.69 (Figure 3.3). Using the prescribed ELISA-Veratox protocol, the 25 g sub-samples of the 75% spiked sub-samples had nine false negatives, the 50% spiked sub-samples had six false negatives and the 25% spiked sub-samples had 15 false negatives compared to the 250 g sub-samples, which had only one false negative in the 25% spiked sub-sample (Table 3.3).

The CV for the 250 g sub-samples decreased from 52.4% in the 25% spiked sub-sample to 30.5% for the 100% sub-spiked sample (Figure 3.3). An $R^2=82.98$ ($P=0.058$) was obtained for the 250 g sub-samples (Figure 3.3). The use of 250 g sub-samples reduced variation when compared with results from the 25 g sub-samples.

3.3.3 Increasing replications

No significant reduction in standard deviation ($R^2=0.96$, $P=0.001$) (Figure 3.4) or CV with a $R^2=0.91$ and $P=0.005$ (Figure 3.5) occurred for up to 25 replications of 25 g sub-sample sizes. In fact standard deviation and CV increased with each increase in replication.

The number-of-replication study using the 25 g sub-sample size showed a slight improvement on the standard deviation for the 50% and 100% (Figure 3.6 a) spiked samples. A reduction in the CV from 85.5 to 67.11 occurred after 10 replications in the 50% spiked samples. The 75% spiked samples had a reduction in CV from 157.93 to 118.20 after 15 replications, and from 61.23 to 46.92 after 10 replications in the 100% spiked samples. The standard deviation was lower where high fumonisin readings were obtained in small samples, compared to low fumonisin readings in small samples (25 g).

In the number-of-replication study where the increased sample size (250 g) were used for spiked samples, standard deviation and CV for the 100% spiked samples were reduced up to 15 replications (Figure 3.7a and 3.7b). Thereafter increased replications appeared to increase variation. The standard deviation and CV for the 75% spiked samples were reduced up to 10 replications (Figure 3.7a and 3.7b). The standard

deviation after five replications was 9.05 and 1.83 after 10 replications. The CV was reduced from 164.13 (five replications) to 33.36 (10 replications) (Figure 3.7a and 3.7b). The same trend was observed for the 50% spiked samples where the standard deviation was reduced from 2.42 after five replications to 1.88 after 15 replications and the CV was reduced from 53.02 after 5 replications to 42.07 after 10 replications (Figure 3.7a and 3.7b). The standard deviation of 25% spiked samples was reduced from 1.24 after 5 replications to 0.79 after 20 replications (Figure 3.7a) and the CV reduced from 71.12 to 53.99 after 20 replications (Figure 3.7b). Variation is reduced by increasing replications, however this reduction was not meaningful, as indicated by the data above. It appears that at lower fumonisin concentrations more replications are needed while at higher fumonisin concentrations less replications would suffice.

3.3.4 Determining variation between laboratories

ELISA-Veratox fumonisin levels correlated significantly ($R^2=0.91$, $P=0.001$) with HPLC ($P=0.032$) fumonisin levels obtained at the MRC (Table 3.4). Although fumonisin concentrations correlated significantly, the fumonisin levels using the ELISA technique were consistently higher ($\pm 33\%$) than fumonisin concentrations using the HPLC technique (Figure 3.8). Fumonisin levels obtained using the ELISA-Veratox technique at the ARC-GCI did not correlate with the HPLC fumonisin level results of SGS ($R^2=0.534$ and $P=0.354$), neither did the fumonisin HPLC fumonisin level results of MRC correlate with the HPLC fumonisin level results of SGS ($R^2=0.166$ and $P=0.798$).

3.4 DISCUSSION

3.4.1 Increasing sub-sample sizes

The ELISA-Veratox protocol requires that a 25 g sample from a larger sample be milled, and 5 g aliquot used for fumonisin testing. Research previously conducted at the ARC-GCI could not be statistically analysed because of high variation in fumonisin data (Dr. B.C. Flett, personal communication). Results obtained in the present study showed variation to be high in small sub-samples (25 g) with a low fumonisin content. In this study variation was lower in samples with a high fumonisin content (50%, 75% and

100% spiked sub-samples) using bigger sub-samples (250 g). Whitaker & Johansson (2005) explain that sub-samples with a high fumonisin content have a better homogeneous spread of fumonisins thus contributing to lower variation in fumonisin detection. Variation increases in small samples with low fumonisin concentrations because of decreasing homogeneity of the mycotoxin (Whitaker & Johansson, 2005). This increases the possibility that the whole sample will be discarded because the sub-sample was not representative of that sample. An increase in replications using the 25 g samples had little effect on reducing standard deviation and CV percentages remained high.

This study showed that substituting the protocol 25 g sub-sample with a modified 250 g sub-sample before drawing a final 5 g aliquot from the sub-sample, significantly reduced variation because the larger sample was more representative of the total sample. This study proved that by using 25 g sub-samples resulted in 23 out of 25 false negatives. The implication of this finding is that fumonisin levels in a small sub-sample can be easily underestimated or worse, not detected (Whitaker & Johansson, 2005). Maize samples with a fumonisin level higher than the legal limit will be accepted and these fumonisin contaminated samples may be consumed by humans and animals, causing various health issues. By using the modified 250 g sub-sample, more accurate and precise fumonisin levels are quantified, thereby reducing the risk of wrongly classifying maize samples.

3.4.2 Variation within a single maize sample

In the 25 g sub-sample study only the 75% and 100% spiked samples produced a slight reduction in the CV and standard deviation. Whitaker & Johansson (2005) explain that random variation among fumonisin levels within the same maize sample can occur. When the sample fumonisin level increases, the variation between fumonisin levels will also increase, but that the CV will decrease. Mean fumonisin levels in the 25 g sub-samples was 1 ppm (25% spiked sub-sample), 2.9 ppm (50% spiked sub-sample), 3.4 ppm (75% spiked sub-sample) and 5 ppm (100% spiked sub-sample). Fumonisin levels ranged from 0 - 8.1 ppm (25% spiked sub-sample), 0 - 7.1 ppm (50% spiked sub-

sample), 0 - 9 ppm (75% spiked sub-sample) and 1.9 - 10.1 ppm (100% spiked sample). Although variation of fumonisin levels was high for all the sub-samples, only the 75% and 100% spiked sub-samples produced a slight reduction in CV and standard deviation, indicating that the high fumonisin levels in spiked sub-samples slightly reduced the variation of fumonisin levels within these sub-samples.

The 250 g sub-samples had a reduction in the CV from 25% - 100% spiked sub-samples. The 250 g sub-samples were more representative than the 25 g sub-samples which yielded 30 false negatives. Whitaker & Johansson (2005) explains that when a sample is tested there is more than a 50% chance that the sample fumonisin level will test lower than the true fumonisin level. The skewness is greater for small sub-sample sizes and the distribution becomes more symmetrical as the sample size increase. Increasing the sub-sample size to 250 g, significantly reduced variation of fumonisin levels in samples containing high and low fumonisin levels.

3.4.3 Increasing replications

Whitaker & Johansson (2005) reported ten replicated aflatoxin test results from the same sample with CV's that ranged from 31.6% - 196.3%. As the aflatoxin concentration in the sample increased, the variance among aflatoxin level results increased, but the CV decreased (Whitaker & Johansson, 2005). Even though a small sub-sample (25 g) was used in this study, the high percentage of fumonisin levels in the 75% and 100% spiked samples with increasing replicates ensured more accurate fumonisin quantification, explaining the slight reduction in CV and standard deviation (Whitaker & Johansson, 2005). It seems that the high fumonisin levels, rather than replications had an effect on reducing the CV and standard deviation of small sub-samples.

This study showed that increased replication numbers had a more significant effect on reducing variation in the modified 250 g sub-samples. This could be due to the fact that the 250 g sub-samples were more representative of the sample, thereby reducing variation in fumonisin levels in each sub-sample (Whitaker & Johansson, 2005). The

number of replications needed to improve accuracy, will depend on the level of fumonisin infection in a sample. Samples with high fumonisin levels, require less replications than moderately or low contaminated samples (Whitaker & Johansson, 2005).

This study has emphasised the importance of using a greater number of replications together with a modified 250 g sub-sample to reduce variation in detected fumonisin levels. The use of a small sub-sample size (25 g) with increased number of replications will not reduce variation except when the sample has a high fumonisin level. More accuracy and less variation in fumonisin levels will occur when a modified 250 g sub-sample that is more representative of the sample, is used in conjunction with replicates.

3.4.4 Determining variation between laboratories

There is a significant correlation between ELISA fumonisin levels of ARC-GCI and the HPLC fumonisin levels carried out at the MRC. Fumonisin levels obtained using the ELISA technique were consistently 33% higher in this study than fumonisin levels obtained using the HPLC, which was in agreement with Shelby *et al.* (1994) and Desjardins (2006). These authors suggested that ELISA reacted to antibodies of all known members of the fumonisin class of mycotoxins (B¹, B² and B³). Additional fumonisin-like molecular structures were also detected by these antibodies and the result was inflated when compared to other methods of analysis such as HPLC (Shelby *et al.*, 1994; Desjardins, 2006). The same HPLC techniques used by MRC and SGS did not correlate, indicating possible analytical errors or incompetence of analysts. This is an indication that the laboratory where analyses are carried out is an important additional source of variation, even though the same quantification technique is used.

A reduction in variation of fumonisin levels using the ELISA-Veratox protocol will result in increased confidence and accuracy of fumonisin quantification by addressing the fact that ELISA fumonisin levels were consistently 33% higher in this study than fumonisin levels obtained using the HPLC and that the laboratory where analyses are carried out

is an additional source of variation. The higher percentage fumonisin levels of ELISA can be reduced by 33% to obtain fumonisin levels in accordance with HPLC fumonisin levels. To reduce variation associated between laboratories, it is advisable to make use of one reputable laboratory for fumonisin quantifications.

REFERENCES

Anonymous, 1994. Export standards and requirements: maize. [Available on Internet:] www.nda.agric.za [Date of access: 10 October 2004].

Anonymous, 1999. USDA Grain fungal diseases and mycotoxin reference. [Available on Internet:] www.usda.gov/gipsa/pubs/mycobook.pdf [Date of access: 4 June 2004].

Anonymous, 2005. European Mycotoxin Awareness Network. Fact sheets on sampling and legislation. [Available on Internet:] http://193.132.193.215/eman2/fsheet6_3.asp [Date of access: 19 October 2006].

Anonymous, 2006. Veratox quantitative fumonisin test instructional pamphlet. Neogen Corporation, 620 Lesher Place, Lansing, MI 48912.

Desjardins, A.E. 2006. *Fusarium* mycotoxins. Chemistry, Genetics and Biology. The American Phytopathological Society. 260pp.

Dilkin, P., Mallmann, C.A., de Almeida, C.A.A. & Corrêa, B. 2001. Robotic animated clean-up for detection of fumonisins B¹ and B² in corn and corn-based feed by high performance liquid chromatography. *Journal of Chromatography* **925**: 151-157.

Du Toit, A.S., Prinsloo, M.A., Durand, W. & Kiker, G. 1996. Vulnerability of maize production to climate change and adaptation assessment in South Africa. [Available on Internet:] <http://www.sanbi.org/countrystudy/Agriculture%20Report.pdf> [Date of access: 9 October 2006].

Fandohan, P., Hell, K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology* **2**: 570-579.

Shelby, R. A., White, D. G. & Bauske, E. M. 1994. Differential fumonisin production

in maize hybrids. *Plant Disease* **78**: 582-584.

Shepard, G.S. 2000. Liquid chromatographic method for fumonisins in corn. *Methods in Molecular Biology* **157**: 147-157.

Sydenham, E.W., Shepard, G.S., Thiel, P.G., Stockenström, S., Snijman, P.W. & Van Schalkwyk, D.J. 1996. Liquid chromatographic determination of fumonisins B1, B2 and B3 in corn. *Association of Official Analytical Chemists* **79**: 688-696.

Vincelli, P. & Parker, G. 2002. Fumonisin, Vomitoxin, and other Mycotoxins in corn produced by *Fusarium* fungi. [Available on Internet:] <http://www.ca.uky.edu>. [Date of access: 11 February 2005].

Whitaker, T.B. & Wiser, E.H. 1969. Theoretical investigations into the accuracy of sampling shelled peanuts for aflatoxin. *Journal of American Oil Chemists' Society* **46**: 377-379.

Whitaker, T.B., Trucksess, M.W., Johansson, A.S., Giesbrecht, F.G., Hagler, W.M. & Bowman, D.T. 1998. Variability associated with testing shelled corn for fumonisin. *Association of Official Analytical Chemists* **81**: 1162-1168.

Whitaker, T.B. 2003. Standardisation of mycotoxin sampling procedures: an urgent necessity. *Food Control* **14**: 233-237.

Whitaker, T.B. & Johansson, A.S. 2005. Sampling uncertainties for the detection of chemical agents in complex food matrixes. *Journal of Food Protection* **68**: 1306-1313.

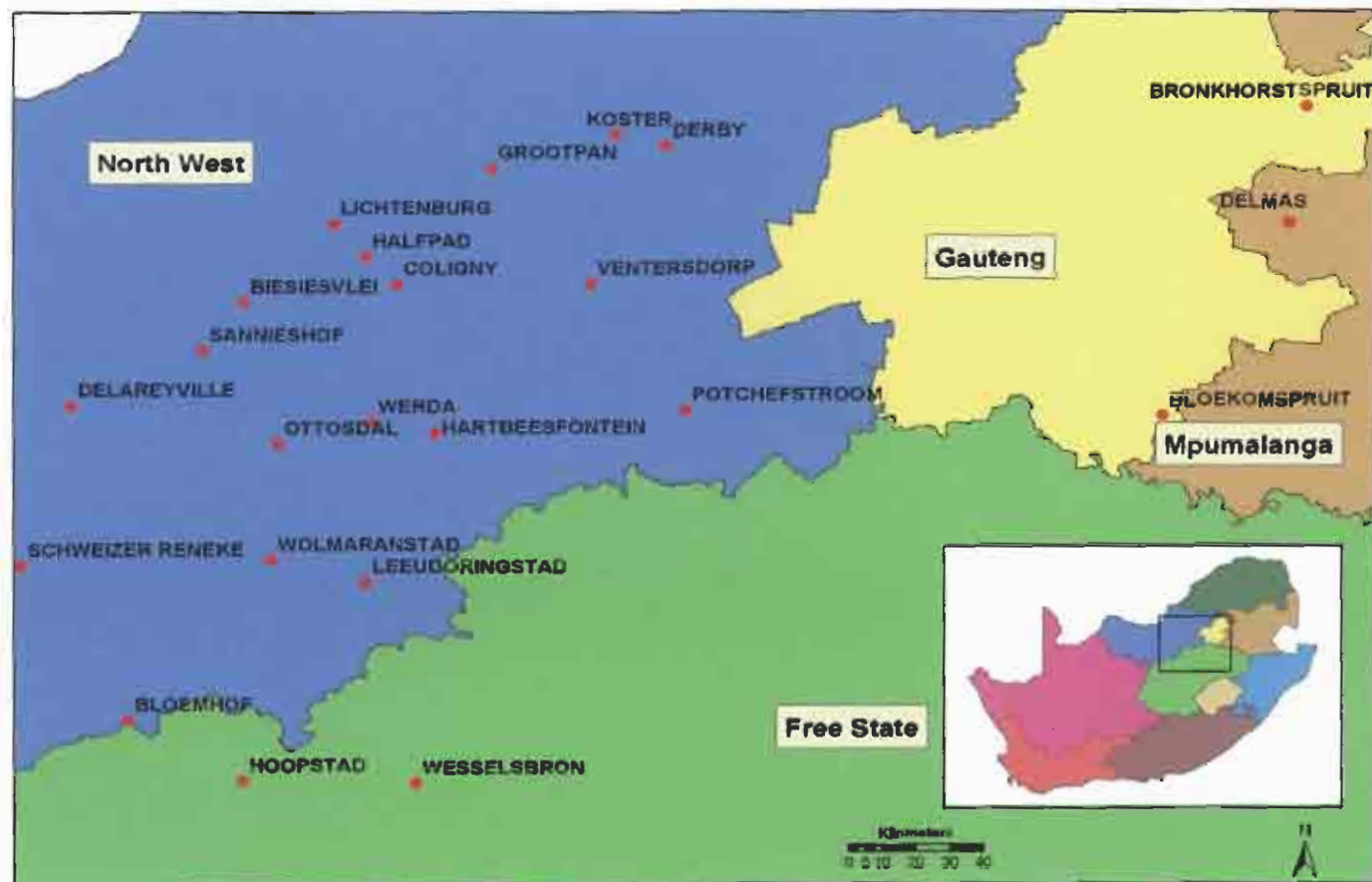


Figure 3.1 Localities from which maize samples were collected from silos in the warm western area and cooler eastern production areas (Chart by W. du Randt).

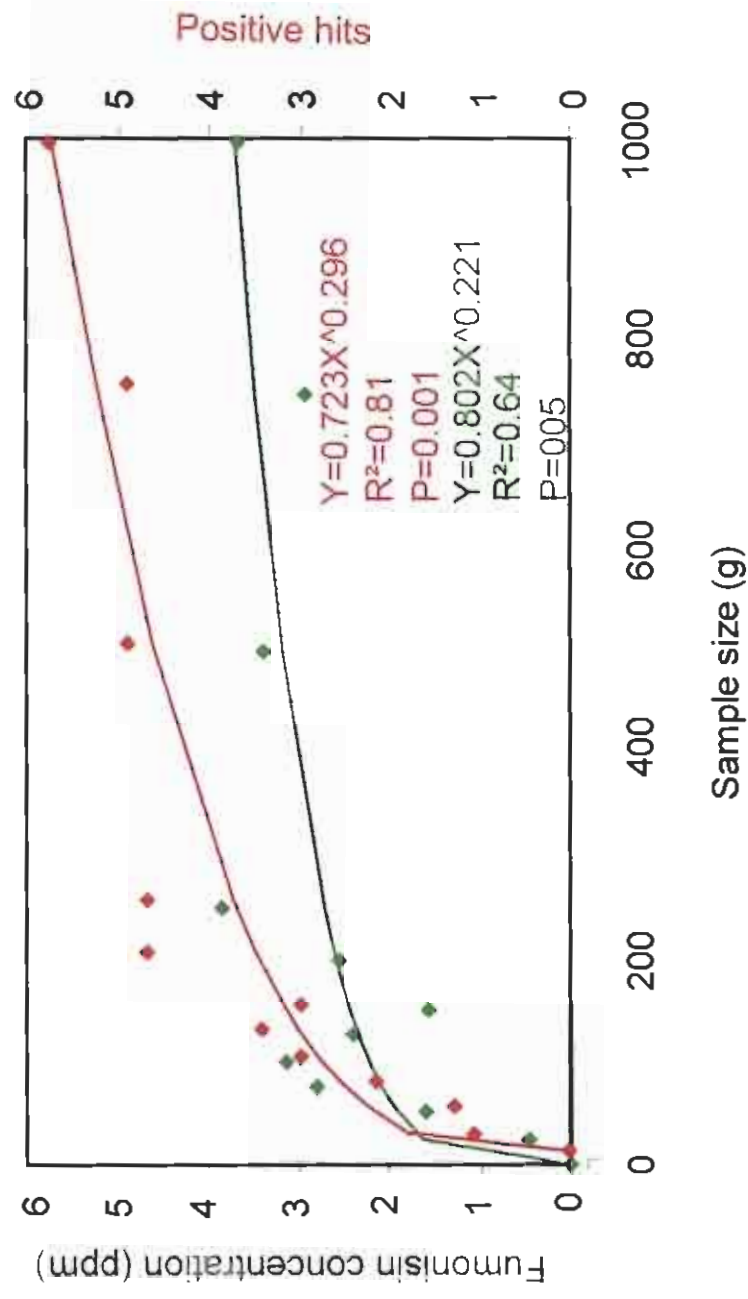


Figure 3.2 Relationship between increasing sub-sample size, number of positive hits and detected fumonisin concentration.

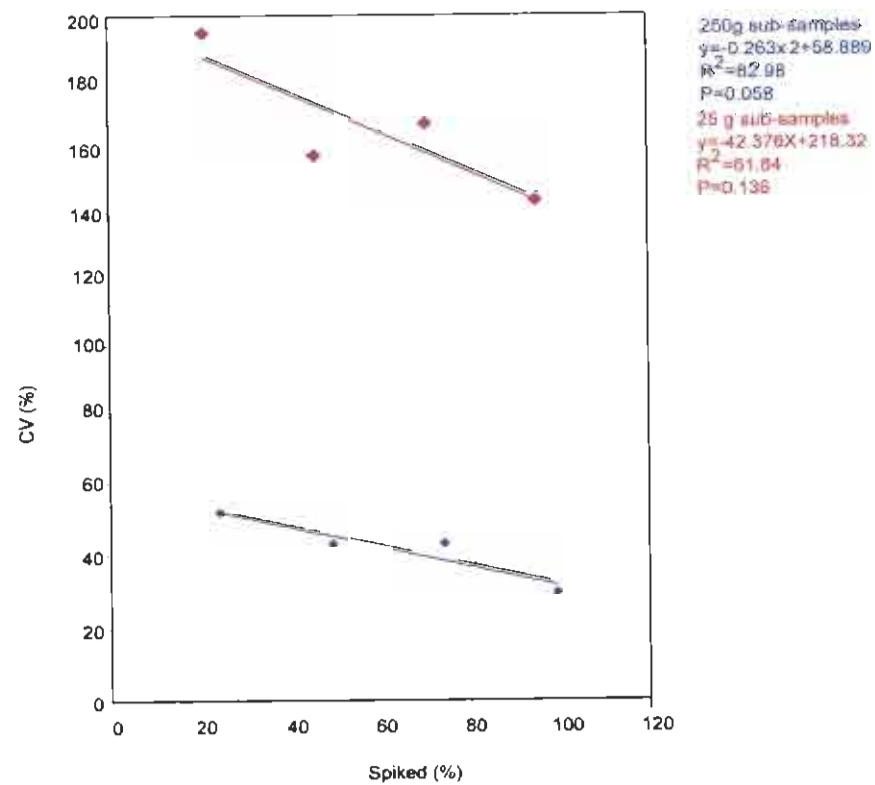


Figure 3.3 The relationship between CV and spiked fumonisin concentrations for 25 g and 250 g sub-samples.

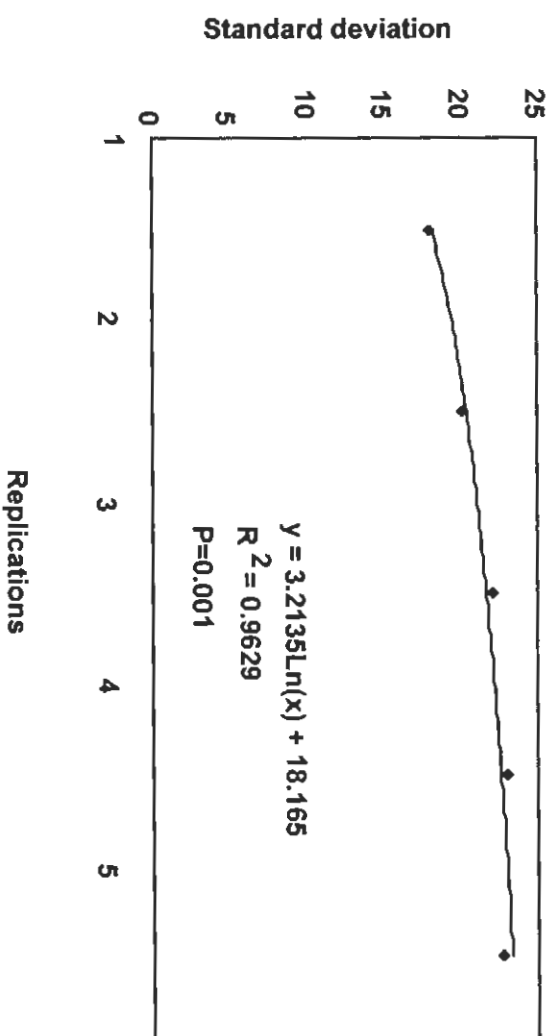


Figure 3.4 The effect of increasing the number of sub-sample replications on standard deviation.

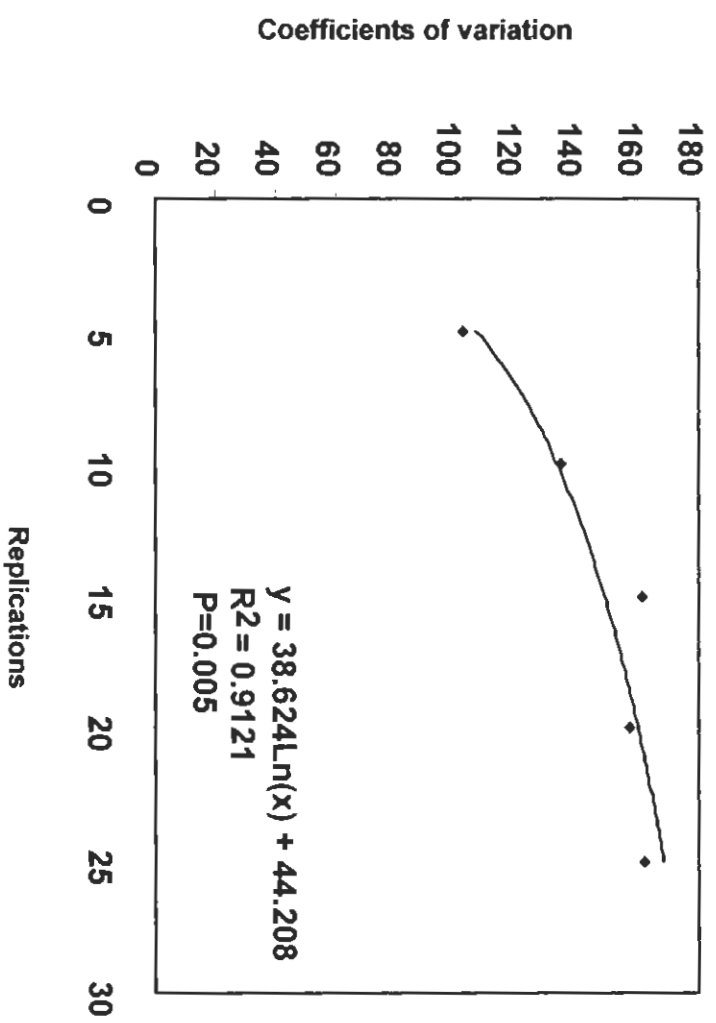


Figure 3.5 Effect of increasing the number of sub-sample replications on the Coefficients of variation

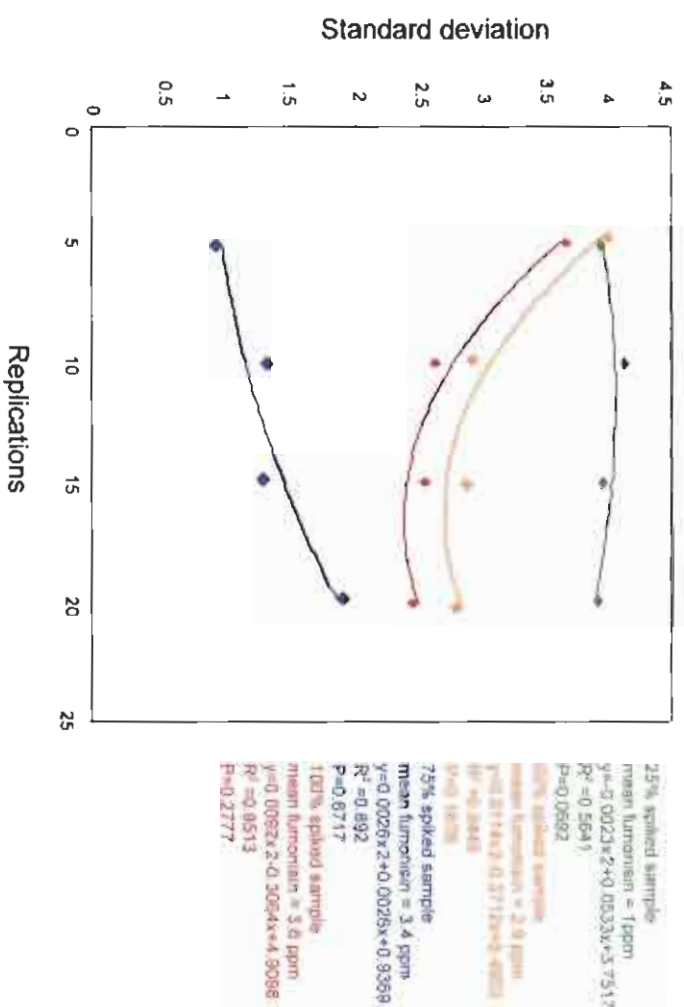
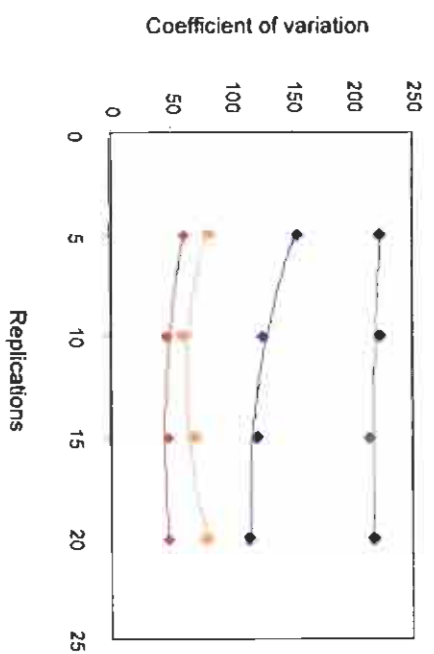


Figure 3.6a Standard deviation of 25%, 50%, 75% and 100% spiked samples where 25 g sub-samples were used over 20 replications.



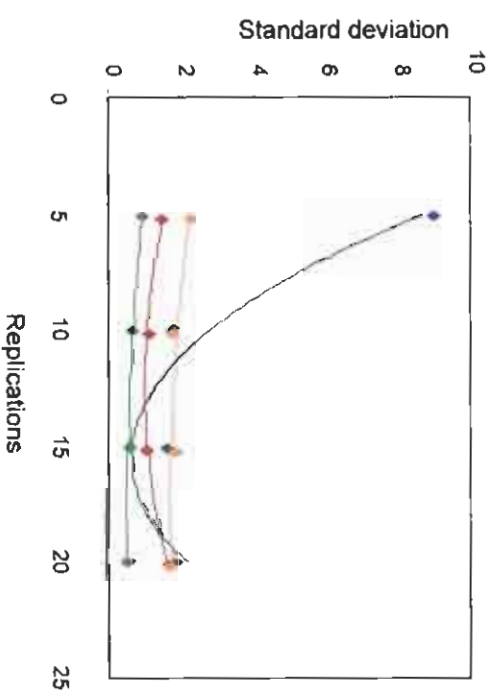
75% spiked sample
mean fumonisin = 1 ppm
 $y = 2.0415x - 2.1448$
 $R^2 = 0.5289$
 $P = 0.3374$

50% spiked sample
mean fumonisin = 2.9 ppm
 $y = 2.471x - 2.4558$
 $R^2 = 0.6434$
 $P = 0.0472$

25% spiked sample
mean fumonisin = 3.4 ppm
 $y = 0.2727x - 2.9749$
 $R^2 = 0.9604$
 $P = 0.0952$

100% spiked sample
mean fumonisin = 3.8 ppm
 $y = 0.1522x - 2.4574$
 $R^2 = 0.9287$
 $P = 0.1452$

Figure 3.6b Coefficient of variation of 25%, 50%, 75% and 100% spiked samples where 25 g sub-samples were used over 20 replications.



25% spiked sample
mean fumonisin = 1.5 ppm
 $y = 0.0022x^2 - 0.0836x + 1.596$
 $R^2 = 0.9806$
 $P = 0.0638$

75% spiked sample
mean fumonisin = 4.5 ppm
 $y = 0.0743x^2 - 2.2885x + 18.297$
 $R^2 = 0.9428$
 $P = 0.2304$

100% spiked sample
mean fumonisin = 5.3 ppm
 $y = 0.0094x^2 - 0.2278x + 2.4351$
 $R^2 = 0.976$
 $P = 0.9195$

Figure 3.7a Standard deviation of 25%, 50%, 75% and 100% spiked samples where the modified 250 g sub-samples were used over 20 replications.

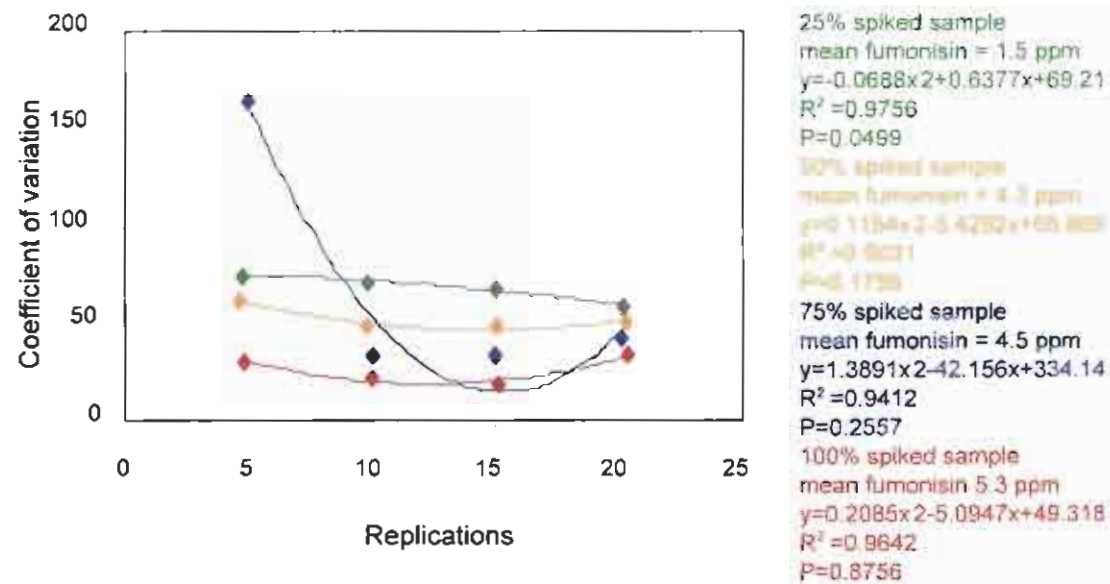


Figure 3.7b Coefficient of variation of 25%, 50%, 75% and 100% spiked samples where the modified 250 g sub-samples were used over 20 replications.

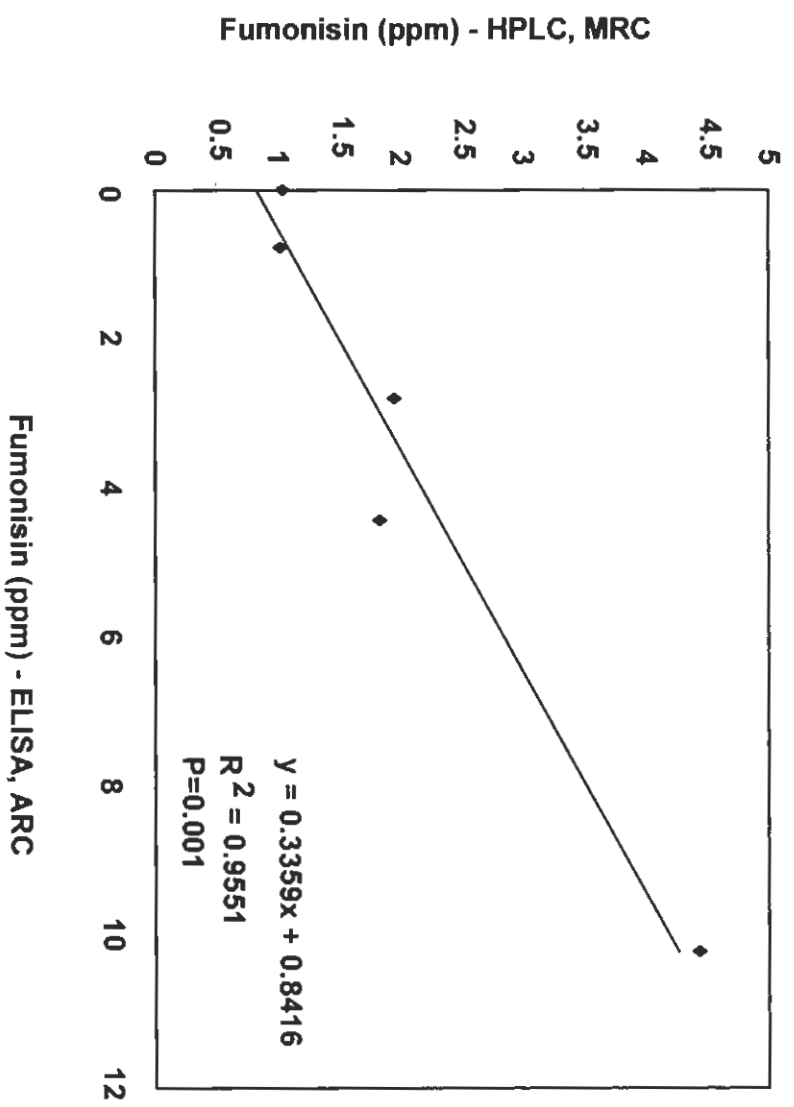


Figure 3.8 Fumonisin concentrations detected by ELISA compared to HPLC fumonisin concentrations.

Table 3.1 Standards for grades of class white maize and class yellow maize (Anonymous, 1994).

Deviation	Maximum permissible deviation (%)					
	White maize			Yellow maize		
	WM1	WM2	WM3	YM1	YM2	YM3
1. Foreign matter	0.30	0.50	0.75	0.30	0.50	0.75
2. Defective maize kernels, above and below the 6.35 mm round-hole sieve	7.00	13.00	30.00	—	—	—
3. Defective maize kernels, that can pass through the 6.35 mm round-hole sieve	-	-	-	4.00	10.00	30.00
4. Defective maize kernels, that can not pass through the 6.35 mm round- hole sieve	-	-	-	9.00	10.00	30.00
5. Other colour maize kernels	3.00	6.00	10.00	2.00	5.00	8.00
6. Deviations referred to in items 1, 2, 3, 4 and 5 collectively: provided that the deviations are individually within the specified limits.	8.00	16.00	30.00	9.00	20.00	30.00
7 Pinked maize kernels	12.00	12.00	12.00	12.00	12.00	12.00

Table 3.2 Maize samples selected from silos in the warmer production areas of South Africa.

Sample Number	Silo Locality	Grade of maize
1	Biesiesvlei	wm2
2	Werda	wm2
3	Hartebeesfontein	wm2
4	Grootpan	wm3
5	Coligny	wm2
6	Delareyville	wm3
7	Derby	ym3
8	Bloekomspruit (Control)	wm3
9	Sannieshof	wm3
10	Koster	ym3
11	Ventersdorp	wm2
12	Hoopstad	wm2
13	Hoopstad	wm3
14	Bloemhof	wm2
15	Wolmaranstad	ym2
16	Wolmaranstad	wm2
17	Schweizer-Reynecke	wm2
18	Leeudoringstad	wm2
19	Wesselsbron	wm2
20	Delmas (Control)	wm1
21	Bronkhorstspuit (Control)	ym1
22	Sannieshof	ym3
23	Lichtenburg	wm3
24	Ottosdal	wm2
25	Halfpad	wm3
26	Potchefstroom	wm3

Table 3.3 Variation within single maize samples

Fumonisin (ppm)											
Replication	25 g samples					250 g samples					
	25% spiked	50% spiked	75% spiked	100% spiked	25% spiked	50% spiked	75% spiked	100% spiked	25% spiked	50% spiked	100% spiked
1	0.0	4.0	9.0	10.1	3.1	8.4	4.7	5.9			
2	0.0	7.1	0.0	2.4	0.0	3.6	7.1	7.0			
3	2.2	10.0	0.0	7.3	2.8	5.6	5.3	5.6			
4	0.0	2.1	0.0	1.9	1.7	2.9	4.6	5.1			
5	0.0	0.0	3.5	8.1	1.2	2.4	5.7	2.9			
6	0.0	3.2	3.7	5.2	1.2	4.1	5.3	7.0			
7	0.0	3.4	12.0	3.3	1.0	4.6	2.1	5.1			
8	0.0	4.0	0.0	5.2	1.6	7.2	5.4	5.6			
9	4.1	2.9	3.2	7.0	1.1	4.8	9.3	5.7			
10	0.0	4.1	2.1	5.4	0.4	2.8	5.2	5.8			
11	0.0	5.2	0.0	2.4	1.6	4.9	5.2	5.8			
12	3.4	2.7	0.0	4.3	2.0	8.3	2.9	7.0			
13	0.0	0.0	1.2	2.9	1.3	3.3	3.5	5.9			
14	0.0	0.0	8.2	8.6	1.0	5.3	5.3	7.0			

Fumonisin (ppm)											
15	0.0	4.5	7.1	6.3	0.1	1.9	5.9	7.0			
16	0.0	0.0	7.1	4.2	2.2	3.9	4.1	3.2			
17	8.1	1.2	9.3	7.3	2.1	2.2	5.8	5.3			
18	1.2	0.0	4.0	2.3	1.7	4.3	2.1	1.2			
19	0.0	3.8	0.0	3.9	1.4	3.7	1.8	2.3			
20	0.0	3.2	0.0	2.5	1.8	2.3	1.8	5.8			
21	3.3	0.0	0.0	4.4	1.7	3.1	1.6	5.4			
Mean	1.0	2.9	3.4	5.0	1.5	4.3	4.5	5.3			
CV	197.59	88.99	115.63	47.43	52.38	43.53	43.45	30.49			

Table 3.4 Comparison between ARC, MRC and SGS toxin levels.

ELISA vs MRC	Correlation coefficient	0.910
	P value	0.032
ELISA vs SGS	Correlation coefficient	0.534
	P value	0.354
ELISA vs SGS	Correlation coefficient	0.166
	P value	0.798

CHAPTER 4

INCIDENCE OF *FUSARIUM* SPP. FROM SECTION LISEOLA AND FUMONISIN PRODUCTION IN MAIZE SAMPLES COLLECTED FROM MAIZE SILOS IN SOUTH AFRICA

4.1 INTRODUCTION

Fusarium verticillioides (Sacc.) Nirenberg is one of the most prevalent seedborne fungi of maize in South Africa (Marasas *et al.*, 1979), the United States of America, China, Europe and South America (Sydenham *et al.*, 1991; Visconti & Doko, 1994; Shephard *et al.*, 1996) and has been reported in 25 other maize-producing countries (Mazzani *et al.*, 2001). This pathogen can cause seedling disease, root rot, stalk rot and ear or kernel rot of maize (Munkvold *et al.*, 1997; Glenn, 2005).

Symptomless infections may occur throughout a maize plant, causing the presence of the fungus to be ignored (Munkvold & Desjardins, 1997). This suggests that some strains of *F. verticillioides* have the ability to induce disease in maize and other strains do not (Bacon & Williamson, 1992). Two common maize pathogens viz. *F. verticillioides* and *Fusarium proliferatum* (Matsushima) Nirenberg have the ability to produce fumonisins that adversely affect humans and animals. Production of fumonisins depends on factors such as geographical region, environmental conditions (Dilkin *et al.*, 2002), insect infestation, pre- and post harvest handling, interactions with other fungi (Fandohan *et al.*, 2003) and the ability of *Fusarium* strains to produce fumonisins. Fumonisin concentrations in a maize field will not necessarily be the same from one year to another (Ono, 1999) because seasonal changes may affect abiotic and biotic factors, which in turn may not influence fumonisin production independently, but as a complex of interactions (Fandohan *et al.*, 2003). Rainfall, humidity and temperature are critical abiotic factors regulating growth of *F. verticillioides* and *F. proliferatum* as well as production of fumonisins. According to Alberts *et al.* (1990) and Scott (1993), information on the minimum, optimum and maximum temperatures for fumonisin production have yet to be ascertained, but the temperature range for fumonisin

production in maize cultures is 20-28°C. Leslie & Summerell (2006) found fumonisin production to improve at $\geq 15^{\circ}\text{C}$ when cultured on maize kernels.

Fumonisin are heat stable and are therefore not destroyed during food processing (Alberts *et al.*, 1990). Because fumonisins cannot be totally eliminated from the food chain (Munkvold & Desjardins, 1997), the Food and Drug Administration (FDA) determined Acceptable Daily Intake levels (Table 4.1) (Anonymous, 2004a) as guidelines for total fumonisins allowed in maize as food and animal feeds in the USA. Surveys of the natural occurrence of *F. verticillioides*, *F. proliferatum* and fumonisins in maize production areas in South Africa are necessary to ensure that maize infected with unacceptable mycotoxin levels can be appropriately utilized or destroyed.

Quantification of *Fusarium* spp. in maize and commodity surveys have included isolation frequency (plate count) and determination of infection levels (percentage of seed that yields fungi after surface disinfection) which were used as an indication of fungal biomass (Schwadorf & Müller, 1989; Saxena *et al.*, 2001). These techniques were time consuming and in the case of infection level, not applicable to meal samples (Schwadorf & Müller, 1989). The use of isolation frequency of pathogens from maize as an indication of fungal biomass was also poorly correlated with fumonisin concentration (personal communication, Dr. B.C. Flett, Ngoko *et al.*, 2001) under natural field conditions.

Ergosterol is the predominant sterol of all fungi except certain aquatic phycomycetes and rust fungi (Schwadorf & Müller, 1989) and plays an essential role as a component of cell membranes and other cell constituents. Ergosterol may be produced by some bacteria in minor quantities compared to fungi and is either absent or a minor constituent in most higher plants and insects (Ness, 1977). Low amounts of ergosterol are found in algae and protozoa, but is generally safe when used as a specific biomarker for fungi (Mille-Lindblom *et al.*, 2004) as the technique is quick and highly sensitive (de Castro *et al.*, 2002). Extracted samples may be stored for extensive periods without pronounced decreases in ergosterol concentration (Mille-Lindblom *et al.*, 2004). Under controlled conditions ergosterol quantification was shown to be a useful method for detection of fungal biomass and appears to be a sensitive early-

indicator of mycotoxin production in foods and commodities such as aflatoxin in rice (Gourama & Bullerman, 1994) and maize (de Castro *et al.*, 2002), fumonisin in maize (Marin *et al.*, 1999) and ochratoxin A in barley and wheat (Saxena *et al.*, 2001).

The purpose of this study was to 1) quantify the incidence and geographic spread of *F. verticillioides*, *Fusarium subglutinans* (Wollenw. et Reinking) Nelson, *F. proliferatum* and fumonisins in maize from different production areas of South Africa 2) determine correlations between the isolation frequency of these *Fusarium* spp. and fumonisin and ergosterol concentrations and 3) correlate fumonisin levels with weather parameters obtained from the sampled locations.

4.2 MATERIALS AND METHODS

4.2.1 Incidence and geographic spread of *Fusarium* pathogens from maize grain silos in South Africa

Kernel subsamples of maize collected from 23 localities were randomly taken for fungal isolations. Two hundred and fifty kernels per subsample were replicated five times for *Fusarium* spp. enumerations. Kernels were surface-sterilized (King & Scott, 1981) and four kernels per 90 mm petri dish were plated onto rose bengal-glycerine-urea (RbGU) selective medium (Van Wyk *et al.*, 1986) for identification of *Fusarium* spp. Petri dishes were placed in the dark at 25°C. After seven days *Fusarium* spp. were identified using the guidelines of Nelson *et al.* (1983) and Leslie & Summerell (2006). Isolates of these fungi were transferred to split plates containing Carnation Leaf Agar (CLA) and Potato Dextrose Agar (PDA) for confirmation. Chloramphenicol was included at 250 mg per 100 ml agar to both CLA and PDA to inhibit bacterial growth (Leslie & Summerell, 2006). CLA/PDA petri dishes were placed under ultraviolet light (12 hours light and 12 hours dark) to promote conidial and teleomorph development to improve isolate identifications. *Fusarium* spp. were identified, quantified on RbGU and CLA/PDA media and expressed as a percentage.

Maps were compiled with ARCGIS Version 9.1 to indicate the geographic distribution

of maize infected with *F. verticillioides*, *F. proliferatum*, *F. subglutinans* and fumonisins.

4.2.2 Relationship between isolation frequency, fumonisin and ergosterol concentrations

Fumonisins were evaluated at the ARC-GCI, using the ELISA - Veratox protocol (Anonymous, 2006). Fumonisin levels were determined in kernels from all 23 localities and replicated 15 times. The average concentration for each locality was calculated and expressed as parts per million (ppm). The relationship between fumonisin concentrations (Table 4.2) and mean isolation percentage determined from plate counts of *F. verticillioides* and *F. proliferatum* was quantified using simple regression analysis (Statgraphics Version 5.0).

Ergosterol, an indication of fungal biomass, was determined on five samples from Bronkhorstspuit, Derby, Hartebeesfontein, Sannieshof and Ventersdorp (Seitz *et al.*, 1979). The relationship between ergosterol concentration and mean percentage isolation frequencies of *F. verticillioides*, *F. proliferatum*, *F. subglutinans* and fumonisins were analysed using simple regression analysis (Statgraphics Version 5.0).

4.2.3 Relationship between fumonisin levels and weather parameters

Minimum and maximum temperatures and rainfall for nine weather stations nearest to the sampled silos (Table 4.2) for November 2003 to July 2004, were collected by the ARC - Institute of Soil, Climate and Water and were used to determine the monthly mean maximum temperature, minimum temperature and rainfall. Statgraphics Version 5.0 was used to correlate weather variables with mean fumonisin data to determine the potential role of weather variables in the production of fumonisins.

4.3 RESULTS

4.3.1 Incidence and geographic spread of *Fusarium* pathogens from maize grain silos in South Africa

F. verticillioides, *F. subglutinans* and *F. proliferatum* were isolated from maize grain sampled at 23 localities, with mean isolation frequencies of 35.1%, 7.4% and 3.2%, respectively (Table 4.2). *F. verticillioides* (Figure 4.1) and *F. subglutinans* (Figure 4.3) was 6.5% were isolated most frequently from maize samples collected in the North West Province, with mean isolations of 36% and 6.5%, respectively. The mean isolation frequency of *F. proliferatum* (Figure 4.2) in the North West Province was 3.4%. In the Free State, *F. verticillioides*, *F. subglutinans* and *F. proliferatum* had mean isolation frequencies of 25%, 1.4% and 4.6%, respectively. The highest incidence of *F. subglutinans* (Figure 4.3) was found in Gauteng, with a mean of 11.2% although *F. verticillioides* was still the most common species, with a mean of 32.7%. *F. proliferatum* had a mean isolation of 2.3% in Gauteng. Mpumalanga yielded mean isolation frequencies of 44.1%, 3.2% and 1.6% respectively for *F. verticillioides*, *F. subglutinans* and *F. proliferatum*.

Fumonisin at the surveyed localities were highest (greater than 2 ppm) at Koster (7.5 ppm) and Derby (6.6 ppm). Areas close to Derby and Koster, mainly Ventersdorp (2.3 ppm), Grootpan (2.8 ppm), Lichtenburg (2.3 ppm) and Sannieshof (2.4 ppm) also had high fumonisin levels (Figure 4.4). Fumonisin levels at the other localities that were sampled ranged from 0 - 2 ppm.

4.3.2 Relationship between isolation frequency, fumonisin incidence and ergosterol concentration

Regression analysis between *Fusarium* spp. plate counts and fumonisins indicated a non-significant relationship between *F. verticillioides* ($R^2=2.2\%$ and $P=0.5$) and *F. proliferatum* ($R^2=0.45\%$ and $P=0.8$) plate counts and fumonisin concentrations.

No significant relationship between fungal plate counts and ergosterol-determined fungal biomass of *F. verticillioides* ($R^2=0.19\%$ and $P=0.9$), *F. proliferatum* ($R^2=31.15\%$ and $P=0.3$) and *F. subglutinans* ($R^2=0.79\%$ and $P=0.9$) was obtained when using a simple regression analysis. Similarly no significant relationship was recorded between fumonisin concentration and ergosterol-determined fungal biomass ($R^2=40.66\%$ and $P=0.2$).

4.3.3 Relationship between fumonisin concentration and weather parameters

A statistically significant correlation was obtained between fumonisin concentration and mean maximum temperatures for May ($P=0.02$) and June ($P=0.01$) 2004. Rainfall and minimum temperatures were not significantly correlated with fumonisin levels.

4.4 DISCUSSION

4.4.1 Incidence and geographic spread of *Fusarium* pathogens from maize grain silos in South Africa

Miller *et al.* (1995) reported the predisposition of maize to infection by *F. verticillioides* by drought. At the time of this survey, dry conditions occurred throughout South Africa (Figure 4.5) December 2003 and January 2004 (Anonymous, 2004b). *F. verticillioides* and *F. proliferatum* were most frequently isolated in the North West Province and *F. subglutinans* in Gauteng. *F. verticillioides* is an important fumonisin producer (Rheeder *et al.*, 2002). The high frequency of *F. verticillioides* in Biesiesvlei (56.2%), Bronkhorstspuit (52.4%), Derby (68.8%), Grootpan (55.4%) and Hartebeesfontein (70.8%) should therefore be seen as a serious problem (Rheeder *et al.*, 1990).

Considering that the total fumonisin allowed ($B^1+B^2+B^3$) for human consumption is set at 2 ppm by the FDA (Table 4.1), the high fumonisin levels from silos in Derby, Koster, Grootpan, Ventersdorp and Lichtenburg is of concern, both for human and animal health. It was estimated by Thiel *et al.* (1992) that the daily intake of fumonisins via maize-based food in South Africa is 14 - 440 mg/kg body weight per day for a

population in a high-risk area, and 5 - 59 mg/kg body weight per day in a low-risk area. Research has shown that maize consumption in developing areas is as high as 46 mg/kg per person per day. In contrast the consumption in developed countries is no higher than 0.7 mg/kg per person per day. The risk of humans being subjected to high fumonisin concentrations from maize from silos in Derby, Koster, Grootpan, Ventersdorp and Lichtenburg is particularly high. Implications to animal health are also of concern. There is a possibility that maize from Derby and Koster could cause leukoencephalomalacia, a brain lesion that can be fatal to horses after only a few days consumption of contaminated feed (Desjardins, 2006).

4.4.2 Relationship between isolation frequency, fumonisin incidence and ergosterol concentration

The non-significant relationships between *F. verticillioides*, *F. proliferatum* and fumonisin levels confirm the reports of Danielsen & Jensen (1998) and Ramirez *et al.* (1996) and indicate that other factors play a role in the production of fumonisin. Visconti (1996) described physiological stresses such as drastic variations in rainfall and relative humidity prior to harvesting as likely to create favourable conditions for fumonisin production. Miller (2001) suggested that drought, rather than temperature stress increased levels of fumonisins.

A number of factors may have resulted in poor statistical relationships between ergosterol, fungal plate counts and fumonisin levels. Ergosterol extracted from fungi growing within a natural substratum may have been recovered from a variety of different fungi (Padgett & Posey, 1993) such as *Diplodia maydis* (Berk.) Sacc. and *F. graminearum* Schwabe, which are common maize pathogens in South Africa. Although *F. verticillioides* is one of the most prevalent fungi associated with maize (Nelson *et al.*, 1993) and together with *F. proliferatum*, are the major fumonisin producers (Marin *et al.*, 1999), it is common for other pathogens such as *Aspergillus* spp., *Penicillium* spp., (Turner *et al.*, 1999, Orsi *et al.*, 2000), *S. maydis* and *F. graminearum* (Williams *et al.*, 1992) to co-infect maize with *Fusarium* spp. (Bush *et al.*, 2003). The quantification of total ergosterol from maize kernels could include various other non-fumonisin-producing

fungi, which results in poor correlations. Similarly *Fusarium* isolates vary in ability to produce fumonisins and fumonisin concentration is thus not a factor of the amount of *Fusarium* present (Jardine & Leslie, 1999).

4.4.3 Relationship between fumonisin levels and weather parameters

Fungal growth as well as toxin production in maize depend on several interacting environmental factors such as low moisture content of the soil, high daily maximum temperatures, high night minimum temperatures and nutrient deficient soils that stress maize plants (Payne, 1992). The significant relationship between maximum temperatures for May and June 2004 and fumonisin production coincides with harvesting of maize in South Africa. This indicates that maximum temperatures in May (23.5°C) and June (18.9°C) could increase fumonisin production during the drying-down and harvesting period. This coincides with the findings of Alberts *et al.* (1990) who stated that the best temperature range for fumonisin production is 20-28°C and Leslie & Summerell (2006), who reported fumonisin to increase at $\geq 15^{\circ}\text{C}$.

Further studies need to be conducted annually to determine *Fusarium* spp. and fumonisin incidence in the maize production areas of South Africa. It is also important that strains and toxicity of the population diversity at each locality be established to determine whether there is a geographical clustering of toxin-producing isolates in maize. Specific toxigenic *Fusarium* strains could be adapted to specific geographic areas, as suggested by Marasas *et al.* (1979) and these areas need to be identified.

REFERENCES

- Alberts, J.F., Gelderblom, W.C.A., Thiel, P.G., Marasas, W.F.O., Van Scalkwyk, D.J. & Behrend, Y. 1990. Effects of temperature and incubation period on production of fumonisins B₁ by *Fusarium moniliforme*. *Applied and Environmental Microbiology* **56**: 1729-1733.
- Anonymous, 2004a. The Food and Drug Administration food and cosmetic compliance program: Mycotoxins in imported food. [Available on Internet:] <http://www.cfsan.fda.gov/~comm/cp07002.html> [Date of access: 27 February 2006].
- Anonymous, 2004b. USDA production estimates and crop assessment division. South Africa: planted corn area at historic lows. [Available on Internet:] <http://www.fas.usda.gov/pecad/highlights/2004/01/safrica/index.html> [Date of access: 22 November 2006].
- Anonymous, 2006. South African Weather Service. [Available on Internet:] <http://www.weathersa.co.za> [Date of access: 22 November 2006].
- Bacon, C.W. & Williamson, J.W. 1992. Interactions of *Fusarium moniliforme*, its metabolites and bacteria with corn. *Mycopathologia* **117**: 65-71.
- Bush, B.J., Carson, M.L., Cubeta, M.A., Hagler, W.M. & Payne, G.A. 2003. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. *Phytopathology* **94**: 88-93.
- Chulze, S.N., Ramirez, M.L., Farnochi, M.C., Pascale, M., Visconti, A. & March, G. 1996. *Fusarium* and fumonisin occurrence in Argentina corn at different ear maturity stages. *Journal of Agricultural Food Chemistry* **44**: 2797-2801.
- Danielsen, S. & Jensen, D.F. 1998. Relationships between seed germination, fumonisin content, and *Fusarium verticillioides* infection in selected maize samples from different regions of Costa Rica. *Plant Pathology* **47**: 609-614.

de Castro, M.F.P.M., Bragagnolo, N. & de Toledo Valentini, S.R. 2002. The relationship between fungi growth and aflatoxin production with ergosterol content of corn grains. *Brazilian Journal of Microbiology* **33**: 22-26.

Desjardins, A.E. 2006. *Fusarium* mycotoxins. Chemistry, genetics and biology. The American Phytopathological Society. APS Press. 260pp.

Dilkin, P., Mallmann, C.A., de Almeida, C.A.A., Stefanon, E.B., Fontana, F.Z. & Milbradt, E.L. 2002. Production of fumonisins by strains of *Fusarium moniliforme* according to temperature, moisture and growth period. *Brazilian Journal of Microbiology* **33**: 111-118.

Fandohan, P., Hell, K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology* **2**: 570-579.

Glenn, A.E. 2005. Natural variation of ascospore and conidial germination by *Fusarium verticillioides* and other *Fusarium* species. *Mycological Research* **110**: 211-219.

Gourama, H. & Bullerman, L.B. 1994. Relationship between aflatoxin production and mold growth as measured by ergosterol and plate count. *Lebensmittel-W. und Technologie* **28**: 185-189.

Jardine, D.J. & Leslie, J.F. 1999. Aggressiveness to mature maize plants of *Fusarium* strains differing in ability to produce fumonisin. *Plant Disease* **83**: 690-693.

King, S.B. & Scott, G.E. 1981. Genotypic differences in maize to kernel infection by *Fusarium moniliforme*. *Phytopathology* **71**: 1245-1247.

Leslie, J.F & Summerell, B.A. 2006. The fusarium laboratory manual. Blackwell publishing. 388pp.

Marasas, W.F.O., Kriek, N.P.J., Wiggins, V.M., Steyn, P.S., Towers, D.K. & Hastie, T.J. 1979. Incidence, geographic distribution, and toxigenicity of *Fusarium* species in South African Corn. *Phytopathology*, **69**: 1181-1185.

Marin, S., Homedes, V., Sanchis, V., Ramos, A.J. & Magan, N. 1999. Impact of *Fusarium moniliforme* and *F. proliferatum* colonisation of maize on calorific losses and fumonisin production under different environmental conditions. *Journal of Stored Products Research* **35**: 15-26.

Mazzani, C., Borges, O., Luzón, Barrientos, V. & Quijada, P. 2001. Occurrence of *Fusarium moniliforme* and fumonisins in kernels of maize hybrids in Venezuela. *Brazilian Journal of Microbiology* **32**: 345-349.

Miller, J.D., Savard, M.E., Schaafsma, A.W., Seifert, K.A. & Reid, L.M. 1995. Mycotoxin production by *Fusarium moniliforme* and *Fusarium proliferatum* from Ontario and occurrence of fumonisin in the 1993 corn crop. *Canadian Journal of Plant Pathology* **17**: 233-239.

Miller, J.D. 2001. Factors that affect the occurrence of fumonisin. *Environmental Health Perspectives* **109**: 321-324.

Mille-Lindblom, C., von Wachenfeldt, E. & Tranvik, L.J. 2004. Ergosterol as a measure of living fungal biomass: persistence in environmental samples after fungal death. *Journal of Microbiological Methods* **59**: 253-262.

Munkvold, G.P. & Desjardins, A.E. 1997. Fumonisins in maize. Can we reduce their occurrence? *Plant Disease* **81**: 556-564.

Munkvold, G.P., Mc Gee, D.C. & Carlton, W.M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. *Phytopathology*, **87**: 209-217.

Nelson, P.E., Tousson, T.A. & Marasas, W.F.O. 1983. *Fusarium species : An illustrated manual for identification*. The Pennsylvania State University Press, University Park and London. 193pp.

Nelson, P.E., Desjardins, A.E. & Plattner, R.D. 1993. Fumonisin, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annual Review of Phytopathology* **31**: 233-252.

Ness, W.R. 1977. The biochemistry of plant sterols. In: Paoletti, R. & Kritchevsky, D. (Eds.). *Advances in Lipid Research*. Academic Press, New York. p233-324.

Ngoko, Z., Marasas, W.F.O., Rheeder, J.P., Shepard, G.S., Wingfield, M.J. & Cardwell, K.F. 2001. Fungal infection and mycotoxin contamination of maize in the humid forest and the western highlands of Cameroon. *Phytoparasitica* **29**: 352-360.

Ono, E.Y.S., Sugiura, Y., Homecin, M., Kamogae, M., Vizzoni, E., Ueno, Y. & Hirooka, E.Y. 1999. Effect of climatic conditions on natural mycoflora and fumonisins in freshly harvested corn in the State of Parana, Brazil. *Mycopathologia* **147**: 130-148.

Orsi, R.B., Corrêa, B., Possi, C.R., Schammas, E.A., Nogueira, J.R., Dias, S.M.C. & Malozzi, M.A.B. 2000. Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *Journal of Stored Products Research* **36**: 75-87.

Padgett, D.E. & Posey, M.H. 1993. An evaluation of the efficiencies of several ergosterol extraction techniques. *Mycological Research* **97**: 1476-1480.

Payne, G.A. 1992. Aflatoxins in maize. *Plant Science* **10**: 432-440.

Ramirez, M.L., Pascale, M., Chulze, S. & Reynoso, M.M. 1996. Natural occurrence of fumonisins and their correlation to *Fusarium* contamination in commercial corn hybrids growth in Argentina. *Mycopathologia* **135**: 29-34.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S., Du Toit, W., Pretorius, A.J., Van Schalkwyk, D.J. 1990. Incidence of *Fusarium* and *Diplodia* species and other fungi in naturally infected grain of South African maize cultivars. *Phytophylactica* **22**: 97-102.

Rheeder, J.P., Marasas, W.F.O. & Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology* **68**: 2101-2105.

Saxena, J., Munimbazi, C. & Bullerman, L.B. 2001. Relationship of mould count, ergosterol and ochratoxin A production. *International Journal of Food Microbiology* **71**: 29-34.

Schwadorf, K. & Müller, H.M. 1989. Determination of ergosterol in cereals, mixed feed components, and mixed feeds by liquid chromatography. *Association of Official Analytical Chemists* **72**: 457-462.

Scott, P.M. 1993. Fumonisins. *International Journal of Food Microbiology* **18**: 257-270.

Seitz, L.M., Sauer, D.B., Burroughs, R., Mohr, H.E. & Hubbard, J.D. 1979. Ergosterol as a measure of fungal growth. *Phytopathology* **69**: 1202-1203.

Shepard, G.S., Thiel, P.G., Stockenstrom & Syndeham, E.W. 1996. Worldwide survey of fumonisin contamination and corn-based products. *Association of Official Analytical*

Chemists **79**: 671-686.

Sydenham, E.W., Shepard, G.S., Thiel, P.G. Marasas, W.F.O. & Stockenstrom, S. 1991. Fumonisin contamination of commercial corn-based human foodstuffs. *Journal of Agricultural Food Chemistry* **39**: 2914-2018.

Thiel, P.G., Marasas, W.F., Sydenham, E.W., Shepard, G.S. & Gelderblom, W.C. 1992. The implications of naturally occurring levels of fumonisins in corn for human and animal health. *Mycopathologia* **117**: 3-9.

Turner, P.C., Nikiema, P. & Wild, C.P. 1999. Fumonisin contamination of food: progress in development of biomarkers to better assess human health risks. *Mutation Research* **443**: 81-93.

Van Wyk, P.S., Scholtz, D.J. & Loss, O. 1986. Selective medium for the isolation of *Fusarium* spp. from soil debris. *Phytophylactica* **18**: 67-69.

Visconti, A. & Doko, M.B. 1994. Survey of fumonisin production by *Fusarium* isolated from cereals in Europe. *Association of Official Analytical Chemists* **79**: 671-678.

Visconti, A. 1996. Fumonisins in maize genotypes grown in various geographic areas. In: Jackson, L.S., de Vries, J.W. & Bullerman, L.B. (Eds.). Fumonisins in food. Plenum Press, New York. p193-204.

Williams, K.C, Blaney, B.J., Dodman, R.L. & Palmer, C.L. 1992. Assessment for animal feed of maize kernels naturally infected predominantly with *Fusarium moniliforme* and *Diplodia maydis*. Fungal isolations and changes in chemical composition. *Australian Journal of Agricultural Research* **43**: 773-782.

Table 4.1 Total fumonisins allowed ($B^1+B^2+B^3$) in maize as food and animal feeds (Anonymous, 2004a).

Humans	2 ppm
Equids and rabbits	5 ppm; no more than 20 % of diet
Swine	20 ppm; no more than 50 % of diet
All other livestock species and pet animals	10 ppm; no more than 50 % of diet

Table 4.2 Isolation frequencies of *Fusarium* spp. and fumonisin concentrations in maize from silos at 23 localities in the maize production areas of South Africa.

Free State							
	<i>F. verticillioides</i> (%)	<i>F. proliferatum</i> (%)	<i>F. subglutinans</i> (%)	Fumonisin* (ppm)	Mean** Rainfall	Mean** min temp	Mean** max temp
Hoopstad	22.6	1.7	2.9	1.8			
Wesselsbron	27.4	1	6.2	0.7			
Mean	25	1.4	4.6	1.2			
Gauteng							
Bronkhorstspuit	52.4	4	14.4	0			
Bloekomspruit	13	0.6	8	0.2			
Mean	32.7	2.3	11.2	0.1			
Mpumalanga							
Delmas	44.1	1.6	3.2	0	40.72	9.86	24.41
North West							
Biesiesvlei	56.2	3.2	2.8	0.9			
Bloemhof	40.6	2.2	5.8	1.7	48.48	10.73	26.54
Coligny	23.4	2.6	9	0.3			
Delareyville	2.2	0	0.2	0			

Derby	68.8	3.2	14.2	7.5			
Grootpan	55.4	3.2	14.8	2.8			
Halfpad	48	1.4	3.6	1.3			
Hartebeesfontein	70.8	4.2	4.8	1.7			
Koster	33.8	1.2	5.8	6.6	64.16	9.65	23.95
Leeudoringstad	23.3	2.2	8.8	1.9			
Lichtenburg	34.5	0.4	10.2	2.3	63.2	10.81	24.92
Ottosdal	45.2	1	2.8	0.5	52.58	9.46	25.32
Potchefstroom	42.2	1.6	3.4	0.8	57.12	11.58	25.28
Sannieshof	22	1.8	5	2.4	54.04	10.51	25.66
Schweizer-Reyneke	23.6	2.8	2	0.1	55.77	11.94	26.35
Ventersdorp	13.4	2.2	9.8	2.3	51.35	10.46	24.46
Werda	17	0.8	7.4	0.4			
Wolmaranstad	28.4	28.3	7	2			
Mean	36	3.46	6.5	1.9			
Mean over all provinces	35.1	3.2	7.4	1.7			

*Average of 15 replications for each locality

**Mean rainfall, minimum and maximum temperature for each locality for November 2003 to July 2004

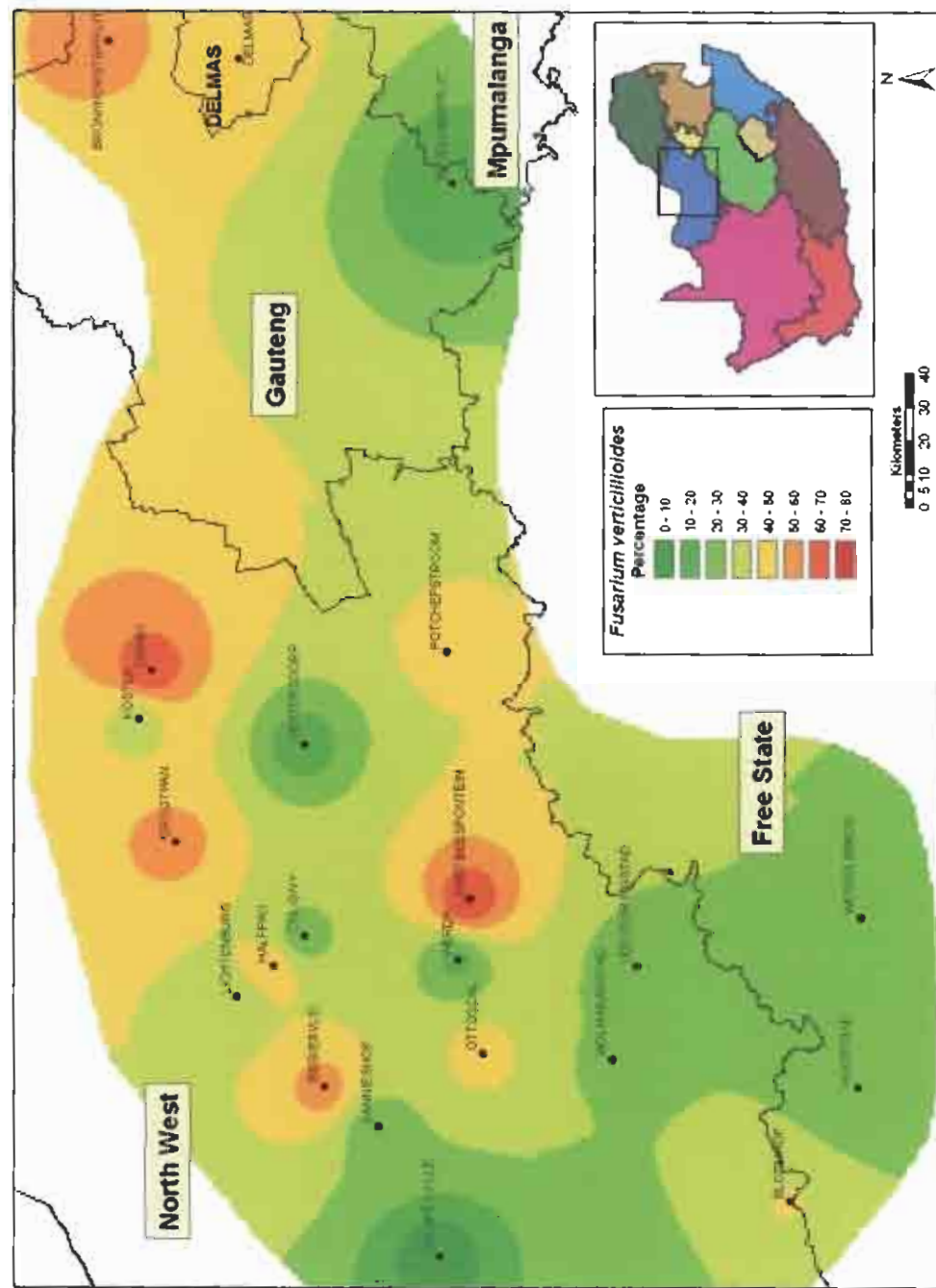


Figure 4.1 Isolation frequencies of *Fusarium verticillioides* in the warmer maize production areas of South Africa.

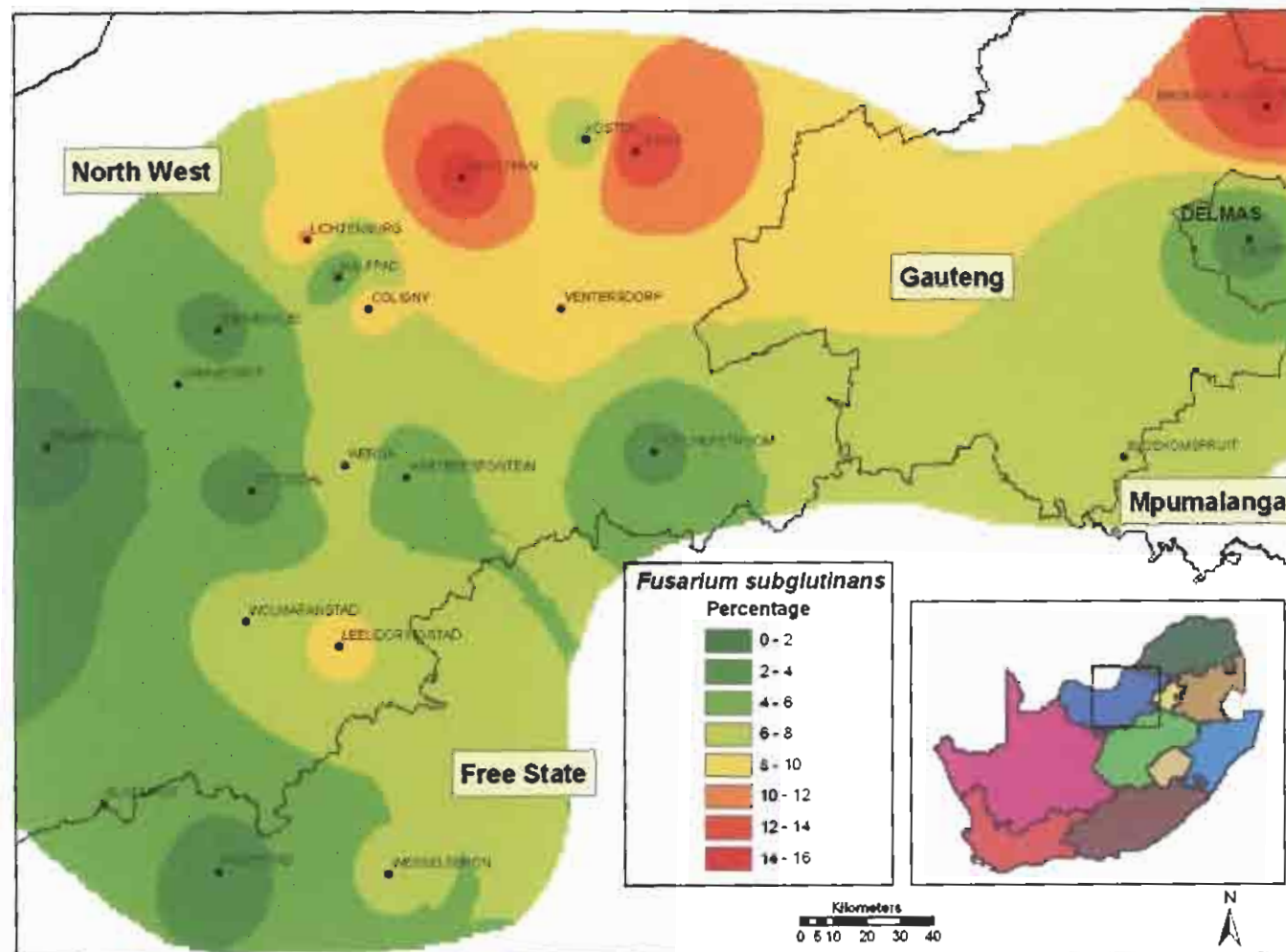


Figure 4.3 Isolation frequencies *Fusarium subglutinans* in the warmer maize production areas of South Africa.

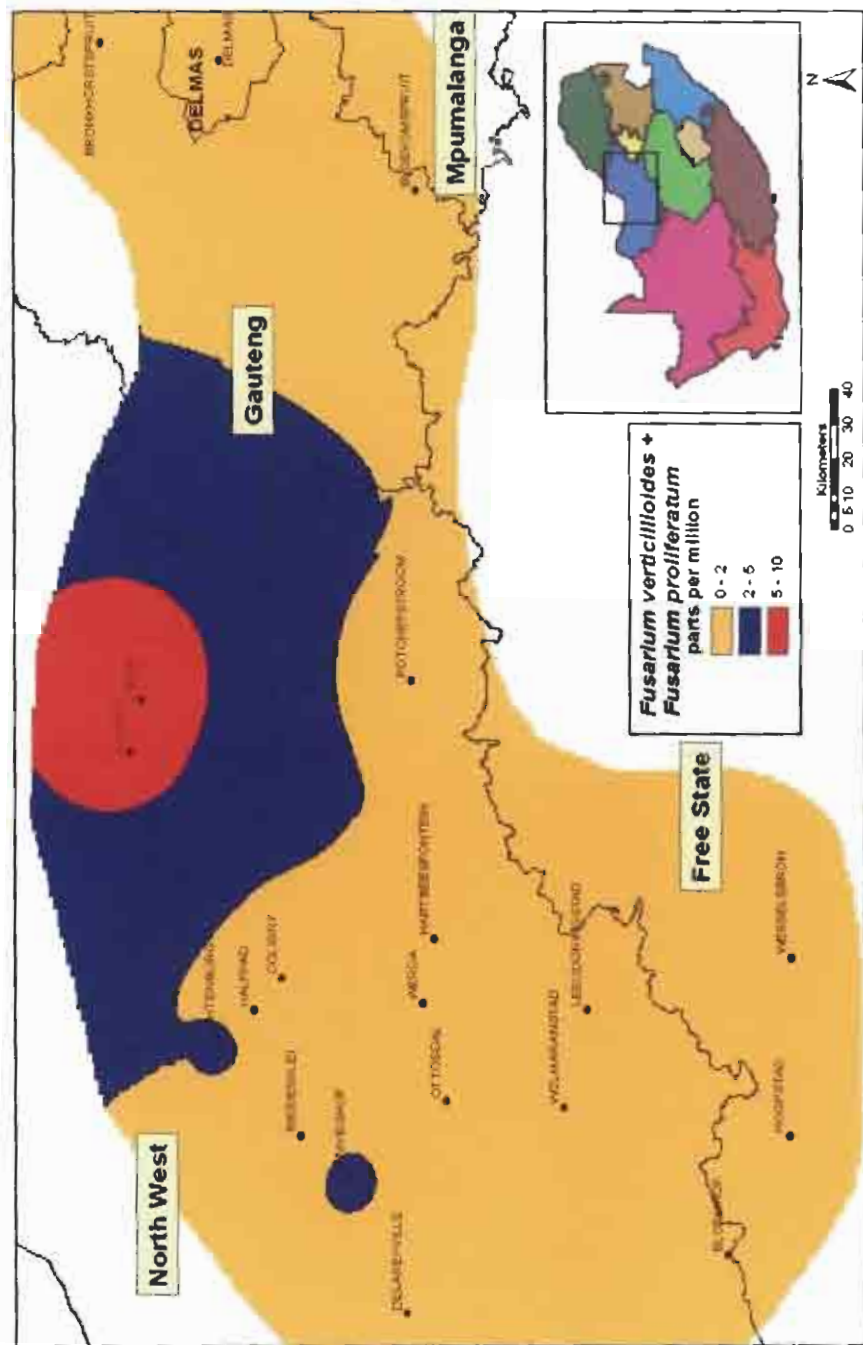


Figure 4.4 Fumonisin occurrence measured in parts per millions in the warmer maize production areas of South Africa.

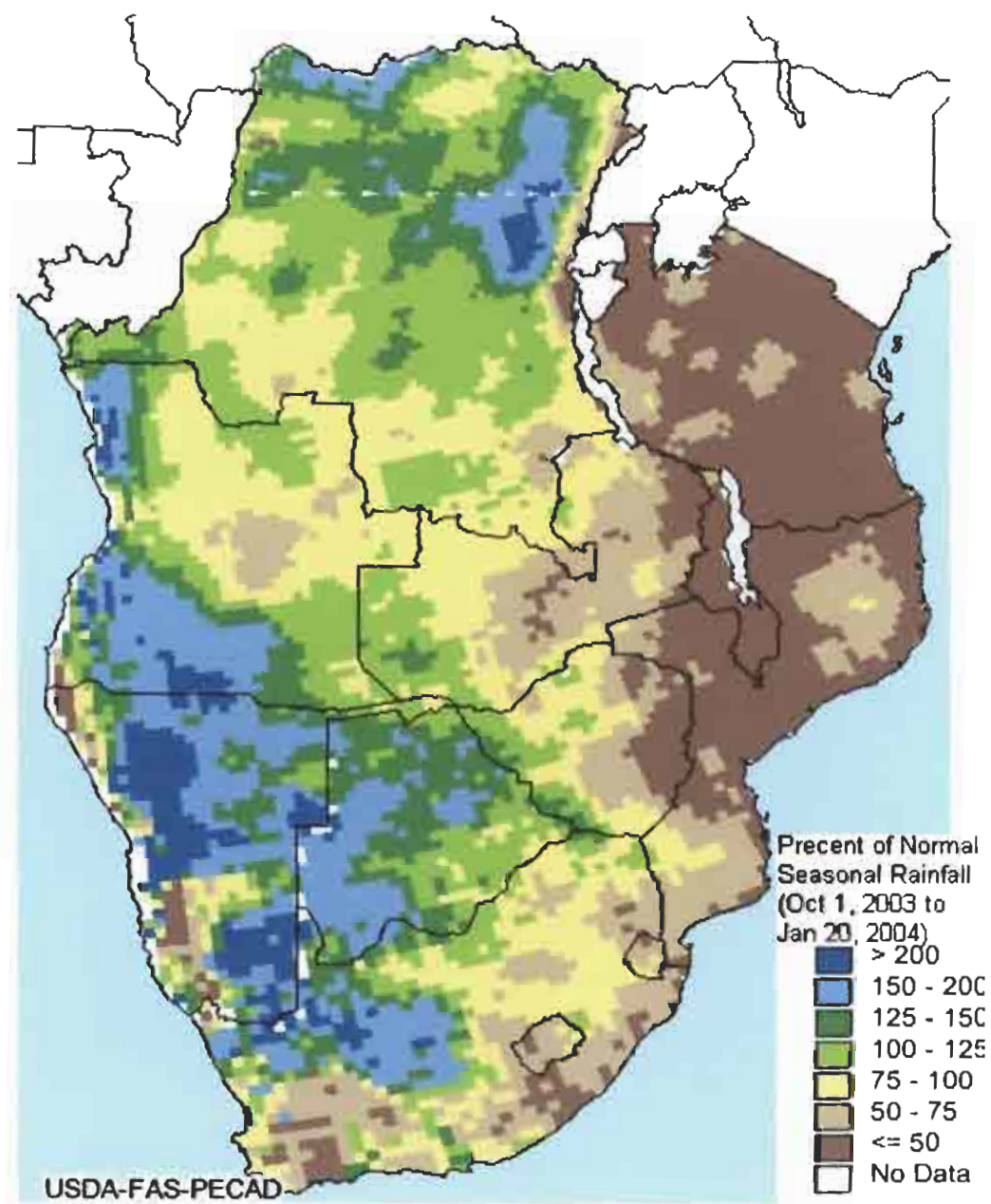


Figure 4.5 Satellite rainfall estimates for January 2003 to January 2004 (Anonymous, 2004b).

CHAPTER 5

VARIATION OF *FUSARIUM* SPP. IDENTIFICATION IN MAIZE SAMPLES DUE TO ENUMERATOR AND MEDIUM DIFFERENCES

5.1 INTRODUCTION

Maize (*Zea mays* L.) is the most important cereal crop produced in southern Africa (Fandohan *et al.*, 2003). *Fusarium* sp. associated with maize ear rot include *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushina) Nirenberg, *F. subglutinans* (Wollenw. et Reinking) Nelson of section Liseola and *F. graminearum* (Schwabe) of section Discolor. *F. verticillioides* is the most common species isolated from diseased maize worldwide (Munkvold & Desjardins, 1997). Many plants have at least one *Fusarium*-associated disease (Leslie & Summerell, 2006) and most plant pathologists, at one time or another will have to identify a culture of a *Fusarium* spp. Molecular methods such as genotyping or DNA sequencing are much faster than present morphological diagnoses because limited culturing of the fungus is required (Leslie & Summerell, 2006). For laboratories that currently lack or are unable to acquire molecular expertise, identifications based on morphology will remain the rule (Leslie & Summerell, 2006). *Fusarium* spp. identifications should be reliable and accurate when using identification keys in manuals (Nelson *et al.*, 1983; Leslie & Summerell, 2006). Primary *Fusarium* spp. that occur on maize are *F. verticillioides* and *F. proliferatum* that have microconidia that form in chains on mono- and polyphialides, respectively, and *F. subglutinans* that has microconidia that are produced in false heads on mono- and polyphialides (Leslie & Summerell, 2006). *F. graminearum* is easier to distinguish from the three above-mentioned *Fusarium* spp. from section Liseola as microconidia are absent and pale, orange macroconidia, usually uniform in shape and size with five to six distinct septa are abundant in sporodochia (Leslie & Summerell, 2006).

Unreliable and inaccurate identification could result in variation in the quantification of *Fusarium* spp. associated with maize ear rot when two or more researchers identify the same *Fusarium* isolates based on morphological characteristics. According to Nita *et al.* (2003) reliability can be defined as “the extent to which the same measurements of

individuals (enumerators) obtained under different conditions yield similar results". The determination of reliability requires consistent identification of the same fungus isolates by different enumerators or techniques (Nutter *et al.*, 1993; Forbes & Korva, 1994). Inter-enumerator reliability was considered a source of variability and was defined as the linear relationship between fungus identifications of the same fungus isolates performed by different enumerators (Nutter *et al.*, 1993; Nita *et al.*, 2003; Ross *et al.*, 2004).

Fusarium spp. vary phenotypically and morphologically when cultured on standard media such as Carnation Leaf Agar (CLA), Spezieller Nährstoffarmer Agar (SNA) and Potato Dextrose Agar (PDA), according to Leslie & Summerell (2006). Physiological characteristics such as growth rate and mycotoxin production also vary on different growth media. Thrane (2001) suggested that the ability of *Fusarium* spp. to produce certain mycotoxins would be an important characteristic in *Fusarium* taxonomy, but that it cannot be used to identify a culture to species level. *F. verticillioides* and *F. proliferatum* are the most important producers of high levels of fumonisins while *F. subglutinans* produces low levels of fumonisins (Rheeder *et al.*, 2002) and *F. graminearum* may produce zearelanone, nivalenol and deoxynivalenol (Leslie & Summerell, 2006).

This study was conducted to 1) determine *Fusarium* spp. variation in maize samples, 2) compare the accuracy of *Fusarium* spp. identification on rose bengal-glycerine-urea (RbGU) as selective and identification medium (Van Wyk *et al.*, 1986) with split plates containing standard CLA and PDA as identification media (Nelson *et al.*, 1983; Leslie & Summerell, 2006) and 3) detect and quantify potential bias among enumerators (inter-enumerator reliability) in direct microscopic identifications of *F. verticillioides*, *F. proliferatum* and *F. subglutinans*.

5.2 MATERIALS AND METHODS

Three enumerators independently identified *Fusarium* spp. isolates from RbGU and CLA/PDA media to determine the accuracy of species identification by different

enumerators (inter-enumerator reliability). Two hundred and fifty kernels from silo localities near Bronkhorstspuit, Derby, Hartebeesfontein, Sannieshof and Ventersdorp were surface-sterilized as described by King & Scott (1981) and four kernels were plated equidistantly onto a petri dish containing RbGU medium (Van Wyk *et al.*, 1986) to isolate and directly identify *Fusarium* species (Flett, 1994). Petri dishes were incubated for seven days in the dark at 25°C (Nelson *et al.*, 1983; Leslie & Summerell, 2006). After seven days fungus colonies were numbered and identified according to the guidelines of Nelson *et al.* (1983) and Leslie & Summerell (2006) by enumerators (designated 1, 2 and 3).

Because of high volume, cultures were not single spored but representative samples of the numbered isolated *Fusarium* spp. were each transferred to CLA/PDA for further identification (Figure 5.1). The broad spectrum antibiotic chloramphenicol was applied at a rate of 250 mg per 100 agar to both CLA and PDA to inhibit bacterial growth (Leslie & Summerell, 2006). CLA/PDA cultures were placed under ultraviolet light to promote the growth of chains and development of the teleomorph stage to aid identification (Leslie & Summerell, 2006). Ultraviolet lighting was programmed to provide 12 hours alternatively of light and darkness. The three enumerators again identified fungi after 14 days using the guidelines of Nelson *et al.* (1983) and Leslie & Summerell (2006) on the CLA/PDA medium.

5.2.1 *Fusarium* spp. variation in maize samples

Five replications of 250 kernels collected from silo localities near Derby, Hartebeesfontein, Sannieshof and Ventersdorp were surface-sterilized and plated onto RbGU and CLA/PDA for enumeration of *F. verticillioides*, *F. proliferatum* and *F. subglutinans* in maize samples by three enumerators. Enumeration data from each silo locality were analysed using multifactor ANOVA to determine the interaction effects of replications, localities, media and enumerators.

Weather data were collected from weather stations nearest to Hartebeesfontein, Derby, Sannieshof and Ventersdorp for November 2003 to March 2004 by the ARC - Institute

of Soil, Climate and Water.

5.2.2 Identification media

Simple linear regression analyses were conducted to determine the relationship between fungus identifications performed by different enumerators on different media. Regression analyses were carried out on fungal enumerations done on RbGU medium and CLA/PDA media for all three enumerators for *F. verticillioides*, *F. proliferatum* and *F. subglutinans*. Regression equation parameters, namely slope and intercept were used to evaluate and compare the accuracy and precision of fungus identifications and the reliability of different media (Nutter *et al.*, 1993).

5.2.3 Inter-enumerator reliability

Inter-enumerator reliability was quantified using simple linear regression analysis of the *Fusarium* spp. (*F. verticillioides*, *F. proliferatum* and *F. subglutinans*) identified by an individual regressed against identifications done by the two other individuals. Regression analyses were conducted using Statgraphics Version 5.

5.3 RESULTS

5.3.1 *Fusarium* spp. variation in maize samples

Analysis of variance showed no significant differences in replications, isolation media used or enumerators in the quantification of *F. verticillioides* from maize kernels. Significant locality differences were obtained ($P \leq 0.05$) (Table 5.1). An unacceptably high CV of 338.31% was obtained on initial data but an ARC-SIN transformation reduced the CV to 8.14%.

Analysis of variance carried out on *F. subglutinans* data showed no significant differences between enumerations on different media or those due to enumerators. Significant locality effects were obtained ($P < 0.05$) with a high CV of 261.76%, which was transformed using an ARC-SIN transformation that reduced the CV to 36.32%.

The interaction between locality and enumerator on the isolation frequency of *F. proliferatum* was significant (Table 5.2). No significant differences were recorded for media and replications.

5.3.2 Identification media

F. verticillioides represented 94%, *F. subglutinans* 4% and *F. proliferatum* 2% of total fungus counts on CLA/PDA media. Positive linear relationships between identifications of *F. verticillioides* made on RbGU and CLA/PDA media were obtained over all three enumerators (Figure 5.2). R^2 values ranged from 89.52% - 99.94%. Significant relationships were obtained between identifications of *F. proliferatum* on RbGU media and CLA/PDA media made by enumerators 1 ($R^2=92.99\%$, $P=0.0080$) and 2 ($R^2=89.67\%$, $P=0.0145$), but not enumerator 3 with a $R^2=68.92\%$ and $P=0.0818$ (Figure 5.3). A significant linear relationship was observed for *F. subglutinans* isolations on RbGU ($R^2=97.32$, $P=0.0019$) and CLA/PDA ($R^2=94.73\%$, $P=0.0052$) media for enumerators 1 and 2 respectively (Figure 5.4). Enumerator 3 RbGU and CLA/PDA media isolations related poorly with a $R^2=71.02\%$ and $P=0.0731$. Results indicate a significant positive relationship between the use of RbGU and CLA/PDA media to identify the fungus *F. proliferatum*, as indicated for enumerators 1 and 2.

5.3.3 Inter-enumerator reliability

Enumerators 1 and 2 demonstrated the highest agreement for identification accuracy of *F. verticillioides* on CLA/PDA (Figure 5.5) ($R^2=99.49\%$; $P=0.0002$) and RbGU media ($R^2=98.96\%$; $P=0.0005$) (Figure 5.6). The relationship between identifications of *F. verticillioides*, *F. proliferatum* and *F. subglutinans* made by different enumerators yielded R^2 values that were generally higher on CLA/PDA than on the RbGU medium.

Inter-enumerator linear regressions for *F. verticillioides* on CLA/PDA were significant ($R^2=99.49\%$; $P=0.0002$) for enumerator 1 and 2, enumerator 1 and 3 ($R^2=99.00\%$, $P=0.0004$) and enumerator 2 and 3 ($R^2=97.66\%$, $P=0.0015$) (Figure 5.5). Linear relationship slopes (ranging from 0.87 - 1.11) and intercepts (ranging from -1.82 to 4.06) indicated low variation between enumerators. Inter-enumerator linear regressions for

F. verticillioides on RbGU media (Figure 5.6) were significant for enumerator 1 and 2 ($R^2=98.96\%$, $P=0.0005$), enumerator 1 and 3 ($R^2=94.54\%$; $P=0.0055$) and enumerator 2 and 3 ($R^2=93.43\%$, $P=0.0073$). Low variation between enumerators were indicated by linear relationship slopes ranging from 0.90 to 1.19 and intercepts ranging from -5.59 to 5.82.

Insignificant inter-enumerator correlations existed for *F. proliferatum* (Figure 5.7) on CLA/PDA media for enumerator 1 and 2 ($R^2=14.90\%$; $P=0.5209$) and enumerator 2 and 3 ($R^2=0.17\%$; $P=0.9464$). Enumerator 1 and 3 ($R^2=80.73\%$; $P=0.0382$) displayed significant inter-enumerator correlations for *F. proliferatum* on CLA/PDA media. Low variation between enumerators for fungus identifications of *F. proliferatum* were indicated by linear relationship slopes ranging from 0.03 to 0.50 and intercepts ranging from 0.25 to 2.63. Inter-enumerator linear regressions for *F. proliferatum* on RbGU media (Figure 5.8) were not significant for enumerator 1 and 2 ($R^2=1.55\%$; $P=0.8418$), enumerator 1 and 3 ($R^2=65.99\%$; $P=0.0947$) or enumerator 2 and 3 ($R^2=17.01\%$; $P=0.4901$). Linear relations slopes ranging from 0.17 to 1.53 and intercepts ranging from -3.26 to 3.07, indicated low variation between enumerators.

Significant inter-enumerator linear regressions for *F. subglutinans* on CLA/PDA existed (Figure 5.9) with a $R^2=98.38\%$ and $P=0.0011$ for enumerator 1 and 2, a $R^2=95.95\%$ and $P=0.0035$ for enumerator 1 and 3 and a $R^2=98.38\%$ and $P=0.0009$ for enumerator 2 and 3. Extremely low variation between enumerators were indicated by linear relationship slopes ranging from 0.96 to 1.04 and intercepts ranging from -0.46 to 0.74. Inter-enumerator linear regressions for *F. subglutinans* on RbGU media (Figure 5.10) was significant with a $R^2=93.76\%$ and $P=0.0067$ for enumerator 1 and 2, a $R^2=83.57\%$ and $P=0.0289$ for enumerator 1 and 3 and a $R^2=88.69\%$, $P=0.0167$ for enumerator 2 and 3. Slopes (ranging from 0.72 to 1.36) and intercepts (ranging from -3.44 to 1.30) indicated low variation between enumerators.

5.4 DISCUSSION

5.4.1 *Fusarium* spp. variation in maize samples

Replications, identification media used and fungus enumerators had no significant effect on the quantification of *F. verticillioides*. This indicated that enumerators were consistent in their identification and quantification of *F. verticillioides* and that the media used were suitable for accurate and effective quantification of the pathogen. Samples collected from localities differed significantly in the frequency of *F. verticillioides*, with Hartebeesfontein and Derby having the highest mean, Sannieshof the second highest mean and Ventersdorp having the lowest mean (Table 1). Rheeder *et al.* (1990a) reported the distribution of *Fusarium* spp. to be linked to certain temperature requirements for fungus growth. The mean maximum temperature in Hartebeesfontein (28.7°C), Derby (29.0°C), Sannieshof (29.2°C) and Ventersdorp (27.3°) and the respective frequencies of *F. verticillioides* agree with Miller (2001) who suggested that *F. verticillioides* grows well above 26°C and Marin *et al.* (1995) who concluded that *F. verticillioides* grew better at 30°C.

No significant differences were observed for RbGU and CLA/PDA media or three enumerators in the quantification of *F. subglutinans*. However, the interaction between localities and replications was significant. Enumerators were consistent in their identification and quantification of *F. subglutinans* and the two identification media used for enumerations were accurate and effective. The interaction between localities and replications indicated that replications of *F. subglutinans* isolations were not consistent and the low isolation frequency of 4% of the total fungus enumerations requires more intense sampling procedures. Localities differed significantly in terms of *F. subglutinans* enumerations, with Derby having the highest and Sannieshof, Hartebeesfontein and Ventersdorp having the lowest mean isolation frequencies (Table 1).

No significant differences were observed for RbGU and CLA/PDA media and replications for the *F. proliferatum* enumerations indicating that the two identification media used for enumerations were accurate and effective and replications did not differ significantly.

The significant locality x enumerator interaction for *F. proliferatum* implies that enumerators struggled to distinguish between the *F. proliferatum* and *F. verticillioides* isolates over localities. The enumerator requires the necessary expertise to identify between monophialides and polyphialides to distinguish between *F. verticillioides* and *F. proliferatum* (Nelson *et al.*, 1983). Since *F. verticillioides* and *F. proliferatum* are isolated from the same localities or environmental conditions (Rheeder *et al.*, 1990b) and have almost the same distinguishing characteristics (Leslie & Summerell, 2006), this may have resulted in the locality and enumerator interaction.

5.4.2 Identification media

When assessment procedures that have potential for high variation such as toxin determinations and/or fungus plate identifications are used for *Fusarium* spp. identifications, it is necessary to limit these sources of variation. In this study enumerators 1 and 2 showed good repeatability of enumerations of *F. verticillioides* on both RbGU and CLA/PDA media, respectively, with enumerator 3 showing less repeatability. Enumerations of *F. subglutinans* and *F. proliferatum* for both RbGU and CLA/PDA media had a significant linear relationship for enumerators 1 and 2 respectively while enumerator 3 showed poor repeatability.

According to Nutter *et al.* (1993) enumerators and methods (media) that have the highest precision would be those in which the regression coefficients (slopes) are closest to 1.0, with y-intercepts closest to 0. Slopes deviating significantly from 1.0 indicate constant bias. The intercepts and slopes for enumerators 1 - 3 in the enumerations of *F. verticillioides*, *F. proliferatum* and *F. subglutinans* on RbGU and CLA/PDA media are an indication of low variation within enumerators. The use of RbGU and CLA/PDA media were eliminated as a source of variation in *Fusarium* identifications.

5.4.3 Inter-enumerator reliability

Enumerators 1 and 2 showed significant agreement for identifications of *F. verticillioides* and *F. subglutinans* and low variation between enumerators were detected for

enumerations on both media. Each increment increase in the identification of *F. verticillioides* by enumerator 1, using CLAPDA media, resulted in estimates by enumerator 2 and 3 increasing by a factor of 0.87 and 0.97, respectively. Similarly each increment increase in the identification of *F. verticillioides* by enumerator 1 using RbGU resulted in estimates by enumerator 2 and 3 increasing by factors of 0.90 and 1.09, respectively. This agrees with the conclusions of Nutter *et al.* (1993) that the difference between identifications made by one enumerator compared to identifications made by another enumerator decrease as fungus incidence increases, especially with *F. verticillioides* that presented 94% of all total fungus counts. Increment increases in the identification of *F. subglutinans* were 0.96, 1.00, 0.70 and 0.97 on CLAPDA and RbGU by enumerators 2 vs 3, respectively. This is indicative of extremely low variation between the three enumerators (Nutter *et al.*, 1993).

It is also stated by Nutter *et al.* (1993) that the difference between identifications made by a specific enumerator compared to identifications by another enumerator increases as fungus incidence decreases (Nutter *et al.*, 1993). This was observed in this study with identifications of *F. proliferatum* that represented 2% of all total fungus counts, where no significant inter-enumerator correlations were recorded between any of the three enumerators.

This study indicated that *Fusarium* spp. enumerations by three different enumerators were reliable and accurate, and were not a source of variation in the quantification of *Fusarium* spp. associated with maize ear rot.

REFERENCES

- Fandohan, P., Hell, K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology* **2**: 570-579.
- Flett, B.C. 1994. Evaluation of maize hybrids for kernel colonization by *Fusarium moniliforme* and *F. subglutinans*. *South African Journal of Plant and Soil* **11**: 41-44.
- Forbes, G.A. & Korva, J.T. 1994. The effect of using a Horsfall-Barrat scale on precision and accuracy of visual estimation of potato leaf blight severity in the field. *Plant Pathology* **43**: 113-120.
- King, S.B. & Scott, G.E. 1981. Genotypic differences in maize to kernel infection by *Fusarium moniliforme*. *Phytopathology* **71**: 1245-1247.
- Leslie, J.F. & Summerell, B.A. 2006. The *fusarium* laboratory manual. Blackwell Publishing. 386pp.
- Marin, S., Sanchis, V. & Magan, N. 1995. Water activity, temperature, and pH effects on growth of *Fusarium moniliforme* and *Fusarium proliferatum* isolates from maize. *Canadian Journal of Microbiology* **41**: 1063-1070.
- Miller, J.D. 2001. Factors that affect the occurrence of fumonisin. *Environmental Health Perspectives* **109**: 321-324.
- Munkvold, G.P. & Desjardins, A.E. 1997. Fumonisins in maize. Can we reduce their occurrence? *Plant Disease* **81**: 556-564.
- Nelson, P.E., Tousson, T.A. & Marasas, W.F.O. 1983. *Fusarium* species : An illustrated manual for identification. The Pennsylvania State University Press, University Park and London. 193pp.

Nita, M., Ellis, A. & Madden, V. 2003. Reliability and accuracy of visual estimation of phomopsis leaf blight of strawberry. *Phytopathology* **93**: 995-1005.

Nutter, F.W., Jr., Gleason, M.L., Jenco, J.H. & Christians, N.C. 1993. Assessing the accuracy, intra-rater repeatability, and intra-rater reliability of disease assessment systems. *Phytopathology* **83**: 806-812.

Rheeder, J.P., Marasas, W.F.O. & Van Wyk, P.S. 1990a. Fungal associations in corn kernels and effects on germination. *Phytopathology* **80**: 131-134.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S., Du Toit, W., Pretorius, A.J. & Van Schalkwyk, D.J. 1990b. Incidence of *Fusarium* and *Diplodia* species and other fungi in naturally infected grain of South African maize cultivars. *Phytophylactica* **22**: 97-102.

Rheeder, J.P., Marasas, W.F.O. & Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* species. *Applied and Environmental Microbiology* **68**: 2101-2105.

Ross, A.J., Wallace, B. & Davies, J.B. 2004. Technical note: measurement issues in taxonomic reliability. *Safety Science* **42**: 771-778.

Thrane, U. 2001. Developments in the taxonomy of *Fusarium* species based on secondary metabolites. In: Summerell, B.A., Leslie, J.F., Backhouse, D., Bryden, W.L. & Burgess, L.W. (Eds.). *Fusarium*: Paul E. Nelson memorial symposium. APS Press, St Paul, Minnesota: 29-49.

Van Wyk, P.S., Scholtz, D.J. & Loss, O. 1986. Selective medium for the isolation of *Fusarium* spp from soil debris. *Phytophylactica* **18**: 67-69.

Table 5.1 Effect of locality on *Fusarium verticillioides* and *Fusarium subglutinans* fungus enumerations on maize kernel samples.

Locality	<i>F. verticillioides</i> (mean)	<i>F. subglutinans</i> (mean)
Hartebeesfontein	62.92a	6.52c
Derby	62.76a	13.42a
Sannieshof	30.54b	7.42c
Ventersdorp	15.77c	9.84b
CV %	8.14	36.32

Means within columns followed by different letters differ according to Fisher's LSD ($P \leq 0.05$) based on ARC-SIN transformed data. CV% is based on ARC-SIN transformed data.

Table 5.2 The effect of locality and enumerator interaction on *Fusarium proliferatum* fungus enumerations on maize kernel samples.

Locality	Enumerator 1	Enumerator 2	Enumerator 3	Mean
Hartebeesfontein	4.3	0.9	5.2	3.5a
Derby	4.9	4.3	5.6	4.9b
Sannieshof	2.0	2.4	3.5	2.6a
Ventersdorp	2.46	3.4	3.8	3.2a
Mean	3.4a	2.8a	4.5b	3.6
CV %	174.3			
LSD	1.8			

Means within rows or columns followed by different letters differ according to Fisher's LSD ($P \leq 0.05$).

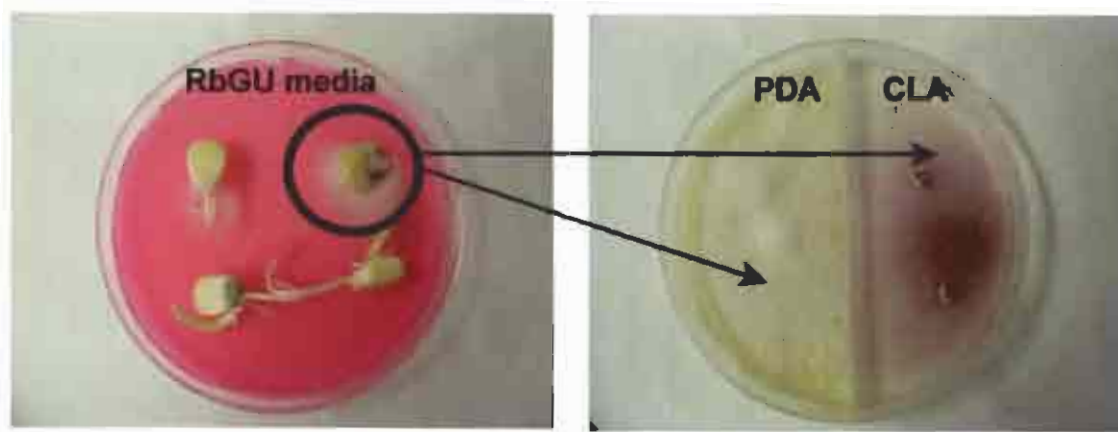


Figure 5.1 Samples of each isolate were transferred from RbGU media to CLA/PDA media.

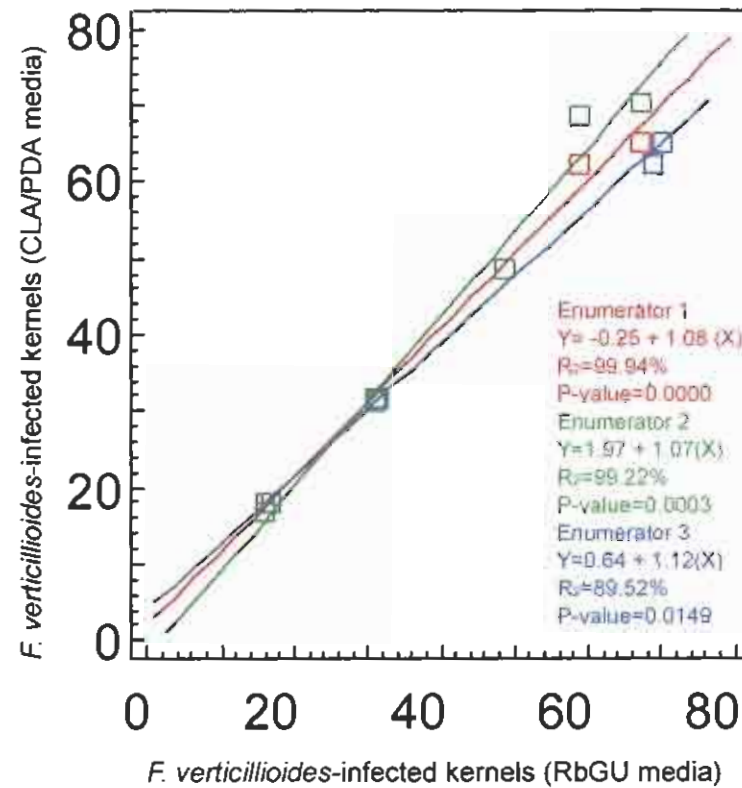


Figure 5.2 Fungus identifications (%) for *F. verticillioides* made on RbGU media compared with identifications of the same fungus isolates on CLA/PDA media for all three enumerators.

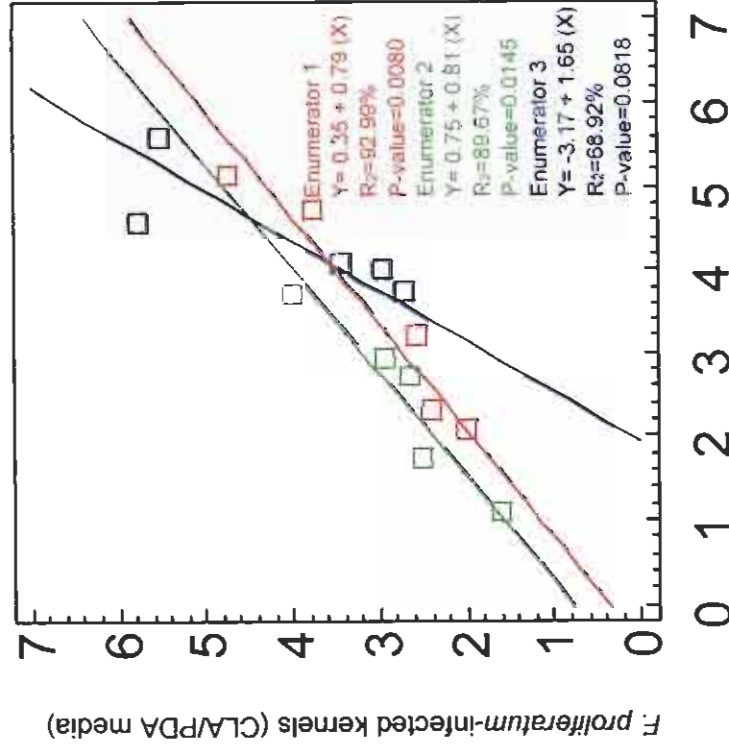


Figure 5.3 Fungus identifications (%) for *F. proliferatum* made on RbGU media compared with identifications of the same fungus isolates on CLA/PDA media for all three enumerators.

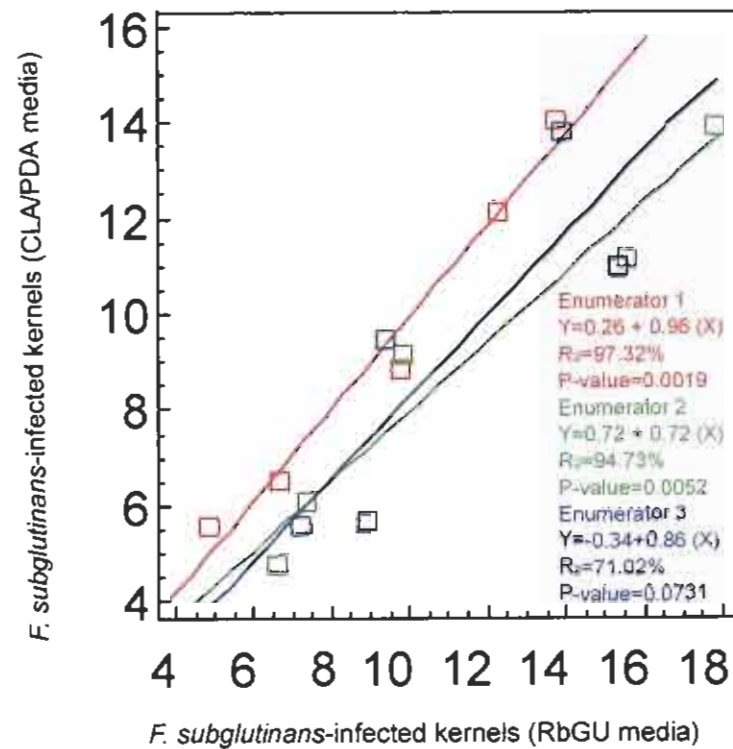


Figure 5.4 Fungus identifications (%) for *F. subglutinans* made on RbGU media compared with identifications of the same fungus isolates on CLA/PDA media for all three enumerators.

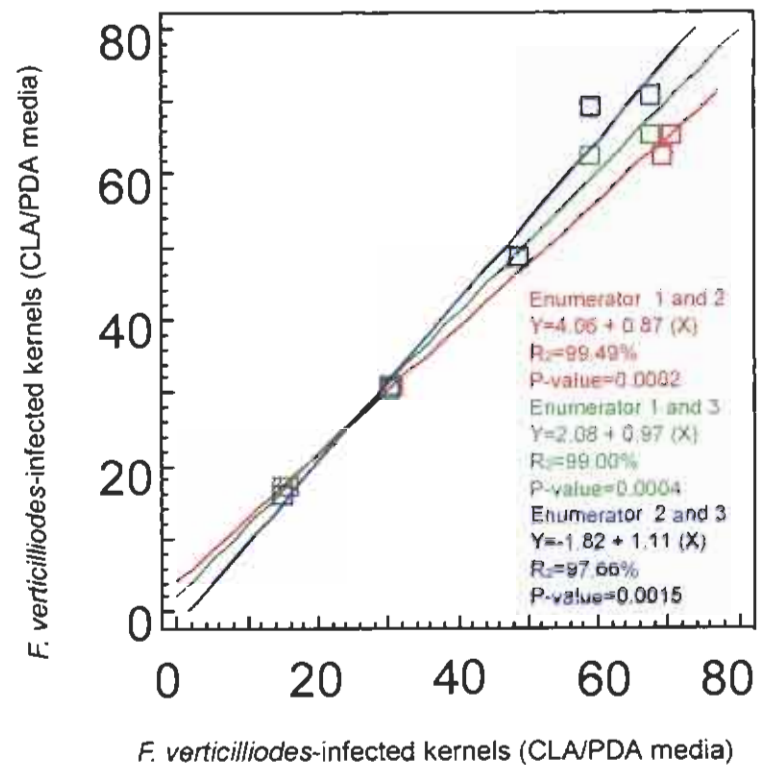


Figure 5.5 Determination of inter-enumerator reliability for fungus identifications (%) for *F. verticillioides* on CLA/PDA media for all three enumerators.

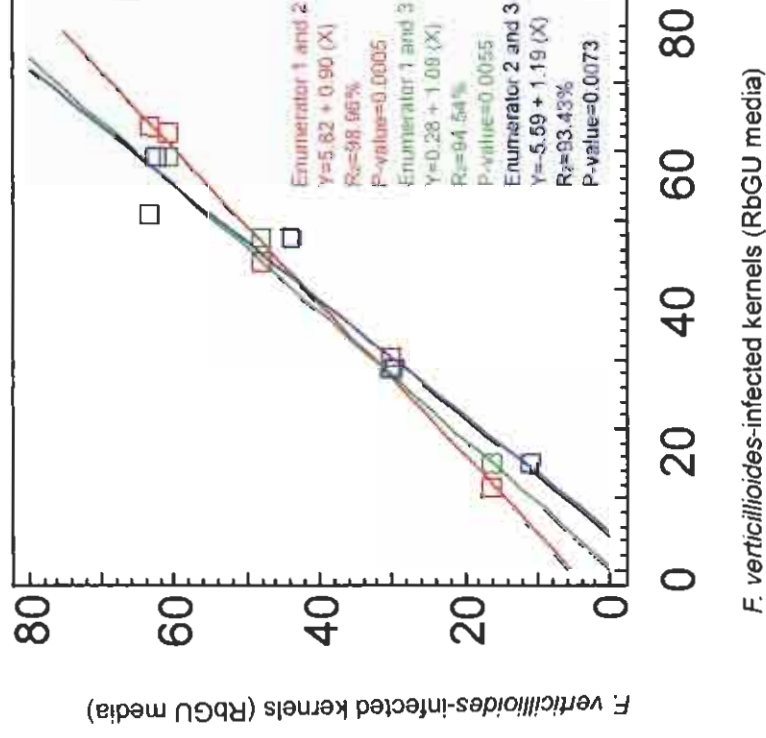


Figure 5.6 Determination of inter-enumerator reliability for fungus identifications (%) for *F. verticillioides* on RbGU media for all three enumerators.

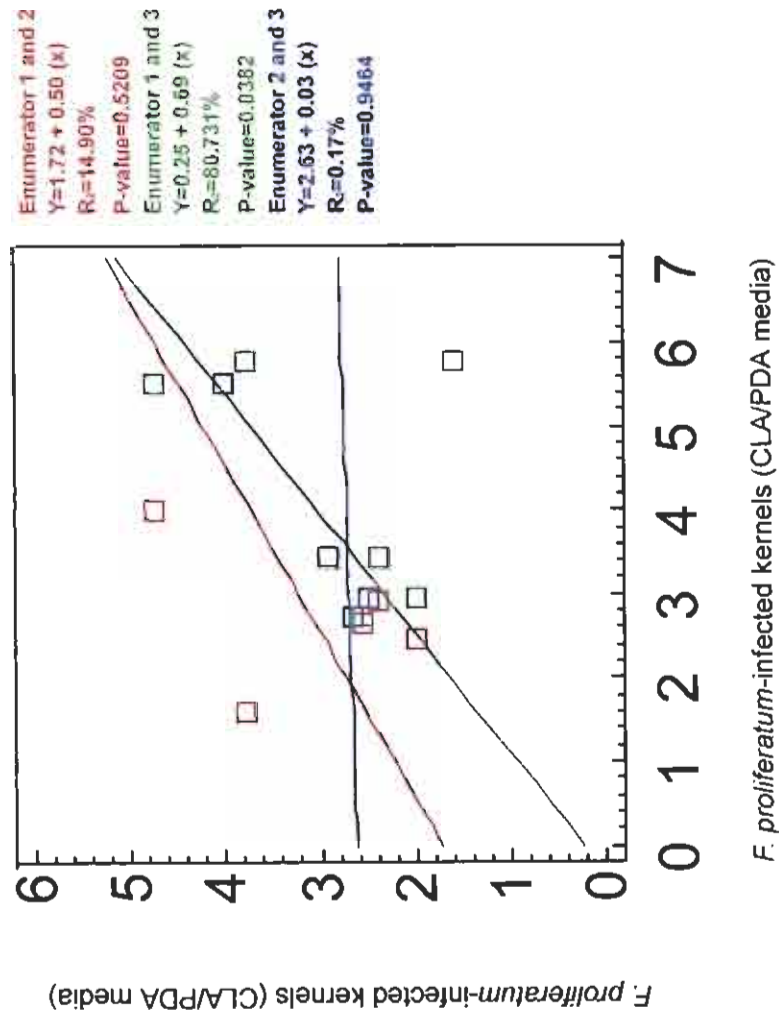


Figure 5.7 Determination of inter-enumerator reliability for fungus identifications (%) for *F. proliferatum* on CLA/PDA media for all three enumerators.

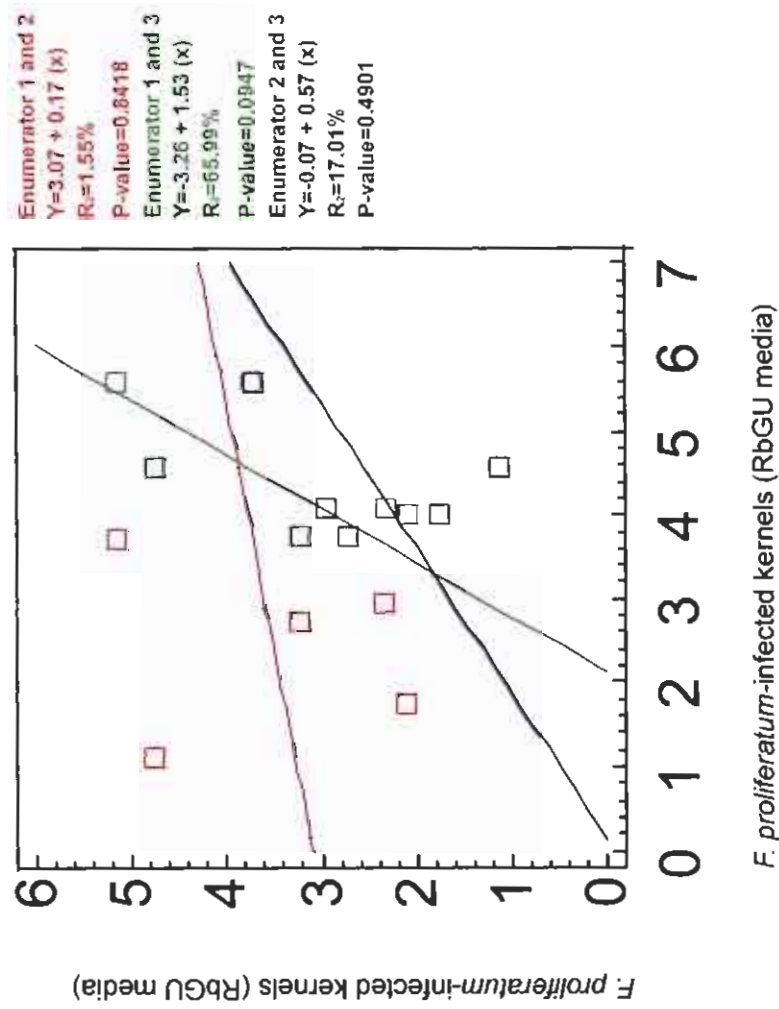


Figure 5.8 Determination of inter-enumerator reliability for fungus identifications (%) for *F. proliferatum* on RbGU media for all three enumerators.

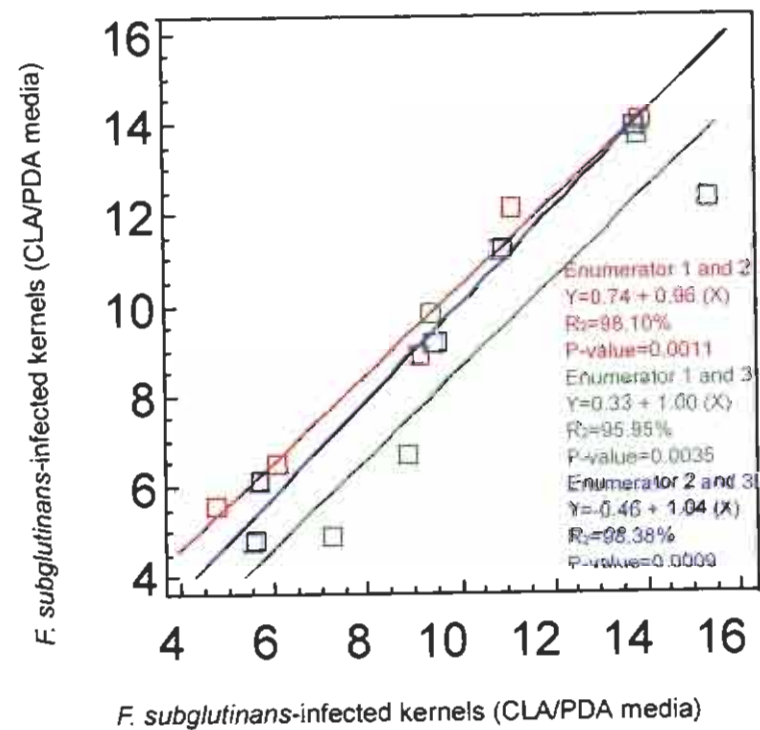


Figure 5.9 Determination of inter-enumerator reliability for fungus identifications (%) for *F. subglutinans* on CLA/PDA media for all three enumerators.

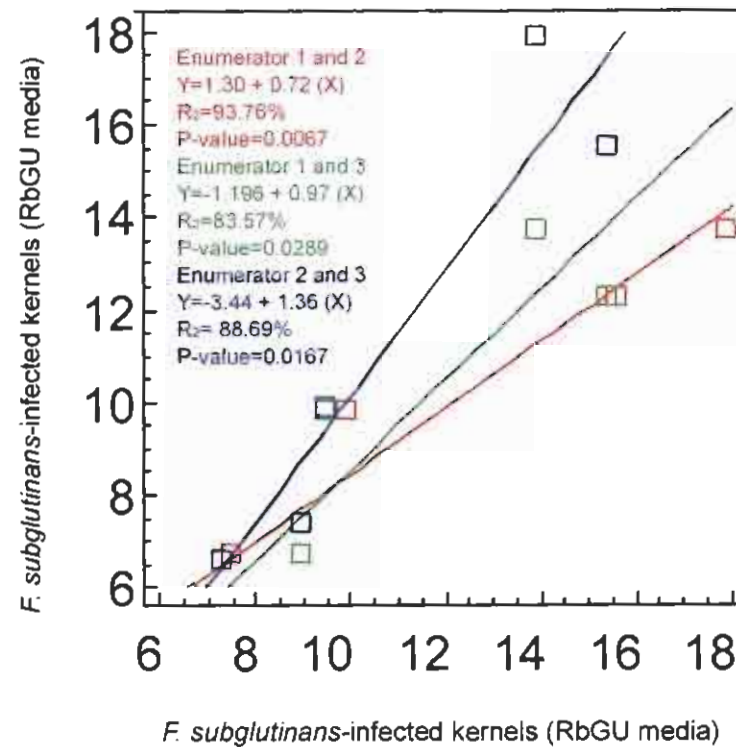


Figure 5.10 Determination of inter-enumerator reliability for fungus identifications (%) for *F. subglutinans* on RbGU media for all three enumerators.

CHAPTER 6

GENERAL DISCUSSION

To date substantial mycotoxin research using the ELISA-Veratox protocol has been carried out, but high variation in fumonisin quantification and the identification of associated spp. confounds statistical analysis of data. Studies were therefore conducted to quantify and address these sources of variation.

Screening of human and animal foodstuffs for the presence of fumonisins is of the utmost importance. The time frame from harvesting, receiving maize samples in the laboratory and testing for fumonisin levels was a major concern in quantifying the health risk associated with mycotoxin-contaminated foodstuffs as toxin levels may change with time. A significant amount of literature is available on the role of maize grain storage on fumonisin levels, but little information is available on storage of maize meal on fumonisin levels.

In Chapter 2, changes in fumonisin concentrations in milled maize samples in storage prior to being tested was determined from grain from three localities (Sannieshof, Ventersdorp and Lichtenburg) that were selected based on high fumonisin levels. Grain from each locality was milled for fumonisin quantification, allowing six replicates of each sample to be tested every two months for a year. Samples from Sannieshof and Lichtenburg had fumonisin levels that initially increased and subsequently decreased over time. The Ventersdorp sample showed little variation in fumonisin levels. A significant locality x storage time interaction was observed. Further research is needed to establish the effect that moisture, insect infestation, temperature and fungal incidence will have on stored grain and milled maize. Until further studies prove otherwise, it is advisable to test for fumonisins as soon as a maize grain or meal sample is obtained in order to obtain accurate fumonisin results.

A major concern in quantifying health risks associated with fumonisin-contaminated maize foodstuffs is the reliability of fumonisin detection methods. Variation in results from a single maize sample using a single evaluation method, could yield false positives and/or negatives. It was established in Chapter 3 that by substituting the prescribed 25

g sub-sample with a modified 250 g sub-sample, before a final 5 g aliquot is drawn from the sub-sample, significantly reduced variation in a single maize sample. This suggested that the 250 g sub-sample was more representative of the total sample. Information gained from this study will improve the accuracy of results using the ELISA technique to determine fumonisin levels and reduce the risk of wrongly classifying maize samples thereby improving food security for humans and animals. It is therefore recommended that the standard sub-sample size drawn for fumonisin detection be increased within the ELISA sampling protocol to 250 g.

According to Whitaker & Johansson (2005) random variation in fumonisin levels within the same maize sample can occur. They further indicated that when the sample fumonisin level increases, the variation between fumonisin levels will also increase, but the CV will decrease. Maize grain from Leeudoringstad with a low fumonisin level was spiked with maize grain from Vredefort that was naturally, highly contaminated by fumonisins. Fumonisin-contaminated grain was added at rates of 25%, 50%, 75% and 100% to study the effect that fumonisin levels had on variation within a sample. Fumonisin levels were established by using 25 g sub-samples as well as 250 g sub-samples. In the 25 g sub-sample study, only the 75% and 100% spiked samples produced a slight reduction in CV% and standard deviation, indicating that at high fumonisin levels there appears to be a reduction in CV% and standard deviation. In the 250 g sub-sample study, the 25%, 50%, 75% and 100% spiked samples had reduced CV% and standard deviation. This supports the view that by using 250 g sub-samples, variation in fumonisin levels in maize samples can be significantly reduced.

Number of replicate studies had no effect on reducing the CV and standard deviation in 25 g sub-sample studies. Modified 250 g sub-samples together with increased replications had a more significant effect in reducing variation. It was established that the number of replications needed to improve accuracy, will depend on the level of fumonisin infection in a sample. Fewer replications are required for samples with high fumonisin levels, than for samples with moderately or low fumonisin levels. The implication of this is that a larger sub-sample size (250 g) together with more replications in the case of low fumonisin levels is needed, although this may not be

feasible in practice because of time and money constraints.

ELISA fumonisin levels quantified at the ARC-GCI correlated significantly with HPLC levels determined at the MRC, although fumonisin levels using the ELISA technique were consistently higher by 33% in this study. This could have resulted in maize samples being wrongly downgraded in the past and fumonisin levels obtained with the ELISA technique can now be lowered by 33% in order to obtain more accurate and precise fumonisin levels and classify the original sample more accurately.

The same HPLC techniques used by MRC and SGS did not correlate indicating possible analytical errors or incompetence of analysts. The laboratory where analyses are carried out is therefore indicated as an important additional source of variation, even though the same quantification technique is used. It is advisable to make use of a reputable and certified laboratory for fumonisin quantifications.

It was established in Chapter 4 that *F. verticillioides* and *F. proliferatum* were most frequently isolated in the North-West Province and *F. subglutinans* in Gauteng. *F. verticillioides* can cause seedling blight, root and crown rot, stalk and ear rot on maize (Munkvold *et al.*, 1997; Glenn, 2005) and is an important fumonisin producer (Fandohan *et al.*, 2003). Because of the abovementioned factors, the high frequency of *F. verticillioides* in Biesiesvlei, Bronkhorstspuit, Derby, Grootpan and Hartebeesfontein is considered a serious problem. Fumonisin levels allowed for human consumption is set at 2 ppm by the FDA, and levels higher than 2 ppm from silos in Derby, Koster, Grootpan, Ventersdorp and Lichtenburg is of concern for both human and animal health. Contamination of food by *Fusarium* spp. and fumonisins has become a serious food safety problem throughout the world. Some information is available on factors contributing to fumonisin production and on those able to reduce fumonisin levels in food (Fandohan *et al.*, 2003), however more information is needed about environmental influences on the geographical spread of *Fusarium* spp. and fumonisin incidence. In South Africa millions of people consume maize and maize products on a daily basis, and may possibly be exposed to fumonisins without being aware of it. Studies into environmental influences on the geographical distribution of *Fusarium* spp. and

fumonisin concentration are warranted and their incorporation into integrated disease management systems may prevent maize products with high fumonisin levels being consumed by humans and animals.

A non-significant relationship was established between *F. verticillioides*, *F. proliferatum* and fumonisin levels indicating that other factors may play a role in the production of fumonisin. It is common for other pathogens such as *Aspergillus* spp., *Penicillium* spp., (Turner *et al.*, 1999, Orsi *et al.*, 2000), *S. maydis* and *F. graminearum* (Williams *et al.*, 1992) to co-infect maize with *Fusarium* spp. (Bush *et al.*, 2003). This could possibly result in the poor statistical relationship between ergosterol and fungal plate counts, suggesting the latter to be more accurate for *Fusarium* spp. assessment. Ergosterol techniques will give an accurate indication of specific fungi only in the absence of other infections.

The significant relationship between maximum temperatures for May and June 2004 and fumonisin production coincides with harvesting of maize in South Africa. This is an indication that maximum temperatures in May (23.5°C) and June (18.9°C) could increase fumonisin production during the drying-down and harvesting period. This information could be useful in the decision-making process and management of *Fusarium* spp. incidence and fumonisin production by maize producers by planting earlier in order to harvest earlier, or artificially drying maize.

In Chapter 5 no significant differences were recorded for replications, media or enumerators for *F. verticillioides* enumerations but significant locality effects were obtained. It is therefore concluded that enumerators were consistent in *F. verticillioides* identification and quantification. RbGU as well as CLA/PDA media used for enumerations were both accurate and effective. No significant differences were recorded for media or enumerators for *F. subglutinans* enumerations and again, significant locality effects were obtained. *F. proliferatum* enumerations had an interaction between locality and enumerator with no significant differences for media and replications. The significant locality x enumerator interaction for *F. proliferatum* implies that enumerators struggled to distinguish between the *F. proliferatum* and *F.*

verticillioides isolates over localities. The enumerator requires the necessary expertise to identify between monophialides and polyphialides to distinguish between *F. verticillioides* and *F. proliferatum* (Nelson *et al.*, 1983). Since *F. verticillioides* and *F. proliferatum* were isolated from the same localities or under similar environmental conditions (Rheeder *et al.*, 1990) and have almost the same distinguishing characteristics (Leslie & Summerell, 2006), this may have resulted in the locality and enumerator interaction.

According to Nutter *et al.* (1993) enumerators and methods (media) that have the highest precision would be those in which the regression coefficients (slopes) are closest to 1.0, with y-intercepts closest to 0. Slopes deviating significantly from 1.0 indicate constant bias. The intercepts and slopes for enumerators 1 - 3 in the enumerations of *F. verticillioides*, *F. proliferatum* and *F. subglutinans* on RbGU and CLA/PDA media is an indication of low variation within enumerators. The use of RbGU and/or CLA/PDA media were eliminated as a possible source of variation in *Fusarium* identifications.

In the inter-enumerator reliability study, enumerators 1, 2 and 3 showed significant agreement for identifications of *F. verticillioides* and *F. subglutinans* and low variation between enumerators were detected for enumerations on both media. No significant inter-enumerator correlations were recorded between any of the three enumerators for *F. proliferatum* enumerations, although slopes and intercepts were indicative of extremely low variation between enumerators. This study indicated that *Fusarium* spp. enumerations by three different enumerators were reliable and accurate and were not a source of variation in the quantification of *Fusarium* spp. associated with maize ear rot.

To conclude, the current dissertation indicated that protocol for fumonisin detection need to be addressed. Larger sub-sample sizes (250 g) together with more replications will result in more accurate detection of fumonisin levels. In addition, these tests should in addition be performed at certified laboratories as variation between laboratories were identified as a source of variation affecting accurate fumonisin level detection. It is not

advisable to store maize meal before testing for fumonisin levels, as levels fluctuated over storage time. Further studies on this issue are necessary. Studies have shown that an earlier planting date and/or artificial drying of maize kernels are possible solutions in reducing fumonisin levels at harvest.

REFERENCES

- Bush, B.J., Carson, M.L., Cubeta, M.A., Hagler, W.M. & Payne, G.A. 2003. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. *Phytopathology* **94**: 88-93.
- Fandohan, P., Hell, K., Marasas, W.F.O. & Wingfield, M.J. 2003. Infection of maize by *Fusarium* species and contamination with fumonisin in Africa. *African Journal of Biotechnology* **2**: 570-579.
- Glenn, A.E. 2005. Natural variation of ascospore and conidial germination by *Fusarium verticillioides* and other *Fusarium* species. *Mycological Research* **110**: 211-219.
- Leslie, J.F. & Summerell, B.A. 2006. The fusarium laboratory manual. Blackwell publishing. 388pp.
- Munkvold, G.P. & Desjardins, A.E. 1997. Fumonisins in maize. Can we reduce their occurrence? *Plant Disease* **81**: 556-564.
- Nelson, P.E., Tousson, T.A. & Marasas, W.F.O. 1983. *Fusarium species : An illustrated manual for identification*. The Pennsylvania State University Press, University Park and London. 193pp.
- Nutter, F.W., Jr., Gleason, M.L., Jenco, J.H. & Christians, N.C. 1993. Assessing the accuracy, intra-rater repeatability, and intra-rater reliability of disease assessment systems. *Phytopathology* **83**: 806-812.
- Orsi, R.B., Corrêa, B., Possi, C.R., Schammass, E.A., Nogueira, J.R., Dias, S.M.C. & Malozzi, M.A.B. 2000. Mycoflora and occurrence of fumonisins in freshly harvested and stored hybrid maize. *Journal of Stored Products Research* **36**: 75-87.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S., Du Toit, W., Pretorius, A.J. & Van Schalkwyk, D.J. 1990. Incidence of *Fusarium* and *Diplodia* species and other fungi in naturally infected grain of South African maize cultivars. *Phytophylactica* **22**: 97-102.

Turner, P.C., Nikiema, P. & Wild, C.P. 1999. Fumonisin contamination of food: progress in development of biomarkers to better assess human health risks. *Mutation Research* **443**: 81-93.

Whitaker, T.B. & Johansson, A.S. 2005. Sampling uncertainties for the detection of chemical agents in complex food matrixes. *Journal of Food Protection* **68**: 1306-1313.

Williams, K.C, Blaney, B.J., Dodman, R.L. & Palmer, C.L. 1992. Assessment for animal feed of maize kernels naturally infected predominantly with *Fusarium moniliforme* and *Diplodia maydis*. Fungal isolations and changes in chemical composition. *Australian Journal of Agricultural Research* **43**: 773-782.