MODELLING THE INCIDENCE OF *FUSARIUM* AND *ASPERGILLUS* TOXIN PRODUCING SPECIES IN MAIZE AND SORGHUM IN SOUTH AFRICA

By

Belinda Janse van Rensburg

A thesis submitted in accordance with the requirements for the degree of
Philosophiae Doctor

In the Faculty of Natural and Agricultural Sciences
Department of Plant Sciences (Centre for Plant Health Management)
University of the Free State
Bloemfontein, South Africa

Promoter
Prof. N.W. McLaren

Co-promoters
Prof. B.C. Flett
Prof. A. Viljoen

June 2012

TABLE OF CONTENTS **Page**

CHAPTER 1

Incidence and severity of cob rot and grain mould fungi and production of aflatoxins and fumonisins in commercial maize and sorghum in South Africa.

CHAPTER 2

Fumonisins associated with the colonisation of commercial South African maize grain by *Fusarium* spp.

CHAPTER 3

Aflatoxin and fumonisin on sorghum grain from commercial production areas of South Africa.

CHAPTER 4

Use of weather variables to quantify the potential risk of grain colonisation by fumonisin-producing *Fusarium* spp. and fumonisin synthesis in commercial maize in South Africa.

CHAPTER 5

Effect of a fungicide spray regime for foliar diseases on incidence of fumonisin producing *Fusarium* spp. and fumonisins on selected maize cultivars.

LIST OF TABLES

LIST OF FIGURES

DECLARATION

'I declare that the thesis hereby submitted by me for the degree Philosophiae Doctor at the University of the Free State is my own independent work and has not previously been submitted by me at another University/Faculty. I further cede copy right of the thesis in favour of the University of the Free State'.

Belinda Janse van Rensburg

ACKNOWLEDGEMENTS

This PhD started with a dream when I was first employed by the Agricultural Research Council – Grain Crops Institute in 1994 as a research assistant. Dreams alone are not enough and it took hard work, perseverance, encouragement and faith to fulfil this dream. Therefore I want to thank the following people and organisations who helped to make this dream a reality.

- Maize Trust and the ARC for funding of this research and use of facilities.
- My promoters Prof. N.W. McLaren (UFS), Prof. B.C. Flett (ARC-GCI, NWU) and Prof. A. Viljoen (US). Thank you for mentoring and encouraging me throughout this study. I value all your inputs and hope to have a long working relationship with you in the future.
- Thank you to the personnel of the ARC for technical assistance:

Laboratory:	Ms. M. Mahlobo, Ms. M. Kwele, Ms. A. Schoeman
Field work:	Mr. K. Croukamp, Mr. J. Baas, Mr. D. Bruwer, Ms. G. Khali, Mr. J. Steyn, Mr. W. Jansen
Maps:	Ms. W. du Randt
Weather data:	Ms. M. Fritz

- Ms. S. Tweer at PANNAR for planting and maintenance of two trials in Greytown as well as data collection.
- Thank you to my husband Sarel who encouraged me. I appreciate your support and help with the children during trying times.
- Most of all I want to thank God for all the blessings I have received.

PREFACE

This thesis is a compilation of five independent manuscripts. The first chapter is a literature overview on ear rot producing *Fusarium* and *Aspergillus* spp. of maize and sorghum. This chapter includes a discussion of the pathogens involved as well as the mycotoxins they produce. Fungal and mycotoxin detection methods are compared and discussed. The possible role of an epidemiological model that can act as an instrument to constantly monitor and assess the risk of fumonisin and aflatoxin contamination in maize and sorghum grain, making it possible to drive agronomic decisions during cultivation and thus enhance management opportunities was discussed. The potential role of fungicide spray programs for the reduction of mycotoxins was also discussed.

In Chapter 2, the natural occurrence of fumonisin producing *Fusarium* species and fumonisin contamination was quantified in various maize production areas of South Africa. In an attempt to elucidate and explain the genotype x environment interactions associated with fumonisin contamination of maize, fumonisin, fungal biomass and plating out method were correlated to determine possible relationships between the measured variables.

In Chapter 3, the level of infection of sorghum produced in South Africa, with aflatoxin- and fumonisin-producing fungi, and their concomitant toxins, were determined. This provided an indication of sorghum grain quality and safety for human and animal consumption.

In Chapter 4, the development of an epidemiological model to assist in the prediction of fumonisin producing *Fusarium* spp. and the resultant fumonisin contamination under various environmental conditions were assessed. Critical phenological growth stages of the maize plant and critical weather variables were discussed.

At present no fungicides are registered for control of Fusarium ear rots of maize. The effect of a fungicide spray regime for foliar diseases on the incidence of fumonisin producing *Fusarium* spp. and fumonisin on selected maize cultivars at various localities was investigated in Chapter 5.

The work presented in this thesis will contribute to a better understanding of maize and sorghum ear rots, caused by fumonisin producing *Fusarium* spp. and aflatoxin producing *Aspergillus* spp.. All 5 chapters are complimentary to each other and this enabled the development of a prediction model that can identify areas or maize batches with potentially dangerous levels of fungi and their mycotoxins. This, too, could enable maize producers to identify the need to implement mycotoxin management strategies. This could help to reduce grain contamination and prevent infected grain from being used for food or feed, thus improving human and animal food health.

GENERAL INTRODUCTION

Maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) constitute an important component of the diet of millions of people in South Africa. Maize is produced throughout the country under diverse environmental conditions. During 2011 approximately 12 million tons of maize grain was produced in South Africa (Anonymous, 2011). Half of the production consisted of white maize, for human food consumption (FAOSTAT, 2009). Sorghum is grown widely in the semi-arid tropics under hot, dry conditions and ranked third in cereal production following maize and wheat with a production of 125,000 tonnes in 2011 in South Africa (Anonymous, 2011).

Maize and sorghum are susceptible to infection by mycotoxigenic fungi such as *Aspergillus flavus* (Link:Fr)*., A. parasiticus* (Speare)*, Fusarium andiyazi* (Marasas, Rheeder*,* Lamprecht, Zeller & Leslie), *F. thapsinum* (Klittich, Leslie, Nelson &Marasas)*, F. verticillioides* (Saccardo) Nirenberg and *F. proliferatum* (Matsushina) Nirenberg. *A. flavus* and *A. parasiticus* produce aflatoxins and *F. verticillioides* and *F. proliferatum* are prolific fumonisin producers. *F. andiyazi* does not produce fumonisin and *F. thapsinum* only produces trace amounts of fumonisin (Leslie & Summerell, 2006).

Monitoring of fungal infection and prediction of high levels of aflatoxin and fumonisin could help authorities and consumers make decisions to reduce the potential impact of these dangerous metabolites. Therefore, the objectives of this study were to quantify the natural occurrence of aflatoxin producing *Aspergillus* spp. and fumonisin producing *Fusarium* spp. of maize and sorghum grain in selected production areas of South Africa. Similarly aflatoxin and fumonisin contamination was also quantified.

Aflatoxins are potentially hazardous to humans and animals displaying strong immunosuppressive, mutagenic, teratogenic and carcinogenic effects (Hussein & Jeffrey, 2001). Aflatoxin B_1 has been reported to be the most toxic and has been classified as a group 1 toxin by the International Agency for Research on Cancer ie. a human carcinogen (IARC, 1993a). Fumonisins occur naturally in maize and feeds associated with field outbreaks of mycotoxicoses in animals (Thiel *et al*., 1991).

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 have been statistically correlated with human oesophageal cancer (Rheeder *et al*., 1992) and are regarded as Class 2B carcinogens (IARC, 1993b) which means it is probably carcinogenic to humans.

There is a paucity of information on the status of aflatoxin producing *Aspergillus* spp. and fumonisin producing *Fusarium* spp. in commercial sorghum grain of South Africa. According to Chandrasheka & Satyanarayana (2006) sorghum grain is less susceptible than other grain such as maize and groundnuts to infection by *A. parasiticus* and aflatoxin contamination due to its physical characteristics and biochemical composition. The lack of publications on the occurrence of aflatoxin producing *Aspergillus* spp. and aflatoxin in sorghum may be because sorghum only represents 3.5% of the world cereal production, but for continents or countries with food insecurities such as Africa and India, this is an important issue which needs to be addressed. Mohammed *et al*. (2010) tested sorghum grain samples imported into Saudi Arabia and found *F. verticillioides* to be the main fumonisin producer from orghum grains and reported levels of up to 19.10 ppm. From these reports it is evident that fumonisin B_1 can be of concern in sorghum and indicates a need to determine the status of fumonisin contamination in South African sorghum samples.

Although Shephard (2005) reported that *A. flavus* and A*. parasiticus* occur sporadically in both commercial and home-grown maize in South Africa and are not common ear rot pathogens under local conditions, Ncube (2008) recorded high levels of *A. flavus* and aflatoxin in subsistence maize sampled from northern Kwa Zulu Natal and Mpumalanga using the ELISA (Enzyme Linked Immuno Sorbent Assay) technique. On the other hand, the global susceptibility of maize to fumonisin producing *Fusarium* spp. is well documented in literature (Marasas, 2001; Leslie &Summerell, 2006; Desjardins, 2006; Boutigny *et al*., 2011). The distribution and predominance of these *Fusarium* spp. and their concomitant fumonisin production varies, depending on season, geographic locality, climatic factors such temperature and moisture, host genotype and agricultural practices (Nyaka*et al*., 2010). It is therefore important to understand all the above mentioned variables and how they

interact with each other in order to develop an epidemiological model unique to environmental conditions of South Africa.

Data from the above-mentioned research was used together with site specific weather data in the development of an epidemiological model. An epidemiological model can act as instrument to constantly monitor and assess the risk of fumonisin contamination in maize grain, making it possible to drive agronomic decisions during cultivation that would enhance management opportunities. This will help reduce grain contamination and prevent such grain being used for food or feed, thus improving human and animal food safety measures.

Currently no fungicides are registered for the control of Fusarium maize ear rot and we wanted to investigate whether our model can be applied to the development of a fungicide spray regime. We applied a spray regime used for foliar diseases to determine the effect on the incidence of fumonisin producing *Fusarium* spp. and fumonisins on selected maize cultivars. Although fungicides that control leaf diseases in maize may play a role in reducing maize ear rot diseases and their resultant mycotoxins, it may not be economically justifiable as possible additional fungicide applications would be required during silking of the maize plant.

REFERENCES

Anonymous, 2011.Index Mundi, South Africa sorghum production by year.[Available on internet:] http://wwwhttp://www.indexmundi.com/agriculture.[Date of access 11/01/12].

Boutigny, A.-L., Beukes, I., Small, I., Zûhlke, S., Spittelier, M. Janse van Rensburg, B., Flett, B.&Viljoen, A. 2011. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant Pathology* **61**: 522–531.

Chandrashekar, A. & Satyanarayana, K.V. 2006. Disease and pest resistance in grains of sorghum and millets. *Journal of Cereal Science* **44**: 287-304.

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

FAOSTAT data 2009. Food and Agriculture Organization of the United Nation Databases. [Available on internet:] http://www.faostat.fao.org. [Date of access 08/02/10].

Hussein, S.H. & Jeffrey, M.B. 2001. Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology* **167**: 101-134.

IARC. 1993a. International Agency for Research on Cancer: Monograph on the evaluation of carcinogenic risk to human. C. Lyon (F): IARC.

IARC. 1993b. World Health Organization, International Agency for Research on Cancer.Toxins derived from *Fusarium moniliforme*: Fumonisin B_1 and B_2 and fusarin. C. Lyon (F): IARC.

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

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: a historical perspective. *Environmental Health Perspectives* **109**: 239-243.

Mohamed, Y., Abdel-Rheem, E.S., Ali, B., Mohamed, M., Kamel, A.E. & Kevin, H. 2010. Mycotoxin producing fungi occurring in sorghum grains from Saudi-Arabia. *Fungal Diversity* **44**: 45-52.

Ncube, E. 2008. Mycotoxin levels in subsistence farming systems in South Africa. MSc (Agric.), In the Faculty of AgriSciences, University of Stellenbosch, Stellenbosch, South Africa.

Nyaka, S.C., Shankar, A.C.U., Niranjana, S.R., Wulff, E.G., Mortensen, C.N. & Prakash, H.S. 2010. Detection and quantification of fumonisin from *Fusarium verticillioides* in maize grown in southern India. *World Journal of Microbiological Biotechnology* **26**: 71-78.

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.

Shephard, G.S. 2005. Aflatoxin and food safety: recent African perspectives. Pp.15-17. *In*: Abbas, H.K. (Ed.). Aflatoxin and food safety. CRC Press, Taylor and Francis group, Boca Raton.

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.

CHAPTER 1

Literature review

Incidence and severity of cob rot and grain mould fungi and the production of aflatoxins and fumonisins in commercial maize and sorghum in South Africa.

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 per hectare, ease of cultivation, adaptability to different agro-ecological zones, versatile food uses and storage characteristics (Fandohan *et al.*, 2003). World production covers an area of 110 million ha. that yields approximately 230 million tons of grain per annum. More than half of this is produced in the USA, i.e. 144 million tons on an area of 25 million ha., with a market value of approximately US $20 thousand billion. The American economy (as in the RSA) is highly dependent on maize production (du Toit, 1999). Approximately 8 million tons of maize grain is produced in South Africa annually on approximately 3.1 million ha. of land under diverse environments. Half of the production consists of white maize, for human food consumption (FAOSTAT data, 2009). In developed countries, maize is consumed mainly as a second-cycle product, in the form of meat, eggs and dairy products. In developing countries, maize is consumed directly and serves as a staple diet for some 200 million people. Most people regard maize as a breakfast cereal. However, in a processed form it is also found as fuel (ethanol) and starch. Starch in turn involves enzymatic conversion into products such as sorbitol, dextrine, sorbic and lactic acid, and appears in household items such as beer, ice cream, syrup, shoe polish, glue, fireworks, ink, batteries, mustard, cosmetics, aspirin and paint (du Toit, 1999).

Maize is a warm weather crop and is not grown in areas where mean daily temperature is below 19°C. Although the minimum temperature requirement for germination is 10°C, germination and emergence will be faster and less variable at soil temperatures of 16 to 18°C. Development of maize early in the season increases linearly with an increase in soil temperature from 15 to 17°C. Exceptionally high temperatures and low humidity during flowering have an adverse effect on pollination and fertilization, resulting in poor seed set. The critical supra-maximal temperature affecting yield is approximately 32°C. Frost can damage maize at all growth stages and a frost-free period of 120 to 140 days is required to prevent damage. While the growing point is below the

soil surface, new leaves will form and frost damage will be limited. Leaves of mature plants are easily frosted and grain fill can be adversely affected (Du Toit, 1999).

Sorghum production in South Africa

Sorghum (*Sorghum bicolor*) (L.) Moench) is a small seeded grass (Chandrashekar & Satyanarayana, 2006) that originated from Africa and Asia (Belton & Taylor, 2004). It subsequently spread to other temperate and sub-tropical regions (da Silva *et al*., 2004). Sorghum is an important crop in warmer climates (Saubois *et al*., 1999), especially in the drier sub-tropical areas. It is cultivated in dry, hot areas (38–40°C) with an average annual rainfall of 400-750 mm, although it can be grown where rainfall is much higher. Sorghum also has the ability to withstand waterlogging. In terms of hectares, sorghum ranks fifth among the world's cereals following wheat, maize, rice and barley (FAOSTAT data, 2006). On a global basis, sorghum represents 3.5% of total cereal production. Roughly 90% of the world's sorghum area can be found in developing countries, mainly Africa and Asia (FAOSTATdata, 2009). More than 55% of the world's sorghum production comes from semi-arid tropical zones (Reddy & Raghavender, 2006). In the United States and South America sorghum is primarily used for animal feed while in developing countries such as Africa and Asia, small-scale farmers use sorghum mainly for human consumption (da Silva *et al*., 2004) either directly or in the form of an alcoholic beverage. Worldwide, approximately 27 million tons of sorghum was consumed as food each year during the 1992-1994 period, almost the entire yield of Africa and Asia (FAOSTATdata, 2009).

Approximately 48% of world sorghum grain production is fed to livestock, human food use constitutes about 52%. Despite a lower demand for sorghum as food, compared to grains such as maize and wheat, the income elasticities for livestock products (and hence the derived demand for feed) are generally positive and high. Demand for animal feed is concentrated in developed countries and in middle-income countries of Latin America and Asia, where

demand for meat is high and the livestock industry is correspondingly intensive. Over 85% of sorghum feed use occurs in three countries (United States, Mexico and Japan) which together absorb nearly 70% of world production (FAOSTAT data, 2009).

Fumonisin producing *Fusarium* spp.

The susceptibility of maize and sorghum to various moulds is well documented. Major fungal genera encountered on maize in tropical and sub-tropical regions are *Fusarium*, *Aspergillus* and *Penicillium* (Turner *et al.,* 1999; Orsi *et al.,* 2000; Navi *et al*., 2005) where it is common for *Aspergillus* and *Penicillium* spp. to co-infect with *Fusarium* spp. (Bush *et al.*, 2004).

To date, fumonisins have been identified in *Fusarium* spp. and *A. niger*, although closely related compounds are produced by *Alternaria* spp. (Desjardins, 2006). The ability to produce fumonisin is not dispersed throughout the *Fusarium* spp., fumonisin production appears to be absent from the *F. solani* spp. complex and from all the trichothecene producing *Fusarium* spp. High levels of fumonisin production have been found consistently among strains of *F. verticillioides* and *F. proliferatum* of the *Gibberella fujikuroi* spp. complex (Desjardins, 2006).

The name *F. verticillioides* (previously named *F. moniliforme*) should only be used for strains that have the *G. moniliformis* teleomorph. Strains grouped under *F. moniliforme* in the past would most likely have included *F. thapsinum* from sorghum, *F. sacchari* from sugar cane, *F. magniferae* from mango, or *F. fujikuroi* from rice (Leslie & Summerell, 2006). The genus Fusarium includes economically important plant pathogens that can infect roots, stalks, ears and grain (King & Scott, 1981, Leslie *et al*., 2005) and cause billions of dollars of losses worldwide annually (Jurgenson *et al.*, 2002). *F. verticillioides* (Sacc.) Nirenberg (synonym: *F. monilifome* Sheldon) is considered a major pathogen of the *Gramineae,* particularly in tropical and sub-tropical 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.

Taxonomy

F. verticillioides (Saccardo) Nirenberg and *F. proliferatum* (Matsushina) Nirenberg, belong to teleomorph *Gibberella moniliformis* and *Gibberella intermedia*, respectively (Leslie & Summerell, 2006). These spp. are in *Fusarium* section *Liseola,* based on morphological characteristics (Nelson *et al.*, 1983). The anamorph sp. *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). Based on the structure in or on which conidiogenous hyphae are borne, *Fusarium spp.* are classified under the Hyphomycetidae sub-class of the Deuteromycetes (Agrios, 2005). *F. verticillioides* and *F. proliferatum* have small, hyaline microconidia that are abundant and primarily single-celled, oval to club shaped, with a flattened base (Glenn, 2005). Microconidia of *F. verticillioides* and *F. proliferatum* are abundantly produced in long, catenate chains 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 monophialides (Nelson *et al.,* 1983). Macroconidia in *F. verticillioides* are present but according to Nelson *et al.* (1983) are rare whereas macroconidia are abundant in *F. proliferatum.* On potato dextrose agar (PDA) *F. verticillioides* cultures will initially have white mycelia but may develop violet pigments with age (Figure 1.1). Pigmentation in the agar varies, ranging from no pigmentation or grayish orange to violet grey, dark violet or dark magenta in others (Leslie & Summerell, 2006). *F. proliferatum* cultures on PDA will initially be white, but may become purple violet with age. Sporodochia may be present. Violet pigments are usually produced in the agar, but overall pigmentation may vary from nearly colourless to almost black.

Fusarium andiyaze and *Fusarium thapsinum* are pathogenic to sorghum and both have been included into the *F. moniliforme* morphology in the past because of their similar characteristics to *F. verticillioides*. *F. andiyaze* does not produce fumonisin or moniliformin, whereas *F. thapsinum* can produce high levels of moniliformin, but little more than trace amounts of fumonisin (Leslie *et al*., 1996, Leslie *et al*., 2005). Macroconidia of *F. andiyaze* and *F. thapsinum* are produced in orange sporodochia, although sporodochia are rare in *F. thapsinum*. Similarly to *F. verticillioides*, microconidia are produced in abundance in chains from monophialides. Cultures of *F. andiyaze* and *F. thapsinum* will initially have a floccose, white powdery mycelium which may become violet on PDA. Violet pigmentation in the agar may vary from pale to dark purple for *F. andiyaze*. *F. thapsinum* pigmentation in PDA agar is quite variable. Most strains produce a distinctive yellow pigment that is diagnostic and is the basis of the spp. epithet (Leslie & Summerell, 2006). Other strains may produce either no pigment or violet pigments in the agar.

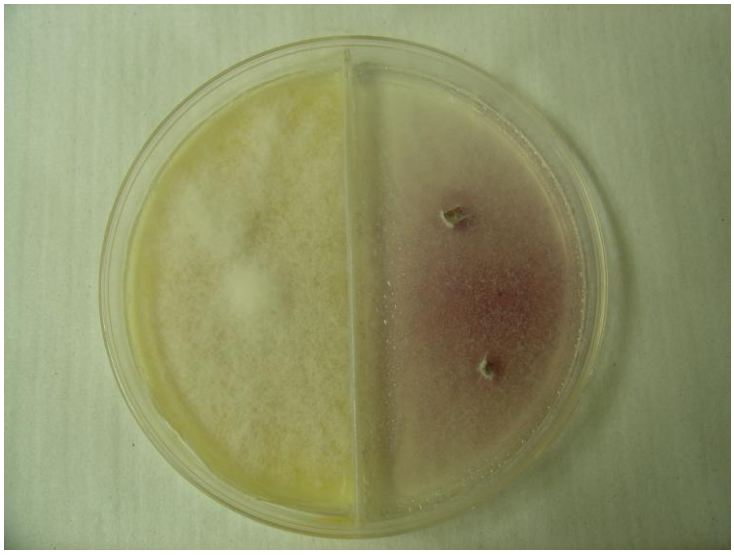


Figure 1.1 White mycelia of *F. verticillioides* on PDA (left) and pigmentation on carnation leaf agar (right) (photo: B. Janse van Rensburg).

Symptoms

Symptoms can vary depending upon genotype, environment and disease severity. One symptom type noted in the field (Flett *et al*., 1996) is the growth of white-pink cottony mould on kernels alongside stalkborer channels (Figure 1.2). Similar symptoms are often associated with insect or bird damage on ears. *F. verticillioides* can also infect Individual or groups of kernels scattered randomly on the ear (Figure 1.3). Another symptom type is a pink discolouration of undamaged kernels (Figure 1.4).

Figure 1.2 White-pink mould on kernels alongside stalkborer channels (photo: Prof. B.C. Flett).

Figure 1.3 ***F. verticillioides*** **infection of kernels scattered on the ear (photo: B. Janse van Rensburg).**

Figure 1.4 **Pink discolouration of undamaged kernels (photo: Prof. B.C. Flett).**

Symptomless infection

F. verticillioides is one of the most common fungi found symptomlesly colonising 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 all host tissues, including kernels, establishing long-term associations with the host, without disease symptoms being observed for extended periods (Jardine & Leslie, 1999). Endophytic hyphae of *F. verticillioides* are neither latent nor 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 takes place and serves as a source of renewed toxin synthesis *in planta* (Bacon *et al.,* 2001). *F. verticillioides* may remain undetected in kernels until germination, when it infects the emerging seedlings (Bacon & Hinton, 1996). Detection and control of endophytic infections in maize ears are difficult because kernels appear to be sound. Symptomless infection of kernels is often very high, but fumonisin levels may be very low (Bush *et al.*, 2004). Presence of fumonisin in visually sound maize intended for human consumption supports the hypothesis by Bacon *et al.* (2001) that low concentrations of fumonisin are synthesised by symptomless, endophytic fungi. Under plant stress conditions, the symptomless endophytic relationship may convert to a disease- and/or mycotoxin producing interaction (Abbas *et al*., 2006). Yield can be reduced by endophytic *F. verticillioides* infected plants, due to deterioration of the stalk parenchyma tissue and gradual dehydration of the plant (Foley, 1962).

Epidemiology

F. verticillioides is more common in regions with hot and dry growing conditions especially before or during pollination. *F. verticillioides* grows well

at temperatures above 26°C and the calculated optimal and maximum temperatures for growth are 31°C and 35°C respectively (Murillo-Williams & Munkvold, 2008). The suggested minimum range for growth is 22°C to 24°C. Marin *et al*. (1999) reported a temperature of 30°C and 0.97 a_w (water activity) to be the optimum conditions for *F. verticillioides* growth (*in vitro*). Low temperature and water stress reduce fungal growth (Jurado *et al*., 2008) but an increase in water stress increased *FUM 1* expression (Jurado *et al*., 2008; Marin *et al*., 2010) which is the first step in fumonisin synthesis. De La Campa *et al*. (2005) developed a fumonisin prediction model and reported 4 critical weather periods relative to silking that explained 76% of variability of fumonisin. They were 4 to 10 days before silking, from 4 days before silking to 2 days after silking, 2 to 8 days after silking and 8 to 14 days after silking. In the first critical period 4 to 10 days before silking, the data suggest that temperatures <15°C and >34°C reduce fumonisin (TMIN and TMAX, respectively), and rain increases fumonisin; however, the effects of rain were negated by temperatures >34°C, as indicated by a negative interaction between the two variables. Extreme temperatures and dry weather before silking likely delayed or reduced sources of inoculum during this period before silking.

Stalks infected during the growing season are 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* overwinters saprophytically on maize residues on the soil surface or in the soil following mechanical incorporation. *F. verticillioides* does not produce chlamydospores, but can produce thickened hyphae that apparently prolong its survival (Munkvold & Desjardins, 1997). Cotten & Munkvold (1998) reported *F. verticillioides* to survive for up to 630 days under Iowa conditions and up to 900 days under cool, dry conditions (Liddell & Burgess, 1985). Ariño *et al*. (2007) studied the natural occurrence of *Fusarium* spp. and fumonisin production in conventionally and organically produced maize in Spain. The organic farming system included crop rotation, plough tillage and compost fertilization while the conventionally grown maize included no-tillage,

fungicide, herbicide, insecticide and fertilizer treatments. Infection by *Fusarium* spp. was nearly 50% higher in conventionally grown maize than in organically grown maize. In contrast to these findings, Flett & Wehner (1991) reported no effect on maize ear rot *Fusarium* spp. infection under different tillage systems. Marocco *et al*. (2008) reported no significant effects on the incidence of fumonisin when comparing no-till to conventional tilling in a monoculture production setting.

F. verticillioides has a saprophytic as well as parasitic stage and may infect maize at all stages of plant development, either via the silk channel, infected seed, or wounds (Reid *et al.*, 1999). *F. verticillioides* can be transmitted to uninfected plants by inoculum from field stubble (Munkvold & Desjardins, 1997) or airborne conidia (micro- and macroconidia) abundant in maize fields during a growing season. Small, hyaline, mostly single celled microconidia are abundantly produced and are well adapted for wind, rain and vectoral dispersal (Glenn, 2005).

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). According to Vincelli & Parker (2002) green silks are relatively resistant to infection and colonisation, whereas senescing, green-brown and brown silks can be colonised by the fungus. The fungus then grows down the silk channel and into developing kernels.

Another proposed infection pathway by Oren *et al.* (2003) is systemically from seed. Systemic infection can be initiated from fungal conidia or mycelia that are either carried within the seeds or on the seed surface. The fungus develops within the young plant and moves from the roots to the stalk and finally to the ear 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). 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).

F. verticillioides growth in a maize plant causes the release of volatile substances that attract lepidopterous and coleopteran pests, thereby increasing infestation of maize ears by these pests (Cardwell *et al.*, 2000; Schulthess *et al.*, 2002). Feeding activities of lepidopterous insects may spread spores to silks, kernels, stems and feeding channels, increasing colonisation by the fungus (Vincelli & Parker, 2002). Bt-transformed maize contains genes from *Bacillus thuringiensis* encoding for insecticidal crystal proteins. Reduced insect damage on Bt maize stalks can reduce infection by *Fusarium* spp. through plant injuries and possibly reduce fumonisin levels as a result. Munkvold *et al.* (1999) and Hammond *et al.* (2004) conducted field experiments by using transgenic maize (Bt) hybrids and near-isogenic, nontransgenic hybrids that were infested with neonatal European Corn Borer larvae. They reported an increase of Fusarium ear rot severity and fumonisin concentrations in kernels of nontransgenic hybrids. Transgenic hybrids expressed less insect feeding on kernels and less Fusarium ear rot and fumonisin contamination. The higher fumonisin concentrations in nontransgenic hybrids were attributed to high European Corn Borer populations during the early reproductive stages of the maize plants. Magg *et al.* (2002) found that the use of Bt maize hybrids compared to their isogenic counterparts, slightly reduced the contamination of maize kernels with mycotoxins produced by *Fusarium* spp. under European conditions while Naéf & Defago (2006) reported no consistent difference in colonisation of maize by *Fusarium* spp. between Bt and non-Bt stalks. Birds that cause physical injury to stalks and ears are also suspected to promote infection by *Fusarium* spp. (Papst *et al.*, 2005).

Grain characteristics

Presently South African maize lines vary in degree of susceptibility to *F. verticillioides* ear rot infection (Small *et al*., 2012). It is exceedingly difficult to predict the response of a genotype at any location, as differences in environmental conditions, planting date, harvest date, insect injury and isolate differences can greatly affect the intensity of *Fusarium* spp. infection and fumonisin production. The maize genotype and grain characteristics such as colour, endosperm type, chemical composition and stage of development may influence fungal infection and subsequent fumonisin production (Fandohan *et al.*, 2003). Infection of maize kernels by airborne conidia or by conidia vectored by insects is exacerbated by incomplete or loose coverage of kernels by the husk leaves, early silk senescence and kernel splitting. Silk cut (preharvest occurrence of one or more lateral splits in the kernel pericarp) expose kernel tissue to pre- or postharvest attack by fungi and insects (Odovy *et al*., 1997).

Conflicting reports about pericarp thickness and wax content as resistance mechanisms to *Fusarium* spp., and fumonisin exist. Ivić *et al*. (2008) reported that pericarp thickness does not contribute to *Fusarium* spp. ear rot resistance under Croatian environmental conditions while contrary to this, Sampietro *et al*. (2009) reported pericarp and it's wax content to be resistance mechanisms to fumonisin accumulation in most genotypes screened in Argentina. Blandino & Reyneri (2007) compared waxy and normal dent hybrids in Italy in field experiments and concluded that waxy hybrids showed a higher average contamination by fumonisin than normal hybrids with the same or similar genealogy, although they showed similar European Corn Borer incidence and Fusarium ear rot incidence and severity. It is supposed that the presence of starch, almost exclusively amylopectin, can stimulate a greater toxinogenesis of *Fusarium* spp., therefore making waxy hybrids/genotypes more susceptible to fumonisin contamination (Blandino & Reyneri, 2007).

Resistance mechanisms of sorghum against pathogens and pests involve both the physical and chemical composition of the grain. Sorghum varieties are generally classified as hard or soft, based on the relative proportion of the outer, hard, translucent endosperm to the inner, soft and opaque endosperm (Mazhar & Chandrashekar, 1993). These sorghum varieties differ in the proportion of the relative areas of corneous and floury endosperm that influences grain hardness (Waniska *et al*., 2001). Hence, varieties with hard sorghum grains are more resistant to fungal attack than varieties with soft grains (Kumari *et al*., 1992). The pigmentations of pericarp and testa are caused by phenolic compounds (Hahn & Rooney, 1985). Sorghum with a red pericarp contains phenolics that prevent fungal growth on the grain surface. White grains with a corneous, hard endosperm resist fungal colonisation internally, but are unable to suppress late infection and sporulation by grain mould fungi on the pericarp (Bandyopadhyay *et al*., 2002). All sorghums contain phenols and flavanoids, but not all sorghums contain tannins. Sorghum thus can be classified based on tannin presence. They are classified as type I, no tannins; type II, tannins in pigmented testa; or type III, tannins in pigmented testa and pericarp (Waniska *et al*., 2001). Sorghums with phenols, especially tannins which are able to inhibit fungal enzyme activity, may confer a degree of resistance against invasion by mould fungi (Hahn *et al*., 1983). Stack & Pedersen (2003) demonstrated that sorghum hybrids with a tannin-content testa layer had the lowest incidence and severity of grain mould. Doherty *et al*. (1987) also reported that grain mould and insect resistant caryopses contain higher free phenolic compounds and tannins than susceptible cultivars (Doherty *et al*., 1987).

The physical characteristics and fat content of sorghum grains plays a role in the accumulation of aflatoxin. Ratnavathi & Sashidhar (2003) reported that certain white sorghum genotypes low in fat, with average starch and high protein content showed maximum aflatoxin resistance. This could be attributed to the corneous nature of the endosperm in combination with the low fat content of the genotypes. They have also noted that grains with high

polyphenol levels are more resistant than those with a floury endosperm and low levels of polyphenols.

Pathogenesis-related proteins such as chitinase and β-1,3-glucanases can be induced in sorghum plants exposed to various stresses such as fungal infection, insect infestation and mechanical wounding. The increase in the level of stress induced proteins in sorghum plants is thought to limit the spread of pathogens or other opportunistic microorganisms (Krishnaveni *et al*., 1999). Antifungal proteins could be more effective when they act synergistically. Guo *et al*. (1997) found almost equal concentrations of ribosome-inactivating protein (RIP) in both resistant and susceptible maize kernels and noted that other proteins may act synergistically with RIP to confer resistance to *A*. *flavus*. Although these proteins play an important role in grain mould resistance, antifungal proteins on their own only confer partial resistance (Rooney *et al.*, 2002). The use of grain mould resistant cultivars is the preferred and most feasible method of controlling and minimizing damage to grains by grain mould fungi (Menkir *et al*., 1996) as no extra effort would be required to control the disease (Marley & Ajayi, 1999).

Chemical control/biological control

No fungicides are registered in South Africa for the control of *F. verticillioides* maize ear rot, but agrochemicals are available and registered for the control of maize leaf diseases as well as maize stem borers. No literature could be found on the effect of these agrochemicals on *F. verticillioides* infections and fumonisin production in South Africa. Folcher *et al.* (2009) studied the control of *Lepidoptera* catepillars with agrochemical treatments and their consequences on *Fusarium* spp. mycoflora and mycotoxin levels in France. Treatments involved either an insecticide (deltamethrine) or an insecticide (deltamethrine)-fungicide (tebuconazole) association. They found that the insect populations were controlled by the insecticide, but there was no reduction in *Fusarium* spp. mycoflora. A significant reduction in mycotoxin (trichothecenes, fumonisin and zearalenone) levels were reported from the

insecticide treatments. In a similar experiment in Italy, De Curtis *et al*. (2011) applied three different fungicides (tebuconazole, tetraconazole and prochloraz+cyproconazole) combined with an insecticide lambda-cyhalothrin. They found that the treatment with insecticide alone reduced the insect damage severity consistently, and that the concentration of fumonisin was reduced in only three of the six hybrids they used. Fungicide treatments combined with the insecticide showed a significant reduction of both Fusarium ear rot incidence and fumonisin contamination.

Reports on the biocontrol of *F. verticillioides* on maize roots with bacteria seem to be promising. *Bacillus* spp. offer several advantages over other bacteria because of their ability to form endospores and because of the broad-spectrum of activity of their antibiotics. Cavaglieri *et al*. (2005) identified the strain *B. subtilis* CE1 to have potential biological control activity against *F. verticillioides* on maize roots whereas Pereira *et al.* (2010) reported that seed treatment with *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* may improve quality of maize grain obtained at harvest by reducing mycotoxin content. The use of natural compounds as antagonists of *F. verticillioides* and fumonisin are also being applied. For instance, thymol which is a cyclic terpene was reported to be the most active inhibitor of fumonisin B_1 biosynthesis when compared to limonene, methol and menthone (Dambolena *et al*., 2008). Similarly, stereoisomer (-)-methol, followed by (+)-menthol were reported to be the most active compounds of methanol in the inhibition of fumonisin B_1 biosynthesis (Dambolena *et al*., 2010a). Menniti & Neri (2010) reported that *trans*-2-hexenal postharvest fumigation is effective in *F. verticillioides* control (also in asymptomatic kernels) but not in reducing fumonisin production. Essential oils are also being studied for their antifungal properties and Dambolena *et al.,* (2010b) found *Ocimum gratissimum* essential oil from Kenya, which has a high content of eugenol (antioxidant), to induce a significant inhibitory effect on fumonisin B_1 production.

Fumonisins

Fumonisins are named after the fungus *F. moniliforme* (renamed *F. verticillioides*) from which fumonisin B_1 was isolated in 1988 by Bezuidenhout *et al*. (1988). These authors elucidated the structures of the fumonisins by mass spectrometry and ^{1}H and ^{13}C n.m.r. spectroscopy as the diester of propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane as well as in each case the C-10 deoxy analogue. In all cases both the C-14 and C-15 hydroxy groups are involved in ester formation with the terminal carboxy group of propane-1,2,3-tricarboxylic acid. The discovery of fumonisin B_1 was followed by the isolation and structural characterisation of fumonisin B_2 and and fumonisin B_3, which lack one of the three hydroxyl groups on the backbone (Bezuidenhout *et al*., 1988; Plattner *et al*., 1992). These three B-series fumonisins (Figure 1.5) account for the majority of fumonisins that occur in grain samples that are naturally contaminated with *F. verticillioides*, *F. proliferatum* and most other fumonisin-producing spp.. The distribution of fumonisin is global and their presence has been confirmed in at least twenty-five countries (Mazzani *et al.*, 2001). FB_1 typically accounts for 70–80% of total fumonisins produced, while FB_2 usually makes up 15–25% and FB_3, 3–8% (Dilkin *et al.*, 2002; Rheeder *et al.*, 2002). Fumonisin B_2 was detected in cultures of *Aspergilus niger* for the first time by Frisvad *et al.* (2007). Later, it was shown that *A. niger* strains were able to produce FB_2 and FB_4 on grapes and raisins (Morgensen *et al*., 2010) as well as FB_2 on coffee (Noonim *et al*., 2009). A new FB_6 has been isolated, together with FB_2, from stationary cultures of the fungus *A. niger* NRRL 326 (Månsson *et al*., 2010).

Higher levels of fumonisin are usually found in maize kernels produced in the warmer regions of the world (Shelby *et al*., 1994). Damaged, *Fusarium* rotted kernels typically contain higher fumonisin levels than intact, healthy grain (Vincelli & Parker, 2002). The presence of high levels of fumonisin in maize seeds might have deleterious effects on seedling emergence (Doehlert *et al.*,

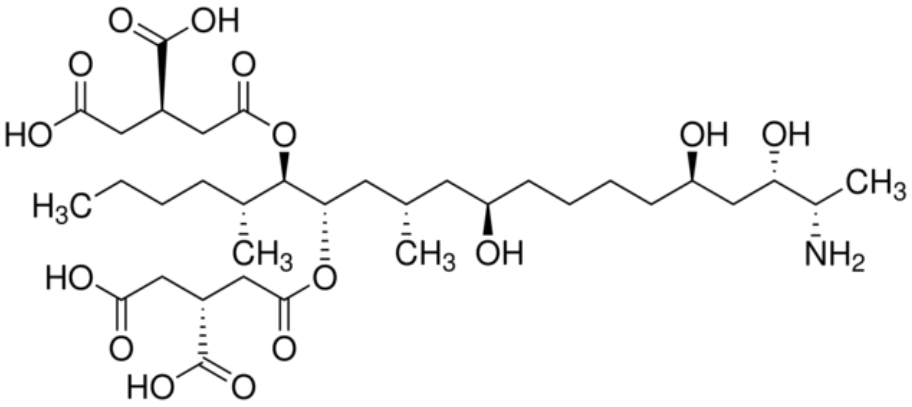
1994). Rheeder *et al.* (2002) reported up to 17.90 ppm fumonisin from isolates of *F. verticillioides* from South Africa and 31.00 ppm from isolates of *F. proliferatum* from Spain. *F. subglutinans* also frequently infects maize worldwide, but is known to produce low fumonisin levels (Rheeder *et al*., 2002). There is a paucity of information regarding the status of fumonisin producing *Fusarium* spp. in commercial sorghum grain of South Africa. In a study by Rabie & Marais (2000), no fumonisins were recorded in sorghum malt samples from South Africa. Bhat *et al*. (2000) reported the widespread natural occurrence of fumonisin in the sorghum-growing regions of Andhra Pradesh, India. Fumonisin contamination was higher in rain-affected and mouldy samples. In fifty Brazilian sorghum samples, *F. verticillioides* was isolated in only 15.1%, with 38% of them being contaminated with fumonisin B_1 at levels ranging from 0.05 to 0.36 ppm (dos Reis *et al*., 2010). In India field trials were conducted at 4 locations to determine fumonisin B_1 production in elite sorghum cultivars. Fumonisin contamination ranged from 0.01-1.40 ppm grain and varied over localities and genotypes (Das *et al*., 2010). Mohammed *et al*. (2010) tested sorghum grain samples imported to Saudi Arabia and found *F. verticillioides* to be the primary fumonisin producer with levels up to 19.10 ppm using the HPLC technique. From these reports it is evident that fumonisin B_1 is of concern in sorghum and indicates a need to determine the status of fumonisin contamination in South African sorghum samples.

The effect of fumonisins on humans and animals

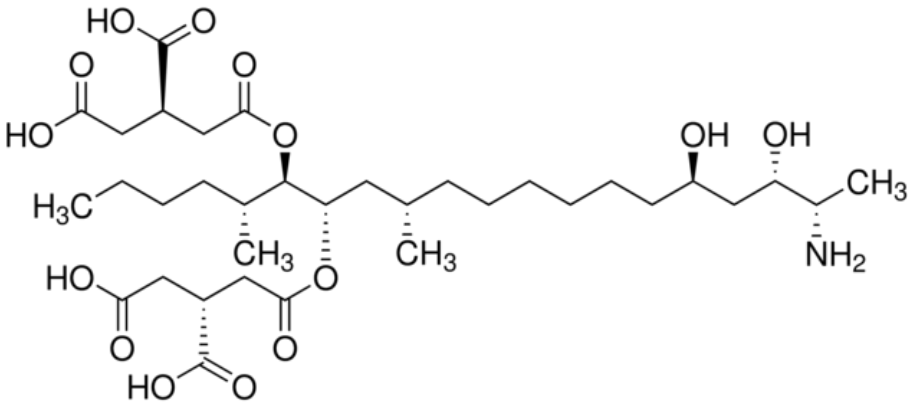
According to Thiel *et al.* (1991) both FB_1 and FB_2 occur naturally in maize and feeds associated with field outbreaks of mycotoxicoses in animals. Although fumonisins have a relatively simple chemical structure, their inhibition of sphingolipid metabolism can have diverse and complex effects in animal 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. Fumonisins also causes pulmonary oedema in swine (Harrison *et al.*, 1990) and is hepatotoxic and carcinogenic to rats (Gelderblom *et al.*, 1988).

Fumonisin B_1



Fumonisin B_2



Fumonisin B_3

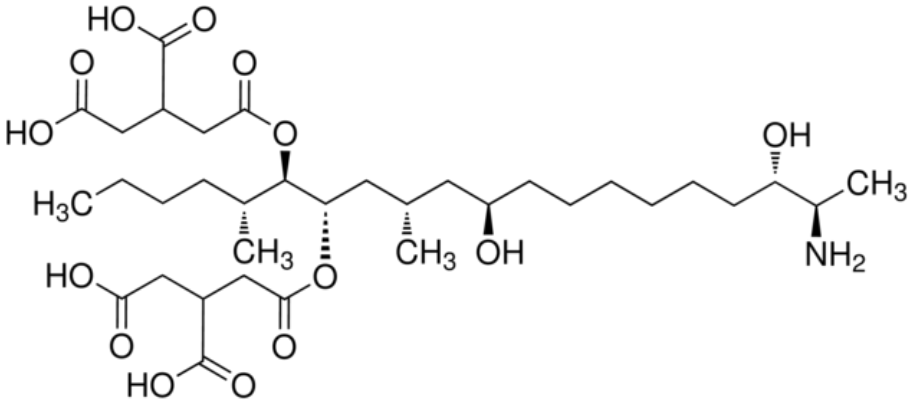


Figure 1.5 Chemical structure of fumonisins B_1, B_2 and B_3. (Source: www. sigmaaldrich.com).

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. Studies by Stack (1998), Placinta *et al.* (1999), Hendricks (1999) and Marasas *et al*. (2004) have shown a strong correlation between consumption of fumonisin-contaminated tortillas and neural-tube defects in humans. The potential carcinogenic risk of fumonisin B_1 to humans was evaluated and classified by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) (Anonymous, 2002) as a "Group 2B carcinogen" which means it is probably carcinogenic to humans. Alberts *et al*. (1990) reported that FB_1 is not destroyed by cooking and could therefore, easily enter the human food chain. Gelderblom *et al*. (2002, PROMEC, Medical Research Council, Tygerberg, South Africa, personal communication) communicated that stored (4°C) maize meal samples in air-tight containers lost about 30% of fumonisin B_1 over a period of 13-20 years. Fandohan *et al.* (2006) reported a general decrease in fumonisin levels when maize grain was stored in a bamboo granary over a eight month period. In contrast Ngoko *et al*. (2001) reported an increase in fumonisin levels in infected maize kernels after a four month storage period in Cameroon. This necessitates the importance of regularly screening human and animal foodstuffs for the presence of fumonisins.

Aflatoxin producing *Aspergillus* spp.

*Aspergillus s*ub-genus *Circumdati* Section *Flavi*, also referred to as the *Aspergillus flavus* group, has attracted worldwide attention for its industrial use and toxigenic potential. Section *Flavi* is divided into two groups of spp.. One includes the aflatoxigenic spp. *A. flavus, A parasiticus* and *A. nomius*, and the other includes non-aflatoxigenic spp. *A. oryzae*, *A. sojae* and *A. tamarii* (Rodrigues *et al*., 2007). Shephard (2005) reported that *A. flavus* and

A. *parasiticus* only occur sporadically in both commercial and home-grown maize in South Africa and are not ear rot pathogens under local conditions. Extensive surveys by the South African Maize Board since 1986 have consistently demonstrated a very low incidence of aflatoxin contamination in local commercial maize. More recently surveys of South African commercial maize have been performed by the South African Grain Laboratory (SAGL). Although South African maize is virtually free from aflatoxin contamination, improper harvest and storage practices can give rise to fungal growth and consequently high levels of aflatoxin (Shephard, 2005).

Reports of aflatoxin in sorghum are mainly from sorghum malt and beer samples. Nkwe *et al*. (2005) tested 46 sorghum malt samples from Gaborone, Botswana for *Aspergillus*- and *Fusarium* spp. as well as aflatoxin and fumonisin. They reported 63% and 37% infection by *F. verticillioides* and *A. flavus* in malt samples respectively, with fumonisin B_1 only present in three samples and no aflatoxin was detected. In the southern region of Malawi aflatoxin was present in all 27 malted sorghum and five traditional beer samples prepared from malted sorghum (Matumba *et al*., 2011). These authors also collected 13 sorghum and seven thobwa (traditional opaque sweet beverage) samples from the same region and reported a 15% and 43% aflatoxin contamination respectively. The sorghum malt prepared for beer brewing had a significant higher total aflatoxin content than any other type of samples. They reported the average aflatoxin content in beer to be 22.32 ug/l. The lack of publications on the occurrence of aflatoxin producing *Aspergillus* spp. and aflatoxin in sorghum may be because sorghum only represents 3.5% of the world cereal production, but certainly for countries with food insecurities such as Africa and India, this is an important issue which should be addressed.

Taxonomy

A. flavus Link:Fr. and *A. parasiticus* Speare are morphologically similar and are the only spp. that produce aflatoxin (Payne, 1999). *A. flavus* which has

smooth spores may be distinguished from *A. parasiticus* with its rough-walled conidia (Klich & Pitt, 1988). *A. flavus* is the predominant spp. on maize and sorghum. *A. flavus* can be found worldwide but is predominantly a tropical to sub-tropical fungus which is more common in cultivated than uncultivated soil (Klich & Pitt, 1988). Conidial heads are globose to radiate to columnar and light yellow green to olive brown. Vesicles are globose or sub-globose, and the larger vesicles have both metulae and phialides. Conidia are globose to sub-globose, smooth to slightly rough and 3-7 µm in diameter. Sclerotia, when present are dark red to black, globose, subglobose or vertically elongate and 400-700 µm in diameter (Payne, 1999). *A. parasiticus* is reported to be frequently isolated from seeds, other plant parts and insects and occasionally from cultivated soils (Klich & Pitt, 1988). Conidial heads are usually radiate with finely roughened to very rough, colourless vesicles, spherical or slightly elongate. Conidia are globose and distinctly rough-walled.

Symptoms

Only a few kernels on a maize ear are usually infected. Infected kernels often have masses of yellow green spores (Figure 1.6) on and between them (Payne, 1999). Older colonies of the fungus may turn dark green to brown but retain a yellow colour. Although any part of the ear may be infected with *A. flavus,* the tip is the most common infection site. Sporulation is evident on kernels that are injured, however, the fungus may be present in kernels with no visible sporulation. These kernels will often appear dull and discoloured. A study by Smart *et al*. (1990) showed that *A. flavus* has parasitic abilities and in the colonisation of the rachis, the fungus causes a collapse of aerenchyma cells and vascular bundles. It's aggressiveness is more pronounced once the maize kernels have been penetrated. The fungus rapidly colonised the scutellum tissue and invaded cells both inter- and intracellularly. They found evidence that the fungus dissolved cells of the scutellum in its path. The collapse of the aerenchyma cells and vascular bundles in advance of the fungus indicate that the fungus may be producing a toxin or cell wall degrading enzyme.

Figure 1.6 *Aspergillus* spp. growth on maize kernels (Photo: P. Lipps)

Epidemiology

A. flavus is a thermotolerant fungus, it is therefore more likely than many other fungi and bacteria to survive at temperatures of up to 48ºC (Brown *et al*., 1999) and under dry conditions (-35 MPa) (Payne, 1999). Aflatoxins are produced between temperatures of 12 and 42°C and the optimum temperature is 25-35°C (Diener & Davis, 1966). The optimum water activity for growth of *A. flavus* is high (approximately 0.99 a_w). The maximum is at least 0.998 a_w whereas the minimum water activity for growth was reported by Pitt & Miscamble (1995) to be approximately 0.82 a_w.

Aflatoxin production is particularly favoured by very moist conditions. Maximum moisture content for aflatoxin production in maize kernels is 25% at 30°C and the minimum relative humidity for aflatoxin production varies between 83% and 88% although Widstrom *et al*. (1990) found high maximum and high minimum daily temperatures, especially during periods with high nett evaporation, to be more important to the development of aflatoxin than humidity or average precipitation.

Sources of inoculum for *A. flavus* and *A. parasiticus* are sporogenic sclerotia (Calvo *et al*., 1999), conidia and mycelia that overwinter saprophytically in soil and plant debris (Yu *et al*., 2005). Conidia are airborne and readily dispersed by air movements (Diener *et al.,* 1987). Insects physically move conidia adhering to their bodies to plant parts during feeding and deposit them via defecation. Hot, humid conditions favour the release of spores on plant residues, and spores are spread to silks by wind or insects. Spores may then land on the silk tissue, germinate and enter the cob prior to pollination and subsist on senescent silks within the husks indefinitely (Payne, 1999). Insect damage predisposes the kernels to fungal penetration and plants that are drought stressed appear to be more susceptible to infection by *A. flavus* (Diener *et al*., 1987).

Grain Characteristics

Colonisation of maize kernel surfaces by *A. flavus* is extremely important in the epidemiology of this disease. Marsh & Payne (1984) and Smart *et al*. (1990) found that the fungus colonises the surface of kernels and the glume tissue surrounding the kernels. The fungus subsequently enters intact kernels in a number of ways. It may grow on the surfaces of the rachis and spikelet and invade kernels at the junction of the bracts and rachillas. At this stage, the cells are large, thin-walled and highly vacuolated. Smart *et al*. (1990) also found that the fungus could grow through the rachis into the spikelet through continuous air spaces in these tissues. Taubenhaus (1920) observed that erect maize ears, which tended to collect water, had the highest incidence of infection.

According to Chandrashekar & Satyanarayana (2006) sorghum grain is less susceptible than other grain, such maize and groundnuts, to infection by *A. parasiticus* and aflatoxin contamination due to its physical characteristics and biochemical composition. Physical grain structure such as pericarp thickness and composition, endosperm texture and various chemical constituents such as hydroxycinnamic acid, ferulic acid, polyphenols (Ratnavathi & Sashidhar,

2007) and various endosperm proteins (Ghosh & Ulaganathan, 1996) that are directly antagonistic to pest and pathogens are involved in defense. Sorghum grain hardness has been implicated in reducing mould infestation (Kumari *et al.,* 1994; Audilakshmi *et al*., 1999). The corneous endosperm of sorghum is enriched with kafirins, especially ß-kafirins that form extensive intra-chain disulfide bonds which may contribute both to texture and resistance to fungal infection (Mazhar & Chandrashekar, 1993). Sorghums with red pericarp and/or tannins are devoid of a hard, corneous endosperm. Ratnavathi & Sashidhar (2003) observed the lowest amount of aflatoxin in sorghum genotypes with a red pericarp due to high levels of flavon-4-ols compared with white pericarp sorghums (Waniska *et al*., 2001).

Chemical/biological control

Aflatoxin cannot be readily removed from contaminated foods by detoxification and therefore, biological control to increase crop safety by suppressing aflatoxin production has become an important control strategy. It has been reported that aflatoxin production is inhibited by lactic acid found in *Bacillus subtilis* and many other moulds. Munimbazi & Bullerman (1998) reported growth suppression by six *Bacillus pumilus* isolates on aflatoxin producing moulds and aflatoxin production. Inhibition of aflatoxin production ranged from 98.2-99.0%. Mycelium production was less inhibited with inhibition ranging from 34.3–56.4%. Bottone & Peluso (2003) identified a compound produced by *B. pumilus* which could inhibit *Aspergillus* spp. growth.

There is a great diversity of phenotypes of *A. flavus* in agricultural fields and the common occurrence of atoxigenic strains (Diener & Davis, 1966; Cotty, 1989; Cotty *et al*., 2007). Furthermore, toxigenicity is apparently unrelated to a strains's ability to colonise and/or infect living or dead plant tissue. As a result atoxigenic strains can be used to displace toxigenic strains (Cotty, 1989; Cole & Cotty, 1990; Atehnkeng *et al*., 2008). Cotty & Bayman (1993) tested the competitive ability of an antagonistic *A. flavus* strain to inhibit the aflatoxin contamination in developing cotton bolls. Competitive exclusion

contributed to the effect of the atoxigenic strain on contamination and results suggested that a second unknown mechanism might have been in effect. Biocontrol of aflatoxin producing strains with atoxigenic strains of *A. flavus* are being developed for maize, cottonseed, groundnuts, rice kernels and wheat seeds (Cotty, 1994; Bock & Cotty, 1999; Atehnkeng *et al*., 2008; Cotty *et al*., 2008).

Aflatoxins

Aflatoxins are secondary metabolites produced by *A. flavus* and *A. parasiticus* from the section *Flavi* (Sweeney & Dobson, 1998). The discovery of aflatoxin dates back to the year 1960 following the severe outbreak of the turkey 'X' disease in the United Kingdom, which resulted in the deaths of more than 100 000 turkeys and other farm animals. The cause of the disease was attributed to a feed, containing Brazilian peanuts, which was heavily infested with *A. flavus*. Analysis of the feed using thin-layer chromatography (TLC) revealed that a series of fluorescent compounds, later termed aflatoxins, were responsible for the outbreak (Sargeant *et al*., 1961). The name aflatoxin has been formed from the following combination: the first letter 'A' for the genus *Aspergillus*, the next three letters *'FLA*" for the spp. *flavus*, and the noun "TOXIN' meaning poison (Ellis *et al*., 1991).

Between April and September of 2004, one of the largest known aflatoxicoses outbreaks occurred in Kenya, with 317 reported cases and 125 deaths (Okioma, 2008). A less severe outbreak occurred in 2005, resulting in 16 deaths. Foods samples were collected from households in the affected area and contained high levels of aflatoxin B_1 (20–1000 ppb). This outbreak resulted from aflatoxin contamination of locally grown maize that was stored under damp conditions.

A. flavus lacks the ability to produce G-aflatoxin due to a gap in the cluster that includes a required cytochrome P450-encoding gene, *cypA* (Ehrlich, 2008). *A. parasiticus* produces both B and G toxins (Pitt, 2006) (Figure 1.7).

Only about 50% of naturally occurring *A. flavus* isolates produce aflatoxin, while virtually all known isolates of *A. parasiticus* are toxigenic (Pitt, 2006).

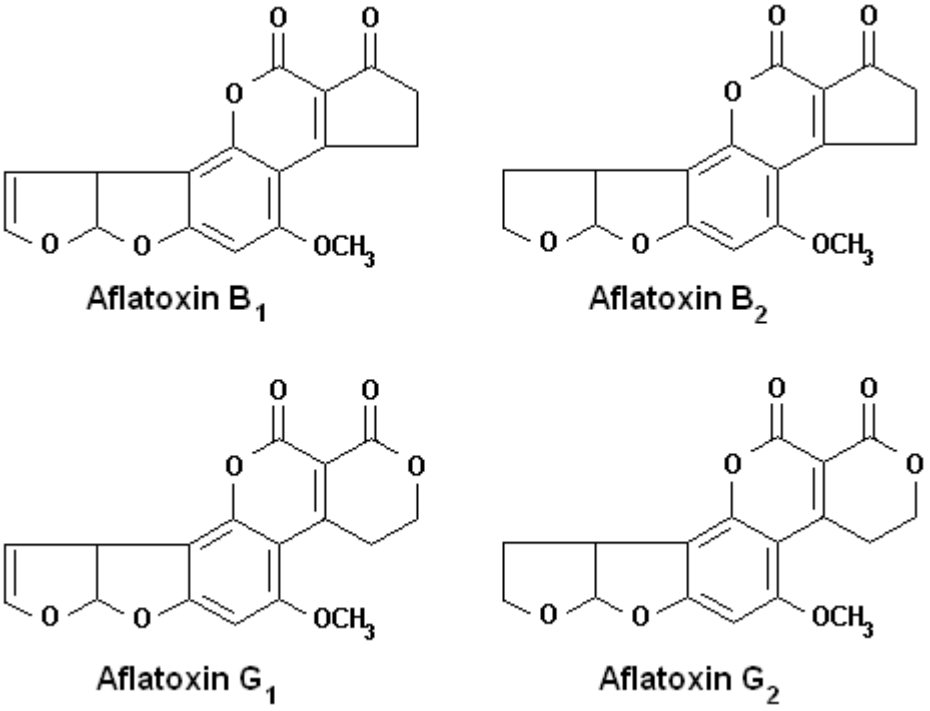


Figure 1.7 The chemical structure of aflatoxin B_1, B_2, G_1 and G_2 (Figure: www.bmb.leeds.ac.uk)

The effect of aflatoxins on humans and animals

Aflatoxins are potentially hazardous to humans and animals displaying strong immunosuppressive, mutagenic, teratogenic (da Silva *et al*., 2004) and carcinogenic effects (Hussein & Jeffrey, 2001). They are the most potent chemical liver carcinogens known (Wu, 2006). Aflatoxin may cause oxidative stress, liver necrosis, haemorrhage and death in broiler chickens, pigs and cattle (Eraslan *et al*., 2005; Osweiler, 2005). Aflatoxin B_1 has been reported to be the most toxic and has been classified as a group 1 human carcinogen by the International Agency for Research on Cancer (Anonymous, 1993). The synergistic effects of aflatoxin and hepatitis B and C (prevalent in China and

sub-Saharan Africa) raise the risk of liver cancer more than tenfold when compared with either exposure alone (Miller & Marasas, 2002). Aflatoxin are also associated with stunting in children (Gong *et al*., 2000) and possibly immune system disorders (Turner *et al*., 2003). The presence of aflatoxin B_1 has been reported in lung tissue of agricultural and textile workers due to respiratory exposure (Richard, 2006).

Detection of fungi and mycotoxins

Fungal detection methods

Quantification of *Fusarium* spp. in maize and commodity surveys have involved isolation frequency (plate count) and determination of infection rate (percentage of seed that yields fungi after surface disinfection) as an indication of fungal biomass (Schwadorf & Müller, 1989; Saxena *et al.*, 2001). Plate counts and determination of infection rate are time consuming and in the case of infection rate, not applicable to meal samples (Schwadorf & Müller, 1989). The use of isolation frequency of pathogens from maize as an indication of fungal biomass is also poorly correlated with fumonisin concentration (Ngoko *et al.*, 2001) under natural field conditions. Furthermore identification of fungi relies on microscopic identification which requires expertise. *Fusarium* spp. vary phenotypically and morphologically when cultured on non-standard media, compared with features associated with growth 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. Ergosterol can 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 ergosterol method has limitations in that ergosterol extract of fungi growing within a natural substratum may be a

recovery of a variety of different fungi embedded therein (Padgett & Posey, 1993). Therefore the quantification of total ergosterol from maize kernels may not give an accurate account of the fumonisin producing fungal mass. Similarly the *Fusarium* strain will determine the amount of fumonisin produced, not necessarily the amount of *Fusarium* present (Jardine & Leslie, 1999).

Aiming to both overcome the obstacles of identification as well as developing rapid tools for detection, nucleic acid based methods have been developed for the identification and quantification of mycotoxigenic fungi. Although conventional PCR techniques are accurate and sensitive, realtime PCR techniques are even more sensitive and eliminate time consuming techniques to analyze the reaction product such as Southern blotting and agarose gel electrophoresis.

The fumonisin biosynthetic genes are clustered (Proctor *et al*., 2003) and one of the fumonisin biosynthetic genes, *fum1*, encodes a polyketide synthase required for fumonisin production (Proctor *et al*., 1999) which catalyses the initial step in fumonisin biosynthesis (Bojja *et al*, 2004). *Fum19* is located about 35 kilobase (kb) downstream from the *fum1* gene and encodes an ABC (ATB-binding cassette) transporter involved in extracellular export of fumonisin (Proctor *et al*., 2003). A TaqMan PCR developed by Waalwijk *et al*. (2008a, 2008b) targets a conserved region in the polyketide synthase gene *fum1,* for the quantification of fumonisin producing fungi such as *F. verticillioides, F. proliferatum, F. nygamai, F. globosum* and *F. thapsinum*.

Aflatoxins are synthesized by a polyketide metabolic pathway (Bhatnagar *et al*., 2003). Mapping of overlapping cosmid clones of *A. parasiticus* and *A. flavus* genomic DNA established that the genes in the aflatoxin biosynthetic pathway are clustered (Bhatnagar *et al*., 2003). The aflatoxin gene cluster in *A. parasiticus* and *A. flavus* consists of 25 genes spanning approximately 70 kb (Bhatnagar *et al*., 2006). The *nor*-1 gene codes for the norsorlorinic acid reductase, one of the first genes in the aflatoxin biosynthetic pathway

(Woloshuk & Prieto, 1998). Mayer *et al*. (2003) developed a method for the quantification of *nor*-1 gene containing strains such as *A. parasiticus* and *A. flavus* in foods. Passone *et al*. (2010) developed a realtime PCR using the *nor*-1 gene for the detection and quantification of *A. parasiticus* and *A. flavus* in peanuts.

DNA-based detection methods such as realtime PCR are more sensitive and specific (Passone *et al*., 2010). The realtime PCR assay appears to be a promising tool in the quantification of fumonisin producing *Fusarium* spp. as well as aflatoxin producing *Aspergillus* spp. in both maize and sorghum.

Detection and quantification of mycotoxins

Most mycotoxins are chemically stable and tend to survive storage, processing and survive cooking at high temperatures reached during baking of bread or breakfast cereal production. This emphasises the importance of toxin free grain at harvest (Turner *et al*., 2009). In contrast the presence of a recognised toxin-producing fungus does not necessarily mean that the associated toxin will also be present, as many factors are involved in toxin formation. Similarly the absence of any visible mould will not guarantee freedom from toxins as the pathogen may have already died while leaving the toxin intact (Turner *et al*., 2009). Since most mycotoxins are toxic in very low concentrations, sensitive and reliable methods for their detection are essential.

Analytical methods for mycotoxin quantification in feeds and foodstuff generally require toxin extraction from the matrix with an adequate extraction solvent, a clean-up step to eliminate interference from the extract and finally, detection/determination of the toxin by suitable analytical instruments/ technologies (Pascale & Visconti, 2008).

Sample extraction and clean-up methods

Mycotoxins are commonly extracted from ground cereals by shaking or blending with mixtures of water or other polar solvents such as methanol or acetonitrile (Pascale, 2009). Purification of the extract is an essential step in the analysis of mycotoxins, especially when chromatographic techniques are used for determination at trace levels. Immunoaffinity columns (IAC), Solid phase extraction (SPE) and multifunctional clean-up columns are frequently used to clean up the extracts of raw cereals as well as cereal-processed products (Pascale, 2009).

Immunoaffinity columns (IAC)

These columns are prepared by binding antibodies (monoclonal or polyclonal) specific for a given mycotoxin to a specially activated solid-phase support and packing the support suspended in aqueous buffer solution into a cartridge (Scott & Trucksess, 1997). The mycotoxin in the extract will bind to the antibody while impurities are removed with water or an aqueous solution. The mycotoxin is desorbed with a miscible solvent such as methanol (Scott & Trucksess, 1997). IACs are commercially available for the detection of aflatoxin, ochratoxin, fumonisins, zearalenone, deoxynivalenol, T-2 and HT-2 toxins (Pascale, 2009). Pascale & Visconti (2008) reported the use of IACs in multi-mycotoxin detection to be accurate and precise.

Solid phase extraction (SPE)

The basic principle of SPE technology is a variation of chromatographic techniques based on small disposable cartridges packed with silica gel, or bonded phases which are in the stationary phase. SPE is a separation process which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Analytical laboratories use solid phase extraction to concentrate and purify samples for analysis. Solid phase extraction can be

used to isolate analytes of interest from a wide variety of matrices, including urine, blood, water, beverages, soil, and animal tissue. The sample is loaded in one solvent, generally under reduced pressure and rinsed, where most of the contaminants are removed, and eluted in another solvent (Turner *et al*., 2009). These cartridges have a high capacity for binding of small molecules.

Multi-functional clean-up columns

According to Pascale (2009) MycoSep™ columns are one of the most commonly used and commercially available columns for removing analytical interferences from raw extracts. These columns contain adsorbents such as charcoal, celite and ion-exhange resins which is pushed into a test tube (containing the extract) thereby forcing the extract to filter upwards through the packing adsorbent material. The interferences adhere to the adsorbents in the column and the purified extract passes through a frit to the surface of the column (Wilson & Romer, 1991). These columns are often used for simultaneous and rapid clean-up of type A- and type B-tricothecenes, as well as aflatoxins, ochratoxin, zearalenone and fumonisins (Pascale, 2009).

Analytical methods

Classic detection methods

Different classical methods are commonly used for quantitative determination of mycotoxins, including high performance liquid chromatography (HPLC) coupled with ultraviolet (UV), liquid chromatography/mass spectrometry (LC/MS), gas chromatography coupled with electron capture detection and thin-layer chromatography (TLC). In addition, commercial immunometric assays, such as enzyme-linked immunosorbent assays (ELISA) or membrane-based immunoassays are frequently used for screening purposes (Chu, 1996; Ono *et al*., 2000; Alakonya *et al*., 2008).

Advantages and disadvantages of classic detection methods

Traditionally the most popular method used for mycotoxin analysis is TLC, which offers the ability to screen large numbers of samples economically. According to Pascale & Visconti (2008) TLC can be used for the simultaneous analysis of multiple mycotoxins. As with HPLC, several methods have been developed to obtain the best results with each separate class of mycotoxin and both one-dimensional and two-dimensional analyses are frequently used (Lin *et al*., 1998). However some of the disadvantages of TLC is that there is an inherent need for sample preparation, the technique may have a poor sensitivity and precision towards some mycotoxins. It is quantitative only when used with a densitometer. Presently analysis of mycotoxins relies heavily on HPLC (Pascale & Visconti, 2008) where normal and reversed-phase columns are used for separation and purification of toxins depending on their polarity. In essence most of the protocols used for HPLC detection of mycotoxins are very similar (Turner *et al*, 2009) and official methods are available. This method has the advantage of good sensitivity, selectivity and repeatability, although the equipment is expensive with specialist expertise required, as some toxins may need derivitisation. With more advanced techniques such as liquid chromatography/mass spectrometry no derivitisation is required, however these techniques are very expensive, specialist expertise is required and the sensitivity relies on the ionization technique used. The Enzyme Linked Immuno Sorbent Assays (ELISA) has become very popular due to their relatively low cost and easy application (Goryacheva *et al*., 2007). Commercially available ELISA kits for detection of mycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target (Stanker *et al*., 2008). The complex formed will then interact with a chromogenic substrate to give a measurable result. They can be portable, rapid and are highly specific as well as simple to use. The disadvantage of these kits lies in the fact that they are for single use, which can increase costs of bulk screening. Additionally, competitive ELISA suffers from having a limited detection range due to the narrow sensitivity of the antibodies, be they

mono- or polyclonal. The development of antibodies for most mycotoxins, due to their small size, require development of a carrier molecule, usually a protein (e.g. bovine serum albumin), to achieve immunogenicity. The conjugation process can also be responsible for decreases in assay selectivity.

New technologies for mycotoxin analysis include lateral flow devices, fluorescence polarization immunoassay (FPIA), infrared spectroscopy, capillary electrophoresis, fibre-optic immunosensors, biosensors, molecularly imprinted polymers and chip technology to name a few. Further investigation is required to validate new technologies and to determine their applicability to real samples, especially at levels close to legal limits (Pascale & Visconti, 2008).

Multi mycotoxin analysis

Most of the analytical methods used, such as TLC and HPLC are selective for quantification of target mycotoxins and are incapable of dealing with a large number of analytes in complicated food matrices (Sulyok *et al*., 2010). Modern methods such as HPLC coupled to (tandem-) mass spectrometry offer higher selectivity, which enables multi-analyte determination without dedicated sample clean-up in principle (Sulyok *et al*., 2010). The drawback of not applying a sample-clean up, is that signal suppression due to matrix effects is far more likely to occur. Despite these difficulties and the proliferation of mycotoxin regulations around the world, there is a need for multi mycotoxin analysis in a single commodity. Some examples include the simultaneous quantification of 17 kinds of *Aspergillus*, *Fusarium* and *Penicillium* mycotoxin contaminants by ultra-performance liquid chromatography combined with electrospray ionization triple quadrupole tandem mass spectrometry (UPLC-MS/MS) with sample clean up (Ren *et al*., 2007). A first-of-its-kind multi-analyte column for simultaneous detection of aflatoxins, ochratoxin A, fumonisin, deoxynivalenol, zearalenone, T-2 and HT-2 toxins was developed by Vicam (Anonymous, 2008) and is used with LC-MS. The application of an LC-MS/MS based multi-mycotoxin method for the

semi-quantitative determination of mycotoxins occurring in different types of food infected by fungi are described by Sulyok *et al*. (2010). Takino *et al*. (2011) have developed a method for the simultaneous determination of deoxynivalenol, T-2 toxin, HT-2 toxin and zearalenone in wheat and biscuit by liquid chromatography/electrospray ionisation/tandem mass spectrometry coupled with immunoaffinity extraction.

Many factors will play a role in selecting a detection and/or quantification method. Due to the varied structures of mycotoxins it is not possible to use one standard technique to detect all mycotoxins, as each will require a different method. What works well for some molecules could be inappropriate for others of similar properties, or for the same molecule in a different environment/matrix. Likewise, practical requirements for high-sensitivity detection and the need for a specialist laboratory setting create challenges for routine analysis. Therefore, depending on the physical and chemical properties, procedures that have been developed around existing classical analytical techniques, which offer flexible and broad-based methods of detecting compounds, will be applied.

Prediction models

An epidemic can be described as a “change in disease intensity in a host population over time and space” (Madden *et al*., 2007). Mathematical modeling of crop disease is a rapidly expanding discipline within plant pathology (Van Maanen & Xu, 2003). The first models of the temporal development of epidemics were developed by Van der Plank (1960, 1963) and have since formed the basis for disease modeling. In epidemiology, modeling aims to understand the main determinants of epidemic development in order to develop sustainable strategies for strategic and tactical management of diseases. Therefore, an epidemiological model can act as instrument to constantly monitor and assess the risk of fumonisin and aflatoxin contamination in maize and sorghum grain, making it possible to drive

agronomic decisions during cultivation that would enhance management opportunities (Schaafsma & Hooker, 2007).

Madden and Wheelis (2003) states that “very few plant diseases have been eradicated thus far and that some inoculum of a pathogen can usually be found in a given area for the important diseases of a given crop”. It is well documented that *F. verticillioides* is the most common fungus associated with maize (Nelson, 1992; Logrieco *et al*., 2002) world-wide. There have been numerous attempts to develop risk assessment models for *Fusarium* contamination of grain.

Various articles have been published regarding Fusarium head blight of wheat caused by *F. graminearum* (De Wolf *et al*., 2003; Rossi *et al*., 2003; Del Ponte *et al*., 2005; Musa *et al*., 2007). According to De Wolf *et al* (2003) Fusarium head blight is well suited for risk assessment modelling because of the severity of epidemics, compound losses resulting from mycotoxin contamination and relatively narrow time periods of pathogen sporulation, inoculum dispersal and host infection. This can be seen from the online forecasting model FusaProg (Musa *et al*., 2007) which is a threshold-based tool to control *F. graminearum* with optimized timing of fungicide applications and forecasts of deoxynivalenol contents during flowering. DONCast, a prediction model from Canada, has been validated extensively and commercialised for wheat (Shaafsma & Hooker, 2007). An adaption of this model has been proposed for maize. DONCast predicts the variation in toxin levels associated with year and agronomic effects from simple linear models using wheat samples from farm fields. The DONCast model account for up to 80% of the variation in deoxynivalenol and is being used commercially for the past 10 years in Canada.

On the other hand, robust field-based models to predict Fusarium ear rot and fumonisin B_1 from maize grain have been elusive, most probably due to the complexity of interactions between numerous abiotic and biotic disease factors (Parsons & Munkvold, 2012). The concentration and severity of

fumonisin produced by *Fusarium* spp. ear rot varies with meteorological conditions, genotype and location (de La Campa *et al*., 2005). In general favourable conditions for *F. verticillioides* infection include high temperatures (Munkvold, 2003), drought stress (Munkvold, 2003; Logrieco *et al*., 2002) and insect damage stress (Munkvold, 2003).

Shelby *et al.* (1994) reported that fumonisin concentrations were inversely proportional to rainfall before silking of maize plants in the USA. Stewart *et al.* (2002) developed a mathematical simulation of the growth of *F. graminearum* and *F. verticillioides* in maize ears after artificial inoculation of silks. This model is limited, only simulating fungal growth and not mycotoxin accumulation. De la Campa *et al*. (2005) developed a preliminary model (Phillipines and Argentina) and identified four weather periods near silking as critical to fumonisin concentration at harvest. This model accounted for 82% of the variability of total fumonisin across all locations in two years of study, but did not consider meteorological conditions during grain maturation when fumonisins are synthesized. A risk assessment model (FUMAgrain) developed by Maiorano *et al*. (2009) for fumonisin synthesis by *F. verticilliodes* in maize grain in Italy gives an initial risk alert at the end of flowering based on meteorological conditions. A second alert follows at maturation stage from assessments made from maize grain moisture, European Corn Borer damage to maize ears and fumonisin synthesis risk. FUMAgrain could simulate fumonisin synthesis in maize with an R^2=0.70 for calibration and R^2=0.71 for validation. These authors emphasized the importance of meteorological conditions at flowering and the growth of *F. verticillioides* and fumonisin synthesis during grain maturation. Maiorano *et al*. (2009) and Bush *et al*. (2004) indicated that 35% grain moisture during the dry down stage can be regarded as a “switch-on” for fumonisin synthesis.

A prediction model using variables such as cultivar, climate, management practice, soil type, phenological stages of the host plant and pathogen variation will help all role players in the industry by identifying areas or maize/sorghum batches with potentially dangerous levels of fungi and their

mycotoxins and enabling them to implement mycotoxin management strategies. This will help reduce grain contamination and prevent such grain being used for food or feed, thus improving human and animal food safety measures.

Conclusions

Maize is regarded as one of the major staple foods in the world, and constitutes the most important agricultural crop produced in southern Africa followed by sorghum. These crops are often affected by ear rot pathogens belonging to the genus *Fusarium* and to a lesser extent *Aspergillus* and the fumonisins and aflatoxins respectively produced. Fumonisins are mycotoxins produced mainly by *F. verticillioides* and *F. proliferatum* particularly in maize and sorghum (da Silva *et al*., 2004). Fumonisins are known to be carcinogenic and are the cause of toxicoses in animals and humans.

Aflatoxins are produced by *A. flavus* and *A. parasiticus* and contamination of maize with *Aspergillus* spp. has always been regarded as serious in countries with high temperatures that are prone to drought such as the USA (Payne, 1999), Brazil (Piedade *et al*., 2002) and Benin (Setamou *et al*., 1997). Although Shephard (2005) and the South African Maize Board have indicated low incidences of aflatoxin in home-grown and commercial maize respectively, Ncube (2008) reported aflatoxin (B_1+B_2+B_3 with the ELISA technique) levels in the Limpopo Province of South Africa higher than the maximum tolerated 5 ppb for aflatoxin B_1 in South Africa, the European Union and USA (FAO, 2003). Therefore, the potential of aflatoxin in maize grain infected with *Aspergillus* spp. should not be underestimated.

It is important to monitor fungal infection, as well as fumonisin and aflatoxin production in maize and sorghum to ensure safe food for human and animal consumption. Legislation for maximum tolerable levels of *Fusarium* mycotoxins in maize and sorghum products have not been determined or entrenched in South Africa. Maize and sorghum are staple food crops in

South Africa and although commercially grown maize and sorghum may contain lower levels of fumonisin than home grown maize and sorghum (Burger *et al*., 2010) the higher daily consumption of a greater part of the population would warrant lower maximum tolerable levels than the 2 ppm set by the European Commission (EC).

The effect that these fumonisins and aflatoxins may have both on subsistence and commercial farming sectors is uncertain. It is important that they be monitored and that high-risk areas as well as consumers that may be exposed to these toxins be identified. An epidemiological model using variables such as cultivar, climate, management practices and pathogen variation can act as an instrument to constantly monitor and assess the risk of fumonisin and aflatoxin contamination in maize and sorghum grain, making it possible to drive agronomic decisions during cultivation that would enhance management opportunities. This will help reduce grain contamination and prevent such grain being used for food or feed, thus improving human and animal food safety measures. The aim of this study is to identify important factors to be included into an epidemiological model to predict disease and mycotoxin incidence in maize and sorghum in South Africa.

References

Abbas, H.K., Cartwright, R.D., Shier, W.T. 2006. Aflatoxin and fumonisin contamination of corn (maize, *Zea mays*) hybrids in Arkansas. *Crop Protection* **25**: 1-9.

Alakonya, A.E., Monda, E.O. & Ajanga, S. 2008. Variation in *in-vitro* fumonisin B_1 production by different *Fusarium verticillioides* isolates in Kenya. *American-Eurasian Journal of Agriculture & Environmental Science* **4**: 368-371.

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 B_1 by *Fusarium moniliforme*. *Applied and Environmental Microbiology* **56**: 1729-1733.

Agrios, G.N. 2005. Plant pathology. Fith Edition. Academic Press. 922pp.

Anonymous. 1993. WHO-IARC. Monograph on the evaluation of carcinogenic risk to human. C. Lyon (F): IARC.

Anonymous. 2002. WHO-IARC. Monograph on the evaluation of carcinogenic risk to humans. [Available on internet:] http://monographs.iarc.fr/ENG/Monographs/vol82/volume82.pdf [Date of access 22/08/12].

Ariño, A., Juan, T. Estopañan, G. & González-Cabo, J.F. 2007. Natural occurrence of *Fusarium* spp., fumonisin production by toxigenic strains, and concentrations of fumonisins B_1 and B_2 in conventional and organic maize grown in Spain. *Journal of Food Protection* **70**: 151-156.

Atehnkeng, J., Ojiambo, P.S., Ikotun, T., Sikora, R.A., Cotty, P. J. & Bandyopadhyay, R. 2008. Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize. *Food Additives and Contaminants* **25**: 1264-1271.

Audilakshmi, S., Stenhouse, J.W., Reddy, T.P, Prasad & M.V.R. 1999. Grain mould resistance and associated characters of sorghum genotypes. *Euphytica* **107**: 91-103.

Bacon, C.W. & Hinton, D.M. 1996. Symptomless entophytic colonisation of corn by *Fusarium moniliforme*. *Canadian Journal of Botany* **74**: 1195-1202.

Bacon, C.W., Porter, J.K., Norred, W.P. & Leslie, J.F. 1996. Production of fusaric acid by *Fusarium* spp.. *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.

Bandyopadhyay, R., Little, C.R., Waniska, R.D. & Butler, D.R. 2002. Sorghum grain mould: through the 1990s into the new millennium. Pp.173-183. *In*: Leslie, J.F. (Ed.), Sorghum and millets diseases 1st ed. Iowa State Press, Iowa.

Belton, P.S. & Taylor, J.R.N. 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology* **15**: 94-98.

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 Chemical Society, Chemical Communications* **11**: 743-745.

Bhat, R.V., Shetty, H.P.K & Vasanthi, S. 2000. Human and animal health significance of mycotoxins in sorghum with special reference to fumonisins. Pp107-115. *In*: Chandrashekar, A., Bandyopadhyay, R & Hall, A.J. (Eds.). Technical and institutional options for sorghum grain mould management: proceedings of an international consultation, 18-19 May, ICRISAT, Pantacheru, India.

Bhatnagar, D., Cary, J.W., Ehrlich, K, Yu, J. & Cleveland, T.E. 2006. Understanding the genetics of regulation of aflatoxin production and *Aspergillus flavus* development. *Mycopathologia* **162**: 155-166.

Bhatnagar, D., Ehrlich, K.C., Cleveland, T.E. 2003. Molecular genetic analysis and regulation of aflatoxin biosynthesis. *Applied Microbiological Biotechnology* **61**: 83-93.

Blandino, M. & Reyneri, A. 2007. Comparison between normal and waxy maize hybrids for *Fusarium*-toxin contamination in NW Italy. *Maydica* **51**: 127-134.

Bock, C.H. & Cotty, P.J. 1999. The effect of harvest date on aflatoxin contamination of cottonseed in Arizona. *Plant Disease* **83**: 279-285.

Bojja, R.S., Cerny, R.L., Proctor, R.H. & Du, L. 2004. Determining the biosynthetic sequence in the early steps of the fumonisin pathway by use of three gene disruption mutants of *F. verticillioides*. *Journal of Agricultural Food Chemistry* **52**: 2855-2860.

Bottone, E.J. & Peluso, R.W. 2003. Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against Mucoraceae and *Aspergillus* spp.: preliminary report. *Journal of Medical Microbiology* **52**: 69-74.

Brown, R.L., Chen, Z.Y., Cleveland, T.E. & Russin, J.S. 1999. Advances in the development of host resistance in corn to aflatoxin contamination by *Aspergillus flavus*. *Phytopathology* **89**: 113-117.

Burger, H-M, Lombard, M.J., Shephard, G.S., Rheeder, J.R., Van der Westhuizen, L, & Gelderblom, W.C.A. 2010. Dietary fumonisin exposure in a rural population of South Africa. *Food and chemical toxicology an international journal published for the British Industrial Biological Research Association* **48**: 2103-2108.

Bush, B.J., Carson, M.L., Cubeta, M.A., Hagler, W.M. & Payne, G.A. 2004. Infection and fumonisin production by *Fusarium verticillioides* in developing maize kernels. *Phytopathology* **94**: 88-93.

Calvo, A.M., Hinze, L.L., Gardner, H.W. & Keller, N.P. 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. *Applied Environmental Microbiology* **65**: 3668-3673.

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.

Cavaglieri, L., Orlando, J., Rodriguez, M.I., Chulze, S. & Etcheverry, M. 2005. Biocontrol of *Bacillus subtilis* against *Fusarium verticillioides in vitro* and the maize root level. *Research in Microbiology* **156**: 748-754.

Chandrashekar, A. & Satyanarayana, K.V. 2006. Disease and pest resistance in grains of sorghum and millets. *Journal of Cereal Science* **44**: 287-304.

Chu, F.S. 1996. Recent studies on immunoassays for mycotoxins. *Immunoassays for Residue Analysis* **621**: 294-313.

Chu, F.S. & Li, G.Y. 1994. Simultaneous occurrence of fumonisin B_1 and other mycotoxins in mouldy corn collected from the People's Republic of China in regions with high incidences of oesophageal cancer. *Applied and Environmental Microbiology* **60**: 847-852.

Cole, R.J. & Cotty, P.J. 1990. Biocontrol of alfatoxin production by using biocompetitive agents. Pp. 62-66. *In*: Robens, J.A. (Ed.). A perspective on aflatoxin in field crops and animal food products in the United States. United States Department of Agricultural Research Services.

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

Cotty, P.J. 1989. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology* **79**: 808-814.

Cotty, P.J. 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. *Phytopathology* **84**: 940-945.

Cotty, P.J., Antilla, L. & Wakelyn, P.J. 2007. Competitive exclusion of aflatoxin producers: Farmer-driven research and development. Pp 241-254. *In*: Vincent, C., Goettel, M.S. & Lazarovits, G. (Eds.). Biological control, a global perspective. Wallingford (UK).

Cotty, P.J. & Bayman, P. 1993. Competitive exclusion of a toxigenic strain of *Aspergillus flavus* by an atoxigenic strain. *Phytopathology* **83**: 1283-1287.

Cotty, P.J., Probst, C. & Jaime-Garcia, R. 2008. Etiology and management of aflatoxin contamination. Pp 287-299. *In*: Leslie, J.F., Bandyopadhyay, R. & Visconti, A. (Eds.). Mycotoxins: Detection methods, management, public health and agricultural trade. Wallingford (UK).

Da Silva, J.B., Dilkin, P., Fonseca, H. & Corrêa, B. 2004. Production of aflatoxins by *Aspergillus flavus* and of fumonisins by *Fusarium* spp. isolated from Brazilian sorghum. *Brazilian Journal of Microbiology* **35**: 182-186.

Dambolena, J.S., López, A.G., Cánepa, M.C., Theumer, M.G., Zygadlo, J.A., & Rubinstein, H.R. 2008. Inhibitory effect of cyclic terpenes (limonene, menthol and thymol) on *Fusarium verticillioides* MRC 826 growth and fumonisin B_1 biosynthesis. *Toxicon* **51**: 37-44.

Dambolena, J.S., López, A.G., Rubinstein, H.R. & Zygadlo, J.A. 2010a. Effects of methol stereoisomers on the growth, sporulation and fumonisin B_1 production of *Fusarium verticillioides*. *Food Chemistry* **123**: 165-170.

Dambolena, J.S., Zunino, M.P., López, A.G., Rubinstein, H.R., Zygadlo, J.A., Mwangi, J.W., Thoithi, G.N., Kibwage, I.O., Mwalukumbi, J.M. & Kariuki, S.T. 2010b. Essential oils composition of *Ocimum basilicum* L. and *Ocimum gratissimum* L. from Kenya and their inhibitory effects on growth and fumonisin production by *Fusarium verticillioides*. *Innovative Food Science and Emerging Technologies* **11**: 410-414.

Das, I.K., Vijay Kumar, B.S., Ratnavathi, C.V., Komala, A., Annapurna, A. & Seetharama, N. 2010. Toxigenicity of *Fusarium* isolates and fumonisin B_1 contamination in rainy season sorghum (*Sorghum bicolor*). *The Indian Journal of Agricultural Sciences* **80**: 724-729.

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.

De Curtis, F., De Cicco, V., Haidukoskwi, M., Pascale, M., Somma, S. & Moretti, A. 2011. Effects of agrochemical treatments on the occurrence of *Fusarium* ear rot and fumonisin contamination of maize in Southern Italy. *Field Crops Research* **123**: 161-169.

De la Campa, R., Hooker, D.C, Miller, D. Schaafsma, A.W. & Hammond, B.G. 2005. Modeling effects of environment, insect damage, and Bt genotypes on fumonisin accumulation in maize in Argentina and the Philippines. *Mycopathologia* **159**: 539–552.

Del Ponte, E.M., Fernandes, J.M.C. & Pavan, W. 2005. A risk infection simulation model for Fusarium head blight of wheat. *Fitopatologia Brasileira* **30**: 634-642.

De Wolf, E.D., Madden, L.V. & Lipps, P.E. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. *Phytopathology* **93**: 248-435.

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.

Diener, U.L., Cole, R.J., Sanders, T.H., Payne, G.A., Lee, L.S. & Klich, M.A. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annual Review of Phytopathology* **25**: 249-270.

Diener, U.L. & Davis, N.D. 1966. Aflatoxin production by isolates of *Aspergillus flavus*. *Phytopathology* **56**: 1390-1393.

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 B_1 on maize seedling growth. *Mycopathologia* **127**: 117-121.

Doherty, C. A., Waniska, R. D., Rooney, L. W., Earp, C. F. & Poe, J. H. 1987. Free phenolic compounds and tannins in sorghum caryopsis and glumes during development. *Cereal Chemistry* **64**: 42-46.

Dos Reis, T.A., Zorzete, P., Pozzi, C.R., da Silva, V.N., Ortega, E. & Corrêa, B. 2010. Mycoflora and fumonisin contamination in Brazilian sorghum from sowing to harvest. *Journal of the Science of Food and Agriculture* **90**: 1445-1451.

Du Toit, 1999. Production of maize in the summer rainfall area. Agricultural Research Council-Grain Crops Institute, CD.

Ehrlich, K.C. 2008. Genetic diversity in Aspergillus flavus and its implications for agriculture. Pp. 233-247. *In*: Varga, J. & Samson, R.A. (Eds.). *Aspergillus* in the genomic area. Wagenin Academic Publishers, The Netherlands.

Ellis, W.O., Smith, J.P., Simpson, B.K. & Oldham, J.H. 1991. Aflatoxins in food: occurrence, biosynthesis, effects on organisms, detection and methods of control. *Critical Reviews in Food Science and Nutrition* **30**: 403-439.

Eraslan, G., Akdogan, M., Yarsan, E., Sahindokuyucu,F., Essiz,D. & Altintas, L. 2005. The effects of aflatoxins on oxidative stress in broiler chickens. *Turkish Journal of Veterinary and Animal Sciences* **29**: 701-707.

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.

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

FAO. 2003. Food and Agriculture Organization of the United Nation Corporate Document Repository: Worldwide regulations for mycotoxins in food and feed in 2003. [Available on internet:] http://www.fao.org/docrep/007/y5499e/y5499e00.htm. [Date of access 08/02/10].

FAOSTAT data. 2006. Food and Agriculture Organization of the United Nation Databases. [Available on internet:] http://www.apps.fao.org. [Date of access 05/05/08].

FAOSTAT data 2009. Food and Agriculture Organization of the United Nation Databases. [Available on internet:] http://www.faostat.fao.org. [Date of access 08/02/10].

Fisher, N.L., arasas, 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.

Flett, B.C. & Wehner, F.C. 1991. Incidence of *Stenocarpella* and *Fusarium* cob rots in monoculture maize under different tillage systems. *Journal of Phytopathology* **133**: 327-333.

Flett, B.C., Bensch, M.J., Smit, E. & Fourie, H. 1996. A field guide for identification of maize diseases in South Africa. Agricultural Research Council-Grain Crops Institute, South Africa.

Folcher, L., Jarry, M., Weissenberger, A., Gérault, F., Eychenne, N., Delos, M. & Regnault-Roger, C. 2009. Comparative study of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (*Zea mays* L.) fields. *Crop Protection* **28**: 302-308.

Foley, D.C. 1962. Systematic infection of corn by *Fusarium moniliforme*. *Phytopathology* **52**: 870-872.

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.

Frisvad, J.C., Smedsgaard, J., Samson, R.A., Larsen, T.O. & Thrane, U. 2007. Fumonisin B_2 production by *Aspergillus niger*. *Journal of Agricultural Food Chemistry* **14**: 9727-32.

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.

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

Ghosh, M. & Ulaganathan, K. 1996. Mature seeds of sorghum contain proteins toxic to aflatoxin-producing *Aspergillus flavus*. *Journal of Stored Products Research* **32**: 339-343.

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

Gong, Y.Y., Cardwell, K., Hounsa, A., Egal, S., Turner, P.C., Hall, A.J. & Wild, C.P. 2000. Dietary aflatoxin exposure and impaired growth in young children from Benin and Togo: cross sectional study. *British Medical Journal* **325**: 20-21.

Goryacheva Y., De Saeger S., Eremin, S.A. & Van Peteghem, C. 2007. Immunochemical methods for rapid mycotoxin detection: evolution from single to multiple analyte screening: a review. *Food Additives and Contaminants A* **24**: 1169-1183.

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.

Guo, B.Z., Chen, Z.Y., Brown, R.L., Lax, A.R., Cleveland, T.E., Russin, J.S., Mehta, A.D., Selitrennikof, C.P. & Widstrom, N.W. 1997. Germination

induces accumulation of specific proteins and antifungal activities in corn kernels. *Phytopathology* **87**: 1174-1178.

Hahn, D. H. & Rooney, L. W. 1985. Effect of genotype on tannins and phenols of sorghum. *Cereal Chemistry* **63**: 4-8.

Hammond, B.G., Campbell, K.W., Pilcher, C.D., Degooyer, T.A., Robinson, A.E., McMillen, B.L., Spangler, S.M., Riordan, S.G., Rice, L.G. & Richard, J.L. 2004. Lower fumonisin mycotoxin levels in the grain of Bt corn grown in the United States in 2000-2002. *Journal of Agricultural Food Chemistry 52*: 1390-1397.

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E. & Cole, J.R. 1990. Pulmonary oedema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostical Investigation* **2**: 217-221.

Hendricks, K., 1999. Fumonisins and neural tube defects in south Texas. *Epidemiology* **10**: 198-200.

Holcomb, M., Thompson, H.C. Jr. &Cooper, W.M. 1996. SFE extraction of aflatoxins (B_1, B_2, G_1, and G_2) from corn and analysis by HPLC. *Journal of Supercritical Fluids* **9**: 118-121.

Hussein, S.H. & Jeffrey, M.B. 2001. Toxicity, metabolism and impact of mycotoxins on humans and animals. *Toxicology* **167**: 101-134.

Ivić, D., Čabrić, M., Palaveršić, B. & Cvjetković, B. 2008. No correlation between pericarp thickness and Fusarium ear rot (*Fusarium verticillioides*) in Croation maize hybrids and lines. *Maydica* **53**: 297-301.

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.

Jurado, M., Marin, P., Magan, N. & González-Jaén, M.T. 2008. Relationship between solute and matric potential stress, temperature, growth, and

FUM1 gene expression in two *Fusarium verticillioides* strains from Spain. *Applied and Environmental Microbiology* **74**: 2032-2036.

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 B_1. *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.

Klich, M.A. & Pitt, J.I. 1988. A Laboratory guide to common *Aspergillus* spp. and their teleomorphs. North Ryde, NSW: CSIRO Division of Food Processing.

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.

Krishnaveni, S., Muthukrishnan, S., Liang, G.H., Wilde, G. & Manickam, A. 1999. Induction of chitinases and β-1,3-glucanases in resistant and susceptible cultivars of sorghum in response to insect attack, fungal infection and wounding. *Plant Science* **144**: 9-16.

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

Kumari, S., Chandrashekar, A. & Shetty, H. S. 1992. Proteins in developing sorghum endosperm that may be involved in resistance to grain moulds. *Journal of the Science of Food and Agriculture* **60**: 275-282.

Kumari, R.S. & Chandrashekar, A. 1994. Isolation and purification of three antifungal proteins from sorghum endosperm. *Journal of the Science of Food and Agriculture* **64**: 357-364.

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

Leslie, J.F., Marasas, W.F.O., Shephard, G.S., Sydenham, E.W., Stockenstrőm & Thiel, P.G. 1996. Duckling toxicity and the production of fumonisin and moniliformin by isolates in the A and F mating populations of *Gibberella fujikuroi*. *Applied and Environmental Microbiology* **62**: 1182-1187.

Leslie, J.F & Summerell, B.A. 2006. The *Fusarium* laboratory manual. Blackwell publishing.

Leslie, J.F., Zeller, K.A., Lamprecht, 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.

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.

Liddell, C.M. & Burgess, L.W. 1985. Survival of *Fusarium moniliforme* at controlled temperature and relative humidity. *Transactions of the British Mycological Society* **84**: 121-130.

Lin, L., Zhang, J., Wang, P., Wang, Y. & Chen, J. 1998. Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. *Journal of Chromatography A*. **815**: 3-20.

Logrieco, A., Mule, G., Moretti, A. & Bottalico, A. 2002. Toxigenic *Fusarium* species and mycotoxins associated with maize ear rot in Europe. *European Journal of Plant Pathology* **108**: 597-609.

Madden. L.V., Hughes, G. & van den Bosch, F. 2007. The study of plant disease epidemics. The American Phytopathological Society.

Madden, L.V. & Wheelis, M. 2003. The threat of plant pathogens as weapons against U.S. crops. *Annual Review of Phytopathology* **41**: 155-176.

Magg, T., Bohn, M.m Klein, D., Merditaj, V. & Melchingher, A.E. 2002. Relationship between European corn borer resistance and concentration of mycotoxins produced by *Fusarium* spp. in grains of transgenic Bt maize hybrids, their isogenic counterparts, and commercial varieties. *Plant Breeding* **121**: 146-154.

Maiorano, A., Reyneri, A., Sacco, D., Magni, D. & Ramponi, C. 2009. A dynamic risk assessment model (FUMAgrain) of fumonisin synthesis by *Fusarium verticillioides* in maize grain in Italy. *Crop Protection* **28**: 243-256.

Månsson, M., Klejnstrup, M.L., Phipps, R.K., Nielsen, K.F., Frisvad, J.C., Gotfredsen, C.H. & Larsen, T.O. 2010. Isolation and NMR characterization of fumonisin B_2 and a new fumonisin B_6 from *Aspergillus niger*. *Journal of Agricultural Food Chemistry* **58**: 949-53.

Marasas, W.F.O. 1982. Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. Pp. 29-40. *In*: Pfeiffer, C.J. (Ed.). Cancer of the Osophagus. CRC Press Inc, Boca Raton, Florida.

Marasas, W.F.O., Jaskiewizc, 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., Riley, R.L., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martinez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E & Merril, Jr., A.H. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among population consuming fumonisin-contaminated maize. *Journal of Nutrition* **134**: 711-716.

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.

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

Marin, P., Magan, N., Vásquez, C. & González-Jaén, M.T. 2010. Differential effect of environmental conditions on the growth and regulation of the fumonisin biosynthetic gene *FUM1* in the maize pathogens and fumonisin producers *Fusarium verticillioides* and *Fusarium proliferatum*. *Federation of European Microbiological Societies* **73**: 303-311.

Marley, P.S. & Ajayi, O. 1999. Sorghum grain mould and the influence of head bug *Eurystylus oldi* in West and Central Africa. *Journal of Sustainable Agriculture* **13**: 35-44.

Marocco, A., Gavazzi, C., Pietri, A & Tabaglio, V. 2008. On fumonisin incidence in monoculture maize under no-till, conventional tillage and two nitrogen fertilizations levels. *Journal of the Science of Food and Agriculture* **88:** 1217-1221.

Marsh, S.F. & Payne, G.A. 1984. Preharvest infection of corn silks and kernels by *Aspergillus flavus*. *Phytopathology* **74**: 1284-1289.

Matumba, L., Monjerezi, M., Khongwa, E.B. & Lakudzala, D.D. 2011. Aflatoxins in sorghum, sorghum malt and traditional opaque beer in southern Malawi. *Food Control* **22**: 266-268.

Mayer, Z., Bagnara, A., Färber, P. & Geisen, R. 2003. Quantification of the copy number of *nor*-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. *International Journal of Food Microbiology* **82**: 143-151.

Mazhar, H. & Chadrashekar, A. 1993. Differences in kafirin composition during endosperm development and germination in sorghum cultivars of varying hardness. *Cereal Chemistry* **70**: 667-671.

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.

Menkir, A., Ejeta, G., Butler, L. & Melake-Berhan, A. 1996. Physical and chemical kernel properties associated with resistance to grain mould in sorghum. *Cereal Chemistry* **73**: 613-617.

Menniti, A.M & Neri, R.G. 2010. Activity of natural compounds on *Fusarium verticillioides* and fumonisin production in stored maize kernels. *International Journal of Food Microbiology* **136**: 304-309.

Miller, J.D. & Marasas, W.F.O. 2002. Ecology of mycotoxins in maize and groundnuts. Supplement to LEISA (Low External Input and Sustainable Agriculture) Magazine, 23-24.

Mohammed, Y., Abdel-Rheem, E.S., Ali, B., Mohamed, M, Kamel, A.E. & Kevin, H. 2010. Mycotoxin producing fungi occurring in sorghum grains from Saudi-Arabia. *Fungal Diversity* **44**: 45-52.

Morgensen, J.M., Frisvad, J.C., Thrane, U. & Nielsen, K.F. 2010. Production of Fumonisin B_2 and B_4 by *Aspergillus niger* on grapes and raisins. *Journal of Agricultural Food Chemistry* **27**: 954-958.

Munimbazi, C. & Bullerman, L.B. 1998. Inhibition of aflatoxin production of *Aspergillus parasiticus* NRRL 2999 by *Bacillus pumilis*. *Mycopathology* **140**: 163-169.

Munkvold, G.P. 2003. Epidemiology of Fusarium diseases and their mycotoxins in maize. *European Journal of Plant Pathology* **109**: 705-713.

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

Munkvold, G.P., Hellmich, R.L & Rice, L.G. 1999. Comparison of fumonisin concentrations in kernels of transgenic Bt maize hybrids and nontransgenic hybrids. *Plant Disease* **83**: 130-138.

Murillo-Williams, A. & Munkvold, G.P. 2009. Systemic infection by *Fusarium verticillioides* in maize plants grown under three temperature regimes. *Plant Disease* **92**: 1695-1700.

Musa, T., Hecker, A., Vogelsang, S. & Forrer, H.R. 2007. Forecasting of Fusarium head blight and deoxynivalenol content in winter wheat with FusaProg. Bulletin OEPP/EPPO **37**: 283-289.

Naéf, A. & Defago, G. 2006. Population structure of plant-pathogenic *Fusarium* spp. in overwintered stalk residues from Bt-transformed and non-transformed maize crops. *European Journal of Plant Pathology* **116**: 129-143.

Navi, S.S., Bandyopadhyay, R., Reddy, R.K., Thakur, R.P. & Yang, X.B. 2005. Effect of wetness duration and grain development stages on sorghum grain mould infection. *Plant Disease* **89**: 872-878.

Ncube, E. 2008. Mycotoxin levels in subsistence farming systems in South Africa. Master of Science (Agric) in the faculty of AgriSciences, University of Stellenbosch, Stellenbosch, South Africa.

Nelson, P.E. 1992. Taxonomy and biology of *Fusarium moniliforme*. *Mycopathologia* **117**: 29-36.

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

Ngoko, Z., Marasas, W.F.O., Rheeder, J.P., Shephard, 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.

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

Nkwe, D. O., Taylor, J.E. & Saime, B.A. 2005. Fungi, aflatoxins, fumonisin B_1 and zearalenone contaminating sorghum-based traditional malt, wort and beer in Botswana. *Mycopathologia* **160**: 177-186.

Noonim, P., Mahakarnchanakul, W., Nielsen, K.F., Frisvad, J.C. & Samson, R.A. 2009. Fumonisin B_2 production by *Aspergillus niger* in Thai coffee beans. *Food Additives and Contaminants* **26**: 94-100.

Odovy, G.N., Spencer, N. & Remmers, J. 1997. A description of silk cut, a stress-related loss of kernel integrity in preharvest maize. *Plant Disease* **81**: 439-444.

Okioma, M.N. 2008. The 2004 and 2005 aflatoxin tragedies in Kenya – a case study. Pp. 127-131. *In*: Leslie, J.F., Bandyopadhyay, R. & Visconti, A. (Eds.). Mycotoxins: detection methods, management, public health and agricultural trade. CAB International.

Ono, E. Y. S., Kawamura, O., Ono, M.A., Ueno, Y. & Hirooka, E.Y. 2000. A comparative study of indirect competitive ELISA and HPLC for fumonisin detection in corn of the state of Paraná, Brazil. *Food and Agriculture Immunology* **12**: 5-14.

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

Osweiler, G. 2005. Alfatoxins and animal health. Iowa State University, Ames, Iowa.

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

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.

Parsons, M.W. & Munkvold, G.P. 2012. Effects of planting date and environmental factors on fusarium ear rot symptoms and fumonisin B_1 accumulation in maize grown in six North American locations. **In Press:** *Plant Pathology.* (Doi: 10.1111/j.1365-3059.2011.02590.x).

Pascale, M. 2009. Detection methods for mycotoxins in cereal grains and cereal products. Institute of Sciences of Food Production, National Research Council, Bari, Italy.

Pascale, M. & Visconti, A. 2008. Overview of detection methods for mycotoxins. Pp. 171-183. *In:* Leslie, J.F. Bandyopadhyay, R. & Visconti, A. (Eds.). Mycotoxins. Detection methods, management, public health and agricultural trade. Cromwell Press, Trowbridge, UK.

Passone, M.A., Rosso, L.C., Ciancio, A. & Etcheverry, M. 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production. *International Journal of Food Microbiology* **138**: 276-281.

Payne, G.A. 1999. Ear and kernel rots. Pp. 44-45. *In*: White, D.G. (Ed.). Compendium of corn diseases. Third Edition. APS Press, The American Phytopathological Society, Minnesota, USA.

Pereira, P., Nesci, A., Castillo, C. & Etcheverry, M. 2010. Impact of bacterial biocontrol agents on fumonisin B_1 content and *Fusarium verticillioides* infection on field-grown maize. *Biological Control* **53**: 258-266.

Piedade, F.S., Fonseca, H., Gloria, E.M., Calori-Domingues, M.A., Piedade, S.M.S. & Barbin, D. 2002. Distribution of aflatoxins in contaminated corn fractions segregated by size. *Brazilian Journal of Microbiology* **33**: 12-16.

Pitt, J.I. 2006. Fungal ecology and the occurrence of mycotoxins. Pp. 33-41. *In:* Njapau, H., Trujillo, S. van Egmond, H.P. & Park, D.L. (Eds.). Mycotoxins and Phycotoxins. Advances in determination, toxicology and exposure management. Wageningen Academic Publishers, The Netherlands.

Pitt, J.I. & Miscamble, B.F. 1995. Water relations of *Aspergillus flavus* and closely related species. *Journal of Food Protection* **58**: 86-90.

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.

Plattner, R.D., Weisleder, D., Shackelford, D.D, Peterson, R. & Powell, R.G. 1992. A new fumonisin from solid cultures of *Fusarium moniliforme. Mycopathologia* **117**: 23-28.

Proctor, R.H., Brown, D.W., Plattner, R.D., Desjardins, A.E. 2003. Co-expression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genetical Biology* **38**: 237-249.

Proctor, R.H., Desjardins, A.E., Plattner, R.D. & Hohn, T.M. 1999. A polyketide synthase gene required for biosynthesis of fumonisin mycotoxins in *Gibberellla fujikuroi* mating population A. *Fungal Genetics and Biology* **27**: 100-112.

Rabie, C.J. & Marais, G.J. 2000. Toxigenic fungi and mycotoxins in South African foods and feeds. Report for the Department of Health. CSIR Bio/Chemtech, Pretoria, South Africa.

Ratnavathi, C.V. & Sashidhar, R.B. 2003. Substrate suitability of different genotypes of sorghum in relation to *Aspergillus* infection and aflatoxin production. *Journal of Agricultural and Food Chemistry* **51**: 3482-3492.

Ratnavathi, C.V. & Sashidhar, R.B. 2007. Inhibitory effects of phenolics extracted from sorghum genotypes on *Aspergillus parasiticus* (NRRL 2999) growth and aflatoxin production. *Journal of the Science of Food and Agriculture* **87**: 1140-1148.

Reddy, B.N. & Raghavender, C.R. 2006. Effects of fungal infection and insect infestation interaction, moisture content, type of earhead and grain on mycotoxin production in field sorghum. Pp. 43-52. *In:* Njapau, H., Trujillo, S., van Egmond, H.P. & Park, D.L. (Eds.). Mycotoxins and Phycotoxins. Advances in determination, toxicology and exposure management. Wageningen Academic Publishers, The Netherlands.

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.

Ren, Y., Zhang, Y., Shao, S., Cai, Z., Feng, L., Pan, H. & Wang, Z. 2007. Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1143**: 48-64.

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

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

Richard, J.L. 2006. Mycotoxins and mycotoxicoses: A 2004 update. Pp. 21-30. *In:* Njapau, H. Trujillo, S. van Egmond, H.P. & Park, D.L. (Eds.). Mycotoxins and Phycotoxins. Advances in determination, toxicology

and exposure management. Wageningen Academic Publishers, The Netherlands.

Rodrigues, P., Soares, C., Kozakiewicz, Z., Paterson, R.R.M., Lima, N. & Venâcio, A., 2007. Identification and characterization of *Aspergillus flavus* and aflatoxins. Pp. 527-534. *In:* Méndez-Vilas, A. (Ed.). Communicating current research and educational topics and trends in applied microbiology.

Rooney, W.L., Collins, S.D., Klein, R.R., Mehta, P.J., Frederiksen, R.A. & Rodreguez-Herrera, R. 2002. Breeding sorghum for resistance to anthracnose, grain mould, downy mildew and head smuts. Pp. 273-279. *In*: Leslie, J. F. (Ed.), Sorghum and millets diseases 1st ed. Iowa State Press, Iowa.

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 leukoencephalomalacie and a pulmonary edema syndrome in swine. *Applied Environmental Microbiology* **56**: 3225-3226.

Rossi, V., Giosue, S., Pattori, E., Spanna, F. & Del Vecchio, A. 2003. A model estimating the risk of Fusarium head blight on wheat. Bulletin OEPP/EPPO **33**: 421-425.

Sampietro, D.A., Vattuone, M.A., Presello, D.A., Fauguel, C.M. & Catalán, C.A.N. 2009. The pericarp and its surface wax layer in maize kernels as resistance factors to fumonisin accumulation by *Fusarium verticillioides*. *Crop Protection* **28**: 196-200.

Sargeant, K., Sheridan, A., O'Kelly, J & Carnaghan, R.B.A. 1961. Toxicity associated with certain samples of groundnuts. *Nature* **192**, 1095-1097.

Saubois, A., Pointelli Laforet, E., Nepote, M.C. & Wagner, M.L. 1999. Mycological evaluation of a sorghum grain of Argentinia, with emphasis on the characterization of *Fusarium* spp.. *Food Microbiology* **16**: 435-445.

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.

Schaafsma, A.W. & Hooker, D.C. 2007. Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. *International Journal of Food Microbiology* **119**: 116-125.

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.

Scott, P.M. & Trucksess, M.W. 1997. Application of immunoaffinity columns to mycotoxin analysis. *Journal of AOAC International* **80**: 941-949.

Setamou, M., Cardwell, K.F., Schulthess, F. & Hell, K. 1997. *Aspergillus flavus* infection and aflatoxin contamination of preharvest maize in Benin. *Plant Disease* **81**: 1323-1327.

Shelby, R.A., White, D.G., & Bauske, E.M. 1994. Differential fumonisin production in maize hybrids. *Plant Disease* **78**: 582-584.

Shephard, G.S. 2005. Aflatoxin and food safety: recent African perspectives. Pp. 15-17. *In*: Abbas, H.K. (Ed.). Aflatoxin and food safety. CRC Press, Taylor and Francis group, Boca Raton.

Shephard, 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.

Small, I.M., Flett, B.C., Marasas, W.F.O., McLeod, A., Stander, M.A. & Viljoen, M.A. 2012. Resistance in maize inbred lines to *Fusarium verticillioides* and fumonisin accumulation in South Africa. *Plant Disease* **96**: 881-888.

Smart, M.G., Shotwell, O.L. & Caldwell, R.W. 1990. Pathogenesis in *Aspergillus* ear rot of maize: Aflatoxin B_1 levels in grain around round-inoculation sites. *Phytopathology* **80**: 1283-1286.

Stack, M.E. 1998. Analysis of fumonisin B_1 and its hydrolysis product in tortillas. *Journal of Analytical Chemistry* **81**: 737-740.

Stack, J. P. & Pedersen, J. F. 2003. Expression of susceptibility of *Fusarium* head blight and grain mould in A_1 and A_2 cytoplasm of *Sorghum bicolor*. *Plant Disease* **87**: 172-176.

Stanker, L. H., Merrill, P. Scotcher, M.C. &. Cheng, L.W. 2008. Development and partial characterization of high-affinity monoclonal antibodies for botulinum toxin type A and their use in analysis of milk by sandwich ELISA. *Journal of Immunological Methods* **336**: 1-8.

Stewart, D.W., Reid, L.M., Nicol, R.W. & Schaafsma, A.W. 2002. A mathematical simulation of growth of *Fusarium* in maize ears after artificial inoculation. *Phytopathology* **92**: 534-541.

Sulyok, M., Krska, R. & Schumacher, R. 2010. Application of an LC-MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. *Food Chemistry* **119**: 408-416.

Sweeney, M.J. & Dobson, D.W. 1998. Mycotoxin production by *Aspergillus, Fusarium* and *Penicillium* species. *International Journal of Food Microbiology* **43**: 141-158.

Takino, M., Tanaka, H., Tanaka, T. 2011. Multi mycotoxin analysis in food products using immunoaffinity extraction. *Methods of Molecular Biology* **747**: 259-266.

Taubenhaus, J.J. 1920. A study of black and yellow moulds of ear corn. *Texas Agricultural Experimental Station Bulletin* **270**: 3-38.

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

Turner, P.C., Moore, S.E., Hall, A.J., Prentice, A.M. & Wild, C.P. 2003. Modification of immune function through exposure to dietary aflatoxin in Gambian children. *Environmental Health Perspectives* **111**: 217-220.

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.

Turner, N.W., Subrahmanyam, S. & Piletsky, S.A. 2009. Analytical methods for determination of mycotoxins: a review. *Analytica Chimicta Acta*. **632**: 168–180.

Van der Plank, J.E. 1960. Analysis of epidemics. Pp.229-289. *In*: Horsfall J.G. & Cowling, E.B. (Eds.). Plant Pathology: An Advanced Treatise. Academic Press, New York, USA.

Van der Plank, J.E. 1963. Plant Diseases: Epidemics and Control. Academic Press, New York, London.

Van Maanen, A. & Xu, X.M. Modelling plant disease epidemics. 2003. *European Journal of Plant Pathology* **109**: 669-682.

Vicam. 2008. Vicam Myco 6 in 1 LC/MS/MS. [Available on internet:] http://www.vicam.com/multi-analyte-test-kits/myco6in1 [Date of access 01/09/11].

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].

Waalwijk, C., de Vries, I., Köhl, J., Xu, X., van der Lee, T.A.J. & Kema, G.H.J. 2008b. Development of quantitative detection methods for *Fusarium* in cereals and their application. Pp. 195-205. *In:* Leslie, J.F., Bandyopadhyay, R. & Visconti, A. (Eds.). Mycotoxins. Detection methods, management, public health and agricultural trade. Cromwell Press, Trowbridge, UK.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B.C., de Vries, I. & Khema, G.H.J. 2008a. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal* **1**: 37-45.

Waniska, R.D., Venkatesha, R.T., Chandrashekar, A., Krishnaveni, S., Bejoano, F.P., Jeoung, J., Jayaraj, J, Muthukrishnan, S. & Liang, G.H. 2001. Antifungal proteins and other mechanisms in the control of sorghum stalk rot and grain mould. *Journal of Agricultural Food Chemistry* **49**: 4732-4742.

Widstrom, N.W., McMillian, W.W., Beaver, R.W. & Wilson, D.M. 1990. Weather-associated changes in aflatoxin contamination of preharvest maize. *Journal of Production Agriculture* **3**: 196–199.

Wilson, T.J. & Romer, T.R. 1991. Use of Mycosep multifunctional cleanup column for liquid chromatographic determination of aflatoxins in agricultural products. *Journal of AOAC International* **74**: 951-956.

Woloshuk, C.P. & Prieto, R. 1998. Genetic organization and function of the aflatoxin B_1 biosynthetic genes. *FEMS Microbiology Letters* **160**: 169-176.

Wu, F. 2006. Economic impact of fumonisins and aflatoxin regulations on global corn and peanut markets. Pp. 83-93. *In:* Barug, D., Bhatnagar D., van Egmond, H.P., van der Kamp, J.W. Osenbruggen, W.A. & Visconti, A. (Eds.). The mycotoxin fact book. Wageningen Academic Publishers, The Netherlands.

Yu, J., Cleveland, T.E., Nierman, W.C. & Bennett, J.W. 2005. *Aspergillus flavus* genomics: Gateway to human and animal health, food safety, and crop resistance to diseases. *Revista Iberoamericana de Micologia* **22**: 194-202.

CHAPTER 2

Fumonisins associated with the colonisation of commercial South African maize grain by *Fusarium* spp.

ABSTRACT

The natural occurrence of fumonisin producing *Fusarium* spp. and fumonisin contamination of maize grain was quantified in selected maize cultivars from various production areas of South Africa. Maize grain samples (1 kg each) were collected from cultivar trials from 16, 20 and 14 localities, during the 2007-2009 planting seasons, respectively. Each sample was analysed using quantitative (q) real-time PCR to determine the respective biomasses of fumonisin-producing *Fusarium* spp. Fumonisin concentrations were quantified by means of High Performance Liquid Chromatography (HPLC). Genotype x Environment (G X E) interactions were quantified using regression analysis ($Y=ax^b$) whereby the relationship between disease/fumonisin potential and observed disease/fumonisin concentration of each cultivar was determined. Kernels from the 2007 samples were also plated onto *Fusarium* selective medium and subsequently, split plates containing PDA & CLA and *Fusarium* spp. were quantified and identified after 14 days. Simple linear regression analysis was used to determine the relationship between qRT-PCR, HPLC and the plating out method. Results indicated high natural infection by fumonisin-producing *Fusarium* spp. and fumonisin concentrations in warmer production areas such as Northern Cape, North West and Free State Provinces. High fumonisin producing fungal biomass and concomitant fumonisin concentrations (above 2 ppm in certain localities) quantified in this study, could negatively impact grain quality food safety and security due to the potentially harmful effects of this mycotoxin on humans and animals. Spearman ranking correlations of cultivars for fumonisin producing *Fusarium* spp. and concomitant fumonisin contamination were poorly correlated over localities/seasons. Regression analyses between disease/fumonisin potential and observed disease/fumonisin concentration in cultivars suggested differential responses of cultivars at the various disease potentials. Regression analysis yielded a significant relationship between qRT-PCR and HPLC data, but not with the plating out of grain data.

Keywords: qRT-PCR, HPLC, G X E interactions

INTRODUCTION

Maize (*Zea mays* L.) is an important crop in South Africa and is produced throughout the country under diverse cultural and weather conditions. The important fumonisin producing ear rot *Fusarium* spp. are *F. verticillioides* and *F. proliferatum* (Rheeder *et al*., 1990). The distribution and predominance of these *Fusarium* spp. and their concomitant fumonisin production varies depending on season, geographic locality, climatic factors such as temperature and moisture, host genotype and agricultural practices (Nyaka *et al*., 2010). At least 28 fumonisin analogues are known, but the most abundant natural forms are fumonisin B_1, B_2 and B_3 (Rheeder *et al*., 2002).

Although fumonisins have a relatively simple chemical structure, their inhibition of sphingolipid metabolism can have diverse and complex effects on animal systems (Desjardins, 2006). Fumonisins cause leukoencephalomalacia (LEM) in horses (Kellerman *et al.*, 1990; Ross *et al.*, 1990), pulmonary oedema in swine (Harrison *et al.*, 1990) and are hepatotoxic and carcinogenic to rats (Gelderblom *et al.*, 1988).

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 where maize infected by *F. verticillioides* was ingested in certain endemic areas of The People's Republic of China. Studies by Stack (1998) and Placinta *et al.* (1999) have indicated a highly significant correlation between consumption of fumonisin-contaminated tortillas and neural-tube defects in humans in Mexico. The potential carcinogenic risk of fumonisin B_1 to humans was evaluated and classified by the International Agency for Research on Cancer (IARC) (Anonymous, 2002a) as "Group 2B carcinogens" which means they are probably carcinogenic to humans.

Few publications are available regarding the incidence of *Fusarium spp*. and their resultant fumonisins from maize production areas in South Africa. A survey of

Fusarium spp. and fumonisins in maize produced by subsistence farmers in South Africa has been published by Ncube *et al*. (2011). Surveys of the natural occurrence of maize ear rots caused by *Diplodia maydis*, *F. moniliforme*, *F. subglutinans* and *F. graminearum* from eight commercial production localities in South Africa have been published by Rheeder *et al*. (1993) although they did not quantify mycotoxins from these samples. A more recent publication by Boutigny *et al.* (2011) describes the quantitative detection of four *Fusarium* spp. and their mycotoxins from two cultivars selected from 14 localities (commercial production) over a two year period. Presently South African maize lines vary in the degree of susceptibility to *F. verticillioides* ear rot infection (Small *et al*., 2012) and cultivars have not yet been identified with resistance to fumonisin producing *Fusarium* spp.

Quantification of *Fusarium* spp. in maize and other commodity surveys have involved isolation (whole seed plate counts) and determination of infection frequency (percentage of seeds that yield fungi after surface disinfection) as an indicator of fungal biomass (Schwadorf & Müller, 1989; Saxena *et al.*, 2001). Plate counts to determine infection rates are time consuming (Schwadorf & Müller, 1989) and the use of isolation frequency of pathogens from maize as an indication of fungal biomass is poorly correlated with fumonisin concentrations under field conditions (Ngoko *et al.*, 2001). DNA-based detection methods, in particular quantitative real-time PCR (qRT-PCR), appear more sensitive and specific. The main advantage of PCR is that organisms can be identified within mixtures of DNA without the culturing of organisms (Abdin *et al*., 2010).

The purpose of this study was (i) to quantify the incidence of fumonisin producing *Fusarium* spp. and to determine the concentrations of fumonisin in commercial maize grain samples from different production localities in South Africa (ii) to study genotype x environment interactions associated with colonisation and fumonisin contamination (iii) and to determine the relationship between fumonisin producing *Fusarium* spp. with morphologically based identifications based on plating out and fumonisin concentrations.

MATERIALS AND METHODS

Maize samples

Maize kernels harvested from National Cultivar Evaluation Trials conducted by the ARC-Grain Crops Institute in Potchefstroom were collected from a range of localities (Tables 2.1-2.3) during the 2007-2009 maize production seasons. All trials were conducted using a randomised complete block design with three replicates. Trials were conducted under dryland conditions and maintained according to "Best Practice" appropriate to the respective production areas with the exception of Vaalharts in the Northern Cape which was flood irrigated. Weather variables, notably mean maximum temperature and rainfall, were monitored at each locality during flowering and grain development (February-March). At harvest, kernels from the three replicates were pooled and thoroughly mixed. One kilogram samples of each cultivar from each locality were collected and stored in a coldroom at 4ºC for a maximum of 1 week prior to milling and further analysis. Sub-samples of 250 g from all the initial 1 kg maize kernel samples were individually milled in a Cyclotech 1093 sample mill with a 1 mm mesh sieve. The mill was thoroughly cleaned with high-pressure air between each sub-sample to minimise cross contamination.

Fungal biomass quantification

Isolation of fumonisin producing Fusarium *spp. DNA for qRT-PCR reactions*

A 0.5 g aliquot from each sub-sample was used to isolate and clean up DNA using the method of Sambrook *et al*. (1989). A DNA solution (500 µl) was precipitated by adding 25 µl of 5M NaCl followed by 1250 µl ice cold absolute ethanol. The samples were incubated in ice for 3 hours. The DNA was peleted by means of centrifugation (14 000 rpm x 10 minutes) and the supernatant was discarded. The DNA was washed with 76% EtOH and 0.2M NaoC for 10 minutes and the wash solution was discarded. DNA was then washed with 76% EtOH and 10mM NH_4OAc for 2 minutes. The wash solution was discarded and the ethanol was evaporated in a laminar flow cabinet. DNA was resuspended by adding 200 µl Melford molecular grade water. DNA samples were stored at -20ºC. Prior to qRT-PCR analysis all

DNA samples were diluted to 10 ng with Melford molecular grade water using a nanodrop to measure DNA concentrations.

Quantification of fumonisin producing Fusarium *spp. using qRT-PCR*

MRC 826 (*F. verticillioides*) strain from the Medical Research Council – Promec Unit was used as the standard in the qRT-PCR technique. This strain is characterized by its ability to produce high concentrations of fumonisins and DNA from this isolate was used to develop a standard curve (Waalwijk *et al*., 2008). qRT-PCR reactions were performed in a MyiQ^{TM}2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten-halogen optical lamp. The primers Taqfum-2F and Vpgen-3R in combination with the FUM-probe 1 as tested by Waalwijk *et al*. (2008) were used in this study. The primer/probe set had the following nucleotide sequence: Taqfum-2F, 5'-ATGCAAGAGGCGAGGCAA-3'; Vpgen-3R, 5'-GGCTCTCRGAGCTTGGCAT-3' and FUM-probe 1,5'-/56-FAM/CAATGCCATCTTCTTG/36-TAMSp/-3'. The sensimix reagent kit (sensimix^{TM}no ref QT 505-05) from Celtic (Bioline) was used for PCR. For each reaction, 4µl of the DNA sample was mixed with 12.5 µl sensimix, 2.125 µl Fum-probe 1 (1 µM), 0.875 µl Taqfum-2F (333 nM), 0.875 µl Vpgen-3R (333 nM) and 4.625 µl Melford molecular grade water. In all the experiments, negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carryover. The 96-well plate was incubated for 10 minutes at 95ºC and thereafter each of the 40 PCR cycles was performed according to the following temperature regime: 95ºC for 10 s, 60ºC for 30 s, and 72ºC for 10 s. The Bio-Rad iCycler^{Tm}iQ Optical System Software Version 3.0a was used to calculate the biomass of fumonisin producing *Fusarium* spp. present in a sample. Regression equations of standard curves from runs were highly significant (R^2=>0.99). Slopes were within the acceptance criterion (between -3.1 and -3.6) and efficiencies ranged from 95 to 105%.

Mycotoxin analyses

Mycotoxins were analysed using the Vicam method (Anonymous, 2002b). A 50 g aliquot from each of the 250 g milled sub-samples was mixed with 5 g of sodium

chloride prior to extraction. Fumonisin were extracted in 100 ml methanol:water (80:20, v/v) for five minutes using an IKA T18 basic Ultra Turrax homogeniser. The extract was then filtered through Whatman No.5 filter paper. A 10ml aliquot was diluted with 40 ml phosphate-buffered saline (1XPBS) (8.0g NaCl, 1.2 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCl, dissolved in 990 ml purified water with pH adjusted to 7.0 with HCl). Diluted samples were extracted through microfiber filters (0.45 µm) and 10ml of the diluted filtrate was passed through FumoniTest affinity columns from Vicam at a flow rate of 1 drop per second. Ten ml of PBS were subsequently passed through the column at a rate of 1 drop per second. The column was then washed with 1.5 ml HPLC grade methanol at a rate of 1 drop per second and the eluate was collected in a glass cuvette. Methanol eluate was dried in a TurboVap LV (Caliper Sciences) with the aid of a slow stream of high purity Nitrogen. Samples were dissolved in 200 µl methanol and purified water (50:50 v/v). Each sample (50 µl) was transferred to 250 µl conical inserts. Each insert was placed into a 2.5 ml glass vial which was then placed into a carousel. The first position of the carousel had a 2.5 ml glass vial with *o*-phthaldialdehyde (OPA from Sigma) which is the derivatisation agent. The Waters 717 plus autosampler was set up to mix 100 µl of the OPA with the 50 µl of sample in the conical insert. This mixture (20 µl) was injected after a delay time of 1 minute.

Fluorescence was performed at excitation and emission wavelengths of 335 and 440 nm respectively using a Waters 2475 multi λ fluorescence detector. The analytical column, Symmetry C18, 5 µm 3.9 x 150 mm from Waters was used. The detection limit of the method used was 0.016 ppm. Recovery data were obtained in triplicate by fortifying clean maize samples (Vicam) with 5 ppm fumonisin B_1 B_2 and B_3. The average recovery rates were 83% (FB_1), 81% (FB_2) and 83% (FB_3).

Isolation, identification and quantification of **Fusarium** *spp. using the plating out method*

The quantification of *Fusarium* spp. using the plating out method was done using two hundred surface-sterilised (King & Scott, 1981) kernels from each of the maize samples from the 2007 season. Kernels were plated onto Rose Bengal-glycerine-urea agar (van Wyk *et al.*, 1986). Four kernels were plated equidistantly on a petri

dish (Flett, 1994) and petri dishes were incubated for seven days in the dark at 25°C (Nelson *et al.*, 1983; Leslie & Summerell, 2006). After seven days, fungal colonies were transferred to CLA/PDA for identification. The broad spectrum antibiotic chloramphenicol was applied at a rate of 250 mg per 1ℓ agar to both CLA and PDA to inhibit bacterial growth (Leslie & Summerell, 2006). CLA/PDA cultures were placed under ultraviolet light to promote the development of spore chains and the teleomorph stage to aid identification (Leslie & Summerell, 2006). Ultraviolet lighting was programmed to provide 12 hours of light and 12 hours of darkness. After 14 days *Fusarium* spp. were identified using the guidelines of Nelson *et al.* (1983) and Leslie & Summerell (2006).

Data analysis

Fungal biomass and fumonisin concentrations were analysed using two factor analysis of variance without replication to determine the effect of locality and season on these variables (Excel, 2007). Simple linear regression analysis using Statgraphics version 5.0 was performed to determine the relationship between mean maximum temperature during January to March of each year and fumonisin concentration in maize grain, the relationship between fumonisin concentration and mean fumonisin producing *Fusarium* spp. biomass (pooled 2007, 2008 and 2009 data) and the relationship between qRT-PCR and the plating out method.

Spearman Ranking Correlations were used to determine the consistency of resistance reactions of cultivars to fungal and fumonisin contamination over localities and seasons. Non-linear regression analysis ($Y=aX^b$) was used to determine the relationship between *Fusarium* colonisation/fumonisin contamination potential at each locality and the observed response of each cultivar (Flett & McLaren, 1994).

RESULTS

Fungal biomass and fumonisin concentration over seasons, localities and cultivars

Fumonisin producing *Fusarium* spp. and fumonisin concentrations were quantified from maize samples from North West, Gauteng, Free State, Mpumalanga, KwaZulu Natal and Northern Cape provinces from 2007-2009 (Figure 2.1a-2.1f).One way analysis of variance indicated highly significant locality effects on fungal biomass (Tables 2.1-2.3). The highest biomasses of these fungi in 2007 were recorded in Rushof (48.77 pg) and Ventersdorp (38.65 pg) while no fumonisin producing *Fusarium* spp. were recorded in Bethlehem, Marquard and Ottosdal. During the 2008 season, high fungal biomasses were recorded in Nampo (119.98 pg) and Bothaville (77.32 pg) with no fungal biomass recorded at seven localities. In the 2009 season, high fungal biomasses were recorded at Wesselsbron (70.87 pg) and Nampo (42.64 pg) with no fungal biomass being recorded at Bethlehem, Delmas and Wonderfontein.

The monthly mean maximum temperature and rainfall from January to March (2007-2009) for each locality were calculated (Tables 2.1-2.3) to determine relationships between weather and infection of kernels by *Fusarium* spp.. No significant relationship between weather parameters and colonisation of grain by fumonisin producing *Fusarium* spp. was recorded, although a tendency was observed for higher fungal infection at warmer localities while low or zero infection was associated with cooler areas.

One way analysis of variance indicated highly significant locality effects on fumonsin concentrations (Tables 2.1-2.3). In 2007 highest fumonisin concentrations were recorded at Ventersdorp (7.54 ppm) and Rushof (5.62 ppm), in 2008 at Bothaville (16.85 ppm), Vaalharts (6.13 ppm) and Nampo (5.60 ppm), and in 2009 Nampo (16.52 ppm) and Wesselsbron (14.39 ppm). No fumonisins were recorded from Tweebuffelsfontein (2007), various locations during 2008 and Delmas and Wonderfontein in 2009 (Tables 2.1-2.3). Mean maximum temperature during February and March for the 2008 season showed a weak positive relationship with

fumonisin synthesis with a R^2=0.64, P=0.047 (Figure 2.2). Similarly a significant relationship between mean maximum temperature for March and fumonisin concentration was recorded during the 2009 season (R^2=0.67; P=0.042) while no relationship was recorded during 2007. Similarly, no significant relationship was recorded between rainfall and fumonisin concentration.

Over all seasons and localities, large variations in both fungal biomass (0-225 pg) and fumonisin concentration (0-33.26 ppm) were recorded with large seasonal x cultivar x locality deviations. During 2007 for example, a high fungal biomass of 21.3 pg in DKC80-12B at Hartebeesfontein, resulted in no fumonisin production while during the same season a fungal biomass of 4.69 pg in PAN6349 yielded a fumonisin concentration of 9.35 ppm at Ventersdorp. Similar deviations were observed during the 2008 and 2009 seasons.

Analysis of variance indicated significant differences in fungal biomass due to cultivar in the 2007 and 2009 seasons but not the 2008 season. In the 2007 season, highest fungal biomass was recorded in cultivar Phb3442 (26.00 pg) with the lowest fungal biomass in LS8521B (0.38 pg). During 2009, the highest fungal biomass was recorded in CRN3505 (43.34 pg) and the lowest in DKC80-12B (8.14 pg). Spearman Ranking Correlations indicated that the responses of cultivars to colonisation of grain by fumonisin producing *Fusarium* spp. were poorly correlated over localities/seasons (r_s 0.42 to 0.64) suggesting that cultivars reacted differently to different environmental/inoculum conditions (disease potentials). Non-linear regression analyses of the relationship between observed cultivar response and colonisation potential (mean fungal biomass associated with all cultivars at a locality during a specific seasons) are presented in Figure 2.3a-f. Based on regression parameters ($a<1$), cultivars CRN 3505 and DKC 80-12B showed a degree of resistance to colonisation despite increasing colonisation potential.

Analysis of variance indicated no significant differences in mean fumonisin concentrations due to cultivar in the 2007 and 2009 seasons (Tables 2.1-2.3). In the 2008 season, CRN3505 had a significantly higher mean fumonisin concentration compared to the rest of the cultivars. As with fungal colonisation, Spearman Ranking Correlations indicated the response of cultivars to fumonisin contamination

to be poorly correlated over localities/seasons (r_s= 0.29 to 0.70). Non-linear regression analyses of the relationship between observed cultivar response and fumonisin potential are presented in Figure 2.3g-l. Observed fumonisin concentrations in cultivars CRN 3505 and PAN 6611 were linearly related to fumonisin potential (a=1), while DKC 80-12B and LS8521B showed a degree of resistance despite fumonisin increasing contamination potential (Flett & McLaren, 1994) (a<1), DKC 78-15B was susceptible (a>1) and cultivar DKC 80-10 yielded a non-significant response to fumonisin potential.

Correlation between qRT-PCR, HPLC, and the plating out method

A simple regression analysis between the plating out method and the qRT-PCR method yielded no significant relationship (R^2=0.04; P=0.429). A highly significant relationship was recorded between mean fungal biomass and mean fumonisin concentrations with a R^2=0.63, P=0.000 (Figure 2.4).

DISCUSSION

F. verticilliodes and *F. proliferatum* are the most prolific fumonisin producing *Fusarium* spp. (Shephard *et al*., 1996) and are generally associated with warm, dry climates (Shephard *et al*., 1996, Fandohan *et al*., 2003; Munkvold, 2003). The natural occurrence of fumonisin producing *Fusarium* spp. in maize samples from 29 localities over a 3 year period showed that infection is common in South African commercial maize samples, especially in the warmer, drier areas such as the North West province, Northern Cape and some areas of the Free State such as Bothaville, Nampo, Rushof and Wesselsbron where the average temperatures ranged from 29°C to 32°C. In cooler areas such as Bethlehem, Bloekomspruit, Cedara, Danielsrus, Delmas, Tweeling, Vrede, Warden and Wonderfontein where mean maximum temperatures ranged from 24°C to 27°C, fungal biomass was absent/low. Murillo-Williams & Munkvold (2008) calculated optimum and supra-maximal temperatures for the growth of *F. verticillioides* at 31°C and 35°C respectively, with minimal fungal growth at 22°C to 24°C.

Similarly highest fumonisin (FB_1+FB_2+FB_3) concentrations were recorded in warmer areas such as Ventersdorp, Rushof, Bothaville, Vaalharts, Nampo, and Wesselsbron with low/absent fumonisin consentrations in the cooler areas and this is supported by Munkvold (2003) and De La Campa *et al.* (2005) who reported optimum temperatures of 30°C and 32°C for fumonisin production.

At present the only two mycotoxins considered under South African national regulations are aflatoxin (in all foodstuffs) and patulin (apple juice) (Rheeder, 2008). No legislation on allowable concentrations for *Fusarium* mycotoxins in South Africa has been formulated (Fandohan *et al*., 2003). The European Union established a limit of 1 ppm for food intended for direct human consumption whereas the Food and Drug Administration of America is currently suggesting a guideline of 2 ppm for direct human consumption when considering food safety in local maize products. High fumonisin concentrations in excess of 2 ppm were recorded at 10 localities over the 3 year period. These concentrations are of concern because of possible mycotoxicoses in animals (Thiel *et al*., 1991) and carcinogenic effects in humans as described by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) (Anonymous, 1993) as well as Marasas *et al.,* (1981; 1988) and Rheeder *et al*., (1992).

In the absence of South African fumonisin regulations, no official testing for fumonisin contamination of grain is conducted in South Africa to prevent high levels of contamination as recorded at some localities, entering the food chain. Internationally, analysis of mycotoxins relies heavily on HPLC (Pascale & Visconti, 2008) as this method has the advantage of good sensitivity, selectivity and repeatability. Due to expensive equipment which requires specialist expertise, as some toxins may need derivitisation, a paucity of equipment and expertise exists in South Africa to screen maize samples. South African grading regulations, for commercial grain at silos aims to reduce the risk of visually contaminated maize being consumed by humans and animals based on observed discoloration and physical defects and resultant downgrading of grain. However, endophytic infections in maize are difficult to detect because kernels appear sound (symptomless) and miss detection. This may result in fumonisins entering the food chain because potential fumonisin levels from endophytic infections have yet to be determined.

This study also indicated that fungal biomass may not be a direct reflection of fumonisin contamination. High fungal biomass with low fumonisin content and low fungal biomass with high fumonisin content were recorded at certain localities/seasons/cultivars. Similarly Bush *et al.* (2003) reported that symptomless kernels are often very highly colonised with low fumonisin concentrations. In contrast Bacon *et al*. (2008) reported that symptomless infections can produce high concentrations of fumonisin.

Surveys of the natural occurrence of fumonisin producing *Fusarium* spp. and fumonisins in commercial maize production areas of South Africa are necessary to assess the potential threat of these toxins to humans and animals. It is therefore important that the level of infection and fumonisin risk be accurately determined to ensure safe food for human and animal consumption. Corresponding high fungal biomass values may also indicate infection levels that may reduce yields and cause grain discoloration, physical breakdown of grain structure and reduction of grain nutritional value (Jurado *et al*., 2006) although these were not quantified in the current study.

Results recorded in the present study indicate that maize cultivars differ in susceptibility to colonisation by fumonisin producing *Fusarium* spp. Cultivars CRN3505 and DKC80-12B showed a degree of resistance to fungal infection. However the economic value of the differences are limited due to the high genotype x environment interactions recorded (2007 and 2009) and indicates the need for local studies on the stability of host response to different isolates or changing environmental conditions. Similar genotype x environment interactions were also reported for *Fusarium* ear rot and fumonisins by Small *et al*. (2012). Inconsistent responses of cultivars over environments could be due to variation in isolates, inoculum levels and adaptation of genotypes to physiological stress factors (Munkvold, 2003; Desjardins, 2006). Host predisposition by physiological stresses and disease favourable conditions prior to harvesting may create conditions for infection by *Fusarium* spp. and fumonisin production (Visconti, 1996). These results illustrate the importance of taking the genotype x environment interaction (G x E) into account when evaluating cultivars for resistance to Fusarium ear rot of maize as well as the need to define evaluation criterion in breeding for *Fusarium* resistance. A

better understanding of the role of environment and the physiology of differential response of cultivars in relation to infection of fumonisin producing *Fusarium* spp. at different localities is essential to the identification of maize production areas with a potential high/low risk of fumonisin synthesis.

No genotype x environment interactions for fumonisin concentrations were recorded for the 2007 and 2009 season, and only CRN3505 had a significant interaction with environment during the 2008 season. Janse van Rensburg, *et al.* (2011) suggested that fumonisin production is related to high temperature and a constant fungal biomass at the dough stage of kernel development at which stage cultivar effects are probably not strongly expressed.

The absence of a relationship in this study between the plating out method and quantification of fumonisin producing *Fusarium* spp. using qRT-PCR may be due to the plating out method reflecting the number of viable spores or mycelia that will germinate on agar as opposed to the actual fungal biomass of grains. Mycelial fragments will give rise to only one colony, even if they consist of many cells (Passone *et al*., 2010). In a qRT-PCR reaction, viable and dead cells can contribute to the number of *fum1* copies and as a result give a more accurate indication of actual fungal biomass. The correlation between qRT-PCR and fumonisin concentration using HPLC was significant, although variation in R^2-values in regression analyses from one season to another may be due to the fumonisin biosynthetic gene, *fum1* being present in the sample, but not always expressed. The qRT-PCR method used in this study will quantify the biomass of fumonisin producing *Fusarium* spp. (pathogenic and/or endophytic) more accurately, thereby giving a better understanding of the potential risk of grain being contaminated with fumonisin. It is not foreseen that this method will be applied at silos because of the expertise, time and cost factors involved, but it can be applied successfully in other applications ensuring food safety and quality to humans and animals.

REFERENCES

Abdin, M.Z., Ahmad, M.M. & Javed, S. 2010. Advances in molecular detection of *Aspergillus*: an update. *Archives of Microbiology* **192**: 409-425.

Anonymous. 2002a. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* **82**: 171-301.

Anonymous. 2002b. Vicam fumonitest instruction manual. Vicam, L.P. 313 Pleasant Street, Watertown, MA 02472, United States of America.

Anonymous. 1993. WHO-IARC. Toxins derived from *Fusarium moniliforme*: Fumonisin B_1 and B_2 and fusarin. C. Lyon (F): IARC.

Bacon, C.W., Glenn, A.E. & Yates, I.E. 2008. *Fusarium verticillioides*: managing the endophytic association with maize for reduced fumonisin accumulation. *Toxin Reviews* **27**: 411-446.

Boutigny, A. L., Beukes, I., Small, I., Zûhlke, S. Spittelier, M., Janse van Rensburg, B., Flett, B. & Viljoen, A. 2011. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant Pathology* **61**: 522–531.

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.

Chu, F.S. & Li, G.Y. 1994. Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of oesophageal cancer. *Applied and Environmental Microbiology* **60**: 847-852.

De la Campa, R., Hooker, D.C, Miller, D. Schaafsma, A.W. & Hammond, B.G. 2005. Modeling effects of environment, insect damage, and Bt genotypes on fumonisin accumulation in maize in Argentina and the Philippines. *Mycopathologia* **159**: 539–552.

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

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

Flett, B.C. 1994. Evaluation of maize hybrids for kernel colonisation by *Fusarium moniliforme* and *F. subglutinans*. *South African Journal of Plant and Soil* **11**: 41-44.

Flett, B.C. & McLaren, N.W. 1994. Optimum disease potential for evaluating resistance to *Stenocarpella maydis* ear rot in corn hybrids. *Plant Disease* **78**: 587-589.

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.

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.

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 B_1, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostical Investigation* **2**: 217-221.

Janse van Rensburg, B., McLaren, N.W., Flett, B.C., Ncube, E., Schoeman, A. & Viljoen, A. 2011. Mycotoxigenic fungi of maize in South Africa. Proceedings of the World Congress of Microbes, Beijing, China, 29 July – 3 August 2011.

Jurado, M., Vázquez, C., Marin, S., Sanchis, V. González-Jaén, M.T. 2006. PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* spp. in maize. *Systematic and Applied Microbiology* **29**: 681-689.

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 fumonisin B_1. *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.

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

Li, F.Q., Yoshizawa, T., Kawamura, O., Luo, X.Y. & Li, Y.W. 2001. Aflatoxins and fumonisin 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. 1982. Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. Pp. 29-40. *In*: Pfeiffer, C.J. (Ed.). Cancer of the Osophagus. CRC Press Inc, Boca Raton, Florida.

Marasas, W.F.O., Jaskiewizc, 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., 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.

Munkvold, G.P. 2003. Epidemiology of *Fusarium* disease and their mycotoxins in maize. *European Journal of Plant Pathology* **109**: 705-713.

Murillo-Williams, A. & Munkvold, G.P. 2009. Systemic infection by *Fusarium verticillioides* in maize plants grown under three temperature regimes. *Plant Disease* **92**: 1695-1700.

Ncube, E., Flett, B.C., Waalwijk, C. & Viljoen, A. 2011. *Fusarium* spp. and levels of fumonisins in maize produced by subsistence farmers in South Africa. *South African Journal of Science* **107**: 1-7.

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

Ngoko, Z., Marasas, W.F.O., Rheeder, J.P., Shephard, 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.

Nyaka, S.C., Shankar, A.C.U., Niranjana, S.R., Wulff, E.G., Mortensen, C.N. & Prakash, H.S. 2010. Detection and quantification of fumonisin from *Fusarium verticillioides* in maize grown in southern India. *World Journal of Microbiological Biotechnology* **26**: 71-78.

Passone, M.A., Rosso, L.C., Ciancio, A. & Etcheverry, M. 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production. *International Journal of Food Microbiology* **138*:*** 276-281.

Pascale, M. & Visconti, A. 2008. Overview of detection methods for mycotoxins. Pp. 171-183. *In:* Leslie, J.F., Bandyopadhyay, R. & Visconti, A. (Eds.).

Mycotoxins. Detection methods, management, public health and agricultural trade. Cromwell Press, Trowbridge, UK.

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.

Rheeder, J.P. 2008. Guidelines on the application of good agricultural practices (GAP) and the HACCP system in mycotoxin prevention and control in South Africa. South African National Health Department (Food Control Directorate), project reference number TCP/SAF/3001 (T).

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

Rheeder, J.P., Marasas, W.F.O. & Van Schalkwyk, D.J. 1993. Incidence of *Fusarium* and *Diplodia* spp. in naturally infected grain of South African maize cultivars: a follow-up study. *Phytophylactica* **25**: 43-48.

Rheeder, J.P., Marasas, W.F.O., Van Wyk, P.S. & Van Schalkwyk, D.J. 1990. Reaction of South African maize cultivars to ear inoculation with *Fusarium moniliforme*, *F. graminearum* and *Diplodia maydis*. *Phytophylactica* **22**: 213-218.

Rheeder, J.P., Marasas, W.F.O. & Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* spp.. *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 fumonisin by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Applied Environmental Microbiology* **56**: 3225-3226.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, Second Edition, Volumes 1, 2 and 3. Cold Spring Harbor Laboratory Press, New York.

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.

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

Shephard,G.S., Thiel, P.G., Stockenstrom, S. & Sydenham, E.W. 1996. Worldwide survey of fumonisin contamination of corn and corn-based products. *Association of Official Analytical Chemists* **79***:* 671-687.

Small, I.M., Flett, B.C., Marasas, W.F.O., McLeod, A., Stander, M. & Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticillioides* and fumonisin accumulation in South Africa. *Plant Disease* **96**: 881-888.

Stack, M.E. 1998. Analysis of fumonisin B_1 and its hydrolysis product in tortillas. *Journal of Analytical Chemistry* **81**: 737-740.

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

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. 1996. Fumonisin in maize genotypes grown in various geographic areas. Pp. 193-204. *In*: Jackson, L.S., de Vries, J.W. & Bullerman, L.B. (Eds.). Fumonisin in Food. Plenum Press, New York.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B.C., de Vries, I. & Khema, G.H.J. 2008. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal* **1**: 37-45.

Table 2.1 Mean fumonisin concentration (FB_1+FB_2+FB_3) using HPLC analysis, *Fusarium* biomass using qRT-PCR, rainfall and maximum temperature during the 2007 maize production season.

2007	DKC80-12B	Pan 6439	DKC 78-15B	Phb 3442	Pan 6611	LS 8521B	CRN 3505	CRN 3549	Phb 32D96	DkC 80-10	Mean*	DKC80-12B	Pan 6439	DKC 78-15B	Phb 3442	Pan 6611	LS 8521B	CRN 3505	CRN 3549	Phb 32D96	DkC 80-10	Mean*	Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, March	Mean max temp, Mar
	Fumonisin concentration (ppm)											*Fusarium* biomass (pg)													
Bainsvlei	nd	0.17	nd	nd	nd	nd	nd	nd	nd	nd	**0.02**^{a}	nd	10.20	nd	nd	nd	nd	15.30	nd	nd	nd	**2.55**^{abc}	0.74	30.78	28.87
Bethlehem	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	0.93	28.24	27.18
Bloekom-spruit	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.04	**0.00**^{a}	nd	nd	nd	5.58	nd	1.47	2.02	nd	nd	nd	**0.91**^{ab}	1.48	28.49	27.98
Bothaville	nd	nd	nd	nd	8.84	nd	2.30	1.74	nd	nd	**1.29**^{a}	nd	5.09	nd	nd	nd	nd	4.66	16.10	nd	nd	**2.59**^{abc}	1.07	31.15	29.84
Delarey-ville	2.30	1.30	nd	1.21	nd	1.73	nd	nd	nd	nd	**0.65**^{a}	7.31	2.56	nd	3.47	nd	nd	5.18	nd	nd	nd	**1.85**^{abc}	1.22	30.65	29.33
Hartebees-fontein	nd	nd	5.30	nd	0.06	nd	nd	nd	nd	nd	**0.54**^{a}	21.30	21.30	199.00	25.00	17.60	nd	nd	1.95	nd	nd	**28.62**^{cde}	1.36	29.67	28.43
Koster	nd	nd	15.24	nd	1.56	nd	0.12	4.41	nd	2.00	**2.33**^{a}	2.11	11.00	23.80	39.40	9.98	nd	33.00	28.80	nd	10.70	**15.88**^{abcd}	0.93	29.89	29.05
Leeudoring-stad	nd	nd	3.49	0.76	nd	nd	10.89	8.37	nd	nd	**2.35**^{a}	4.36	2.83	10.30	92.40	nd	nd	40.60	119.00	1.40	6.89	**27.78**^{bcde}	0.83	30.45	29.35
Marquard	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	4.03	25.36	24.39
Ottosdal	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	1.11	31.23	29.92
Potchef-stroom	nd	nd	nd	nd	nd	2.90	14.88	5.09	nd	nd	**2.29**^{a}	nd	7.14	nd	6.64	55.60	2.40	26.70	103.00	2.03	nd	**20.35**^{abcd}	1.89	30.35	29.52
Rushof	14.31	nd	5.24	13.50	7.25	nd	nd	7.18	8.69	nd	**5.62**^{b}	21.00	1.36	5.33	202.00	58.10	nd	nd	84.60	113.00	2.19	**48.768**^{e}	1.40	30.88	29.78
Tweebuffels-fontein	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	2.59	nd	1.38	nd	nd	nd	1.63	nd	nd	**0.56**^{a}	0.56	28.77	28.01
Ventersdorp	nd	9.35	nd	11.38	18.73	0.84	9.70	6.49	1.18	17.72	**7.54**^{b}	nd	4.69	nd	14.20	135.00	1.79	30.00	80.00	14.10	26.70	**30.65**^{de}	2.63	30.37	29.31
Wesselbron	nd	2.70	nd	nd	nd	nd	nd	3.84	nd	nd	**0.65**^{a}	nd	1.40	nd	nd	nd	nd	nd	nd	nd	nd	**0.140**^{a}	0.28	31.41	30.64
Mean*	**1.11**^{a}	**0.90**^{a}	**1.95**^{a}	**1.79**^{a}	**2.43**^{a}	**0.36**^{a}	**2.53**^{a}	**2.47**^{a}	**0.66**^{a}	**1.32**^{a}		**3.74**^{a}	**4.68**^{ab}	**15.90**^{abc}	**26.00**^{bc}	**18.42**^{abc}	**0.38**^{a}	**10.50**^{abc}	**29.10**^{c}	**8.70**^{abc}	**3.10**^{a}		**1.36**	**29.85**	**28.77**

*Indicates significant differences (LSD P>0.05)

nd = not detected

Limit of detection = 0.016 ppm

Table 2.2 Mean fumonisin concentration (FB_1+FB_2+FB_3) using HPLC analysis, *Fusarium* biomass using qRT-PCR, rainfall and maximum temperature during the 2008 maize production season.

2008	CRN 3505	DKC 80-12B	LS8521B	PAN 6611	DKC 80-10	DKC 78-15B	Mean*	CRN 3505	DKC 80-12B	LS8521B	PAN 6611	DKC 80-10	DKC 78-15B	Mean*	Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, March	Mean max temp, Mar
	Fumonisin concentration (ppm)							*Fusarium* biomass (pg)									
Bethlehem	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	2.64	24.79	23.30
Bloekomspruit	nd	nd	nd	nd	2.30	nd	**0.38**^{a}	nd	2.47	nd	nd	10.90	nd	**2.23**^{a}	3.29	26.48	25.98
Bothaville	25.10	21.84	21.84	12.80	19.09	0.46	**16.85**^{d}	6.53	162.00	172.00	2.93	114.00	6.43	**77.32**^{bc}	1.83	30.38	29.95
Cedara	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	2.78	25.00	24.25
Coligny	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	2.89	28.50	27.45
Danielsrus	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}			
Delmas	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	53.00	74.00	2.30	**21.55**^{ab}	4.30	26.10	24.70
Frankfort	nd	nd	nd	nd	5.88	nd	**0.98**$_{a}$	nd	nd	nd	nd	21.30	nd	**3.55**^{a}	2.90	25.98	23.55
Hartebeesfontein	nd	nd	nd	nd	nd	nd	**0.00**^{a}	3.33	25.20	nd	nd	0.21	nd	**4.79**^{a}	3.27	28.71	27.54
Jim Fouche	nd	nd	nd	4.82	3.19	nd	**1.34**^{ab}	nd	nd	nd	15.80	12.60	nd	**4.73**^{a}	1.98	26.04	24.98
Leeudoringstad	nd	nd	nd	4.80	0.94	nd	**0.96**^{a}	14.00	13.50	4.22	25.70	159.00	6.72	**37.19**^{ab}	3.34	28.61	26.92
Nampo	26.21	5.67	nd	nd	nd	1.73	**5.60**^{bc}	569.00	117.00	nd	4.66	15.00	14.20	**119.98**^{c}	2.79	29.76	27.44
Potchefstroom	nd	nd	nd	nd	15.00	nd	**2.50**^{abc}	21.30	10.60	nd	nd	37.60	3.98	**12.25**^{a}	3.37	27.27	25.22
Rushof	6.85	nd	nd	nd	1.58	nd	**1.41**^{ab}	6.70	nd	3.17	3.24	8.63	nd	**3.62**^{a}	29.43	29.25	28.65
Tweebuffels	nd	nd	0.44	nd	nd	nd	**0.07**^{a}	nd	nd	4.67	nd	nd	nd	**0.78**^{a}	14.11	25.34	23.94
Tweeling	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	3.61	27.49	26.54
Vaalharts	22.53	nd	nd	5.30	8.93	nd	**6.13**^{c}	33.70	nd	nd	12.90	96.00	nd	**23.77**^{ab}	1.98	31.41	29.69
Vrede	nd	nd	nd	nd	Nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	2.29	25.76	25.13
Wonderfontein	nd	nd	nd	nd	Nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	4.21	26.88	25.49
Mean*	**4.13**^{b}	**1.56**^{a}	**1.36**^{a}	**1.32**^{a}	**2.71**^{a}	**0.27**^{a}		**32.40**^{a}	**16.05**^{a}	**9.61**^{a}	**5.63**^{a}	**26.27**^{a}	**2.26**^{a}		**5.01**	**27.43**	**26.15**

*Indicates significant differences (LSD P>0.05)

nd = not detected

Limit of detection = 0.016 ppm

Table 2.3 **Mean fumonisin concentration(FB_1+FB_2+FB_3) using HPLC analysis, *Fusarium* biomass using qRT-PCR, rainfall and maximum temperature during the 2009 maize production season.**

2009	CRN 3505	DKC 80-12B	LS8521B	PAN 6611	DKC 78-15B	DKC 80-10	Mean*	CRN 3505	DKC 80-12B	LS8521B	PAN 6611	DKC 78-15B	DKC 80-10	Mean*	Mean rainfall, Jan, Feb, Mar	Mean max temp, Feb, March	Mean max temp, Mar
	Fumonisin concentration (ppm)							*Fusarium* biomass (pg)									
Bethlehem	nd	nd	nd	nd	3.18	nd	**0.53**^{ab}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	8.95	24.58	24.66
Bloekomspruit	nd	nd	1.12	18.88	nd	2.98	**3.83**^{bc}	2.76	13.50	18.60	50.10	nd	12.30	**16.21**^{ab}	3.78	26.67	26.32
Cedara	nd	2.00	nd	nd	nd	1.50	**0.58**^{ab}	nd	19.40	nd	2.58	nd	2.17	**4.03**^{a}	2.25	25.86	25.57
Coligny	nd	13.93	nd	nd	nd	nd	**2.32**^{abc}	2.23	7.47	nd	nd	2.27	nd	**2.00**^{a}	3.42	30.17	28.78
Delmas	nd	nd	nd	nd	nd	2.50	**0.42**^{ab}	nd	nd	nd	nd	nd	80.59	**13.43**^{ab}	3.83	25.78	25.54
Hoogekraal	6.44	nd	nd	nd	15.04	7.00	**4.75**^{c}	2.71	1.74	7.92	nd	9.18	74.00	**15.93**^{ab}	3.42	29.50	28.45
Leeudoringstad	26.71	1.73	nd	nd	nd	nd	**4.74**^{c}	117.00	6.87	nd	6.84	2.49	95.00	**38.03**^{cd}	0.00	28.76	29.12
Nampo	19.06	12.44	3.56	32.68	14.84	nd	**13.76**^{e}	41.40	17.40	13.70	104.00	36.70	30.00	**40.53**^{d}	6.19	29.49	29.72
Ottosdal	4.49	1.93	8.51	nd	6.35	nd	**3.55**^{bc}	16.70	2.23	38.50	1.83	4.39	21.00	**14.11**^{ab}	4.88	27.16	27.57
Potchefstroom	8.80	nd	0.01	8.11	0.65	nd	**2.93**^{abc}	90.23	12.11	9.21	3.35	7.12	16.10	**23.02**^{bc}	5.91	27.08	26.79
Rushof	0.03	0.10	0.03	0.73	2.66	2.00	**0.93**^{ab}	14.20	nd	nd	15.10	33.10	12.00	**12.40**^{ab}	3.44	27.51	26.46
Vaalharts	9.89	7.94	13.53	17.41	3.91	20.92	**12.27**^{de}	50.20	15.75	22.55	40.45	49.85	28.35	**34.53**^{cd}			
Wesselsbron	33.26	4.58	2.65	17.06	1.89	nd	**9.91**^{d}	225.98	22.90	12.40	22.20	60.75	nd	**57.37**^{e}	3.05	28.62	27.99
Wonderfontein	nd	nd	nd	nd	nd	nd	**0.00**^{a}	nd	nd	nd	nd	nd	nd	**0.00**^{a}	4.05	27.79	27.74
Mean*	**7.76**^{b}	**3.19**^{ab}	**2.10**^{a}	**6.78**^{ab}	**3.47**^{ab}	**2.64**^{ab}		**40.24**^{b}	**8.53**^{a}	**8.78**^{a}	**17.60**^{a}	**14.70**^{a}	**26.54**^{a}		**4.09**	**27.61**	**27.29**

*Indicates significant differences (LSD P>0.05)

nd = not detected

Limit of detection = 0.016 ppm

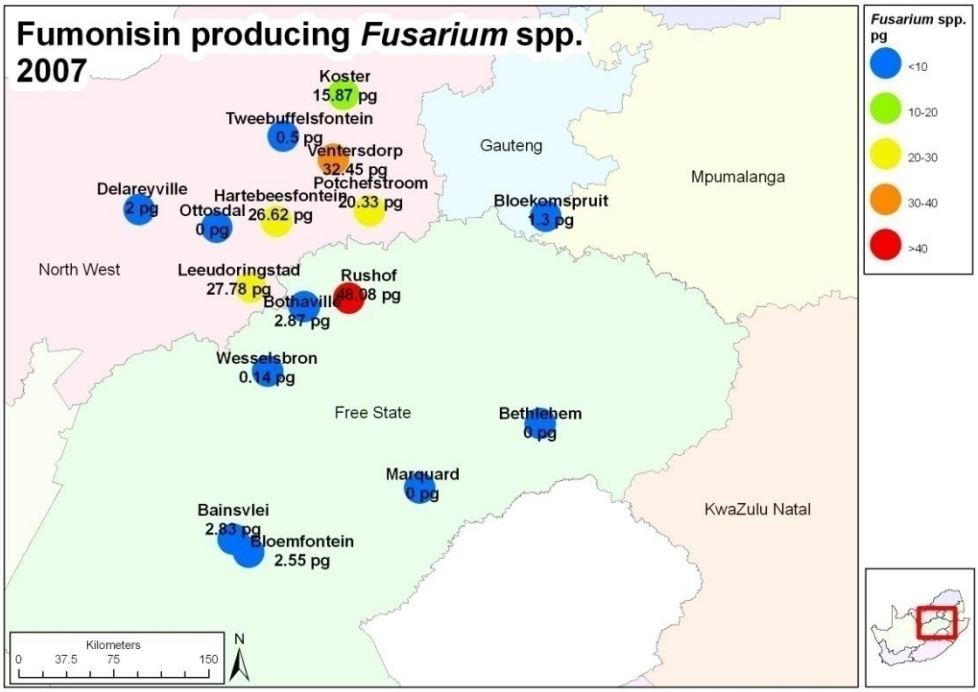


Figure 2.1a Infection of maize by fumonisin producing *Fusarium* spp. in the 2007 commercial maize production areas of South Africa.

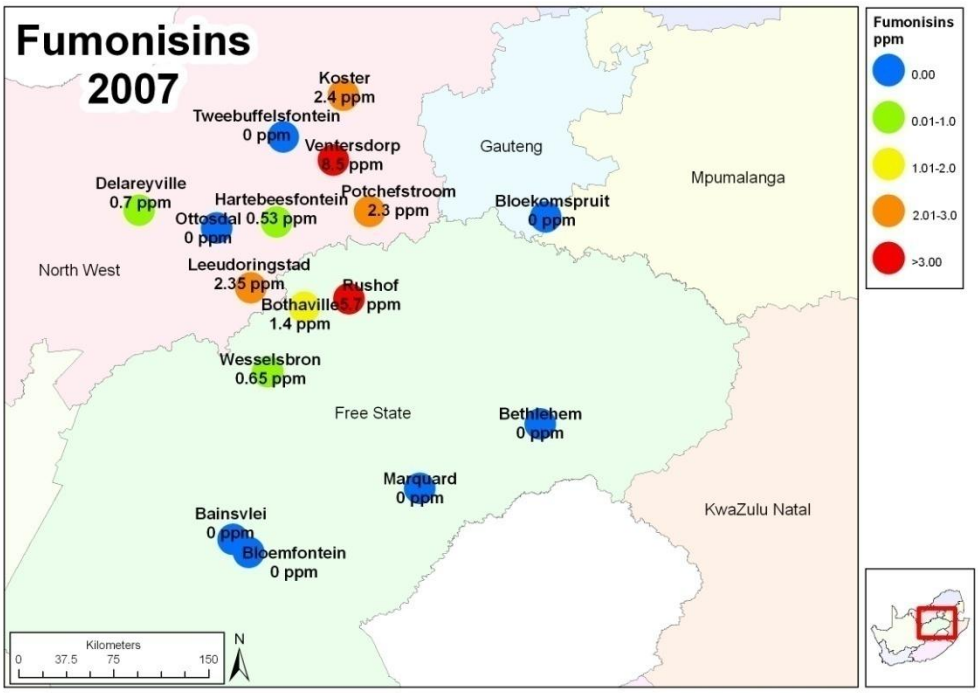


Figure 2.1b Fumonisin levels in the 2007 commercial maize production areas of South Africa.

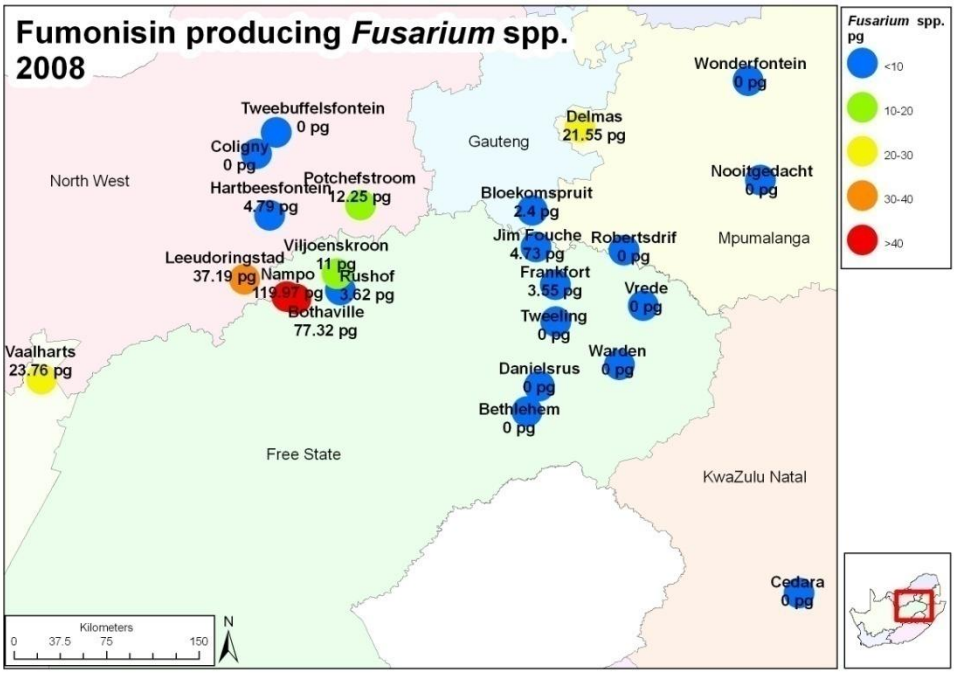


Figure 2.1c Infection of maize by fumonisin producing *Fusarium* spp. in the 2008 commercial maize production areas of South Africa.

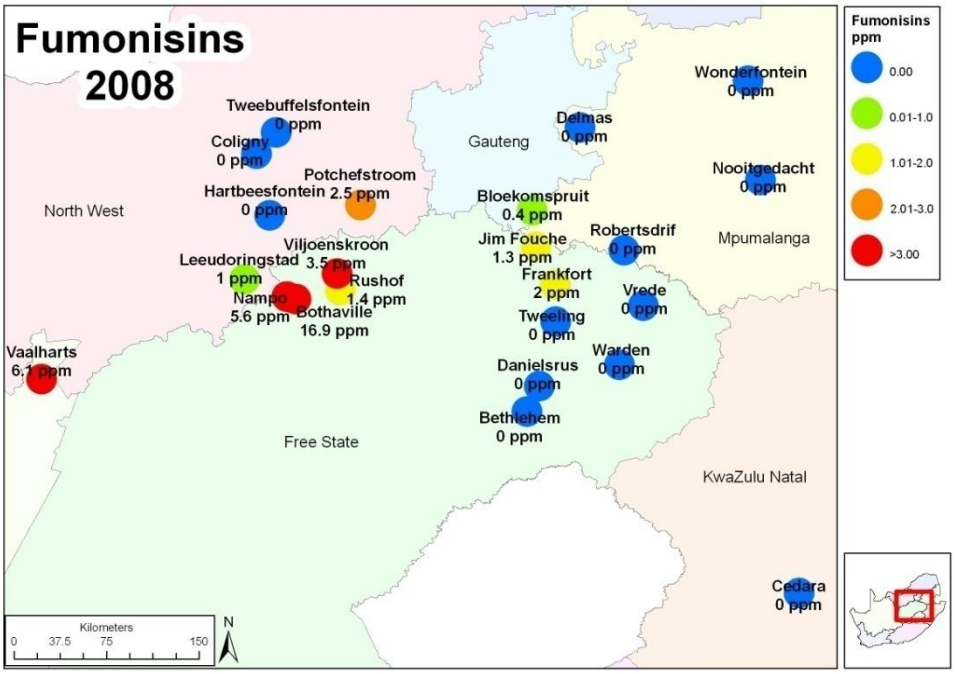


Figure 2.1d Fumonisin levels in the 2008 commercial maize production areas of South Africa.

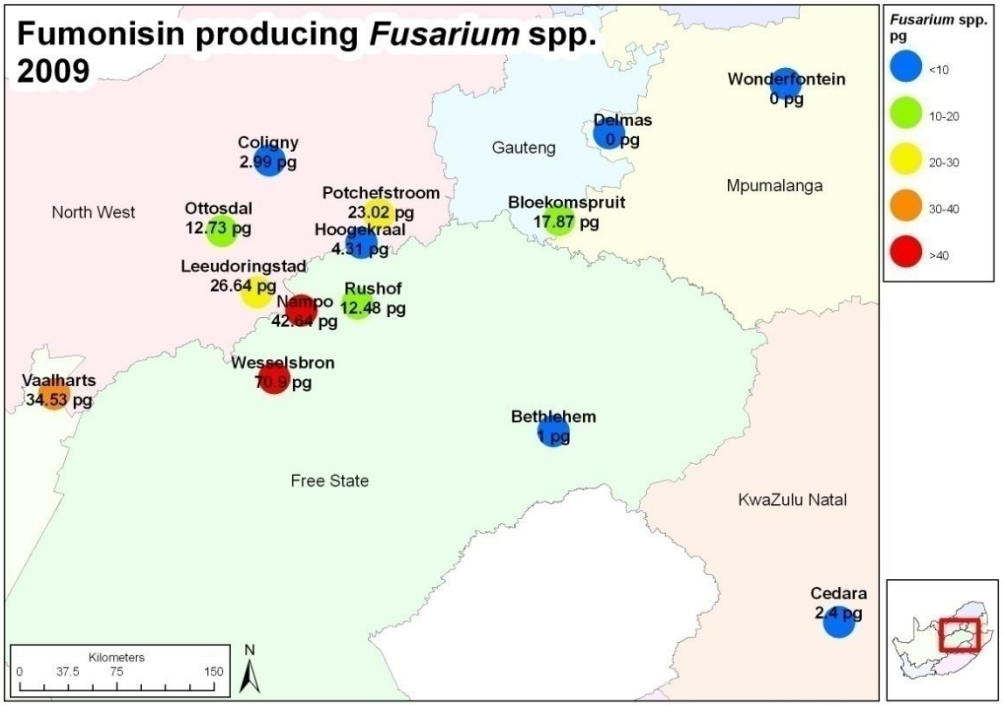


Figure 2.1e Infection of maize by fumonisin producing *Fusarium* spp. in the 2009 commercial maize production areas of South Africa.

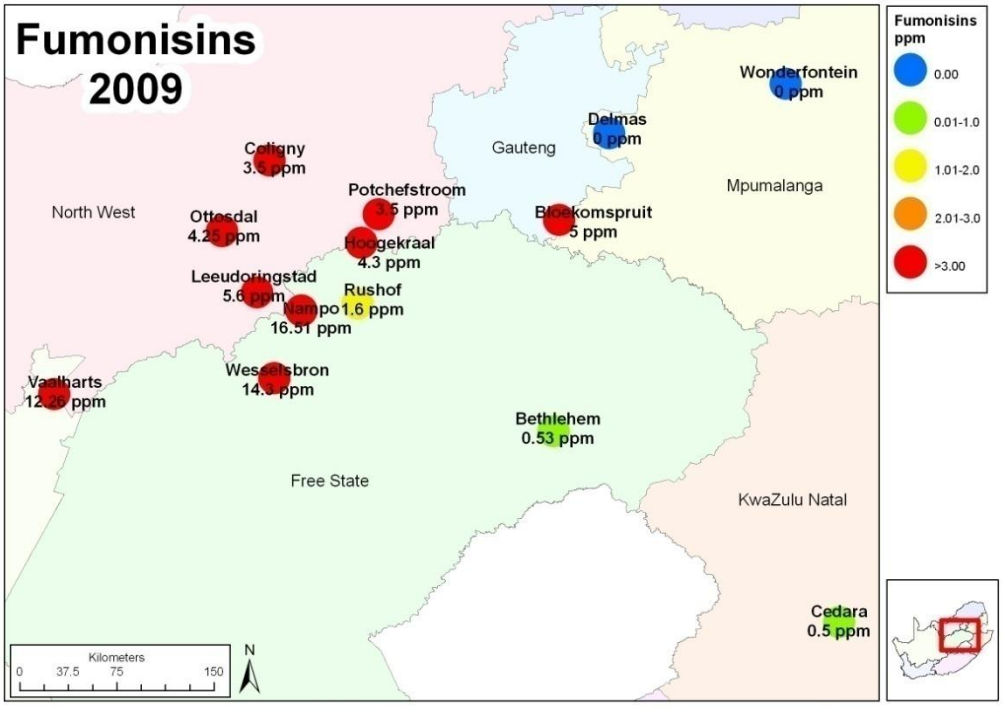


Figure 2.1f Fumonisin levels in the 2009 commercial maize production areas of South Africa.

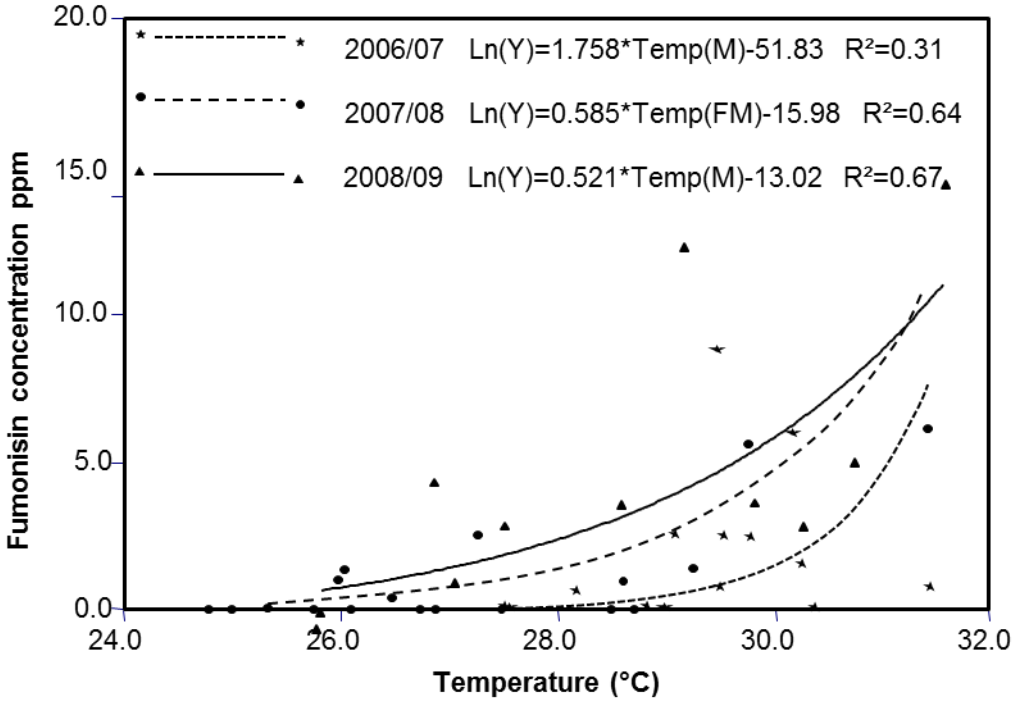


Figure 2.2 Relationship between mean maximum temperature during February/March (2008) or March (2007 and 2009) and fumonisin concentration in maize grain.

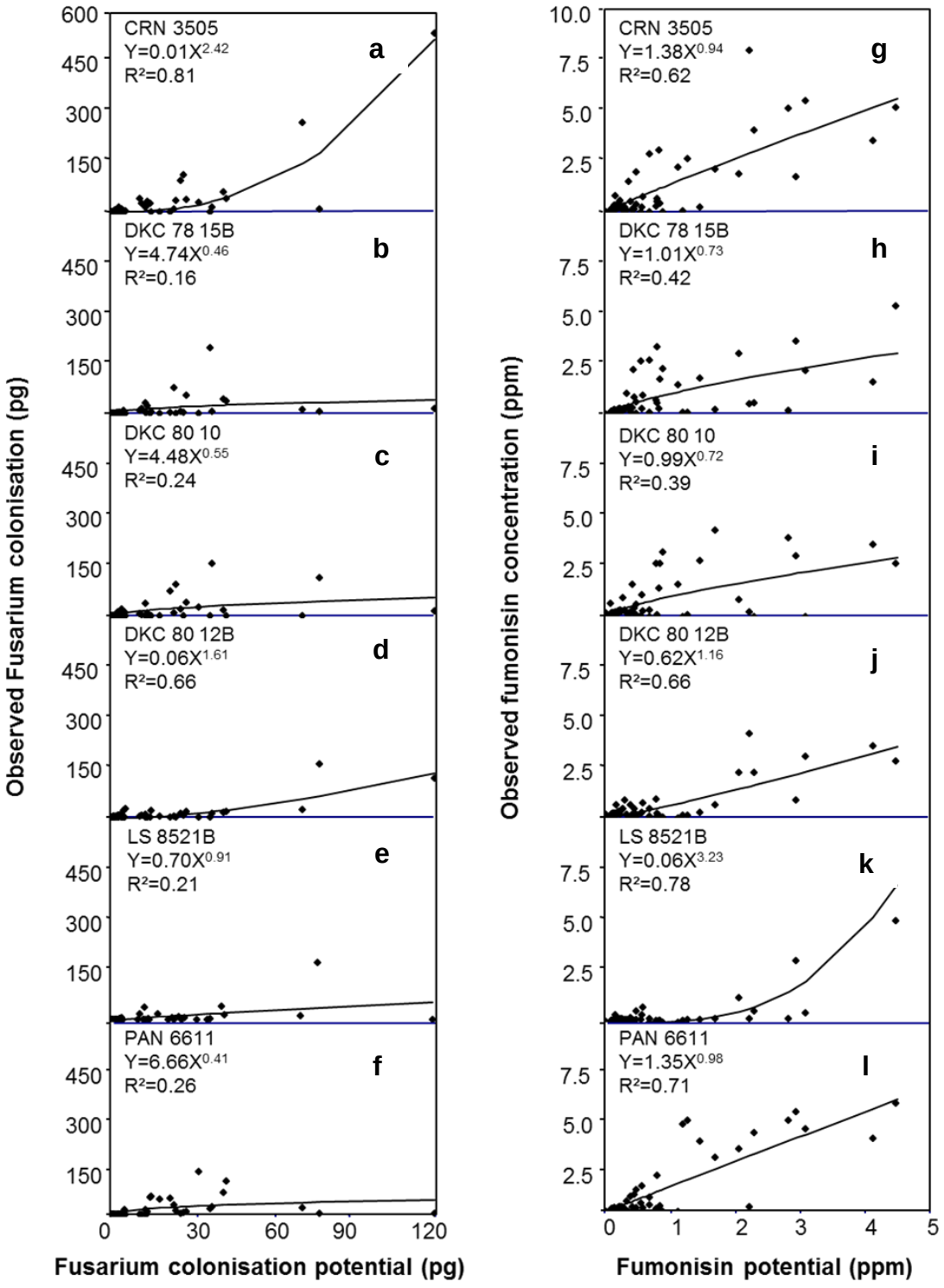


Figure 2.3 **Maize genotype responses to colonisation by fumonisin producing *Fusarium* spp. and fumonisin contamination at various disease/fumonisin potentials.**

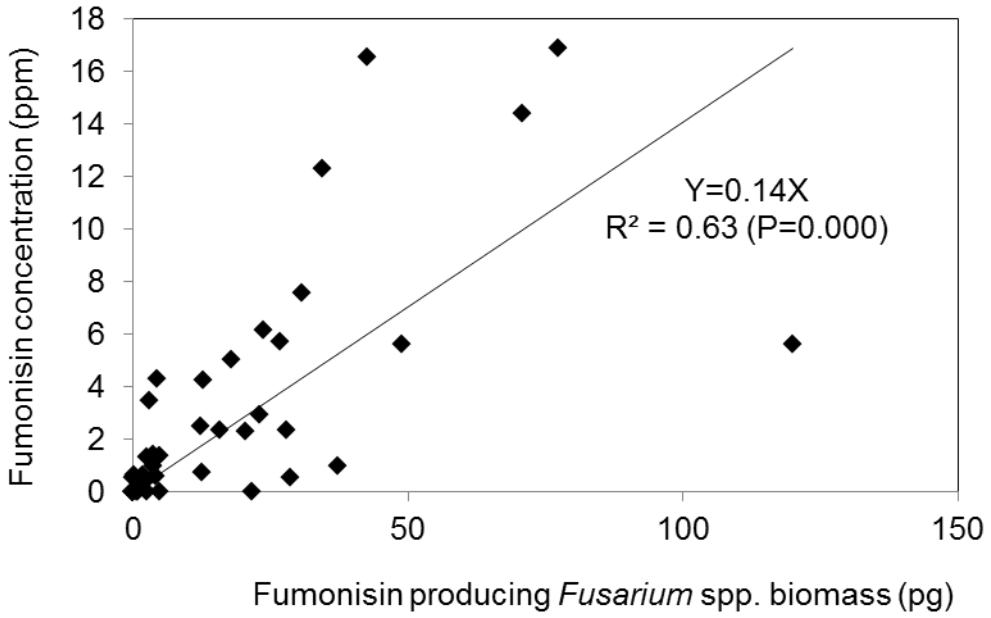


Figure 2.4 Relationship between mean fumonisin concentrations and mean fumonisin producing *Fusarium* spp. biomass.

CHAPTER 3

Aflatoxin and fumonisin on sorghum grain from commercial production areas of South Africa

Janse van Rensburg, B., McLaren, N.W., Viljoen, A. & Flett, B.C. 2011. Aflatoxin and fumonisin on sorghum grain from commercial production areas of South Africa. *South African Journal of Plant and Soil* 28: 236-238.

ABSTRACT

Sorghum plays an important role in food security in Africa where it serves as the staple food for millions of people. The crop, however, is susceptible to infection by mycotoxigenic fungi such as *Aspergillus flavus, A. parasiticus, Fusarium andiyazi, F. thapsinum, F. verticillioides* and *F. proliferatum*. This can lead to a reduction in yield and the production of aflatoxin and fumonisin that are harmful to humans and animals. Sorghum grain samples were collected from five cultivars planted at 21 localities in South Africa from 2007-2009. Each sample was subjected to quantitative (q) real-time-PCR to determine the presence and biomass of aflatoxin-producing *Aspergillus* spp. and fumonisin-producing *Fusarium* spp.. Aflatoxin and fumonisin concentrations were also quantified by means of High Performance Liquid Chromatography (HPLC). HPLC results yielded minute concentrations of aflatoxin with little or no fungal contamination determined with qPCR. The soft grain genotype NS5511 was more prone to aflatoxin contamination than the other cultivars. Fumonisin-producing *Fusarium* spp. were absent from sorghum samples collected in 2007. In 2008, sorghum samples from the Northwest and Kwa-Zulu Natal Provinces had small amounts of fungal contamination. In 2009, low fungal biomasses from samples from Northwest, Kwa-Zulu Natal and the Free State Provinces were recorded. Although small fungal biomasses were present, fumonisin were not produced. HPLC and qPCR results indicate that *Aspergillus* and *Fusarium* spp. and their mycotoxins do not pose a threat to sorghum production in South Africa.

Keywords: *Aspergillus*, *Fusarium*, mycotoxins

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is considered one of the most important food crops in Africa and provides the major source of dietary energy and protein for millions of people (Chandrashekar & Satyanarayana, 2006). The susceptibility of sorghum grain to various fungi is well documented (Williams & McDonald, 1983). Toxins produced by fungi such as *Aspergillus* and *Fusarium* spp. have been known to contaminate sorghum (Singh & Bandyopadhyay, 2000; da Silva *et al*., 2004). *Aspergillus flavus* (Link:Fr.) and *A. parasiticus* (Speare) produce aflatoxin, of which aflatoxin B_1 represents one of the most harmful naturally-occurring carcinogens known (Squire, 1989). Epidemiological studies have shown a strong correlation between the incidence of primary liver cancer in humans and the consumption of aflatoxin-contaminated food (O'Brian *et al*., 2003). Consumption of aflatoxin-contaminated feed by livestock results in oxidative stress, liver necrosis, haemorrhage and death (Osweiler, 2005). *Fusarium verticillioides* (Sacc.) Nirenberg (synonym: *F. monilifome* Sheldon) and *F. proliferatum* (Matsushina) Nirenberg produce fumonisin B_1, B_2 and B_3 (Rheeder *et al*., 2002), with fumonisin B_1 typically accounting for 70 to 80% of all fumonisins produced. Fumonisin B_1 is classified as Group 2B carcinogens, which means that they are potentially carcinogenic to humans. High incidences of human neural tube defects were reported (Marasas *et al.*, 2004) where substantial consumption of fumonisins have been documented (Guatemala, South Africa, China and Texas). Fumonisin also cause leukoencephalomalacia (LEM) in horses (Kellerman *et al.*, 1990; Ross *et al.*, 1990), pulmonary oedema in swine (Harrison *et al.*, 1990) and are hepatotoxic and carcinogenic to rats (Gelderblom *et al.*, 1988). The objective of this study was to determine the amount of infection of sorghum in South Africa with aflatoxin- and fumonisin-producing fungi, and their concomitant toxin concentrations, to obtain an indication of sorghum grain quality and safety for human and animal use.

MATERIALS AND METHODS

Sorghum grain (1-kg samples) were collected from the National Cultivar Trials from five cultivars (PAN8609, PAN8625, PAN8816, PAN8247, NS5511) planted at 21 localities in South Africa by PANNAR and the ARC-Grain Crops Institute. Colonisation by aflatoxin-producing *Aspergillus* spp. and fumonisin-producing *Fusarium* spp. were determined using qPCR. Real-Time PCR reactions were performed in a MyiQ^{TM}2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten-halogen optical lamp. The primer/probe set for aflatoxin-producing *Aspergillus* spp. had the nucleotide sequence: Nortaq 1, 5'-GTCCAAGCAACAG GCCAAGT-3'; Nortaq 2, 5'-TCGTGCATGTTGGTGATGGT-3' and Norprobe 1, 5'-/6-FAM/TGTCTTGATCGGCGCCCG/36 - TAMRA/-3' (Mayer *et al*., 2003). The primer/probe set for the fumonisin-producing *Fusarium* spp. had the nucleotide sequence: Taqfum-2F, 5'-ATGCAAGAGGCGAGGCAA-3'; Vpgen-3R, 5'-GGCTCTCRGAGCTTGGCAT-3' and FUM-probe 1, 5'-/56-FAM/CAAT GCCATCTTCTTG/36 - TAMSp/-3' (Waalwijk *et al.*, 2008). Aflatoxin (B_1, B_2, G_1 and G_2) and fumonisin (B_1, B_2 and B_3) concentrations were determined by means of HPLC on a Waters 1525 HPLC systems (Aura Industries, New York, USA). The HPLC AflaTest® and FumoniTest® of the United States Department of Agriculture – Federal Grain Inspection Service (USDA-FGIS) procedure for sorghum was used (Anonymous, 1999). Breeze chromatography software version 3.30 was used to quantify aflatoxin and fumonisin concentrations present.

RESULTS

No aflatoxin-producing *Aspergillus* spp. were detected by qPCR. However, aflatoxin concentrations, ranging from 0.01 to 2.53 parts per billion (ppb), were present in sorghum samples collected at all locations during either 2007 and/or 2008 (Table 3.1). No aflatoxins were detected from any sample collected in 2009. Fumonisin-producing *Fusarium* spp. were detected in samples from Amersfoort (0.70 pg), Cedara (0.83 pg), Kafferskraal (0.52 pg),

Klerksdorp (0.50 pg), Potchefstroom (0.80 pg) and Weiveld (0.83 pg) but no fumonisins were detected.

DISCUSSION

Passone *et al*. (2010) reported that a single conidium of *A. parasiticus* contains approximately 96±30 pg of DNA. Pham *et al*. (2003) and Abdin *et al*. (2010) detected purified *Aspergillus* DNA from 20 ng–200 fg and 500 pg–50 fg, respectively. In this study, qPCR did not detect fungal biomasses less than 50 fg. It is, therefore, possible that a very low biomasses of *Aspergillus* spp. may have been present that were not detected by real-time PCR. This could explain the low concentration of aflatoxin found in sorghum.

At present the only two mycotoxins considered under South African national regulations are aflatoxin (5 ppb in all foodstuffs) and patulin (apple juice) (Rheeder, 2008). Legislation on tolerable levels for *Fusarium* mycotoxins do not exist (Fandohan *et al*., 2003). The European Union established a limit of 1 ppm for food intended for direct human consumption with fumonisins whereas the Food and Drug Administration of America is currently suggesting a guideline of 2 ppm for direct human consumption when considering food safety in local maize products (FAO, 2003). Currently these guidelines are used when considering food safety. This study revealed that aflatoxin and fumonisin in commercially-produced sorghum in South Africa are below the maximum suggested allowable limits of 5 ppb and 2 ppm, respectively, and should not be considered a threat to animal and human health. The biomass of fumonisin-producing *Fusarium* spp. found in the grain is also not expected to negatively affect yield. Caution should however be taken with stored sorghum because low concentrations of aflatoxin can sharply increase during storage under sub-optimal conditions if contaminated with *Aspergillus* spp. (Hell *et al*., 1995).

REFERENCES

Anonymous. 1999. VICAM AflaTest® Intruction Manual. Vicam, L.P. 313 Pleasant Street, Watertown, MA 02472, USA.

Abdin, M.Z., Ahmad, M.M & Javed, S. 2010. Advances in molecular detection of *Aspergillus*: an update. *Archives of Microbiology* **192**: 409-425.

Chandrashekar, A. & Satyanarayana, K.V. 2006. Disease and pest resistance in grains of sorghum and millets. *Journal of Cereal Science* **44**: 287-304.

Da Silva, J.B., Dilkin, P., Fonseca, H. & Corrêa, B. 2004. Production of aflatoxins by *Aspergillus flavus* and of fumonisins by *Fusarium* spp. isolated from Brazilian sorghum. *Brazilian Journal of Microbiology* **35**: 182-186.

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

FAO. 2003. Food and Agriculture Organization of the United Nation Corporate Document Repository: Worldwide regulations for mycotoxins in food and feed in 2003. [Available on internet:] http://www.fao.org/docrep/007/y5499e/y5499e00.htm. [Date of access 08/02/10].

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

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 B_1, a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostic Investigation* **2**: 217-221.

Hell, K., Cardwell, K.F., Schultz, F.A. & Sétamou, M. 1995. Aflatoxin B_1 contamination of traditional stores in four agroecological zones of Benin. *In*: XIII International Plant Protection Congress. The Hague, The Netherlands. *European Journal of Plant Pathology*. P 1356.

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 B_1. *Onderstepoort Journal of Veterinary Research* **57**: 269-275.

Marasas, W.F.O., Riley, R.T., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-Van Waes, J., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martinez, C., Miller, J.D., Starr, L., Roman, M.C., Voss, K.A., Wang, E., & Merrill, A.H. 2004. Fumonisins disrupts sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. *Journal of Nutrition* **134**: 711-716.

Mayer, Z., Bagnara, A., Färber, P. & Geisen, R. 2003. Quantification of the copy number of *nor*-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of *Aspergillus flavus* in foods. *International Journal of Food Microbiology* **82**: 143-151.

O'brian, G.R., Fakhoury, A.M. & Payne, G.A. 2003. Identification of genes differentially expressed during aflatoxin biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus*. *Fungal Genetics and Biology* **39**: 118-127.

Osweiler, G. 2005. Aflatoxins and animal health. Iowa State University, Ames, Iowa.

Passone, M.A., Rosso, L.C., Ciancio, A. & Etcheverry, M. 2010. Detection and quantification of *Aspergillus* section *Flavi* spp. in stored peanuts by real-time PCR of *nor*-1 gene, and effects of storage conditions on aflatoxin production. *International Journal of Food Microbiology* **138**: 276-281.

Pham, A.S., Tarrand, J.J. & May, G.S. 2003. Diagnosis of invasive mold infection by real-time quantitative PCR. *American Journal of Clinical Pathology* **119**: 38- 44.

Rheeder, J.P. 2008. Guidelines on the application of good agricultural practices (GAP) and the HACCP system in mycotoxin prevention and control in South Africa. South African National Health Department (Food Control Directorate), project reference number TCP/SAF/3001 (T).

Rheeder, J.P., Marasas, W.F.O. & Vismer, H.F. 2002. Production of fumonisin analogs by *Fusarium* spp. *Applied 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.

Singh, D.P. & Bandyopadhyay, R. 2000. Grain mould. Pp. 38-40. *In*: Frederiksen, R.A. and Odvody, G.N. (eds.). Compendium of sorghum diseases. Second edition. APS Press, Minnesota,.

Squire, R.A. 1989. Ranking animal carcinogens: a proposed regulatory approach. *Science* **214**: 887-891.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B.C., De Vries, I. & Khema, G.H.J. 2008. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal* **1**: 37-45.

Williams, R.J. & McDonald, D. 1983. Grain molds in the tropics: problems and importance. *Annual Review of Phytopathology* **21**: 153-178.

Table 3.1 Matrix table indicating concentrations of aflatoxin in sorghum cultivars from different localities and seasons in South Africa.

	qPCR (pg)	HPLC (ppb)					
Locality and season		**PAN8816**	**PAN8247**	**PAN8609**	**PAN8625**	**NS5511**	**Mean**
Amersfoort, 2009	nd*	0.00	0.00	0.00	0.00	0.00	**0.00**
Bethlehem, 2008	nd	0.00	0.04	0.03	0.01	0.00	**0.02**
Bethlehem, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Cedara, 2008	nd	0.00	0.00	0.02	0.00	1.24	**0.25**
Cedara, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Dover, 2007	nd	0.00	0.00	0.00	0.00	2.36	**0.47**
Dover, 2008	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Dover, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Goedgedagt, 2008	nd	0.00	0.00	0.00	0.00	2.42	**0.48**
Goedgedagt, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Gottenburg, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Greenlands, 2008	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Kafferskraal, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Klerksdorp, 2007	nd	0.00	0.00	0.00	1.38	0.00	**0.28**
Klerksdorp, 2008	nd	0.00	0.00	0.00	2.27	2.39	**0.93**
Klipdrift, 2008	nd	0.00	0.00	0.00	0.00	1.22	**0.24**
Klipdrift, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Leeukraal, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Holmdene, 2008	nd	0.00	0.00	0.00	0.00	2.42	**0.48**
Parys, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Perdekop, 2007	nd	0.00	0.00	0.00	0.00	2.35	**0.47**
Perdekop, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Platrand, 2007	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Platrand, 2008	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Platrand, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Potchefstroom, 2007	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Potchefstroom, 2008	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Potchefstroom, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Rietfontein, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Skaapplaas, 09	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Vaalharts, 2008	nd	0.00	0.00	0.00	0.00	2.53	**0.51**
Vaalharts, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Val, 2008	nd	0.00	0.00	0.00	1.24	0.00	**0.25**
Val, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Weiveld, 2008	nd	0.00	0.00	1.23	0.00	0.00	**0.25**
Weiveld, 2009	nd	0.00	0.00	0.00	0.00	0.00	**0.00**
Mean		**0.00**	**0.00**	**0.04**	**0.14**	**0.47**	

*nd (not detected)

CHAPTER 4

Use of weather variables to quantify the potential risk of grain colonisation by fumonisin-producing *Fusarium* spp. and fumonisin synthesis in commercial maize in South Africa.

ABSTRACT

Fumonisins are mycotoxins produced primarily by *Fusarium verticillioides* and *F. proliferatum* on maize. These mycotoxins are secondary, carcinogenic metabolites and have been reported on maize worldwide. Since no commercial cultivar has yet been developed with adequate resistance to *F. verticillioides* and *F. proliferatum* in South Africa, alternative disease management strategies must be used to reduce fumonisin contamination of the local maize crop. An epidemiological model that can enable producers to assess the potential risk of fumonisin contamination in maize grain at a specific locality within a season could aid agronomic decisions and reduce the risk of infection by *Fusarium* spp. Maize samples (1 kg each) were collected from cultivar trials (2007-2009) planted at 15, 19 and 14 localities respectively in the maize production areas of South Africa. Site-specific weather data, including temperature, radiation, humidity, rainfall and evapo-transpiration were provided by the ARC-Institute for Soil Water and Climate's meteorology office. Grain colonisation by fumonisin producing *Fusarium* spp. was determined using quantitative real-time PCR and contamination with fumonisins using HPLC analysis. Fungal biomass ranged from 0-15.7 pg in 2007, 0-20.9 pg in 2008, 0-31.5 pg in 2009. Fumonisin concentrations ranged from 0-2.09 ppm, 0-6.13 ppm and 0-12.26 ppm from 2007-2009, respectively. *Fusarium* colonisation of grain and fumonisin concentrations were related to prevailing weather conditions during early post-flowering and dough stage of grain development, respectively. Both colonisation and fumonisin production were significantly inversely correlated with mean maximum temperature (r=-0.77 and r=-0.60, respectively) and minimum relative humidity (r=-0.83 and r=-0.79, respectively) during the critical growth periods. A preliminary model based on the non-linear, 3-dimentional Lorentzian equation (Sigmaplot 10.0) was developed and evaluated.

Keywords: epidemiology, fumonisin, HPLC, qRT-PCR

INTRODUCTION

Fumonisins are mycotoxins produced primarily by the maize pathogens *Fusarium verticillioides* (Sacc.) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg (Rheeder *et al*., 1990). Fumonisins are secondary carcinogenic metabolites, which occur naturally as contaminants of agricultural products such as maize. The consumption of maize contaminated with fumonisins cause mycotoxicoses in animals such as leucoencephalomalacia in horses (Kellerman *et al*., 1990; Ross *et al*., 1990) and pulmonary oedema in swine (Harrison *et al*., 1990). Fumonisin infected maize has been statistically associated with human oesophageal cancer in South Africa (Marasas *et al*., 1981; Marasas, 1982; Marasas *et al*., 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 humans that ingest maize infected by fumonisin in certain endemic areas of The People's Republic of China. Stack (1998), Placinta *et al*. (1999), Hendricks (1999) and Marasas *et al*. (2004) have shown a strong correlation between the consumption of fumonisin-contaminated tortillas and neural-tube defects in humans. The potential carcinogenic risk of fumonisin B_1 to humans was evaluated and classified by the World Health Organizations International Agency for Research on Cancer (WHO-IARC) as "Group 2B carcinogens" which means they are probably carcinogenic to humans (Anonymous, 1993).

The United States Food and Drug Administration (FDA, 2001) has set guidelines of 2 ppm for degermed dry milled maize products and 4 ppm for whole or partially degermed dry milled maize products for human consumption. Currently South Africa has no legislation or monitoring system regarding allowable fumonisin concentrations and consumers may be at greater risk due to the higher consumption of maize in comparison to European countries and Canada (Marasas *et al*., 2001). Marasas (2001) recorded large variations in probable daily intake (PDI) ranging from 1.2 µg/kg bodyweight (bw)/day in urban South Africans consuming commercial maize, to 354.9 µg/kg bw/day in rural South Africans consuming mouldy, home-grown maize.

Since maize is consumed either directly or indirectly when processed into food and feed products, fumonisins are of concern as contamination has been reported worldwide (Plancinta *et al*., 1999). Pre-harvest control of fumonisin infestation is complicated by unforeseeable weather conditions (Maiorano *et al*., 2009). In contrast, post-harvest conditions associated with commercial storage are potentially controllable (temperature and humidity). The latter does not apply to subsistence and small scale farmers who do not always have proper storage technologies in place.

To date, no commercial cultivar with adequate levels of resistance to infection by *F. verticillioides* and fumonisin production is available in South Africa. Inconsistent responses of cultivars to the pathogen and variations in fumonisin production despite similar pathogen biomasses within grain over environments could be due to variation in isolates and the adaptation of genotypes to physiological stress associated with production locality and weather (Miller, 2001). Emphasis has therefore, shifted to management strategies at field level to achieve quality and safe maize food and products. By studying and understanding the complexity of interactions between numerous abiotic and biotic disease factors (Parsons & Munkvold, 2012) we can aim to prevent or reduce the growth of *F. verticillioides* and *F. proliferatum* at field level, thereby reducing contamination by fumonisins. A disease and fumonisin prediction model could act as an instrument to constantly monitor and assess the risk of mycotoxin contamination of maize grain, making it possible to drive agronomic decisions during cultivation and enhance management opportunities (Schaafsma & Hooker, 2007). This could also benefit collectors (traders that store and sell maize grain) and processors by providing information regarding *F. verticillioides* and *F. proliferatum* infection and mycotoxin contamination with regard to sourcing, testing and management of damaged and contaminated grain.

There have been numerous attempts to develop risk assessment models for *Fusarium* contamination of grain. Stewart *et al.* (2002) developed a mathematical simulation of the growth of *F. graminearum* and *F. verticillioides*

in maize ears after artificial inoculation of silks. This model does not take mycotoxin accumulation into account. De la Campa *et al*. (2005) developed a preliminary model (Phillipines and Argentina) and identified four weather periods near silking as critical to fumonisin concentration at harvest. This model accounted for 82% of the variability of total fumonisin across all locations in two years of study, but did not consider meteorological conditions during grain maturation when fumonisins are synthesized. A risk assessment model (FUMAgrain) developed by Maiorano *et al*. (2009) for fumonisin synthesis by *F. verticilliodes* in maize grain in Italy gives an initial risk alert at the end of flowering based on meteorological conditions. A second alert follows at maturation stage from assessments made from maize grain moisture, European Corn Borer damage to maize ears and fumonisin synthesis risk. FUMAgrain could simulate fumonisin synthesis in maize with an R^2=0.70 for calibration and R^2=0.71 for validation.

The aim of the current study was to develop an epidemiological model that would assist in the prediction of *F. verticillioides* and *F. proliferatum* infection and resultant fumonisin contamination under environmental conditions associated with maize production areas of South Africa. Identification of critical phenological growth stages of the maize plant in relation to infection by *F. verticillioides* and fumonisin production and their relationship with weather variables was emphasized.

MATERIALS AND METHODS

Field trials

Maize kernels were harvested from selected cultivars in the National Cultivar Evaluation Trials at a range of localities during the 2007-2009 maize production seasons (Table 4.1). Cultivar trials were conducted using a randomised complete block design with three replicates under dryland conditions, except for Vaalharts in the Northern Cape which was flood irrigated. Trials were maintained according to “Best Practice” appropriate to the respective production areas. Weather variables, notably daily maximum

and minimum temperature, maximum and minimum humidity, evapo-transpiration and rainfall were monitored at each locality during flowering and grain development (February-March). At harvest kernels from all replications were pooled, thoroughly mixed and a 1 kg sample from each cultivar and locality was collected and stored in a coldroom at 4ºC for a maximum of one week prior to milling and further analysis. Sub-samples of 250 g from all the initial 1 kg maize kernel samples were individually milled in a Cyclotech 1093 sample mill with a 1 mm mesh sieve. The mill was thoroughly cleaned with high-pressure air between each sub-sample to prevent cross contamination.

Fungal biomass and fumonisin concentration

Isolation of fumonisin producing Fusarium *spp. DNA for qRT-PCR reactions*

A 0.5 g aliquot from each sub-sample was used to isolate and clean up DNA, using the method of Sambrook *et al*. (1989). A DNA solution (500 µl) was precipitated by adding 25 µl of 5M NaCl followed by 1250 µl ice cold absolute ethanol. The samples were incubated in ice for 3 hours. The DNA was pelleted by means of centrifugation (14 000 rpm x 10 minutes) and the supernatant was discarded. The DNA was washed with 76% EtOH and 0.2 M NaoC for 10 minutes and the wash solution was discarded. DNA was then washed with 76% EtOH and 10mM NH_4OAc for 2 minutes. The wash solution was discarded and the ethanol was evaporated in a laminar flow cabinet. DNA was resuspended by adding 200 µl Melford molecular grade water. DNA samples were stored at -20ºC. Prior to qRT-PCR analysis all DNA samples were diluted to 10 ng with Melford molecular grade water using a nanodrop to measure DNA concentrations.

qRT-PCR reactions

MRC 826 (*F. verticillioides*) strain from the Medical Research Council - Promec Unit was used as the standard in the qRT-PCR technique. This strain is characterised by its ability to produce high concentrations of fumonisins and

DNA from this isolate was used to develop a standard curve (Waalwijk *et al*., 2008). qRT-PCR reactions were performed in a MyiQ^{TM}2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten-halogen optical lamp. The primers Taqfum-2F and Vpgen-3R in combination with the FUM-probe 1, as tested by Waalwijk *et al*. (2008), were used in this study. The primer/probe set had the following nucleotide sequence: Taqfum-2F, 5'-ATGCAAGAGGCGAGGCAA-3'; Vpgen-3R, 5'-GGCTCTCRGAGCTTGGCAT-3' and FUM-probe 1, 5'-/56-FAM/CAATGCCAT CTTCTTG/36 - TAMSp/-3' . PCR was conducted using the sensimix reagent kit (sensimix^{TM} no ref QT 505-05) from Celtic (Bioline). In each reaction 4 µl of the DNA sample was mixed with 12.5 µl sensimix, 2.125 µl Fum-probe 1 (1µM), 0.875 µl Taqfum-2F (333 nM), 0.875 µl Vpgen-3R (333 nM) and 4.625 µl Melford molecular grade water. In all the experiments, negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carryover. The 96-well plate was incubated for 10 minutes at 95ºC and thereafter, each of the 40 PCR cycles was performed according to the following temperature regime: 95ºC for 10 s, 60ºC for 30 s, and 72ºC for 10 s. The Bio-Rad iCycler^{Tm} iQ Optical System Software Version 3.0a was used to calculate the biomass of fumonisin producing *Fusarium* spp. present in a sample. Regression equations of standard curves from runs were highly significant (R^2=>0.99). Slopes were within the acceptance criterion (between -3.1 and -3.6) and efficiencies ranged from 95 to 105%.

Fumonisin analysis

Mycotoxins were analysed using the Vicam method (Anonymous, 2002). A 50 g aliquot from each of the 250 g milled sub-samples was mixed with 5 g of sodium chloride prior to extraction. Fumonisins were extracted in 100 ml methanol:water (80:20, v/v) for five minutes using an IKA T18 basic Ultra Turrax homogeniser. The extract was then filtered through Whatman No.5 filter paper. A 10 ml aliquot was diluted with 40 ml phosphate-buffered saline (1XPBS) (8.0 g NaCI, 1.2 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCI, dissolved in 990 ml purified water with pH adjusted to 7.0 with HCI). Diluted samples were

extracted through microfiber filters (0.45 µm) and 10 ml of the diluted filtrate was passed through FumoniTest affinity columns from Vicam at a flow rate of 1 drop per second. Ten ml of PBS were subsequently passed through the column at a rate of 1 drop per second. The column was then washed with 1.5 ml HPLC grade methanol at a rate of 1 drop per second and the eluate was collected in a glass cuvette. Methanol eluate was dried in a TurboVap LV (Caliper Sciences) with the aid of a slow stream of high purity Nitrogen. Samples were dissolved in 200 µl methanol and purified water (50:50 v/v). Each sample (50 µl) was transferred to 250 µl conical inserts. Each insert was placed into a 2.5 ml glass vial which was placed into a carousel. The first position of the carousel had a 2.5 ml glass vial with *o*-phthaldialdehyde (OPA from Sigma) which is the derivatisation agent. The Waters 717 plus autosampler was set up to mix 100 µl of the OPA with the 50 µl of sample in the conical insert. This mixture (20 µl) was injected after a delay time of 1 minute.

Fluorescence was performed at excitation and emission wavelengths of 335 and 440 nm respectively using a Waters 2475 multi λ fluorescence detector. The analytical column, Symmetry C18, 5 µm 3.9 x 150 mm from Waters was used. The detection limit of the method used was 0.016 ppm . Recovery data were obtained in triplicate by fortifying clean maize samples (Vicam) with 5 ppm fumonisin B_1 B_2 and B_3. The average recovery rates were 83% (FB_1), 81% (FB_2) and 83% (FB_3).

Statistical analysis

Weather x colonisation/fumonisin production analyses were conducted on data collected during each individual season as well as pooled data from the respective seasons. Weather data subsequent to 50% silking in each season were bulked into periodic running means (0, 5, 10, 14 and 21 days) and correlation matrix analysis was used to determine clusters of significant relationships between bulked weather variables and *Fusarium* biomass and fumonisin production in the post-silking period. Stepwise multiple regression analysis, after transformation of data to ensure linearity was also applied for

variable selection. *Fusarium* biomass, as determined by qRT-PCR was included as a variable in the analysis of fumonisin concentration. Re-analysis with variables identified by correlation and step-wise variable selection was done using the non-linear, 3-dimentional Lorentzian equation (Sigmaplot 10.0) for model development.

Model development from one season to the next was evaluated by comparing predicted values from the 2007 model with data collected during 2008 and 2009 season and vice versa. The relationship between predicted colonisation and observed colonisation was compared using difference measures proposed by Willmott (1982).

RESULTS

Fungal biomass in harvested maize kernels ranged from 0-15.7 pg in 2007, 0-20.9 pg in 2008 and 0-31.5 pg in 2009. Fumonisin concentrations over this period ranged from 0-2.09 ppm, 0-6.13 ppm and 0-12.26 ppm respectively.

Relationship between weather variables and grain colonisation by fumonisin producing Fusarium *spp.*

No significant correlations were recorded between grain colonisation by fumonisin-producing *Fusarium* spp. and all combinations of periodic means of minimum temperature, maximum and minimum humidity, rainfall and evapo-transpiration. Significant correlations were recorded between periodic means of maximum temperature in the post 50% silking period and *Fusarium* spp. biomass with the highest correlation with mean maximum temperature for the 14 day post-silking period corresponding with the periods 76-89 days after planting in 2007 (r=0.81***), 75-88 days after planting in 2008 (r=0.49*) and 70-83 in 2009 (r=0.69**). Stepwise multiple regression analysis, after transformation of data to ensure linearity selected the variables mean maximum temperature and mean minimum humidity (days 1-14 post silking) as having significant relationships with colonisation of maize kernels by fumonisin-producing *Fusarium* spp.

The post-silking 14 day means of maximum temperature and minimum humidity were included into the 3-dimentional Lorentian equation (Sigmaplot 10.0) and yielded highly significant relationships between these variables and colonisation of grains by fumonisin-producing *Fusarium* spp. with R^2=0.88 R^2=0.96 and R^2=0.93 for 2007, 2008 and 2009, respectively (Figure 4.1a-c). Optimum temperatures for grain colonisation determined by the respective equations were 28.97, 32.14 and 30.40°C respectively. Optimum minimum humidities were calculated at 27.29, 31.86 and 29.74% respectively. Despite the high R^2 - values recorded during the three seasons, indicating that, despite good "within season" relationships, there was seasonal variation which is not accounted for by the two weather variables.

Data from the three seasons were sequentially pooled and data from the subsequent season was used as the observed values against which to measure predicted values using indexes of agreement as calculated according to Willmott (1982). The index of agreement between the 2007 model and 2008 data was d=0.09 indicating no predictive value in the model. Pooled data from 2007 and 2008 were used in model development and the predicted values were measured against 2009 observed data. The index of agreement improved to d=0.31 indicating that an increased number of degrees of freedom could improve the reliability of the model. However, the reduced R^2 value (R^2=0.39) of the pooled model compared to individual models further emphasises that one or more modifying variables that take differences between seasons into account, need to be identified. When the 2009 data were included into the pooled model the model improved to R^2=0.53 (Figure 4.1d).

Fumonisin analysis

The application of correlation analysis and stepdown multiple regression analysis on sequential means of weather data, as indicated above, and the inclusion of observed fungal biomass as variable, yielded significant relationships between the latter variable and 14-day mean maximum temperature during the dough stage of grain development with fumonisin concentration in kernels. This period coincided with day's 98-111, 94-107 and 93-106 after planting during 2007, 2008 and 2009 respectively. Respective R^2 values of the individual season models were R^2=0.95, R^2=0.93 and R^2=0.86 (Figure 4.2a-c).

Optimum fumonisin concentrations were obtained with fungal biomasses of 39.94 pg during 2007, 47.65 pg during 2008 and 90.69 pg during 2009. Optimum temperatures for fumonisin production were 30.33, 31.12 and 29.80 ºC for 2007-2009, respectively. The index of agreement between the 2007 model and 2008 data was d=0.004. Models developed from pooled data from 2007, 2008 and the three seasons combined yielded non-significant model parameters and could not be used for comparisons of model improvement.

DISCUSSION

Fusarium colonisation of grain and subsequent fumonisin concentrations were related to prevailing weather conditions during the early post-flowering and grain development stages, respectively. The optimum temperature recorded for colonisation of maize by *Fusarium* spp. is 30ºC (Reid, 1999; Munkvold, 2003) which was confirmed in this study, where optimum temperatures of 28.97, 32.14 and 30.40ºC over the three seasons were recorded. Similarly, high fumonisin concentrations are associated with hot and dry weather, followed by a period of high humidity (Munkvold, 2003; de la Campa *et al*., 2005) which agrees with the relationship recorded in this study with optimum temperatures of 30.33, 31.12 and 29.80°C over the three seasons De la Campa *et al.* (2005) reported that weather during the period 2-8 days post-silking accounted for 42.9% of the variation in fumonisin concentration,

confirming one of the critical periods recorded in this study. De la Campa *et al.* (2005) also recorded a positive effect with increasing maximum temperature and negative effect with rainfall during this early post-silking period which coincides with the relationship with minimum humidity recorded in the current study. The current study, however, suggests two phases in the development of fumonisins in maize kernels i.e. colonisation of maize tissues during the early post-silking stage followed by fumonisin production during the dough stage of grain fill. Our model, therefore, suggests that fumonisin production by a constant biomass of fumonisin producing *Fusarium* spp. on grain is influenced largely by temperature and less so by rainfall, as suggested by de la Campa *et al*. (2005), during the second critical host growth period.

Models developed in the current study did not give consistent prediction values over seasons, which indicates variation that is not accounted for by the two weather variables identified in this study. According to Miller (2001) and Parsons & Munkvold (2012) the most influential factors in relation to Fusarium ear rot and fumonisin risk are temperature, drought stress, insect damage, other fungal diseases and maize genotype. Primary inoculum from maize residues was not quantified in this study, and variation associated with tillage/no-till systems could be an important factor in the recorded colonisation levels although Flett & Wehner (1991) reported no differences in *Fusarium* spp. maize ear rot severity under different tillage systems. Similarly, the possible role of seed as an inoculum source and possible systemic colonisation of maize plants was also not investigated.

Parsons & Munkvold (2012) have indicated thrips (*Frankliniella occidentalis*) to play a key role in the dispersal of *F. verticillioides* and elevated fumonisin concentrations under dry conditions after pollination in the United States of America. In temperate maize areas from Europe, European Corn Borer (*Ostrinia nubilalis*) damage on maize can cause fumonisin contamination at rates of 40 times higher compared to uninfested maize (Alma *et al*., 2005). In South Africa economically important pests of maize are the stem borers *Busseola fusca* and *Chilo partellus* (Van Wyk *et al*., 2008) which can cause

between 10 and 100% yield loss in maize (Kfir *et al*., 2002). De La Campa *et al*. (2005) reported that insect damage to maize kernels could explain 11% of the variability in fumonisins in Argentinia and the Phillipines. The inclusion of *Busseola fusca* infestation levels in future development of the model may be necessary to improve the prediction of fumonisins using the current model.

Although our models are not consistent over seasons regarding prediction, they are consistent regarding time of fungal infection and fumonisin production. In our study, highest correlation between mean maximum temperature in the post 50% silking period and *Fusarium* spp. biomass were recorded 76-89 days after planting in 2007, 75-88 days after planting in 2008 and 70-83 days after planting in 2009. Similarly the correlation between fungal biomass and mean maximum temperature during the dough stage of grain development, were significant for fumonisins and coincided with day's 98-111, 94-107 and 93-106 after planting during 2007, 2008 and 2009 respectively.

The model in the current study was consistent in identifying the variables mean maximum temperature and minimum humidity as driving variables in the colonisation of maize kernels by fumonisin producing *Fusarium* spp. Optimum fungal biomass in relation to fumonisin concentration was similar in 2007 (39.94 pg) and 2008 (39.94 pg) seasons but higher in the 2009 (90.69 pg) season. Waalwijk *et al*. (2008) reported the accumulation of fumonisins in maize kernels to be highly correlated with fungal biomass in the kernels and this explains the mean fumonisin concentration calculated by the model of 11.79 ppm (2007), 9.56 ppm (2008) and 22.45 ppm (2009).

This is an on-going study and further data are needed to ensure the accuracy of regression parameters. Subsequent testing under field conditions to determine the forecasting potential of the model is also required. The current model does not take into account all critical variables, as discussed above and these variables need to be identified and included in future cycles of model improvement and verification.

REFERENCES

Alma, A., Lessio, F., Reyneri, A. & Blandino, M. 2005. Relationships between *Ostrinia nubilalis* (Lepidoptera: Crambidae) feeding activity, crop technique and mycotoxin contamination of corn kernel in northwestern Italy. *International Journal of Pest Management* **51**: 165-173.

Anonymous. 1993. WHO-IARC. Toxins derived from *Fusarium moniliforme*: Fumonisin B_1 and B_2 and fusarin. C. Lyon (F): IARC

Anonymous, 2002. Vicam fumonitest instruction manual. Vicam, L.P. 313 Pleasant Street, Watertown, MA 02472, United States of America.

Chu, F.S. & Li, G.Y. 1994. Simultaneous occurrence of fumonisin B_1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of oesophageal cancer. *Applied and Environmental Microbiology* **60**: 847-852.

De la Campa, R., Hooker, D.C, Miller, D. Schaafsma, A.W. & Hammond, B.G. 2005. Modeling effects of environment, insect damage, and Bt genotypes on fumonisin accumulation in maize in Argentina and the Philippines. *Mycopathologia* **159**: 539–552.

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

Food and Drug Administration. 2001. Guidance for industry: fumonisin levels in human foods and animal feeds; final guidance. [Available on internet:] Http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/ GuidanceDocuments/ChemicalContaminantsandPesticides/ucm109231.htm [Date of access 14/03/12].

Flett, B.C. & Wehner, F.C. 1991. Incidence of Stenocarpella and Fusarium cob rots in monoculture maize under different tillage systems. *Journal of Phytopathology* **133**: 327-333.

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

Harrison, L.R., Colvin, B.M., Greene, J.T., Newman, L.E. & Cole, J.R. 1990. Pulmonary oedema and hydrothorax in swine produced by fumonisin B_1,

a toxic metabolite of *Fusarium moniliforme*. *Journal of Veterinary Diagnostical Investigation* **2**: 217-221.

Hendricks, K., 1999. Fumonisins and neural tube defects in south Texas. *Epidemiology* **10**: 198-200.

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 B_1. *Onderstepoort Journal of Veterinary Research* **57**: 269-275.

Kfir R., Overholt. W.A., Khan. Z.A. & Polaszek, A. 2002. Biology and management of economically important Lepidopteran cereal stem borers in Africa. *Annual Review of Entomology* **47**: 701-731.

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.

Maiorano, A., Reyneri, A., Sacco, D., Magni, D. & Ramponi, C. 2009. A dynamic risk assessment model (FUMAgrain) of fumonisin synthesis by *Fusarium verticillioides* in maize grain in Italy. *Crop Protection* **28**: 243-256.

Marasas, W.F.O. 1982. Mycotoxicological investigations on corn produced in esophageal cancer areas in Transkei. Pp. 29-40. *In*: Pfeiffer, C.J. (Ed.). Cancer of the Oesophagus. CRC Press Inc, Boca Raton, Florida.

Marasas, W.F.O. 2001. Discovery and occurrence of the fumonisins: a historical perspective. *Environmental Health Perspectives* **109**: 239-243.

Marasas, W.F.O., Jaskiewizc, 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, Miller, J.D, Riley, R.T., Visconti, A. 2001. Occurrence, toxicology, metabolism and risk assessment. Pp. 332-359 *In*: Summerell, B.A., Leslie, J.F., Backhouse, D., Bryden, W.L. & Burgess, L.W. (Eds.). Fusarium Paul E. Nelson symposium. St. Paul: APS press.

Marasas, W.F.O., Riley, R.L., Hendricks, K.A., Stevens, V.L., Sadler, T.W., Gelineau-van Waes, J., Missmer, S.A., Cabrera, J., Torres, O., Gelderblom, W.C.A., Allegood, J., Martinez, C., Maddox, J., Miller, J.D., Starr, L., Sullards, M.C., Roman, A.V., Voss, K.A., Wang, E & Merril, Jr., A.H. 2004. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and *in vivo*: a potential risk factor for human neural tube defects among population consuming fumonisin-contaminated maize. *Journal of Nutrition* **134**: 711-716.

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.

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

Munkvold, G.P. 2003. Epidemiology of Fusarium disease and their mycotoxins in maize. *European Journal of Plant Pathology* **109**: 705–713.

Parsons, M.W. & Munkvold, G.P. 2012. Effects of planting date and environmental factors on Fusarium ear rot symptoms and fumonisin B_1 accumulation in maize grown in six North American locations. *Plant Pathology.* Doi: 10.1111/j.1365-3059.2011.02590.x

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.

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**: 1028-1037.

Rheeder, J.P., Marasas, W.F.O., Thiel, P.G., Syndeham, E.W., Shephard, 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., Van Wyk, P.S. & Van Schalkwyk, D.J. 1990. Reaction of South African maize cultivars to ear inoculation with *Fusarium moniliforme*, *F. graminearum* and *Diplodia maydis*. *Phytophylactica* **22**: 213-218.

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.

Sambrook, J., Fritsch, E.F & Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, Second Edition, Volumes 1, 2 and 3. Cold Spring Harbor Laboratory Press, New York.

Schaafsma, A.W. & Hooker, D.C. 2007. Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. *International Journal of Food Microbiology* **119**: 116-125.

Shephard, 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.

Stack, M.E. 1998. Analysis of fumonisin B_1 and its hydrolysis product in tortillas. *Journal of Analytical Chemistry* **81**: 737-740.

Stewart, D.W., Reid, L.M., Nicol, R.W. & Schaafsma, R.W. 2002. A mathematical simulation of growth of *Fusarium* in maize ears after artificial inoculation. *Phytopathology* **92**: 534-541.

Van Wyk, A., Van Den Berg, J. & Van Hamburg, H. 2008. Diversity and comparative phenology of Lepidoptera on Bt and non-Bt maize in South Africa. *International Journal of Pest Management* **54**: 77-87.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B.C., de Vries, I. & Khema, G.H.J. 2008. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal* **1**: 37-45.

Willmott, C.J. 1982. Some comments on the evaluation of model performance. *Bulletin American Meteorological Society* **63**: 1309-1313.

Table 4.1 Localities and cultivars sampled over a three year period for the development of a model to predict colonisation of maize by fumonisin-producing *Fusarium* spp.

Localities 2007	Cultivars at each locality 2007	Localities 2008	Cultivars at each locality 2008	Localities 2009	Cultivars at each locality 2009
Bainsvlei	CRN3505	Bethlehem	CRN3505	Bethlehem	CRN3505
Bethlehem	DKC80-12B	Bloekomspruit	DKC80-12B	Bloekomspruit	DKC80-12B
Bloekomspruit	LS8521B	Bothaville	LS8521B	Cedara	LS8521B
Bothaville	PAN6611	Cedara	PAN6611	Coligny	PAN6611
Delareyville	DKC80-10	Coligny	DKC80-10	Delmas	DKC80-10
Hartebeesfontein	DKC78-15B	Danielsrus	DKC78-15B	Hoogekraal	DKC78-15B
Koster	PAN6439	Delmas		Leeudoringstad	
Leeudoringstad	PHB3442	Frankfort		Nampo	
Marquard	CRN3549	Hartebeesfontein		Ottosdal	
Ottosdal	PHB32D96	Jim Fouche		Potchefstroom	
Potchefstroom		Leeudoringstad		Rushof	
Rushof		Nampo		Vaalharts	
Tweebuffelsfontein		Potchefstroom		Wesselsbron	
Ventersdorp		Rushof		Wonderfontein	
Wesselbron		Tweebuffels			
		Tweeling			
		Vaalharts			
		Vrede			
		Wonderfontein			

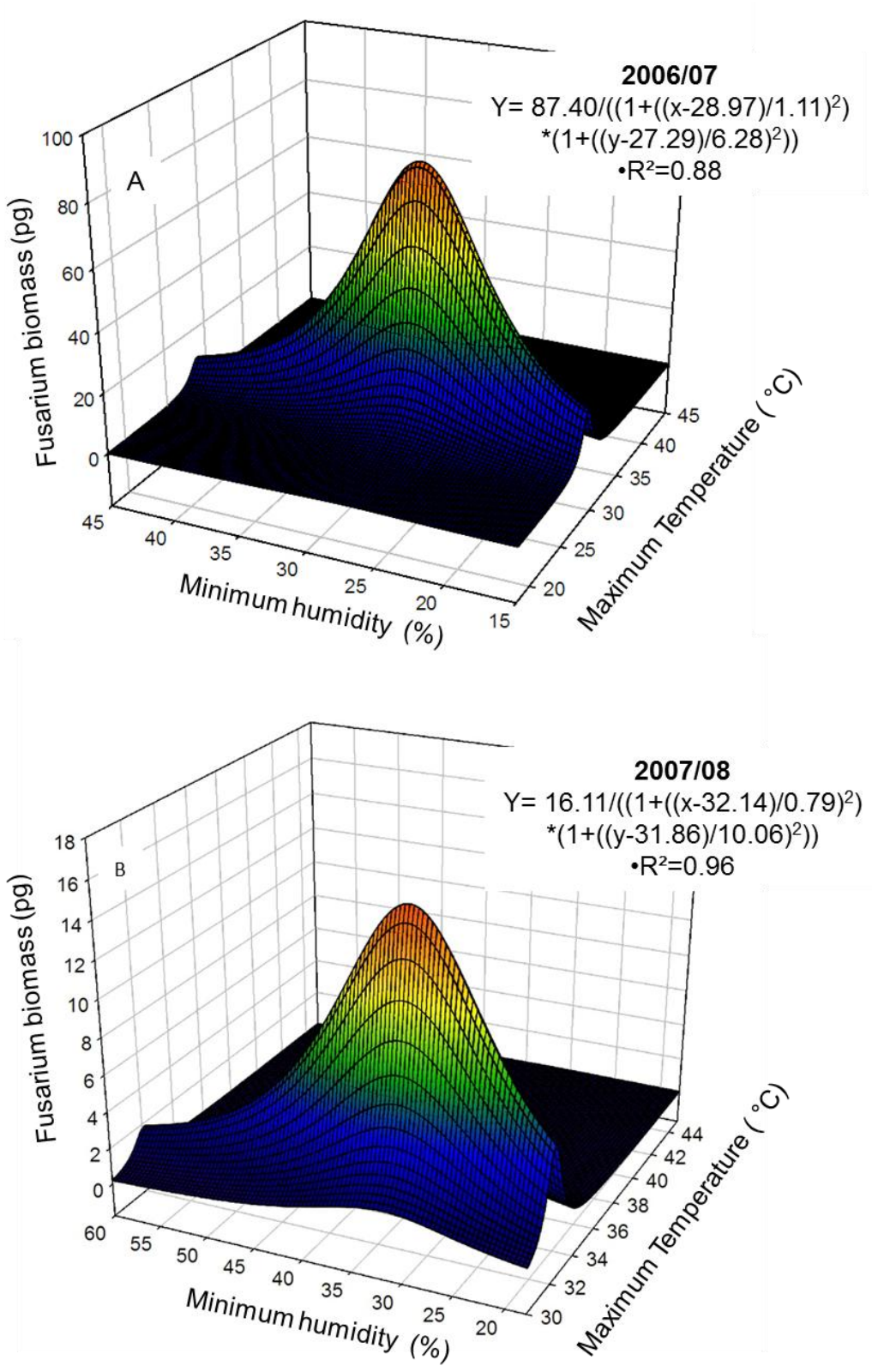


Figure 4.1a-b **Mean maximum daily temperature and mean minimum relative humidity for the 14 day post-silking period and their relationship with *Fusarium* colonisation of maize.**

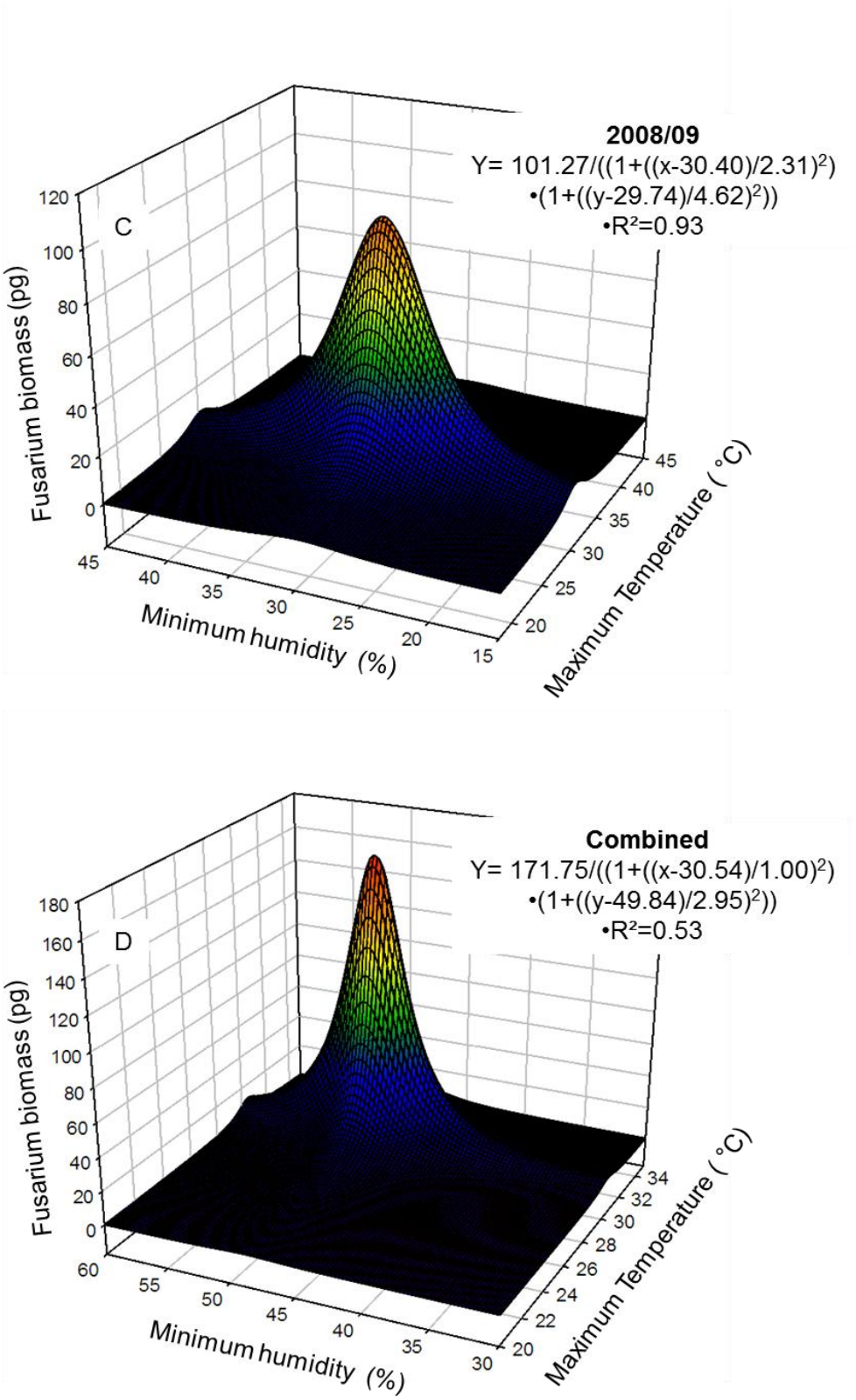


Figure 4.1 c-d **Mean maximum daily temperature and mean minimum relative humidity for the 14 day post-silking period and their relationship with *Fusarium* colonisation of maize.**

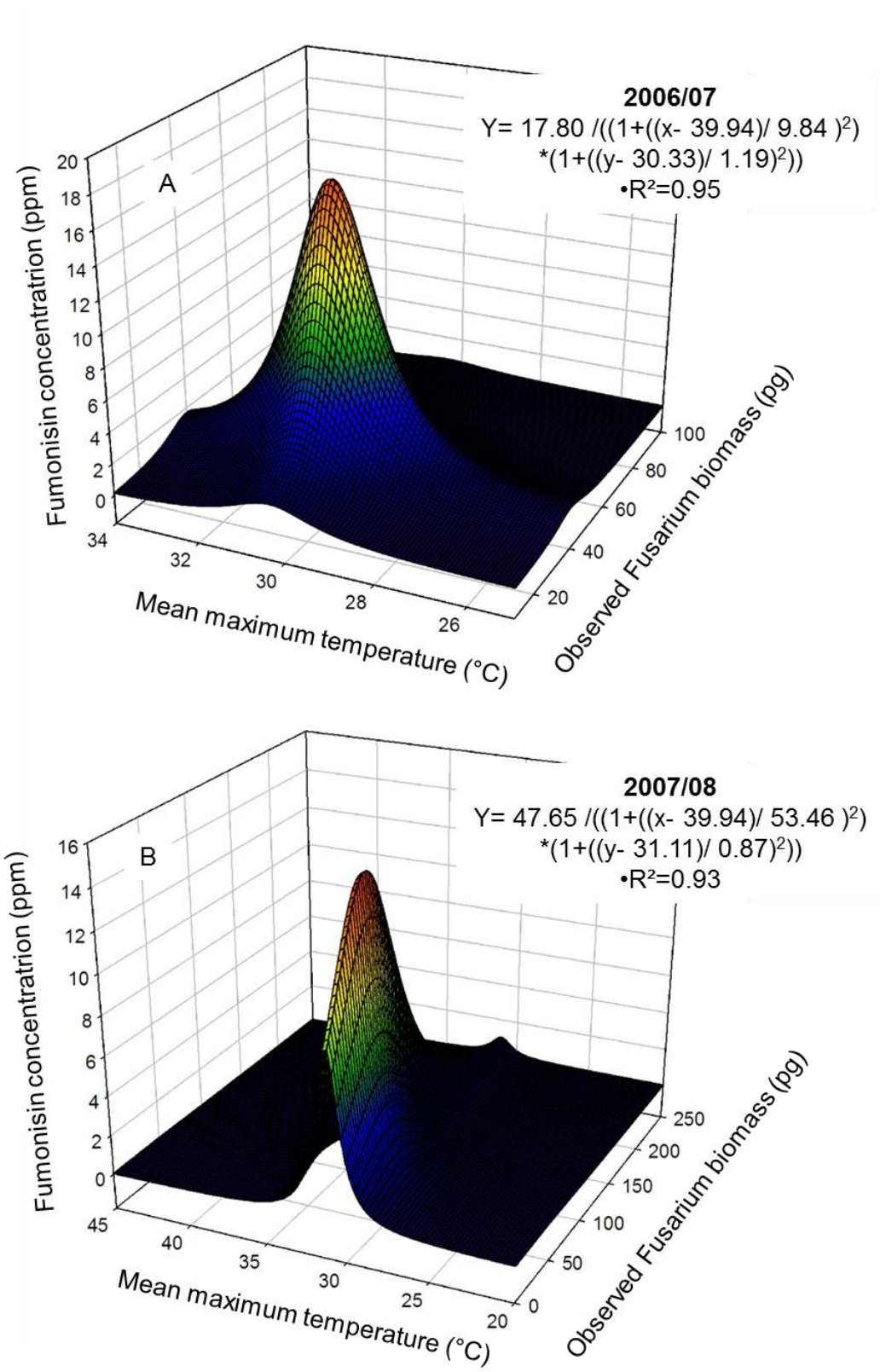


Figure 4.2a-b **Mean maximum daily temperature and observed fungal biomass for the 14 day dough stage period and their relationship with fumonisin synthesis in maize.**

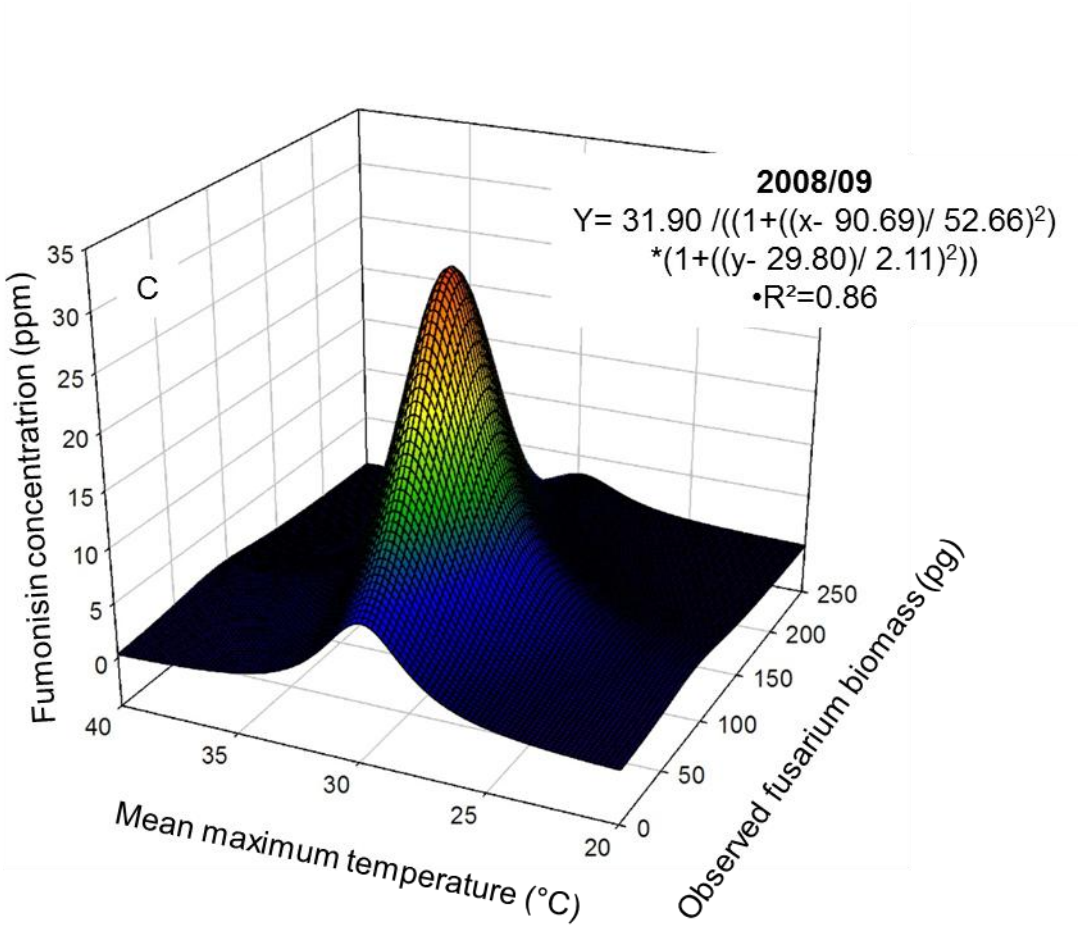


Figure 4.3 c Mean maximum daily temperature and observed fungal biomass for the 14 day dough stage period and their relationship with fumonisin synthesis in maize.

CHAPTER 5

Effect of a fungicide spray regime for foliar diseases on the incidence of fumonisin producing *Fusarium* spp. and fumonisin on selected maize cultivars.

ABSTRACT

Field trials were carried out in five maize production areas of South Africa to study the effect of an existing prophylactic fungicide regime for the control of foliar diseases on the infection of grains by fumonisin producing *Fusarium* spp. and fumonisin production. Azoxystrobin+difenoconazole (strobilurin, 200 g/l+ triazole, 125 g/l), was applied 40-45 days after planting followed by flusilazole+carbendazim (silicone triazole, 125 g/l+benzimidazole, 250 g/l) with petroleum as adjuvant 28-30 days later. Fumonisins were analysed using High Performance Liquid Chromatography (HPLC) and fumonisin producing *Fusarium* spp. were quantified by means of quantitative RealTime-PCR (qRT-PCR). Results from field trials showed that the natural colonisation of maize kernels by fumonisin producing *Fusarium* spp. and fumonisin contamination were high at Vaalharts and Greytown (4.23-10.54 ng and 11.57-32.19 ppm), moderate at Cedara (675.33 pg–3.51 ng and 2.31–7.43 ppm) and low at Potchefstroom and Buffelsvlei (61.63 pg–3.02 ng and 0.12-6.11 ppm). Anova showed no significant differences between sprayed and control treatments on colonisation of grain by fumonisin producing *Fusarium* spp. or fumonisin contamination. A cultivar x locality interaction was recorded. No significant differences were observed between Bt and non-Bt cultivars at Buffelsvlei, Potchefstroom and Cedara for total fumonisin, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3). No differences between Bt and non-Bt cultivars at Greytown was observed for total fumonisin, FB_1 and FB_2, however cultivars DKC80-12B and DKC78-15B had significantly lower concentrations of FB_3 than their isolines DKC80-10 and CRN3505. In contrast to this, total fumonisin, FB_1 and FB_3 from Vaalharts was significantly higher in DKC80-12B than the isoline DKC80-10, whereas these cultivars reacted similarly for FB_2. Further investigation is needed to evaluate the effect of different spray regimes on infection of maize kernels by *Fusarium* spp. and fumonisin contamination in South Africa. Application rates of these fungicides, phenological stage of the maize plant as well as stalk borer infestation also need to be taken into account.

Keywords: Fusarium ear rot, foliar fungicide, fumonisin

INTRODUCTION

Maize (*Zea mays L.*) is the most important crop in South Africa, with approximately 8 million tons produced annually on 3.1 million ha. of land under diverse environments (FAOSTAT data, 2009). Maize is susceptible to many fungal diseases, and the economically important diseases in South Africa are grey leaf spot (*Cercospora zeae-maydis*) (Crous *et al*., 2006), northern corn leaf blight (*Exserohilum turcicum*) (Craven & Morey, 2011), common rust (*Puccinia sorghi*) (Dunhin, 2001), Gibberella ear rot (*Fusarium graminearum sensu lato*) (Boutigny *et al.*, 2011), Fusarium ear rot (*Fusarium verticillioides* and *F. proliferatum*) (Marasas, 1996) and Diplodia ear rot (*Stenocarpella maydis*) (Flett & McLaren, 1994). Foliar diseases can be managed with agricultural practices such as the selection of resistant cultivars, crop rotation and tillage practices that reduce inoculum build up. Fungicides are also available to manage foliar fungal diseases and a number of registered products are available (Nel *et al*., 2003).

Since 2007 the use of fungicides in maize production has increased significantly due to an increase in the incidence of foliar diseases, as well as an increase in the market price of maize which makes fungicide sprays economically viable. New fungicides have recently been registered and extensively marketed (Wise & Mueller, 2011). Changes in crop production practices such as reduced tillage or no-till have increased the amount of maize residue, which in turn can serve as a source of primary inoculum for several important foliar diseases such as grey leaf spot, northern corn leaf blight and common rust (Lipps, 1987; Wise & Mueller, 2011). In the past maize producers relied upon low-input cultural methods (genetic resistance, crop rotation and tillage) to manage these diseases. They scouted their fields and applied fungicides only when diseases symptoms became visible. Today less emphasis is placed on selecting hybrids with high levels of disease resistance and greater emphasis is placed on planting hybrids with high yield potential (Wise & Mueller, 2011). The net result is an increased reliance on fungicides to control maize diseases, with fungicides now being applied

preventatively to maize crops (personal communication: Dr. Rikus Klopper, Plant Pathologist, PANNAR).

Strobilurin, or Quinone-outside inhibitor fungicides (Qol) are widely marketed in maize production for the management of biotic stresses, with the suggestion that these fungicides can increase yield (Hershman *et al*., 2011) even in the absence of disease (Wise & Mueller, 2011). In plants, Qol fungicides may increase water and nitrogen use efficiency, improve chlorophyll retention and delay senescence, thus lengthening the growing period. Ebelhar *et al*. (2010) conducted maize trials in Illinois from 2008 to 2010 and demonstrated that a foliar fungicide application (pyraclostrobin) reduced the incidence of stalk rot in trials where foliar disease pressure was high. Severe foliar disease during the reproductive stage of the maize plant can reduce the photosynthetic area needed by the plant to drive grain fill (Carson, 1999; Wise & Mueller, 2011) thus creating a source-sink imbalance that predisposes the host to root and stalk rots (Dodd, 1980). Nutrient imbalances, poor grain fill, increased stay-green status and the use of late maturity hybrids (Blandino *et al*., 2008) could lead to a major risk of mycotoxin contamination as literature has indicated that delayed grain maturation can give *F. verticillioides* more time to synthesise fumonisin (Maiorano *et al*., 2009).

D'Mello *et al*. (1998) reported that *in-vitro,* sub-lethal doses of carbendazim, tridemorph, difenoconazole and tebuconazole+triadimenol may increase 3-Acetyl DON and nivalenol produced by *F. culmorum* and T-2 toxin produced by *F. sporotrichioides*. Placinta *et al*. (1996) also reported that sub-lethal concentrations of carbendazim may stimulate the production of T-2 and other mycotoxins, but this was dependent on fungicide dosage and temperature. Falcão *et al*. (2011) found that fungicide additions (quintozene and fludioxonil+metalaxyl-M) to culture medium increased mean FB_1 production when compared to the control. In contrast, field studies have shown that some triazole fungicides, alone or in combination, reduced Fusarium head blight of wheat and deoxynivalenol accumulation in Italy (Blandino *et al*., 2008). Similarly De Curtis *et al*. (2011) reported a reduction in Fusarium ear

rot and fumonisin with fungicide application (tebuconazole, tetraconazole and prochloraz+cyproconazole) used in combination with an insecticide (2.5% lambda-cyhlothrin) in Mollise, Italy. On the other hand, although a partial control of Fusarium head blight of wheat has been reported (Matthies & Buchenauer, 2000; Edwards *et al*., 2001; Pirgozliev *et al*., 2002), an increase in deoxynivalenol production with the use of azoxystrobin based fungicides was observed. This was also confirmed by Mankevičienė *et al*. (2008) who reported winter rye plots sprayed with azoxystrobin to have higher deoxynivalenol and T-2 toxin than control plots. According to D'Mello (1998) difenoconazole which is effective against rust diseases is relatively persistent and residues may affect the secondary metabolism of other fungi.

F. verticilliodes and *F. proliferatum*, two economically important ear rot pathogens of maize in South Africa, can produce fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Rheeder *et al*., 2002). They are carcinogenic and are related to a wide range of animal and human health problems. Since no cultivars have been identified with resistance, Fusarium ear rot of maize in South Africa remains difficult to control (personal communication: Prof. B.C. Flett, ARC-GCI, Plant Pathologist). To date, no fungicides have been registered for the control of ear rots in South Africa and this study was aimed at determining whether fungicides applied prophylactically for the control of foliar diseases could have an additional advantageous effect by reducing Fusarium ear rot of maize and resultant fumonisin synthesis.

MATERIALS AND METHODS

Field trials

During the 2010-2011 planting season, randomised split plot experiments were conducted with three replicates at Buffelsvlei, Cedara, Greytown and Potchefstroom under dryland conditions. Vaalharts was flood-irrigated. A pre-emergence herbicide flumetsulam+S-metolachlor (triazolopyrimidine sulfon-anilide, 20 g/l+chloro-acetanilide, 630 g/l) was applied directly after planting at

the rate of 1.5 l/ha. No insecticides were applied. Main plot factors were two spray treatments (with and without fungicide) with seven cultivars, chosen from the National Cultivar Trials (Table 5.1) as sub-plot factors. Cultivars were selected to include both yellow and white maize varieties, Bt and non-Bt and regional adaptations. The fungicide treated main plot was sprayed with azoxystrobin+difenoconazole (strobilurin, 200 g/l+triazole, 125 g/l) at a rate of 500 ml/ha, 40-45 days after planting. This was followed 28-30 days later by a second spray of flusilazole+carbendazim (silicone triazole, 125 g/l+ benzimidazole, 250g/l) at a rate of 750 ml/ha with petroleum as adjuvant (500 ml/ha).

At maturity (12% moisture content) plots were hand harvested, threshed and one kilogram of grain was randomly collected for further analysis. Sub-samples of 250 g from all the 1 kg maize kernel samples collected were individually milled in a Cyclotech 1093 sample mill with a 1 mm mesh sieve. The mill was thoroughly cleaned with high-pressure air between each sub-sample to prevent cross contamination of mycotoxins from the different samples.

HPLC quantification of FB_1, FB_2 and FB_3

Fumonisins were quantified using HPLC analysis (Anonymous, 2002). A 50 g aliquot from each of the 250 g milled sub-samples, was mixed with 5 g of sodium chloride prior to extraction. Fumonisins were extracted in 100 ml methanol:water (80:20, v/v) for 5 minutes using an IKA T18 basic Ultra Turrax homogeniser. The extract was filtered through Whatman No.5 filter paper. A 10 ml aliquot was diluted with 40 ml phosphate-buffered saline (PBS). Diluted samples were extracted through microfiber filters (0.45 µm) and 10 ml of the diluted filtrate was passed through FumoniTest affinity columns (Vicam) at a flow rate of 1 drop per second. Ten ml of PBS were subsequently passed through the column at a rate of 1 drop per second. The column was then washed with 1.5 ml HPLC grade methanol at a rate of 1 drop per second and the eluate was collected in a glass cuvette. Methanol eluate was dried in a TurboVap LV (Caliper Sciences) with the aid of a slow stream of high purity

Nitrogen. Samples were dissolved in 200 µl methanol and purified HPLC-grade water (50:50, v/v). Each sample (50 µl) was transferred to 250 µl conical inserts. Each insert was placed into a 2.5 ml glass vial which was then placed into a HPLC carousel. The first position of the carousel had a 2.5 ml glass vial with OPA (*o*-phthaldialdehyde from Sigma) which was the derivitisation agent. The Waters 717 plus autosampler was set up to mix 100 µl of the OPA with the 50 µl of sample in the conical insert. From this mixture 20 µl were injected after a delay time of 1 minute.

Fluorescence detection was performed at excitation and emission wavelengths of 335 and 440 nm respectively using a Waters 2475 multi λ fluorescence detector. The analytical column, Symmetry C18, 5 µm 3.9 x 150 mm from Waters was used. Retention times using this method were 5 minutes for FB_1, 11 minutes for FB_3 and 13 minutes for FB_2. Total fumonisins were determined as the sum of FB_1, FB_2 and FB_3. The detection limit of the method used was 0.016 ppm . Recovery data were obtained in triplicate by fortifying clean maize samples (Vicam) with 5 ppm fumonisin B_1 B_2 and B_3. The average recovery rates were 83% (FB_1), 81% (FB_2) and 83% (FB_3).

qRT-PCR to quantify fumonisin producing* Fusarium *spp. from harvested grain.

The biomass of fumonisin producing *Fusarium* spp. was determined using qRT-PCR. MRC 826 (*F. verticillioides*) strain from the Medical Research Council–Promec Unit was used as the standard in the qPCR technique and DNA from this isolate was used to develop a standard curve (Waalwijk *et al*., 2008). A 0.5 g aliquot from each milled 250 g sub-sample was used to isolate and clean up DNA, using the method of Sambrook *et al*. (1989).

qRT-PCR reactions were performed in a MyiQ^{TM}2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten-halogen optical lamp. The primers Taqfum-2F and Vpgen-3R in combination with the FUM-probe 1 as tested by Waalwijk *et al*. (2008) were used in this study. The primer/probe set had the following nucleotide

sequence: Taqfum-2F, 5'-ATGCAAGAGGCGAGGCAA-3'; Vpgen-3R, 5'-GGCTCTCRGAGCTTGGCAT-3' and FUM-probe 1, 5'-/56-FAM/CAATGC CATCTTCTTG/36-TAMSp/-3'. For PCR, the sensimix reagent kit (sensimix^{TM} no ref QT 505-05) from Celtic (Bioline) was used. For each reaction 4 µl of the DNA sample was mixed with 12.5 µl sensimix, 2.125 µl Fum-probe 1 (1 µM), 0.875 µl Taqfum-2F (333nM), 0.875 µl Vpgen-3R (333nM) and 4.625 µl Melford molecular grade water. In all the experiments, negative controls containing no template were subjected to the same procedure to exclude or detect any possible contamination or carryover. The 96-well plate was incubated for 10 minutes at 95ºC and thereafter each of the 40 PCR cycles was performed according to the following temperature regime: 95 ºC for 10 s, 60ºC for 30 s, and 72ºC for 10 s. The Bio-Rad iCycler^{Tm} iQ Optical System Software Version 3.0a were used to calculate the biomass of fumonisin producing *Fusarium* spp. present in a sample. Regression equations of standard curves from runs were highly significant (R^2=>0.99). Slopes were within the acceptance criterion (between -3.1 and -3.6) and efficiencies ranged from 95 to 105%.

Statistical analysis

Data analysis was conducted using GenStat (2010), 13th edition. Data was analysed using split-plot ANOVA to determine the effect of main and sub-plot factors and their interaction on fumonisin B_1, fumonisin B_2, fumonisin B_3, and total fumonisin concentrations and fungal biomass. The residuals were tested for deviation from normality. In cases where deviation was significant, this was due to kurtosis and not skewness and we therefore, continued with the interpretation of the results (Glass *et al.,* 1972). Fisher's protected LSD (Least Significant Difference) was calculated at a 5% significance level to compare means of treatment effects. Linear regression analysis (Y=a+bx) was used to quantify the relationship between FB_1, FB_2, FB_3, total fumonisin and fungal biomass.

RESULTS

ANOVA indicated no significant difference between the sprayed and unsprayed treatments (main effects) on the biomass of fumonisin producing *Fusarium* spp. (qRT-PCR) and fumonisin contamination of grain (Table 5.2).

Significant locality main effects on total fumonisin, (P=0.003); FB_1, (P=0.001); FB_2, (P=0.022) and FB_3, (P=0.002) were recorded. Total fumonisins (Table 5.2) were the highest at Vaalharts and Greytown and lowest at Buffelsvlei. FB_1 and FB_2 followed a similar tendency being high at Vaalharts and Greytown while FB_3 was generally low at all localities. The percentage of fumonisin analogues (calculated from total fumonisin), FB_1, FB_2 and FB_3 ranged from 60.81-76.66%; 1.83-33.85% and 3.78-8.92% respectively (Table 5.3). The ratio of FB_1 and FB_2 was generally constant at all localities with the exception of Potchefstroom where FB_1 made up 76.66% of total fumonisin, with FB_2 only 1.83% of total fumonisin. Although total fumonisin was the lowest in Buffelsvlei compared to the other localities, FB_3 synthesis was the highest at this locality.

Significant ($P<0.05$) cultivar sub-plot effects on all fumonisin concentrations in grain were similarly recorded. Over all localities, mean total fumonisin (Table 5.2) was the highest in cultivar CRN3505 (11.15 ppm) and the lowest in LS8521B (6.25 ppm). FB_1 and FB_2 followed a similar tendency with FB_3 being slightly higher in PAN6P-110 (mean of 0.60 ppm) compared to the remaining cultivars (mean range of 0.41-0.49 ppm).

A significant ($P<0.05$) locality x cultivar interaction was recorded. Cultivars DKC80-12B and PAN6P-110 had the highest mean total fumonisins at Vaalharts and Greytown respectively but had significantly lower mean total fumonisin concentration at Potchefstroom, Buffelsvlei and Cedara.

At Potchefstroom, Buffelsvlei and Cedara no significant differences were observed in total fumonisin, FB_1, FB_2 and FB_3 between Bt and non-Bt cultivars. However, at Vaalharts, total fumonisin was the lowest in cultivar

LS8521B and the highest in cultivar DKC80-12B. FB_1 and FB_2 reacted similarly to total fumonisin with FB_3 being the lowest in DKC80-10 and the highest in DKC80-12B. DKC80-12B had significantly higher concentrations of total fumonisin, FB_1, and FB_3 than it's isoline DKC80-10 in Vaalharts.

In contrast, DKC80-12B followed by DKC78-15B had the lowest total fumonisin, FB_1, FB_2 and FB_3 concentrations at Greytown. PAN6P-110 and PAN6611 had the highest total fumonisin, FB_1, FB_2 and FB_3 concentrations. When comparing Bt and non-Bt, low total fumonisin in DKC80-12B and DKC78-15B was similarly low in isolines DKC80-10 and CRN3505. This was also observed for FB_1 and FB_2, although FB_3 was significantly higher in DKC80-10.

Correlation coefficients between FB_1, FB_2, FB_3 and total fumonisin were calculated. Total fumonisin correlated significantly with FB_1 (r=0.99), FB_2 (r=0.98) and FB_3 (r=0.94). FB_1 correlated significantly with FB_2 (r=0.97) and FB_3 (r=0.94). FB_2 correlated significantly with FB_3 (r=0.92).

Linear regression analysis was used to quantify the relationship between FB_1, FB_2, FB_3, and total fumonisin with fungal biomass as determined with qRT-PCR. Fungal biomass correlated significantly with FB_1 (P-value=0.000, R^2=78.77%), FB_2 (P-value=0.000, R^2=83.68%), FB_3 (P-value=0.000, R^2=73.62%) and total fumonisin (P-value=0.000, R^2=81.72%) (Figure 5.1).

DISCUSSION

The leaves of maize plants are vulnerable to infection by various fungal pathogens which in turn may have an impact on stalk integrity and yield loss due to downgrading of grain as a result of poor grain fill. It has been reported that grey leaf spot is capable of reducing maize grain yields by as much as 30-60% (Ward *et al*., 1996).

The aim of the study was to determine whether the current prophylactic fungicide regimes for foliar diseases could reduce the risk of colonisation of

grains by fumonisin producing *Fusarium* spp. and fumonisin contamination. This research showed no difference in the colonisation of grain by fumonisin producing *Fusarium* spp. and fumonisin concentrations between the control and fungicide spray regime. This lack of efficacy may be attributed to the timing of fungicide application relative to the plant growth stages critical to the infection of kernels by ear rot fungi. Azoxystrobin+difenoconazole was applied 40-45 days after planting which corresponds with the pre-tassling period while flusilazole+carbendazim was applied 28-30 days later i.e. soft dough stage. De Curtis *et al*. (2011) emphasised the importance of fungicide application at flowering when maize ears are the most vulnerable to infection by *Fusarium* spp. through the silks (Munkvold *et al*., 1997; Pascale *et al*., 2002; Duncan & Howard, 2010). The application of fungicides at flowering could be key in reducing airborne microconidia as an inoculum source for silk infection and subsequent fumonisin contamination and the timing of fungicides in the current study missed this critical point. Reports have indicated that sub-lethal doses of certain fungicides may increase mycotoxins such as fumonisin and T-2 (Placinta *et al*., 1996; Falcão *et al*., 2011). Fungicides applied in this study, did not elevate fungal or fumonisin concentrations, which is encouraging.

The current study re-iterated that ear rots caused by fumonisin producing *Fusarium* spp. can reach serious proportions in some commercial production areas of South Africa such as Vaalharts and Greytown. Mean total fumonisin ranged from 11.57-32.19 ppm at Vaalharts and 15.27-29.83 ppm in Greytown. These levels far exceed the 2 ppm for FB_1+FB_2+FB_3 intake by humans as set by the United States Food and Drug Association (Anonymous, 2001), a level used as a guideline in the absence of South African legislation. Fumonisin levels also exceeded recommended levels of 5 ppm for equines, pigs, rabbits and pet animals.

The strong interaction between cultivar and locality may be due to cultivar adaptation/behaviour under different environmental conditions. Cultivar responses across localities indicate that CRN3505 had the highest mean total fumonisin content with LS8521B having the lowest. At Potchefstoom and

Buffelsvlei cultivars LS8521B, PAN6P-110, DKC8-12B, DKC78-15B and DKC 80-10 had fumonisin concentrations of <2 ppm. Only PAN661 reacted differently with <2ppm in Potchefstroom and >4 ppm in Buffelsvlei. Potchefstroom and Buffelsvlei are situated approximately 30 km apart and have similar environmental conditions and this may explain the similar cultivar reactions. Similarly at Cedara the cultivars LS8521B, PAN6P-110, DKC8-12B, DKC78-15B are "grouped" together and had fumonisin concentrations of >2-4 ppm with the exception of DKC80-10 (>6-8 ppm). Cultivars reacted differently in Vaalharts and Greytown with unacceptably high fumonisin concentrations.

The absence of significant differences observed between Bt and non-Bt cultivars in Potchefstroom, Buffelsvlei and Cedara could be due to low populations of stalk borers, although this was not quantified. At Greytown cultivars DKC80-12B and DKC78-15B had significantly lower concentrations of FB_3 than their isolines DKC80-10 and CRN3505. In contrast to this, total fumonisin, FB_1 and FB_3 from Vaalharts were significantly higher in DKC80-12B than the isoline DKC80-10, whereas these cultivars reacted similarly for FB_2. Kruger *et al*. (2009) reported the presence of a stem borer population that is resistant to Bt maize at Vaalharts. This stem borer resistance may have contributed to a higher stem borer population and subsequently higher fumonisin concentrations (Munkvold, 1999; Wu, 2006) in DKC8-12B, however it does not explain why the isoline DKC80-10 had lower concentrations of FB_1 and FB_3. The presence of stem borers was not quantified in our study and need to be included in further studies to support the above-mentioned hypothesis.

The qRT-PCR method used to quantify colonisation of grain by fumonisin-producing *Fusarium* spp. correlated significantly with concentrations of fumonisin fractions (total fumonisin, FB_1, FB_2 and FB_3) found in the maize samples. Rheeder *et al*. (2002) reported that FB_1 typically accounts for 70% to 80% of total fumonisin produced, while FB_2 usually makes up 15% to 25% and FB_3, 3% to 8%. We found FB_1 to range from 60.81-76.66%; FB_2 ranged from 1.83-33.85% and FB_3 ranged from and 3.78-8.92% respectively. We

could not explain the low percentage of FB_2 from Potchefstroom and this should be addressed in further studies looking at isolate and environmental differences. In relation to this, we found that samples containing ≥18 pg fungal DNA/mg per sample of FB_1, (≥25 pg fungal DNA/mg per sample) of FB_2 and (≥52 pg fungal DNA/mg per sample) of FB_3 are suspected to exceed the limit of 2 ppm (Figure 5.1). This is also in line with the findings of Waalwijk *et al.* (2008) who reported samples that contained ≥40 pg fungal DNA/mg sample of fumonisin FB_1+FB_2 to exceed the 1 ppm total fumonisin level.

If prophylactic fungicide sprays for control of leaf diseases in maize could reduce maize ear rot diseases and their resultant mycotoxins these would add value by controlling both leaf diseases and mycotoxins. The possible use of fungicide sprays to reduce mycotoxins will only be beneficial where legislation ensures an economic advantage. In countries such as South Africa where mycotoxin legislation is restricted to aflatoxin and patulin (Rheeder, 2008), control of other mycotoxins would not be feasible due to a lack of financial incentive. The introduction of legislation regarding fumonisin in South African maize will challenge maize producers to adapt management practices to reduce *F. verticillioides* infection and resultant fumonisin production. Only then may the application of prophylactic fungicides for the control of foliar diseases become economically justifiable for the specific control of Fusarium ear rot and fumonisin synthesis.

To establish the justifiable use of possible additional fungicide applications for ear rot and mycotoxin control, one must determine the fungicide to be applied, the phenological growth stage for spray application as well as dose rate. The economics of an extra fungicide treatment must be justified by improved grain quality and mycotoxin reduction. We need to be pro-active in our research in order to assist farmers when legislation regarding fumonisin is introduced in South Africa.

REFERENCES

Anonymous, 2001. United States Food and Drug Administration guidance for Industry: Fumonisin levels in human foods and animal feeds; final guidance. [Available on Internet:] http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ChemicalContaminantsandPesticides/ucm109231.htm [Date of access: 13 September 2011].

Anonymous, 2002. Vicam fumonitest instruction manual. Vicam, L.P. 313 Pleasant Street, Watertown, MA 02472, United States of America.

Anonymous, 2011a. PANNAR product catalogue. [Available on Internet:] http://www.pannar.com [Date of access: 6 June 2011].

Anonymous, 2011b. Link Seed product catalogue. [Available on Internet:] http://www.linkseed.co.za [Date of access: 6 June 2011].

Anonymous, 2011c. Monsanto cultivar guide. [Available on Internet:] http://www.monsanto.co.za [Date of access: 6 June 2011].

Blandino, M., Corbellini, M., Scudellari, D. & Vanara, F. 2008. Effect of soil tillage, varieties and fungicide treatment on the deoxynivalenol contamination of soft wheat. *Journal of Plant Pathology* **90**: 71-76.

Boutigny, A.-L., Beukes, I., Small, I., Zûhlke, S., Spiteller, M, Janse van Rensburg, B.J. & Viljoen, A. 2011. Quantitative detection of *Fusarium* pathogens and their mycotoxins in South African maize. *Plant Pathology* **61**: 522–531.

Carson, M.L. 1999. Foliar diseases. Pp. 16-17. *In*: White, D.G. (Ed.). Compendium of corn diseases. Third Edition. APS Press, The American Phytopathological Society, Minnesota, USA.

Craven, M. & Morey, L. 2011. Characterisation of South African short season maize hybrids based on reaction to *Exserohilum turcicum* inoculation. *South African Journal of Plant & Soil* **28**: 163-171.

Crous, P.W., Groenewald, J.Z., Groenewald, M., Caldwell, P., Braun, U. & Harrington, T.C. 2006. Species of *Cercospora* associated with grey leaf spot of maize. *Studies in Mycology* **55**: 189–197.

De Curtis, F.D., De Cicco, V., Haidukowski, M., Pascale, M., Somma, S. & Moretti, A. 2011. Effects of agrochemical treatments on the occurrence

of Fusarium ear rot and fumonisin concentration of maize in southern Italy. *Field Crops Research* **123**: 161-169.

D'Mello, J.P.F., Macdonald, A.M.C., Postel, D., Dijksma, W.T.P., Dujardin, A. & Placinta, C.M. 1998. Pesticide use and mycotoxin production in *Fusarium* and *Aspergillus* phytopathogens. *European Journal of Plant Pathology* **104**: 741-751.

Dodd, J. L. 1980. Grain sink size and predisposition to *Zea mays* to stalk rot. *Phytopathology* **70**: 534-535.

Duncan, K.E. & Howard, R.J. 2010. Biology of maize kernel infection by *Fusarium verticillioides*. *Molecular Plant Microbe Interactions* **23**: 6-16.

Dunhin, B.J. 2001. Host-Pathogen studies of common rust of maize in South Africa. Magister Scientiae Agriculturae at the Faculty of Natural and Agricultural Sciences, Department of Plant Pathology, University of the Free State, Bloemfontein, South Africa.

Ebelhar, S.A., Hart, C.D. & Bradley, C.A. 2010. Corn insecticide seed treatment and foliar fungicide effects on corn response to fertilizer nitrogen. Online proceedings of the Illinois Fertilizer Conference, Department of Crop Science, University of Illinois.

Edwards, S.G., Pirgozliev, S.R., Hare, M.C. & Jenkinson, P. 2001. Quantification of trichothecene-producing *Fusarium* spp. in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied Environmental Biology* **67**: 1575-1580.

Falcão, V.C., Ono, M.A., de Ăvilo, M.T., Vizoni, E, Hirooka, E.Y & Ono, E.Y. 2011. *Fusarium verticillioides*: evaluation of fumonisin production and effects of fungicides on *in vitro* inhibition of mycelial growth. *Mycopathologia* **171**: 77-84.

FAOSTAT data 2009. Food and Agriculture Organization of the United Nation Databases. [Available on internet:] http://www.faostat.fao.org. [Date of access 08/02/10].

Flett, B.C. & McLaren, N.W. 1994. Optimum disease potential for evaluating resistance to *Stenocarpella maydis* ear rot in corn hybrids. *Plant Disease* **78**: 587-589.

GenStat (2010). VSN International, 13th Edition. VSN International, Hemel Hempstead, UK. Web page: GenStat.co.uk

Glass, G.V., Peckham, P.D. & Sanders, J.R. 1972. Consequences of failure to meet assumptions underlying the fixed effects analyses of variance and covariance. *Review of Educational Research* **42**: 237-288.

Hershman, D.E., Vincelli, P. & Kaiser, C.A. 2011. Foliar fungicide use in corn and soybeans. Plant pathology fact sheet PPFS-MISC-05, UK Cooperative extension service, University of Kentucky, College of Agriculture.

Kruger, M., Van Rensburg, J.B.J. & Van den Berg, J. 2009. Perspective on the development of stem borer resistance to Bt maize and refuge compliance at the Vaalharts irrigation scheme in South Africa. *Crop Protection* **28**: 684-689.

Lipps, 1987. Gray leaf spot epiphytotic in Ohio corn. *Plant Disease* **71**: 281.

Maiorano, A., Reyneri, A., Magni, A. & Ramponi, C. 2009. A decision tool for evaluating the agronomic risk of exposure to fumonisins of different maize crop management systems in Italy. *Agricultural Systems* **102**: 17-23.

Mankevičienė, A., Gaurilčikienė, I. & Supronienė, S. 2008. The infestation of winter rye and triticale grain with *Fusarium* fungi as affected by fungicide use. *Cereal Research Communications* **36**: 683-687.

Marasas, W.F.O. 1996. Fumonisins: history, world-wide occurrence and impact. *Advances in Experimental Medicine and Biology* **392**: 1-17.

Matthies, A. & Buchenauer, H. 2000. Effect of tebuconazole (Folicur) and prochloraz (Sportak) treatments on Fusarium head scab, yield and deoxynivalenol (DON) content in grains of wheat following artificial inoculation with *Fusarium culmorum*. *Journal of Plant Disease Protection* **107**: 33-52.

Munkvold, G.P. 1999. Comparison of fumonisin concentrations in kernels of transgenic Bt maize hybrids and nontransgenic hybrids. *Plant Disease* **83:** 130-138.

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

Nel, A., Krause, M. & Khelawanlall, N. 2003. A guide for the control of plant diseases (2nd ed.). Department of Agriculture, Republic of South Africa. Technical advice (Act No. 36 of 1974), Pretoria.

Pascale, M., Visconti, A. & Chelkowsky, J. 2002. Ear rot susceptibility and mycotoxin contamination of maize hybrids inoculated with *Fusarium* spp. under field conditions. *European Journal of Plant Pathology* **108**: 645-651.

Pirgozliev, S.R., Edwards, S.G., Hare, M.C. & Jenkinson, P. 2002. Effect of dose rate of azoxystrobin and metconazole on the development of Fusarium head blight and the accumulation of deoxynivalenol (DON) in wheat grain. *European Journal of Plant Pathology* **108**: 469-478.

Placinta, C.M., Macdonald, A.M.C., D'Mello, J.P.F. & Harling, R. 1996. The influence of carbendazim on mycotoxin production in *Fusarium sporotrichioides*. Pp. 415-416. *In*: Proceedings of the Brighton Crop Protection Conference. British Crop Protection Council, Farnham, UK.

Rheeder, J.P. 2008. Guidelines on the application of good agricultural practices (GAP) and the HACCP system in mycotoxin prevention and control in South Africa. South African National Health Department (Food Control Directorate), project reference number TCP/SAF/3001 (T).

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

Sambrook, J., Fritsch, E.F & Maniatis, T. 1989. Molecular cloning: A Laboratory Manual, Second Edition, Volumes 1, 2 and 3. Cold Spring Harbor Laboratory Press, New York.

Waalwijk, C., Koch, S., Ncube, E., Allwood, J., Flett, B.C., de Vries, I. & Khema, G.H.J. 2008. Quantitative detection of *Fusarium* spp. and its correlation with fumonisin content in maize from South African subsistence farmers. *World Mycotoxin Journal* **1**: 37-45.

Ward, J.M.J., Hohls, T., Laing, M.D. & Rijkenberg, F.H.J. 1996. Fungicide responses of maize hybrids to grey leaf spot. *European Journal of Plant Pathology* **102**: 765-771.

Wise, K. & Mueller, D. 2011. Are fungicides no longer just for fungi? An analysis of foliar fungicide use in corn. [Available on Internet:] http://www.apsnet.org/publications/apsnetfeatures/Pages/fungicide.aspx [Date of access: 10 October 2011].

Wu, F. 2006. Mycotoxin reduction in Bt corn: potential economic, health and regulatory impacts. *Transgenic Research* **15**: 277-289.

Table 5.1 Cultivars from the National Cultivar Trials used to study the effect of fungicide applications on colonisation of maize kernels by *Fusarium* spp. and fumonisin contamination.

	Cultivar	Grain colour	*Growth period	Wet/dry land	Adaptation (production area)	Yield Guard gene	Reference
	PAN6611	Yellow	medium	dryland	East	No	Anonymous, 2011a
	PAN6P-110	White	medium	dryland	East	No	Anonymous, 2011a
	LS8521B	White	medium	dryland	East	Yes	Anonymous, 2011b
Isolines	DKC80-10	Yellow	medium	dryland	East/West	No	Anonymous, 2011b
	DKC80-12B	Yellow	medium	dryland	East/West	Yes	Anonymous, 2011b
Isolines	DKC78-15B	White	medium	dryland	East/West	Yes	Anonymous, 2011b
	CRN3505	White	medium	dryland	East/West	No	Anonymous, 2011c

*Medium growth period = 130 days (West) to 145 (East) days until physiological maturity.

Table 5.2 Locality x cultivar interactions on total fumonisins, FB_1, FB_2 and FB_3 in maize kernels.

Locality/ cultivar	**Potchefstroom**				**Buffelsvlei**				**Vaalharts**				**Greytown**				**Cedara**				**Mean of total**
	Total	**FB_1**	**FB_2**	**FB_3**	**Total**	**FB_1**	**FB_2**	**FB_3**	**Total**	**FB_1**	**FB_2**	**FB_3**	**Total**	**FB_1**	**FB_2**	**FB_3**	**Total**	**FB_1**	**FB_2**	**FB_3**	
PAN6611	1.12	0.69	0.27	0.16	4.78	3.25	1.15	0.39	13.71	8.08	4.88	0.75	27.17	16.44	9.12	0.61	4.59	2.97	1.41	0.20	**10.27**
LS8521B	0.12	0.11	0.01	0.00	0.45	0.25	0.03	0.17	11.57	6.81	3.80	0.96	16.82	11.12	4.79	0.90	2.31	1.53	0.66	0.11	**6.25**
PAN6P-110	0.08	0.08	0.00	0.00	0.33	0.25	0.07	0.02	20.95	13.18	6.61	1.16	29.83	17.32	9.72	1.72	3.13	1.99	0.99	0.10	**10.86**
DKC80-10	6.11	4.30	1.40	0.41	1.87	1.25	0.47	0.15	21.89	12.44	8.24	0.21	17.58	10.65	5.84	1.09	7.43	4.67	2.58	0.19	**10.98**
DKC80-12B	0.09	0.07	0.01	0.01	0.63	0.49	0.17	0.06	32.19	20.20	10.21	1.78	13.97	9.15	4.32	0.50	3.28	2.4	1.04	0.11	**10.03**
DKC78-15B	0.64	0.45	0.17	0.03	1.06	0.72	0.27	0.07	24.60	15.85	7.48	1.27	15.27	9.53	5.15	0.60	2.82	2.21	0.52	0.09	**8.88**
CRN3505	4.46	3.96	0.42	0.08	2.67	1.67	0.85	0.16	26.22	15.38	9.98	0.86	18.23	11.71	5.41	1.11	4.15	2.77	1.13	0.24	**11.15**
Mean	**1.80**	**1.38**	**0.33**	**0.10**	**1.68**	**1.13**	**0.43**	**0.15**	**21.59**	**13.13**	**7.31**	**1.00**	**19.84**	**12.27**	**6.34**	**0.93**	**3.96**	**2.65**	**1.19**	**0.15**	
CV=78.1% and LSD=8.299 for total fumonisins (all localities and cultivars)																					
CV=76.1% and LSD=5.135 for FB^1 (all localities and cultivars)																					
CV=89.8% and LSD=3.027 for FB^2 (all localities and cultivars)																					
CV=89.9.1% and LSD=0.522 for FB^3 (all localities and cultivars)																					

Table 5.3 Mean percentage of fumonisin analogues in relation to total fumonisins at various localities in South Africa.

Locality/ fumonisin analogue	**Potchefstroom**	**Buffelsvlei**	**Vaalharts**	**Greytown**	**Cedara**
Total fumonisins (ppm)	1.80	1.68	21.59	19.84	3.96
*FB_1	76.66	67.26	60.81	61.84	66.92
*FB_2	1.83	25.59	33.85	31.95	30.05
*FB_3	5.55	8.92	4.63	4.69	3.78

*% of total fumonisins

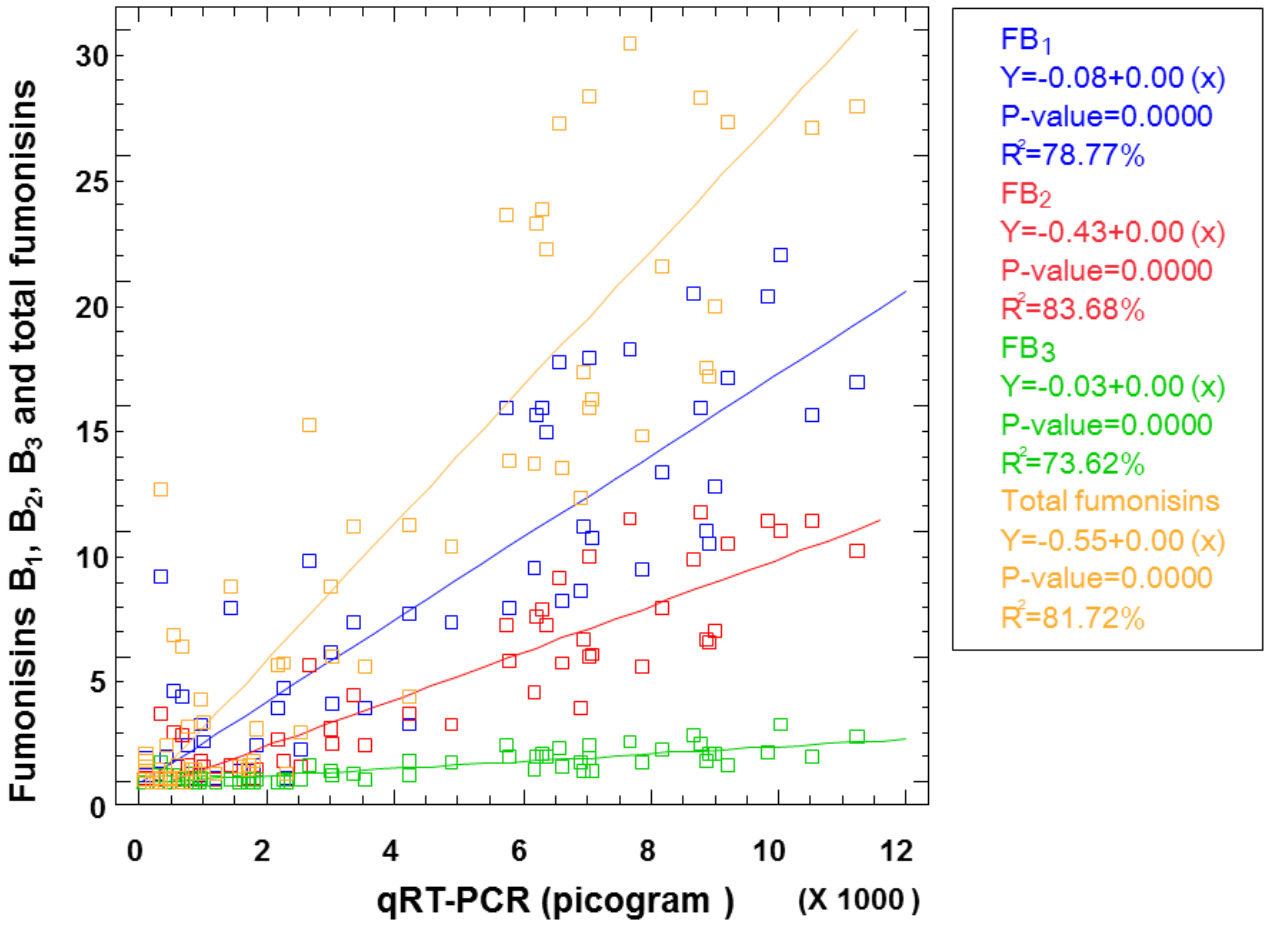


Figure 5.1 The relationship between *Fusarium* biomass, determined using qRT-PCR and FB_1, FB_2, FB_3 and total fumonisins.

SUMMARY

Maize and sorghum are important crops produced in South Africa with 8 million- and 125 000 tonnes being produced respectively, annually. Ear and head rot fungi can negatively affect yield and grain quality of these crops and also produce mycotoxins which negatively impact on human and animal health. This study focussed on the occurrence of aflatoxin producing *A. flavus* and *A. parasiticus* and fumonisin producing *F. verticillioides* and *F. proliferatum* from commercial maize and sorghum grain.

The natural occurrence of fumonisin producing *Fusarium* spp. and fumonisin contamination of maize was quantified in 29 maize production areas of South Africa over a three year period. Higher fungal biomass and fumonisin concentrations were associated with warmer production areas such as Northern Cape, North West and some areas of the Free State where the average temperatures ranged from 29°C to 32°C. In the cooler areas of Mpumalanga, KwaZulu-Natal, Gauteng and some areas of the eastern Free State, where mean maximum temperatures ranged from 24°C to 27°C, fungal biomass and fumonisin levels were absent or low. High fumonisin levels, in excess of 2 ppm recorded at 10 localities over the three year period, are of concern because of possible mycotoxicoses in animals and carcinogenic effects in humans. Corresponding high fungal biomass values may also indicate infection levels that may reduce yields and cause grain discoloration, physical breakdown of grain structure and reduction of grain nutritional value.

Maize cultivars differed in susceptibility to colonisation by fumonisin producing *Fusarium* spp.. This necessitated a better understanding of the role of environment and the physiology of differential responses of cultivars in relation to infection by fumonisin producing *Fusarium* spp. at different localities in order to identify maize production areas with a potential high/low risk of fumonisin synthesis. The qRT-PCR method used in this study quantified the biomass of fumonisin producing *Fusarium* spp. (pathogenic and/or endophytic) in maize kernels more accurately than the plating out method and will replace the plating out method in future research.

To determine the effect of aflatoxin producing *Aspergillus spp.* and fumonisin producing *Fusarium* spp. and their resultant mycotoxins on sorghum production in South Africa, sorghum grain samples were collected from five cultivars planted at 21 localities in South Africa from 2007-2009. HPLC and qPCR results indicate that *Aspergillus spp.* and *Fusarium* spp. and their mycotoxins do not pose a threat to sorghum production in South Africa.

Data from Chapter 2, together with meteorological data, were used in the development of a provisional epidemiological model to predict the risk of maize kernel colonisation by *Fusarium* spp. and fumonisin contamination. *Fusarium* colonisation of grain and fumonisin levels were related to prevailing weather conditions during early post-flowering and grain development stages, respectively. Both colonisation and fumonisin production were significantly inversely correlated with mean maximum temperature and minimum relative humidity during the critical growth periods. Our models were consistent regarding time of fungal infection and fumonisin production in each respective season (2007-2009), although it did not give consistent prediction values over seasons, which indicates variation that is not accounted for by the selected two weather variables. This is an on-going study and the continuous incorporation of data into this model should improve predictive values over seasons.

Since no cultivars/lines have been identified with resistance to Fusarium ear rot of maize in South Africa, the disease remains difficult to control. To date, no fungicides have been registered for the control of ear rots in South Africa and the potential of prophylactic fungicides, generally applied for the control of foliar diseases, to reduce Fusarium ear rot of maize and fumonisin synthesis was investigated. No significant differences between sprayed and control treatments on colonisation of grain by fumonisin producing *Fusarium* spp. or fumonisin contamination were recorded. This lack of efficacy may be attributed to the timing of fungicide applications relative to the plant growth stages critical to the infection of kernels by ear rot pathogens as determined from our epidemiological model. Further investigation into the possibility of using fungicides for the control of maize ear rots are being undertaken including time of application, application dosage and different fungicide regimes. Such applications must contribute to a reduction in maize leaf diseases as well as maize

ear rots and their resultant mycotoxins. Only when fumonisin legislation and incentives are introduced into South Africa, will these fungicide spray programmes be economically justifiable.

Robust, field-based models to predict fumonisin producing Fusarium ear rot in maize grain have been elusive due to the complexity of interactions between numerous abiotic and biotic disease factors. The findings in this study could contribute to an understanding of these complex interactions, thereby creating new management strategies to prevent or reduce the growth of *F. verticillioides* and *F. proliferatum* at field level as well as reducing contamination of grain with fumonisins.

Keywords: aflatoxins, *Aspergillus flavus*, epidemiology, fumonisins, *Fusarium verticillioides*, genotypes, HPLC, maize, qRT-PCR, sorghum.

OPSOMMING

Mielies en sorghum is belangrike gewasse in Suid Afrika en ongeveer 8 miljoen ton mielies en 125 000 ton sorghum word jaarliks geproduseer. Swamme wat kopvrotte van grane veroorsaak, kan lei tot ’n afname in opbrengs en graan kwaliteit. Verdermeer kan die swamme mikotoksiene produseer wat skadelik is vir mense en diere. Hierdie studie het gefokus op die voorkoms van aflatoksien-produserende *A. flavus* en *A. parasiticus* asook fumonisien-produserende *F. verticillioides* en *F. proliferatum* op komersiële mielies en sorghum.

Die natuurlike voorkoms van fumonisien-produserende *Fusarium* spp. en fumonisiene is in 29 mielieproduksie areas van Suid Afrika oor ‘n tydperk van drie jaar gekwantifiseer. Hoër swam biomassa en fumonisien konsentrasies is in warm produksie areas soos die Noord Kaap, Noord Wes en sekere areas van die Vrystaat waargeneem, waar gemiddelde maksimum temperature gevarieër het van 29ºC tot 32ºC. In die koeler areas van Mpumalanga, KwaZulu-Natal en Gauteng, asook sommige gedeeltes van die oos Vrystaat, waar die gemiddelde maksimum temperature gevarieer het van 24ºC tot 27ºC, was swam biomassa en fumonisien of nie waargeneem nie, of baie laag. Fumonisien vlakke bokant 2 dele per miljoen (dpm) in 10 van die lokaliteite (oor die drie jaar tydperk) is kommerwekkend omdat dit ‘n gesondheidsgevaar inhou vir diere en mense. Ooreenstemmende hoë swam biomassa kan ook lei tot oprengsverliese, venietiging van die graanstruktuur en dus ‘n verlaging in voedingswaarde van die graan.

Mielie kultivars het verskillend gereageer ten opsigte van kolonisasie van fumonisien-produserende *Fusarium* spp.. Daarom is dit belangrik om die rol van die omgewing te bepaal asook die fisiologie van onderskeie kultivars ten opsigte van infeksie deur fumonisien-produserende *Fusarium* spp. by verkillende lokaliteite. Sodoende kan mielieproduksie-areas ge-identifiseer word met potensiële hoë/lae risikos vir fumonisien produksie. Die qRT-PCR (“quantitative Real Time-PCR”) metode wat in die studie gebruik is om fumonisien produserende *Fusarium* spp. (patogenies en endofities) in mieliegraan te kwantifiseer, is meer akkuraat as die ou uitplaat metode en sal dus, in toekomstige navorsing gebruik word.

Sorghum graanmonsters is van vyf kultivars by 21 lokaliteite gedurende 2007-2009 versamel om die effek wat *Aspergillus*- en *Fusarium* spp. en hulle mikotoksiene op sorghum produksie in Suid Afrika het te bepaal. Mikotoksien data soos bepaal deur HPLC ("High Performance Liquid Chromatography") en swam biomassa soos bepaal deur qRT-PCR het getoon dat aflatoksien formende *Aspergillus* spp. en fumonisien formende *Fusarium* spp. nie 'n bedreiging vir sorghum produksie in Suid Afrika inhou nie.

Swam biomassa en fumonisien data van Hoofstuk 2 is geinkorporeer met weerkundige data om 'n epidemiologiese model te ontwikkel wat die risiko van mielie besmetting deur fumonisien-produserende *Fusarium* spp. en fumonisien kontaminasie kan voorspel. 'n Verhouding tussen *Fusarium* kolonisasie van graan en fumonisien vlakke met heersende weersomstandighede tydens die vroeë na-blom en graanvorming groeistadiums is waargeneem. Beide kolonisasie en fumonisien produksie was betekenisvol negatief ge-korreleerd met die gemiddelde maksimum temperatuur en minimum relatiewe humiditeit tydens hierdie kritiese groeistadiums. Die modelle was konsekwent rakende die groeistadiums van besmetting en fumonsien produksie vir die onderskeie seisoene (2007-2009). Die modelle was egter nie konsekwent rakende voorspellings waardes oor seisoene nie en dit dui daarop dat ander veranderlikes 'n rol speel wat nie deur die twee weerveranderlikes gedek word nie. Hierdie studie is steeds aan die gang en ons is tans besig om addisionele data te inkorporeer om voorspellings waardes oor seisoene daardeur te verbeter.

Geen kultivars/lyne is tot op hede in Suid Afrika ge-identifiseer wat weerstand bied teen Fusarium kopvrot van mielies nie. Dit bly dus moeilik om die siekte te beheer. Verder is geen swamdoders geregistreerd vir die beheer van kopvrotte in Suid-Afrika nie, en die potensiaal van profilaktiese swamdoder toedienings wat gebruik word vir blaarsiektes van mielies as beheermaatreël teen Fusarium kopvrot infeksie en fumonisien produksie, is ondersoek. Daar was geen betekenisvolle verskille tussen bespuite en onbespuite persele rakende *Fusarium* spp. kolonisasie van graan asook fumonisien produksie nie. Dit kan toegeskryf word aan die tyd van toedienings in verhouding met plant groeistadiums wat krities is vir besmetting van mielies deur kopvrotpatogene, soos deur die epidemiolgiese model bepaal. Verdere studies om

die tye van toediening, verskillende swamdoderprogramme asook swamdoder dosis te ondersoek, word benodig. Hierdie swamdoder-toedienings moet beide blaarsiektes en mielie-kopvrotte en hul gepaardgaande mikotoksiene, verminder. Slegs wanneer fumonsien wetgewing en gepaardgaande intensiewe in Suid Afrika bekendgestel word sal hierdie spuitprogramme ekonomies geregverdigbaar wees.

Tot op hede is daar geen kragtige veld-gebasseerde model wat die voorkoms van Fusarium kopvrot en fumonisiene effektief kan voorspel nie, as gevolg van komplekse interaksies van talle abiotiese en biotiese faktore. Die bevindinge in hierdie studie kan bydra om hierdie komplekse interaksies beter te verstaan, en ons in staat te stel om beheer strategieë te ontwikkel om die besmettingsvlakke van fumonisien-produserende *Fusarium* spp. te voorkom of te verminder in die veld en dus ook kontaminasie van graan met fumonisiene.

Sleutelwoorde: aflatoksiene, *Aspergillus flavus*, epidemiologie, fumonisiene, *Fusarium verticillioides*, genotipes, HPLC, mielies, qRT-PCR, sorghum.