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Resistance of maize cultivars against the infestation of mycotoxigenic fungi

By

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Dedication:

For my wonderful Mum,

Thank you for all that you do and everything that you are to me.

Declaration:

I declare that the thesis which I hereby submit at the University of Pretoria for the award of the degree MSc (Microbiology) is my work and has not been submitted by me for a degree to any other university or institution of higher education.

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List of Abbreviations

AFB1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
AIDS	Acquired Immune Deficiency Virus
ARC	Agricultural Research Council
BEN	Balkan endemic nephropathy
CO_2	Carbon Dioxide
CTAB	Cetyltrimethylammonium Bromide
DEB	DNA Extraction Buffer
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DON	Deoxynivalenol
EDTA	Ethylenediaminetetraacetic acid
EF1 α	Elongation factor 1 α
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	Food and Agricultural Organization
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2

FB_3	Fumonisin B_3
FDA	Food and Drug Administration
HACCP	Hazard Analysis Critical Control Point
HIV	Human Immunodeficiency Virus
ITS	Internal Transcribed Spacer Regions
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kGy	kiloGray
LD_{50}	Lethal dose needed to kill 50% of the test animals
LEM	Leucoencephalomalacia
MSA	Malt Salt Agar
NaCl	Sodium Chloride
NDA	National Department of Agriculture
OTA	Ochratoxin A
PCNB	Pentachloronitrobenzene agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphisms
rRNA	Ribosomal ribonucleic acid
SACU	South African Custom Union
SAGL	South African Grain Laboratories
TAE	Tris-acrylamide-EDTA
TBE	Tris-borate –EDTA
USA	United States of America

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Preface:

Maize is the staple food of South Africa and its cultivation covers the largest area of farmland in the country. It plays an important role in the economy as South Africa is consuming 10 million tons of maize per annum. Most South Africans consume maize in some form or another. This commodity has regularly been associated with mycotoxigenic fungi and in some cases their respective mycotoxins. The problem is not only confined to the borders of South Africa but is a concern worldwide.

Mycotoxins are known to affect both human and animal health. Some of the most well known mycotoxins are the fumonisins, deoxynivalenol and trichothecenes produced by *Fusarium* spp., aflatoxins and ochratoxin A produced by mostly *Aspergillus* spp., as well as patulin and citrinin that are produced by mainly *Penicillium* spp. Mycotoxins acting together and individually can be hepatotoxic, carcinogenic and teratogenic to humans and animals.

The main objective of this study was to screen commercially produced maize cultivars in South Africa that would be either resistant to, or have a slower infection rate when inoculated with the ten selected mycotoxigenic fungi from South African maize. The objective was also to develop a method to detect and identify these mycotoxigenic fungi in the infected maize cultivars, using both basic microbiological and molecular means. These objectives were achieved by a series of experiments that are outlined in the individual chapters.

The first part of this study evaluated the level of infestation of fungi in all the commercially produced maize cultivars in South Africa. A basic fungal enumeration and identification was carried out. This allowed the comparison of the *in vivo* ability of the selected cultivars to endure the natural invasion of mycotoxigenic fungi during cultivation in the same area namely Potchefstroom.

As part of the maize evaluation, the cultivars were artificially inoculated with ten selected mycotoxigenic fungi, which consisted of five field fungi nl. *Alternaria alternata, Fusarium graminearum, Fusarium verticillioides, Phoma sorghina* and *Stenocarpella maydis,* and five storage fungi nl. *Aspergillus flavus, Aspergillus ochraceus, Eurotium repens, Penicillium islandicum* and *Rhizopus oryzae* under storage conditions. The ability of certain maize cultivars to resist the infestation by mycotoxigenic fungi under storage conditions was demonstrated.

The second part of this study was to use basic molecular methods to detect and identify the ten mycotoxigenic fungi in the infected maize. This was done by making use of the sequence variations of the internal transcribed spacer (ITS) and D1/D2 regions of the fungal rRNA gene. Results showed that the ITS region gave better differentiation and in most cases allowed identification of the mycotoxigenic fungi.

The application of the combined use of microbiological and molecular methodology for the detection and identification of mycotoxigenic fungi in maize by testing laboratories in South Africa were outlined in the final chapter. Implementation of a plan to evaluate maize cultivars in the pre-planting, harvesting and storage phases, can provide a holistic overview of the commodity and can be further implemented in the rural areas.

Chapter 1:

Mycotoxigenic fungi and their mycotoxins in maize in South Africa: a review

1. Introduction:

A wide variety of crops are produced in South Africa, of which maize is regarded as the most important (NDA, **2004**). This is due to the use of maize as a source of food for both humans and animals. Maize is cultivated on the largest area of available farmland, followed by wheat and, on a smaller scale, oats, sugar cane and sunflowers (NDA, **2004**). The influence that maize production has on the economy and food security is thus significant, both in the formal agricultural sector and among subsistence farmers. South Africa is the main maize producer in the South African Custom Union (SACU) with an average production of approximately 9.0 million tons per annum. It is estimated that more than 8000 commercial farmers produce the major bulk of South African maize while the rest is produced by many thousands of small-scale producers (South African Government, **2009c**).

In South Africa, maize is normally produced in areas where the rainfall exceeds 350 mm per year (Bennie & Hensley, **2001**). However, production is dependent on an even distribution of rain throughout the growing season to be able to produce good quality maize. The more erratic the rainfall, the more unstable maize production becomes. Dry land production of maize mainly takes place in the Free State (38%), Mpumalanga (23%) and North West (12%) provinces (South African Government, **2009a**). Planting time of maize varies between October and December, and from the eastern to the western production areas. This is dependent on the rainfall pattern, temperature and the duration of the growing season.

The total area that was planted in South Africa for the 2008/2009 production season was 2 427 500 ha of which 1 489 000 ha was white maize and 938 500 ha was yellow maize. Final crop yield in total was 12.7 million tons of which 7.5 million tons was white maize and 5.2 million tons was yellow maize (Crop Estimates Committee, **2009**). Approximately 7.5 million tons (4.4 million tons white maize and 3.1 million tons yellow maize) comprises the total

consumption requirements of South Africa, and the surplus maize is usually exported (NDA, **2004**).

This review focuses on the role that fungi and their mycotoxins play in the quality and safety of South African maize. Various aspects such as environmental conditions, transportation and storage of maize are discussed here due to their influence and contribution towards fungal contamination and mycotoxin production during pre- and postharvest conditions.

2. Maize quality in South Africa

It is estimated that all South Africans consume maize in some form or another due to the use of maize as an ingredient in many different food products (maize meal, grits, cornflakes, snacks) (Sydenham *et al.,* **1991**). The majority of South Africans consume maize meal as their staple food, which is popular as a beneficial porridge called 'pap' or 'braaipap'. Many other products such as pharmaceuticals, confectionary, toothpastes, popcorn, soups, snacks, and sweets include maize in various forms (Tongaat Hulett, **2010**). Maize, consumed by livestock and poultry also end up in the human food chain through meat products, dairy products, cheeses and eggs. The quality of maize in South Africa has thus a direct impact on the health of humans and animals who consume maize products on a regular basis.

The ecological adaptation of maize varieties, available from public breeding programs in eastern and southern Africa, reflects the characteristics of local production environments. About 5% of all varieties released in eastern and southern Africa have been developed for temperate cultivation (Hassan *et al.,* **2001**). White maize is mainly processed for human consumption (60%) while yellow maize is used for livestock consumption (40%) (South African Government, **2009d**).

There are many different varieties of maize cultivated in South Africa. These can be categorized based on certain characteristics of the maize kernel such

as the type of endosperm, pericarp, crown appearance and texture of the endosperm. These characteristics determine what the maize can be used for. For example, floury maize has a thin pericarp, slightly dented crown and soft endosperm, which makes the kernel ideal for human consumption. Table 1 (pg. 51) shows the different types of kernels that are used.

Floury maize is most commonly grown in South Africa, as the kernels are normally used for dry milling and preparation of traditional products, such as homemade beer. This type of maize is also well adapted to grow in dry areas (Salunkhe *et al*., **1985**). Floury kernels are soft when dry and have the advantage of being suitable for hand-grinding. However, due to the high lysine content, kernels tend to become more infected by fungi such as *Fusarium verticillioides* on the mature ears in wet areas, leading to crop loss even before harvesting (Warren, **1978**).

Maize processing in South Africa is heavily biased towards dry milling rather than wet milling, because mainly products such as pap and maize meal are used as staple foods for a majority of the country's population. However, a larger percentage of the annual production of maize in South Africa is used for animal feed (South African Government, **2009d**).

The maize kernel can be dry milled to produce a coarse maize meal or fine flour, which can be used in a variety of ways. In Africa, for example, they are used to make cooked paste which can either be fermented or used as is. Dough is made from the flour by adding water. The dough is then used for preparing unleavened bread.

Several maize properties that play an important role during the dry milling process are influenced during the plant growth period. Even before harvesting, variations in climatic conditions can influence the degree of maturity and moisture content of the maize kernels. This can contribute to insect or fungal infestation, even at this early stage.

In South Africa, natural drying occurs where the maize is left in the field. When the kernels are dried to the appropriate dryness (~13-14%), the maize is harvested. This usually occurs around June/July. In South Africa, rain is sometimes experienced in the months prior to harvesting, which necessitates that the maize be left in the field to dry for longer periods. Due to the rewetting of the maize and, hence, longer period of drying, fungal and insect infestation can occur. If unusually high temperatures are experienced for too long during the drying period, minute cracks on the surface of the kernel can cause shattering of the endosperm into pieces with a particle size below the miller's requirements. If, on the other hand, the initial moisture content of the grain is too high, incomplete separation in degerming, and improper breaking of endosperm particles, can result (FAO, **2004**; Hassan *et al.*, **2001**).

Once this happens, small particles accumulate in the milling system and thus allow fungal development within the facilities and equipment. This, eventually, leads to contamination of the products. It is known that, to date, at least 188 toxigenic fungal species (see Table 2, pg. 52) are associated with foods and feeds in South Africa (Rabie & Marais, **2000**).

Fungal contamination and damage can occur during storage and during processing (Figure 1, pg. 67). There are different stages in the processing pipeline that have diverse effects on the maize kernels before they reach their final destination of consumption either by humans or by livestock. Firstly, maize is produced in the field by farmers where natural damage occurs in the maize. This is normally caused by insect and fungal infestation in the field under natural conditions.

Once the maize is deemed ready for harvesting, pre-processing occurs. The maize is stored in silos after which it is processed by removing kernels from the cob, trimming and milling.

During transporting, damage to the kernels may occur either by bruising or by breaking. Further spoilage can occur if the maize is transported for long distances, such as on ships or between inland and coastal areas. It is known,

for example, that the migration of moisture within the maize can take place if day and night temperatures fluctuate significantly. During the warm days, the moisture is in a damp form, but during the cooler nights the moisture condensates in areas that are the coolest. These include areas where the surfaces of the containers are exposed to cooler temperatures on the outside. This eventually results in moisture pockets to form within the commodity. It is, therefore, not unusual to have specific areas within the same batch of maize where the moisture content is above 14% and other areas where it is below.

During storage, insects, fungi, bacteria, rodents and birds may spoil or cause damage to the maize kernels. Spoilage during storage can reduce both feeding and market value of the maize (Salunkhe *et al.,* **1985**) and can lead to serious economic and health hazards (FAO, **2004**). Higher moisture can also lead to sprouting of the kernels during storage, depending on the storage conditions. In addition, rancidity and over-ripening of the kernels can also be experienced.

During the pressing and packaging stages, maize kernels may also be damaged through excessive peeling, trimming and polishing. If the final moisture content of the maize is above 14%, fungal growth and damage can continue, even after packaging and distribution.

Quality assessments of maize in South Africa are done by South African Grain Laboratories (SAGL), who is appointed by the Maize Trust for this purpose. SAGL is the only ISO/IEC 17025 accredited laboratory to conduct quality analyses for defective kernel identification on whole maize kernels (S. du Preez, personal communication, **2009**).

The quality standards of maize can be divided into 3 classes namely, White maize, Yellow Maize and Other Maize. Each class is based on the colour of the endosperm of the maize kernel, which is then further graded into 3 sub-grades. White Maize, for example, is subdivided into WM1, WM2 and WM3, whereas Yellow Maize is subdivided into YM1, YM2 and YM3. Maize that falls

under Other Maize has no sub-grades (Government Gazette No. 32190, **2009**).

For the purpose of law in South Africa, the quality of maize need to conform to the following (Government Gazette No. 32190, **2009**):

a) Maize should not have an off-odour of being musty or sour.
b) Maize should not have any particles such as glass, metal, coal or dung.
c) Maize should not contain any additives that make it unsuitable for human and animal consumption or for further processing.
d) There should not be insects in the maize.
e) There should not be any particles like stones that can be sieved through a 6,35 mm sieve.
f) There should not be any poisonous seeds or unwanted grains than what is permitted in Foodstuffs, Cosmetics and Disinfectants Act no. 54, 1972 (Government Gazette No. 26849, **2009**).
g) Maize should have a moisture content of 14% and less.
h) The commodity should not exceed the limits of deviation for the standards for grades of Class White Maize and Class Yellow Maize.

To determine whether the maize meets each of these standards to be legally graded, each requirement is either assessed sensorially or analyzed chemically where possible. Therefore, maize kernels that may be infected with mycotoxigenic fungi, but do not show visual signs of damage, could be passed as being of good quality and ending up in the human food chain. Those kernels that do show visual fungal growth or discolorations associated with fungal growth are removed. No microbiological analyses are carried out on the maize kernels (W. Louw, personal. communication, **2009**).

In South Africa, there are two diverse sectors that produce maize. One sector is the production of commercial maize and forms the major bulk of maize production. The other sector is subsistence farming that focuses mainly on

the cultivation of maize for personal or local use. Due to these farmers not having access to fertilizers, pesticides and equipment; sub-standard quality maize is produced. Such maize is prone to fungal decay and insect damage. The consumption of these products influences the health of humans and animals. An additional burden is put on the health of the people in these areas due to the likely consumption of mycotoxins. In South Africa, it is also these rural areas that are greatly affected by HIV and AIDS. Due to the effect of mycotoxins on the immune system, makes the presence of mycotoxins in the main diet of these people all the more risky.

3. Fungi and mycotoxins associated with maize

Mycotoxicoses refer to diseases of humans and animals that are caused by the ingestion of foodstuffs contaminated with mycotoxins. The latter are mainly secondary fungal metabolites that have the ability to negatively influence human and animal health. These are low molecular weight chemical compounds, which are not necessarily detected by antigens and, hence, produce no obvious symptoms initially. This is why mycotoxins are sometimes referred to as insidious poisons (Pitt, **1989**). Mycotoxin contamination of forages and cereals frequently occurs in the field due to the infestation of plants by particular pathogenic fungi or symbiotic endophytes. Contamination may also occur during processing and storage of harvested crops when environmental conditions are favourable for the growth of spoilage fungi. Based on the FAO (**2004**), the mycotoxins that are most important globally are aflatoxins, ochratoxin A, patulin, fumonisins, zearalenone and some trichothecenes such as deoxynivalenol.

Aflatoxins are the most widely known and studied mycotoxins, and are produced by a number of *Aspergillus* species, of which *A. flavus* and *A. parasiticus* are the more important ones. In maize, *A. flavus* is mostly associated with the production of aflatoxins, with aflatoxin B_1, B_2, G_1, and G_2 being regarded as problematic. Of these, aflatoxin B_1 is carcinogenic to the liver and is seen as the most important mycotoxin in this group. It is regulated

in food commodities destined for human consumption by many countries all over the world with maximum levels ranging between 4 and 20 µg/kg (FAO, **2004**). This mycotoxin is known to be converted to aflatoxin M_1 when ingested by cattle, and eventually can end up in milk (Bhat & Vasanthi, **2003**). In South Africa, aflatoxins are not associated with locally produced maize, although imported maize from countries such as Argentina and the USA could contain these mycotoxins.

Ochratoxin A (OTA) is produced by both *Aspergillus* and *Penicillium* species. This mycotoxin is carcinogenic and damages the liver and kidneys (see Table 3, pg. 55). It is a toxin most commonly found in maize, barley and wheat, ranging across northern and central Europe, Canada and South East Asia (JECFA, **2008**). This toxin is also known to be found in meat products (Pitt *et al.*, **2000**) in cases where animals have consumed contaminated feed. The maximum tolerated levels laid down by countries for human consumption range from 3 to 50 µg/kg. The LD_{50} value (lethal dose needed to kill 50% of the test animals) ranges from 0.5 mg/kg for dogs to over 50 mg/kg for mice (Fung & Clark, **2004**).

Fumonisins are seen as the more important mycotoxins associated with maize, especially in South Africa. They are produced by *Fusarium* species such as *F. verticillioides* and *F. proliferatum*. Thess mycotoxins are known to cause leucoencephalomalacia in horses, mules and donkeys, as well as liver damage in rats and lung disorders in pigs. In addition, fumonisins are associated with oesophageal cancer in humans, especially in the Eastern Cape Province of South Africa (Marasas *et al.,* **1988**). Levels of fumonisins legislated by countries ranges between 1000 and 3000µg/kg (FAO, **2004**).

Zearalenone is a mycotoxin mainly produced by *Fusarium graminearum*. This toxin can be found in maize but can also be found in wheat, barley and rye. It is known to have an estrogenic effect in animals and can cause infertility, abortions and other breeding problems in pigs (see Table 3, pg. 55). Zearalenone is regulated in 16 countries worldwide and ranges between 50 and 1000 µg/kg (FAO, **2004**).

Trichothecenes form an important group of mycotoxins that are mainly produced by *Fusarium* species. From this group, deoxynivalenol (DON) and T-2 toxin are best known. Deoxynivalenol causes vomiting, feed refusal and decreased weight gain in farm animals (see Table 3, pg. 55). The limits laid down by countries for deoxynivalenol ranges between 300 and 2000 µg/kg (FAO, **2004**). T-2 toxin causes dermatological, gastrointestinal and neurological symptoms. The oral LD_{50} value of T-2 toxin is 5200 µg/kg in rat and mouse (Oancea & Stoia, **2008**).

Depending on the level of exposure, mycotoxins can either be acutely or chronically toxic to humans and animals (Küiper-Goodman, **1998**). Health effects of mycotoxins may include immunological effects, organ-specific toxicity, cancer and in some cases, death. Agricultural workers are also at risk for dermal and respiratory exposures during crop harvest and storage (Jeebhay, **2002**).

Crops can be contaminated by mycotoxins at three phases including pre-harvest, during harvesting and drying, or during storage and processing (Sauer *et al*., **1992**) The influence of each of these phases to mycotoxin contamination depend on a number of factors such as the specific crop, type of fungi and mycotoxins involved, and environmental conditions. Growth of fungi may begin in the field, increase during the harvesting and drying operations and continue accumulating in storage.

The most critical factors affecting mycotoxin production in grains are moisture content and temperature. For example, as summarised in Table 4 (pg. 64) (Wilson & Abramson, **1992**), for mycotoxin formation to occur in the field, the physical conditions required include moisture, temperature and mechanical damage. Chemically, mineral nutrients, carbon sources and nitrogen sources need to be available for fungal growth and mycotoxin formation. The biological factors that are needed include plant stress to allow the fungus to easily infect the plant without triggering the plant defence mechanism. Invertebrate vectors play an important role in the distribution of fungal spores

to ensure neighbouring plants to be infected. In addition, the fungal strains must be able to produce mycotoxins under existing conditions. All these factors acting together at optimum can assist the fungus to infect and colonise the plant, and produce mycotoxins.

4. Field and Storage Fungi:

The terms field and storage fungi, were first used by Christensen & Kaufmann (**1974**) who divided the fungi that occur on grain into two groups based on their ecological preferences. This was primarily based on their moisture content requirements. A summary of some key elements that differentiate between the field and storage fungi can be seen in Table 5 (pg. 65) (Martin, **1974**). Field fungi are generally known to persist within the plant and seeds whereas the storage fungi attack the seeds from the outside of the plant. Biochemical changes occur in fruits and seeds of plants infected by storage fungi whereas the field fungi produce no significant changes.

Under storage conditions, both field and storage fungi can produce mycotoxins. Field fungi can and do produce mycotoxins while in the field and in some instances are seen as being more relevant than the mycotoxins produced by the storage fungi. One example can be given in maize, fumonisins (mycotoxin produced by field fungi) are regarded as the most important followed by DON and zearalenone (also produced by field fungi) whereas in storage, mycotoxins produced by the *Penicillium* species (storage fungus) are seen as being more important although not much is known about the occurrence of these mycotoxins.

The field fungi associated with maize include mainly species of *Alternaria, Stenocarpella, Cladosporium, Fusarium, Phoma* and *Helminthosporium*. These fungi are mostly plant pathogens and normally invade kernels just before or during harvesting. Characteristic of the field fungi is their requirements for high moisture contents in order to grow and propagate (22 to 25% on wet weight basis or 30-33% on dry weight basis) (Christensen &

Kaufmann, **1965**). Effects caused by these fungi when kernels are invaded include discolouration, loss of germinability, shrivelling, seedling blight, root rot or other diseases when seeds are planted. Field fungi tend to lose their viability in grains at water activities below 0.75 during storage, although some can survive and stay dormant for years in dry grain (Tomkins, **1929**; Pitt & Christian, **1968**; Gock *et al.*, **2003**).

Storage fungi mainly include saprohytic fungi consisting of *Aspergillus* and *Penicillium* species. They normally grow at water activities ranging from 0.65 to 0.9 in the absence of free water. This is a special biological niche to which they have become adapted to in co-existence with the development of mass production of grain in agriculture. Different species of *Aspergillus* within this group have different and sharply delimited moisture content ranges at which they can grow (Sauer *et al.*, **1992**). For example, *A. chevalieri* grows at 0.65 whereas *A. niger* grows at 0.85 water activity. Some species of *Penicillium* and *Aspergillus* are field fungi whereas others are storage fungi (Christensen & Kaufmann, **1974**) (e.g. *Penicillium oxalicum* and *Penicillium funiculosum*). Even some species such as *Aspergillus flavus* can either act as a field fungus (maize produced in the USA) or a storage fungus (South African maize). Overall, storage fungi are not known to invade crops before harvesting due to their saprophytic nature.

A wide variety of both field and storage fungi are associated with South African maize (see Table 6, pg. 66) (Rabie & Marais, **2000**). However, there are only a few that are regarded as playing a significant role in the production of mycotoxins in South African maize. For example, the presence of fumonisins and other *Fusarium* toxins are seen as a major concern. In addition, a wide variety of *Penicillium* species are also associated with South African maize, although little has been done to date to determine the significance of the presence of *Penicillium* toxins in this commodity. There is also a lack of knowledge regarding the presence of fungi and their mycotoxins during the storage and milling processes of South African maize.

Classifying fungi either as field or storage fungi should be done with caution. The role that environmental conditions, climatic change, agricultural practices, maize varieties and fungal adaptation plays can influence the ability of fungi to colonize crops before, during and after harvesting. The types of maize varieties used in South Africa differ from those used elsewhere in the world. For example, South Africa experiences relatively low rainfall, hence the varieties of maize used here need to be fairly drought resistant. Due to the fact that South Africa is regularly confronted with changing rainfall patterns, so does the infestation and extent of fungal damage also sometimes differ from year to year. For example, the effect of El Ninõ in the 1999/2000 season caused a delay in the harvesting of maize due to excessive rain (South African Government, **2009b**). It can then be established that due to the higher rainfall experienced, the drying period in the field had to be extended to reduce the moisture content in the maize. Due to this set back, unusually high levels of fungal infestation where experienced in the crop for that season, making the crop high risk for mycotoxins to be present.

Agricultural practices can also play a significant role in the ability of mycotoxigenic fungi to invade crops such as maize. Crop rotation, for example, can result in residual fungi from the previous crop to invade newly planted maize (Lipp & Deep, **1991**). *Fusarium graminearum* is a fungus that can attack wheat and maize, resulting in the presence of deoxynivalenol and zearalenone in these crops. For example, the excessive use of fertilizers and continuous irrigation can also favor the development of mycotoxigenic fungi (FAO, **2004**). Irrigation creates a favorable environment for those fungi that need high moisture contents to flourish. In addition, subsistence farmers do not use the sophisticated agricultural practices as commercial farmers and, therefore, produce a sub-standard maize product that is prone to fungal and mycotoxin contamination.

Fungi that develop and propagate during storage can originate either from the field or from the surrounding environment during the storage process. Invasion by fungi before harvesting is primarily driven by plant host-fungus and other biological interactions (e.g. insects). On the other hand, growth by

post-harvest fungi is influenced by the crop (available nutrients), physical (temperature, moisture) and biotic (insects, interference competition) factors (Miller, **1995**). It is, therefore, important to take these factors in consideration when preventative measures are needed to control mycotoxin contamination.

4.1. Field fungi:

A systemic relationship between field fungi and their hosts exists. A good example of this systemic relationship with the maize plant has been demonstrated in *Fusarium verticillioides* (Agrios, **1997**). For the plant host to become diseased it must first come in contact with the fungus. In addition, conducive environmental conditions are needed to facilitate the attack of the fungus onto the plant host. The interaction between these three factors is called the "disease triangle" and is directly dependant on the number of characteristics influencing each factor (see Figure 2, pg. 68).

The mechanism by which a plant recognizes the attack from a fungal pathogen is still unclear. When a plant is attacked by a fungus, it is assumed that the fungus first comes into contact with a plant host cell, which triggers a fairly rapid response in both organisms. Depending on the ability of resistance of the plant, the fungus either actively continues or fails with its attack (Agrios, **1997**). The extent of fungal infestation in the plant is thus dependant on the resistance mechanism used by the plant, whether it is genetically or physiologically driven.

It is generally accepted that the greater the degree of infestation in a food commodity, the greater the risk of mycotoxins to be present. The main factors influencing the development of toxins in natural products are seen as:

a) The suitability of the substrate for fungal decay and supporting microbial growth,

b) High moisture content (14% and higher in maize) due to high rainfall or high humidity;

c) Stress conditions suffered by fruits and seeds before or during harvesting or storage;

d) Duration of drying e.g. the shorter the drying period the lower the risk for fungal infestation;

e) Method of harvesting e.g. minimal physical damage to the harvest limits the ability of fungi to penetrate through the outer layers of the kernels;

f) Temperature e.g. mycotoxigenic fungi grow optimally at certain temperatures, but the formation of their mycotoxins can occur at a different temperature. For example, results indicate that aflatoxin formation takes place at relatively high temperatures between 28 to 32°C, whereas the fusarial toxins are produced at low or freezing temperatures (-7 – 25°C), and a cycle of alternate freezing and thawing can greatly enhance the formation of mycotoxins (Martin, **1974**). In addition, the interaction and competition between fungi for available nutrients can also influence the level of mycotoxigenic fungi and mycotoxin formation in food commodities such as maize (Lee & Magan, **2000)**. Marin *et al.* (**1998**) have done studies on the interaction and competition between *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. The interaction between *Aspergillus flavus* and insects has been studied by Cardwell *et al,* (**2000**). Velluti *et al.,* (**2000**) focused their studies on the competition and interaction of *Fusarium proliferatum, Fusarium graminearum, Fusarium moniliforme* and the formation of fumonisin B_1 and zearalenone.

4.2 Storage fungi

There are a number of factors influencing the ability of fungi to invade maize during storage. Some are given below:

4.2.1. Available nutrients

When looking at the structure and composition of a maize kernel, there are areas that are more prone to fungal invasion than others (see Figure 3, pg.69). For example, the maize kernel consists of the pericarp or hull, which encloses the kernel; the starch section consists of the horny (hard) and floury (soft) endosperm; and the germ (embryo) that contains a high proportion of oil (4.5% w/w). The endosperm, the largest portions of the kernel represents about 82.3% of the weight of the grain and consists largely of the starch along with the gluten, which is the bound protein (9.4%). The germ represents 11.5% of the total weight and contains the maize vegetable oil. The hull or pericarp represents about 5.3% and the pedicel or tip cap about 0.8% of the kernel (Hoseney & Faubion, **1992**).

According to Mclean and Berjak (**1987**), the availability of nutrients plays an equal or perhaps more significant role than that of water, at least for the species of *Aspergillus* in maize. *Eurotium* species and *A. flavus* have shown to be able to inhabit the same commodity. Members of *Eurotium* are able to inhibit the growth of *A. flavus* to some extent, but eventually are dominated by the more aggressive *A. flavus.* Both species are able to use simple sugars for their growth but once the substrate sugars have run out, *A. flavus* is able to switch to a different nutrient source whereas *Eurotium* species are not able to do the same. The developmental age of maize kernels also plays a role in the production of mycotoxin in maize in that mycotoxin production depends on the moisture content (freshly harvested maize) compared to stored maize (dried maize) (Warfield & Gilchrist, **1999**).

4.2.2. Moisture content

Storage fungi do not all grow optimally at the same moisture content, and the minimum moisture content recorded to allow fungal growth was 13.5% (Tipples, **1995**). As moisture content increases, so too does fungal growth (Hoseney & Faubion, **1992**). Maize kernels are able to lose or gain moisture,

depending on the water vapour in the air surrounding it. Depending on the humidity in the air, if exposed to dry grain, the grain is able to absorb the moisture until equilibrium is reached between the grain and the surrounding environment. Likewise, if moist grain is exposed to dry air, the grain is able to lose moisture until equilibrium is reached (Brook, **1992**).

When maize is stored in a silo, the temperature is not necessarily homogenous throughout the facility. Some areas may be exposed to a higher temperature (maize closer to the wall of the silo) whereas the other maize (at the centre of the silo) would be exposed to a lower temperature during daytime. In this respect, the moisture from the kernels evaporates and increases the humidity between the spaces of the kernels. When the temperature cools down, the moisture condenses onto the maize kernels and is absorbed. This results in moisture pockets developing inside the commodity where certain areas can have high enough moisture to allow fungal growth (de Lima, **1990**).

4.2.3. Temperature:

Temperature is an imperative factor in the growth and development of fungi. Through the evolutionary development of fungi, an ability was established among fungi to grow at a wide range of temperatures. Fungi can basically be divided into four groups based on their preferences to grow at different temperatures.

Psycrophilic fungi grow at temperatures between -5^0C and 15^0C e.g. *Humicola marvinii*, isolated from fell fields in Antartica (Weinstein *et al.*, **1997**); *Cladosporium herbarum, Mucor* sp.*, Phoma* sp.*, Penicillium roqueforti* isolated from frozen chicken pies (Kuehn & Gunderson, **1963**). Psychrophilic fungi are usually found in the arctic and Antarctic regions (Robinson, **2001**).

Psychrotrophic fungi grow at 20^0C and higher e.g. *Phoma herbarum* (Robinson, **2001**) and *Verticillium lecanii* (Fenice *et al.*, **1998**).

Mesophiles grow between 5 and 10^{0}C with a maximum above 25^{0}C. Mesophilic fungi are the type of fungi most ubiquitous in nature (Kendrick, **2000**) e.g. *Alternaria alternate, Aspergillus ochraceus, Penicillium islandicum* (Anastasi *et al.*, **2002**); *Aspergillus flavus, Fusarium* sp. isolated from fruit filled pastries (Kuehn & Gunderson, **1962**).

Thermophilic fungi are able to grow between 20^{0}C to 50^{0}C e.g. *Chaetomium thermophile* (Tansey, **1972**)*, Talaromyces thermophilus, Thielavia terrestris* (Maheshwari *et al.*, **2000**); *Byssochlamys* sp. (Davis *et al.*, **1975**).

Thermotolerant fungi grow from 20^{0}C but have an optimum temperature at about 55^{0}C e.g. *Aspergillus fumigatus* (Davis *et al.*, **1975**); *Aspergillus candidus, Paecilomyces varioti* (Wareing, **1997**) from maize.

Mycotoxigenic fungi are mostly mesophilic e.g. *Alternaria alternata, Aspergillus flavus, Aspergillus ochraceus.* Although there are some fungi that are thermophilic e.g. *Talaromyces thermophilus, Acremonium halabemensis, Mucor pusillus, Sporotrichum thermophile, Byssochlamys* sp. (Davis *et al.*, **1975**). *Aspergillus fumigatus* is regarded as being thermotolerant (Davis *et al.*, **1975**).

Temperature and moisture together largely determine the length of safe storage life. Other conditions, such as proportion of kernels initially infected by fungi and the degree to which they are infected (Bailey, **1992**) also play a part in the duration of storage. Previous storage conditions (Magan & Lacey, **1984)**, cleanliness and soundness of grain; insect and mite infestation; and age, interacting with the other conditions also determine the duration of stability (Christensen & Qasem, **1960**).

Temperature and moisture have a direct relationship. For example, according to Sanchis & Magan (**2004**), *Aspergillus flavus* and *Aspergillus parasiticus* are able to grow optimally at higher temperatures (35^{0}C) and lower water activity (0.95), whereas aflatoxin production occurs optimally at a lower temperature

(33^{0}C) and higher water activity (0.99). When looking at *Fusarium verticillioides* and *Fusarium proliferatum*; the relationship between temperature and moisture is different from that of *A. flavus* and *A. parasiticus*. Regarding temperature for growth, *F. verticillioides* and *F. proliferatum* grow between 4-37^{0}C with an optimum at 30^{0}C and a water activity of 0.90. For fumonisin production in each of these fungi the required temperature range is 10-37^{0}C with an optimum between 15-30^{0}C. The optimum water activity is 0.93.

The temperature needed for fungal growth on the one hand and mycotoxin production on the other could differ substantially. According to Schindler *et al.,* (**1967**), *Aspergillus flavus* grows optimally at a temperature between 29 and 35^{0}C, although the maximum aflatoxin production is at 24^{0}C. Another example is shown by Martins & Martins (**2002**) and Jimenez *et al.* (**1996**) where the production of zearalenone by *Fusarium graminearum* is dependent on the temperature and the incubation period in maize. Zearalenone was produced at a higher level when incubated at 28^{0}C for 16 days followed by 12^{0}C for about 20 days.

Fungi grow slowly, and some not at all, below 10^{0}C, but can cause serious damage at 29^{0}C under favourable moisture conditions. The temperature inside a mass of grain can change spontaneously by respiration as it is an excellent insulator. Cereal grains produce water, carbon dioxide and heat during respiration. As water content and temperature increases, when cereals are stored below 14% moisture at 20^{0}C, so too does respiration (Pomeranz, **1992**). Due to the different storage structures used to store grain, the temperature of the maize varies. For example, grain next to the steel wall of a bin exposed to the sun may reach 45^{0}C during the day, but the temperature of the grain that is 1.22 m away from the wall would remain unchanged. Overheating in a grain mass may proceed undetected for substantially long periods and completely destroy small amounts of grain (Sharma, **1989**).

Temperatures are seldom uniform throughout any bin or lot. When cold grain is added to a bin of warm grain, moisture moves up into the cold grain. A bin

of cold grain next to a bin of warm grain causes condensation on the wall of the warm bin (Wicklow *et al.*, **1998**). The moisture contents of the kernels increase, when cold grain is exposed to warm air outside. During artificial cooling, alteration of moisture contents is likely.

Insects and bacteria can also have an effect on maize kernels in storage, which have a direct relationship with temperature. The infestation of the insect and its subsequent metabolism in the commodity causes moisture pockets to form due to the temperature gradients that develop. These moisture pockets create ideal conditions for fungi to develop in the commodity. Grain infesting insects are highly sensitive to temperature (Sukprakarn, **2004**). Their optimal temperature is about 29^0C and at this temperature their life cycles may be as short as 30 days.

Bacteria are unable to grow in environments where there is no free water available. In stored grain, water availability is at a minimum. Water becomes more available as spoilage fungi increase the temperature in the grain. Once this happens, thermophilic bacteria are able to become involved in the spoilage process, but by then the grain is most likely spoiled beyond the use for human and/or animal consumption (Christensen & Kaufmann, **1974**).

Generally, mycotoxins are stable compounds and are not easily destroyed by general cooking processes. One of the techniques that is applied to reduce mycotoxin levels is the use of high temperatures. It is generally noted that the higher the temperature, the greater the reduction in mycotoxin levels although still unable to completely eliminate them (Bullerman & Bianchini, **2007**). There are some mycotoxins that are quite thermostable like fumonisin B_1 (Dupuy *et al.*, **1993**). A review by Humpf *et al.* (**2004**) has shown that the milling and cleaning processes of maize is able to remove a portion of the fumonisin. Extrusion also reduces the mycotoxin by a fraction, but the uncertainty of the reduction lies in whether it was achieved by the heat or by the binding of the mycotoxin to another substrate in the process. Fumonsins are fairly stable at a temperature of 100-120^{o}C although the increase of heat during the cooking process causes a reduction of the mycotoxin due to the interaction of pH,

duration, temperature, sugar and water content. Although the cooking process causes the breakdown of fumonisin, it is still able to retain its biological activity (Munkvold & Desjardins, **1997**). Aflatoxins are resistant to processing and cooking (Wild & Gong, **2010**). According to Boudra *et al.,* (**1995**) the complete detoxification of ochratoxin A in wheat was not achieved by the heating treatment. A review by Amézqueta *et al.* (**2009**) has shown that ochratoxin A can be reduced in the different procedures in the making of beer, extrusion of breakfast cereal manufacturing and baking of cakes.

4.2.4. Spoutlines

A spoutline is the cone that develops at the top of the bin where the lighter material of the maize forms the core and the heavier denser material fall to the sides of it (Hoseney & Faubion, **1992**). They occur at the top of the bin load and are formed as grain falls into the container or bin. A vertical core results of which, the center is a solid mass. This almost completely prevents any air circulation between the inner and outer stored kernels of the slope. This leads to the accumulation of heat and prevents the escape of any heat that may be produced by the activity of fungi, insects, mites or other causes. Grain in the core turns from brown to black, moisture condensation appears on the surface and eventually heating spreads throughout the bin. These often shorten storage life considerably.

5. Identification of mycotoxigenic fungi

The identification of mycotoxigenic fungi rely on both microbiological and molecular methods. Most fungal cultures are identified by their morphological characteristics by using stereo microscopy, light microscopy and various compendiums and reference books based on fungal identification (Murray *et al.,* **1995**). Due to the time constraints for fungal structures to develop, it is not always possible to identify fungi to species level.

To determine which mycotoxigenic species can be found on maize, sometimes molecular methods are required in cases where fruiting structures are absent. Previous work that has been done by Jurado *et al.* (**2006**) was to detect the contamination of mycotoxigenic *Fusarium* species in maize based on molecular techniques. Bruns & Shefferson (**2004**) showed that internal transcribed spacer regions (ITS) are used to detect different fungal species. What makes the ITS region so popular is its highly conserved priming sites that make it easier to amplify from virtually all fungi. ITS regions are stretches of DNA between the 18S, 5.8S and 28S rRNA regions (White *et al.*, **1990**). There is also the added advantage of the growing ITS sequence data that helps in identification of the various fungi. Use of this information can also allow the development of species specific primers to detect certain fungi in a much shorter period than using morphological means (Mulé *et al.*, **2004**).

6. Effect of fungi and their mycotoxins on humans and animals

There are a diversity of mycotoxins that exist with different affects in humans and animals. Their mechanisms of toxicity, origins, chemical structures and pathways of synthesis are all different (Pitt *et al.*, **2000**). For example, the trichothecenes group, are a structurally similar group but are able to produce a wide range of toxic effects. These mycotoxins are produced by *Fusarium* species, *Trichoderma* species and *Cephalosporium crotocinogenum* (Wijnands & Van Leusden, **2000**). For example, looking at deoxynivalenol, in man it causes acute toxicity, vomiting, and irritation to the respiratory tract. In animals, it causes immunotoxicity in pigs and mice, stunted growth in pigs and chickens, and causes damage at a genetic level to mice (Rotter *et al.*, **1996**).

South African research over the last four decades has contributed significantly to the global understanding of mycotoxins and their affect on humans and animals. Examples include the discovery of ochratoxin A produced by *Aspergillus ochraceus* by Steyn *et al.* (**1965**); the isolation and identification of aflatoxin M_1 and M_2 by Holzapfel *et al.* (**1966**). Marasas *et al.* (**1976**)

discovered that leucoencephalomalacia (LEM) in horses was caused by a mycotoxin produced from *Fusarium verticillioides* in homegrown maize. In **1977**, Marasas *et al.* first reported the natural occurrence of zearalenone and deoxynivalenol, a 12, 13-epoxytrichotechene, in South Africa. Aucock *et al.* (**1980**) showed the effect of maize infected with *Fusarium graminearum* and contaminated with zearalenone on pigs.

Rabie *et al.*(**1985**) identified the first mycotoxin, rhizonin, which is produced by the zygomycetous fungi belonging to the genus, *Rhizopus*. Bezuidenhout *et al.* (**1988**) discovered fumonisins which is regarded as very important group of mycotoxins worldwide in maize. In **1988**, Marasas *et al.,* provided evidence that there is an association between *Fusarium verticillioides* and oesophagal cytological abnormalities in living individuals in the Eastern Cape area.

Mycotoxins are intricate raw compounds that are quite stable at room temperature. Some of the major mycotoxins are aflatoxins, deoxynivalenol, fumonisins, ochratoxin A, patulin and zearalenone.

Aflatoxins have been associated with the Turkey X disease in England in **1961** (Wannop). Aflatoxins consist of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 (Fung & Clark, **2004**). Aflatoxin B_1 is the most potent metabolite out of all, although the severity of the effect of the mycotoxin differs in animals regarding their breed, dose, age, species, nutritional well-being and length of exposure (Richard, **2007**). They are able to cause liver cancer in animals (Marasas *et al.,* **2008**) and in humans (Kuiper-Goodman, **1995**). The biological mode of action is that aflatoxins are able to stoutly attach to DNA and RNA. By doing this it is able to impede DNA replication or transcription of RNA into proteins, thus resulting in liver necrosis (Mishra & Das, **2003**). In a ruminant (cow), aflatoxin B_1 is changed into aflatoxin M_1 and transferred to milk (Hayes, **1980**) which can be carcinogenic. The European Union (EU) limit of aflatoxin B_1 is 2 µg/kg and total aflatoxins is 4 µg/kg (Richard, **2007**), whereas the LD_{50} value is 7.2 mg/kg in male rats (Hayes, **1980**). When comparing aflatoxins it is estimated that aflatoxin B_1 is twice as toxic as aflatoxin G_1.

Fusarium graminearum mostly produces deoxynivalenol. It is a trichothecene commonly known as vomitoxin or DON, by (Richard, **2007**). When ingested, this toxin causes reduced feed intake by swine, it is immunosuppressive and it causes kidney problems. In humans, similar effects as in animals are experienced (D'Mello *et al.*, **1999**). The effect of deoxynivalenol on pigs varies according to age, sex and source of contamination (Rotter *et al.*, **1996**). Prelusky *et al.,* (**1987**) have shown that deoxynivalenol is able to be transmitted to eggs via oral administration of the mycotoxin, whereas Rotter *et al.,* (**1996**) showed that cows are not affected by deoxynivalenol. In fact, the cows were able to detoxify in 24 hours. Deoxynivalenol is able to inhibit protein synthesis by interfering and stopping DNA and RNA synthesis (Rotter *et al.,* **1996**). The LD_{50} value for deoxynivalenol in mice range from 49-70 mg/kg orally (Forsell *et al.*, **1987**) and for broiler chickens it is 140 mg/kg (Huff *et al.*, **1981**).

Bezuidenhout *et al.,* (**1988**) discovered and characterized fumonisins that are produced by *Fusarium verticillioides.* Fumonisin B_1 is the most copiously produced fumonisin (Kumar *et al.*, **2008**) and is linked to high incidences of esophageal cancer in Transkei (Sydenham *et al.*, **1990**). According to Gelderblom *et al.* (**1996**), fumonisins are hepatotoxic and has a carcinogenic effect in rats. It also causes porcine pulmonary edema, severe mortality in broiler chickens (Javed *et al.*, **1993**) and duodenitis/proximal jejunitis in horses older than 2 years (D'Mello *et al.*, **1999**). The widely known affect of fumonisins is leucoencephalomalacia in horses, mules and donkeys (Marasas *et* al, **1976**). In humans, birth defects can be caused by consumption of fumonison B_1 by pregnant woman (Hendricks, **1999**). Fumonisins interfere with sphingolipid metabolism (Merrill *et al.*, **2001**) that can result in liver disease and tumors in the liver and kidneys (Richard, **2007**). This mycotoxin is fairly thermostable (Kuiper-Goodman, **1995**). The different strains of *Fusarium* differ in the amount of toxin produced (Richard, **2007**). Another study conducted by Richard *et al.,* (**1996**), shows that there is no carry over of the toxin from the cow into the milk. As yet there is no known LD_{50} value for fumonisins (Moss, **2000**).

Aspergillus ochraceus, A. carbonarius and *Penicillium verrucosum* are able to produce ochratoxin A (OTA) (Miller, **1995**; Sweeney & Dobson, **1998**) and are produced exclusively in storage. Ochratoxin A is nephrotoxic (Pfohl-Leszkowicz, **2007**), hepatotoxic, teratogenic in lab animals and carcinogenic to single stomach animals (Sherif *et al.*, **2009**), which makes cattle more resistant to naturally contaminated maize (Miller, **1995**). The toxicity of ochratoxin A is dependent on animal species, sex and the manner in which it is administered (Pfohl-Leszkowicz, **2007**). Ochratoxin A is the key cause of Balkan endemic nephropathy (BEN) and is related to urinary tract tumors (Hult *et al.*, **1982**). In high concentrations, ochratoxin A can cause damage to the liver, as it has a slow elimination process from the body and can build up in body tissues and fluids (Richard, **2007**). Ochratoxin A has been found in breast milk and can be passed on to babies who are being breast-fed (Jonsyn *et al.*, **1995**). This toxin has an elevated affinity for plasma protein and is able to hinder protein, DNA and RNA synthesis (Fung & Clark, **2004**). The LD_{50} value of ochratoxin A via oral administration is 0.2 mg/kg body weight in dogs to 30.3 mg/kg in male rats (Pfohl-Leszkowicz, **2007**).

Patulin is produced most commonly by *Penicillium expansum* and some *Aspergillus* species. The mycotoxin causes congestion and edema of pulmonary, hepatic and intestinal blood vessels and tissues (Fung & Clark, **2004**). Patulin has an immunosuppressive affect and hinders DNA synthesis (Sharma, **1993**). The LD_{50} value of patulin ranges from 15-25 mg/kg with different animals, likely due to the different ways the toxin was administered (Fung & Clark, **2004**).

Fusarium graminearum and *F. culmorum* are the major producers of zearalenone (Miller, **1995**). This mycotoxin mostly affects swine by causing infertility, abortions and other breeding problems (Kumar *et al.*, **2008**). The major effects of zearalenone are estrogenic and affect the urogenital system (Siame & Nawa, **2008**) as it binds to estrogenic receptors and results in hormonal changes (Richard, **2007**). The LD_{50} value of zearalenone is 20-100 g/kg body weight in mice (D'Mello *et al.*, **1999**).

Usually these mycotoxins can cause devastating health affects in different organ systems in humans. There is a relationship between consuming mycotoxin contaminated food and infirmity such as hepatic, gastrointestinal and carcinogenic disease. A variety of mycotoxicoses can occur such as superficial skin disease (e.g. tinea) to invasive organ pathology (e.g. pulmonary aspergillosis) (Fung & Clark, **2004**).

Mycotoxins are able to work interactively to produce different toxic affects in humans and animals. Owing to the diverse configurations of the mycotoxins and their diverse mechanisms of action, they can produce additive, synergistic or antagonistic reactions. According to Riley (**1998**) deoxynivalenol and other trichothecenes work together to suppress immune response to pathogens in animals. A literature review conducted by Speijers & Speijers (**2004**) shows that ochratoxin A and citrinin can act together to produce synergistic, additive and antagonistic affects in different animals. Regarding the interaction of mycotoxins, further studies are needed.

In third world countries, were food is scarce, children are distinctly vulnerable to be exposed to mycotoxins due to a greater degree of food shortage. Due to the fact that children grow and develop, they have a larger scale of cell production which results in a greater change in their bodies (Sherif *et al.*, **2009**). According to Anderson *et al.,* (**2000**) and Barton *et al.,* (**2005**), children from a young age, exposed to chemicals that act through a mutagenic mode of action, have an increased susceptibility towards the toxin and more time to develop a chronic disease. For children the damage lies in the extent of exposure and the danger of each mycotoxin (Sherif *et al.*, **2009**).

The effect of such mycotoxins can cause a huge medical burden on a country. Due to the increase of critically ill, immuno-compromised and hospitalized patients, increases the chances of adventitious pathogens in humans to cause infections (Garber, **2001**). The HIV epidemic and different diseases of the immune system add to the threat of mycotoxin exposure. In third world countries, e.g. in Africa, the issues of food safety is balanced against food security. Most people do not have food to consume and the little food that

they do have via subsistence farming is highly contaminated. More stringent regulations are also not the answer as it could hamper the availability of food resources to feed the masses (Marasas *et al.*, **2008**).

7. Mycotoxin regulations in maize

There are many important factors that are considered when looking at planning legislation for mycotoxins. These factors are either based on science or the socio-economic environment in a country. According to a document of the FAO (**2004**) these factors would include the following:

a) Be aware of the law regarding regulations in the countries in which trade contracts exist
b) The accessibility of toxicological information
c) The availability of the correct analytical methods to analyze the mycotoxins
d) The awareness of the location and accumulation of mycotoxins in a lot of grain
e) The accessibility of information regarding the incidence of mycotoxins in the different grain commodities
f) The requirements of adequate food supply

There are 119 countries worldwide that have mycotoxin regulations, although sometimes these regulations are very detailed while others are very basic. Overall, most countries cover only a few mycotoxins such as: aflatoxin B_1, total aflatoxins of B_1, B_2, G_1, G_2, M_1; the trichothecenes: deoxynivalenol, deacetoxyscirpenol, T 2-toxin and HT2-toxin; the fumonisins B_1, B_2, B_3; agaric acid, ergot alkoloids, ochratoxin A, patulin, phomopsins, sterigmatocystin and zearalenone. In North America, Canada and the USA have legislations on mycotoxins. In Africa, 15 countries have mycotoxin legislations, which represent about 59% of the human population on the continent. Latin America has 19 countries with legislation and cover approximately 91% of their population. Twenty-six countries have mycotoxin regulations in place in Asia/Oceania and covers 88% of the population. Europe has the highest

number of countries with regulations (39 countries) that covers about 99% of the continent's population. On average about 86.7% of the world population is protected by mycotoxin regulations, with Africa at the bottom of this list (FAO, **2004**).

South Africa is one of the countries on the African continent that has legislation in place. In total, 7 mycotoxins are regulated in Africa on food commodities destined for human consumption. These include total aflatoxins, aflatoxin B_1, M_1, G_1, ochratoxin A, patulin and zearalenone. Aflatoxin B_1 is regulated by a total of 11 countries in Africa. There are regulations regarding 5 mycotoxins when it comes to animal feed, including total aflatoxin, aflatoxin B_1, G_1, ochratoxin A and zearalenone. Once again aflatoxin B_1 is the most highly regulated in 5 countries. In South Africa, aflatoxin B_1, total aflatoxin, and patulin are regulated, as well as the presence of ergot schlerotia (Government Gazette No. 26849, **2009**). Feed destined for animals are regulated by the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no.36 of 1947 (Government Gazette No. 32935, **2010**).

Regulations in South Africa do not necessarily cover all mycotoxins relevant to food and feed commodities. For example, it is known that mycotoxins such as deoxynivalenol, ochratoxins, zearalenone and a number of others have been found to be associated with South African foodstuffs (Rabie & Marais, **2000**). There is thus still a need to expand the range of mycotoxins in the regulations of South Africa, but a fine balance is needed to ensure that food security is not compromised due to too strict legislations on food safety.

The current regulations in South Africa indicate that no food commodity destined for human consumption may contain more than 10 µg/kg (ppb) total aflatoxins, of which only 5 ppb may be aflatoxin B_1. It also states that fruit juices should not contain more than 50 ppb patulin. In addition, no grain may contain more than 0.02% ergot schlerotia (Government Gazette No. 26849, **2009**).

Feed destined for animals is regulated by the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no.36 of 1947 (Government Gazette No. 32935, **2010**). In this legislation aflatoxin B_1, deoxynivalenol, fumonisins, zearalenone and ochratoxin A are regulated. Maize products intended for the feedlot have a maximum level of aflatoxin B_1 of 300000 mg/kg (ppm) relative to farm feed with a moisture content of 120 g/kg, the highest level in the legislation for feedstuffs. The limits allowed for deoxynivalenol range from a minimum of 1 ppm in pigs and pets to a maximum of 5 ppm in cattle, the same maximum set for zearalenone in sows and pigs. A total of 50 ppm is set for horses and pets regarding fumonisin B_1. Ochratoxin A has the lowest maximum level allowed which is 0.2 ppm in poultry.

8. Conclusion

Maize is the staple food of South Africans and will continue to be so for many years to come. Subsistence farming is common in South Africa with homegrown crops the only source of food, irrespective of quality considerations. Human mycotoxicoses in South Africa is aggravated by the high rates of hepatitis B and HIV infection. In certain cases, maize is prepared in traditional ways that sometimes contribute to mycotoxin production. Due to the high maize intake levels, dietary mycotoxin loads can also be high. The control of mycotoxins, therefore, becomes very important to ensure food security and food safety in these regions.

The control of mycotoxins internationally is becoming much more stringent. For South Africa to be able to access international markets, it will have to comply with international regulations regarding each of the mycotoxins for the different countries. South Africa therefore does not only have an obligation to its own people for the control of mycotoxins but also to the international community.

In rural areas in South Africa, food is difficult to come by and people turn towards subsistence farming which can sometimes produce food with a high

risk of containing mycotoxins. South Africa needs to balance the issues of food safety with that of food security. As stated by Marasas *et al.* (**2008**), people in rural areas would rather choose the consumption of contaminated foods over that of starvation.

The production of mycotoxins, while crops are still cultivated in the field, is not always humanly possible to control. Environmental conditions can cause unexpected conditions that can favor fungal development and mycotoxin formation. However, when the crop is harvested, stored and processed, conditions can be more easily controlled through general HACCP procedures (FAO, **2001**).

In order to ascertain the correct conditions under which to prohibit the production of mycotoxins, further research is required to comprehend the factors under which mycotoxigenic fungi grow and produce their respective mycotoxins. For example, *Fusarium* spp. are the most prominent field fungi to be found in South Africa but much is still to be learned when it comes to the metabolic pathways that are involved in the production of their mycotoxins, as well as the various temperature requirements that activates the different metabolic pathways that are able to synthesize diverse mycotoxins (Sanchis & Magan, **2004**). There are also fungi such as *Stenocarpella maydis* that produce unknown mycotoxins. Also, the role of *Penicillium* spp. in the production of mycotoxins in South African maize is still not known.

9. References:

Agrios, G.N. 1997. Plant Pathology. 4th Ed. Academic press. 635 pp.

Amézqueta, S., González-Peñas, E., Murillo-Arbizu, M., López de Cerain, A. 2009. Ochratoxin A decontamination: A review. *Food Control.* 20: 326–333.

Anastasi, A., Varese, G.C. & Marchisio, V.F. 2005. Isolation and identification of fungal communities in compost and vermicompost. *Mycologia*. 97(**1**): 33-44.

Anderson, L.M., Diwan, B.A., Fear, N.T. & Roman, E. 2000. Critical windows of exposure for children's health. Cancer in humans, epidemiological studies and neoplasms in experimental animal models. *Environmental Health Perspectives.* 108 (**S3**): 573-594.

Aucock, H.W., Marasas, W.F.O., Meyer, C.J. & Chalmers, P. 1980. Field outbreaks of hyperoestrogen (vulvo-vaginitis) in pigs consuming maize infected by *Fusarium graminearum* and contaminated with zearalenone. *Journal of South African Veterinary Association*. 51(**3**): 163-166.

Bailey, J.E. 1992. Whole grain storage, Chapter 5, Pg. 157. In Storage of Cereals Grains and their Products. 4th edition. Ed D.B Sauer. American Association of Cereal Chemists. 615 pp.

Barton, H.A., Cogliano, V.J., Flowers, L., Valcovic, L., Setzer, R.W. & Woodruff, T.J. 2005. Assessing susceptibility from early-life exposure to carcinogens. *Environmental Health Perspectives.* 113(**9**): 1125-1133.

Bennie, A.T.P. & Hensley, M. 2001. Maximizing precipitation utilization in dryland agriculture in South Africa – a review. *Journal of Hydrology.* 241:124-139.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C.P., Horak, R.M., Marasas, W.F.O., Spiteller, G. & Vleggaa,r R. 1988. Structure elucidation of the Fumonisins, mycotoxins from *Fusarium moniliforme. Journal Chemistry Society. Chemical Communication.* 743-745.

Bhat, R.V. & Vasanthi, S. 2003. Mycotoxin food safety risk in developing countries, Section 3, Pg. 7. In Food safety in food security and food trade. 10th Issue. Ed. Unnevehr, L.J. International Food Policy Research Institute. 38 pp.

Boudra, H., Le Bars, P. & Le Bars, J. 1995. Thermostability of ochratoxin A in wheat under two moisture conditions. *Applied and Environmental Microbiology.* 61(**3**):1156-1158.

Brook, R.C. 1992. Drying cereal grains. Chapter 6. Pg. 183. In Storage of cereal grains and their products. 4th edition. Ed. DB Sauer. American Association of Cereal Chemists. 615 pp.

Bruns, T.D. & Shefferson, R.P. 2004. Evolutionary studies of ectomycorrhizal fungi: recent advances and future directions. *Canadian Journal of Botany.* **82**: 1122–1132.

Bullerman, L.B. & Bianchini, A. 2007. Stability of mycotoxins during food processing. *International Journal of Food Microbiology.* 119: 140-146.

Cardwell, K.F., Kling, J.G., Maziya-Dixon, B. & Basque-Perez, N. 2000. Interactions between *Fusarium verticillioides, Aspergillus flavus* and insects infestation in four maize genotypes in improved maize population in lowland Africa. *Phytopathology*. 90:276-284.

Christensen, C.M. & Kaufmann, H.H. 1965. Deterioration of stored grains by fungi. *Annual Review of Phytopathology.* 3: 69-85.

Christensen, C.M. & Kaufmann, H.H. 1974. Microflora in Storage of Cereal grains and their Products Volume 5. 2nd Ed. Ed C.M. Christensen. American Association of Cereal Chemists. 549pp.

Christensen, C.M. & Qasem, S.A. 1960. Influence of various factors on the deterioration of stored corn by fungi. *American Phytopathological Society*. 50:703-709.

Crop Estimates Committee. Department of Agriculture. 2009. www.doa.agric.za/links/cropestimates

Davis, N.D., Wagener, R.E., Morgan-Jones, G. & Diener, U.L. 1975. Toxigenic Thermophilic and Thermotolerant fungi. *Applied Microbiology.* 29(**4**): 455-457.

de Lima, C.P.F. 1990. Airtight storage: principle and practice. Pg. 13. In Food preservation by modified atmosphere. Eds. Calderon, M. & Barkai-Golan, R. CRC Press.

D'Mello, J.P.F., Placinta, C.M. & Macdonald, A.M.C. 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. *Animal Food Science and Technology.* 80: 183-205.

Dupuy, J., Le Bars, P., Boudra, H. & Le Bars, J. 1993. Thermostability of Fumonisin B1, a mycotoxin from *Fusarium moniliforme*, in corn. *Applied and Environmental Microbiology.* 59(**9**): 2864-2867.

Fenice, M., Selbmann, L., Di Giambattista, R. & Federici, F. 1998. Chitinolytic activity at low temperature of an Antarctic strain (A3) of *Verticillium lecanii. Research Microbiology.* 149: 289-300.

Food and Agricultural Organisation (FAO). 1989. Introduction. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Food and Agricultural Organisation (FAO). 1989. Fungal damage in durable foodstuffs with special reference to storage in the tropics. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Food and Agricultural Organisation (FAO). 1989. Solutions to the identified problems. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Food and Agricultural Organisation (FAO). 2004. Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition paper. 163 pp.

Food and Agriculture Organisation (FAO). 2001. Manual on the application of the HACCP system in mycotoxin prevention and control. FAO Food and Nutrition paper 73. 120pp.

Food and Agriculture Organisation (FAO). 1993. Maize in human nutrition. FAO Food and Nutrition paper 25. 168pp.

Forsell, J.H., Jensen, R., Tai, J.H., Witt, M, Lin, W.S. & Pestka, J.J. 1987. Comparison of acute toxicities of deoxynivalenol (vomitoxin) and 15-acetyldeoxynivalenol in the B6C3F1 mouse. *Food and Chemical Toxicology.* 25 (**2**): 155-162.

Fung, F. & Clark, R.F. 2004. Health effects of mycotoxins: a toxicological overview. *Journal of Toxicology*. 42(**2**):217-234.

Garber, G. 2001. An overview of fungal infections. *Drugs*. 61 (**1**): 1-12.

Gelderblom, W.C.A., Smuts, C.M., Abel, S., Snyman, S.D., Cawood, M.E., van der Westhuizen, L. & Swanevelder, S. 1996. Effect of Fumonisin B1 on protein and lipid synthesis in primary rat hepatocytes. *Food and Chemical Toxicology.* 34: 361-369.

Gock, M.A., Hocking, A.D., Pitt, J.I. & Poulos, P.G. 2003. Influence of temperature, water activity and pH on growth of some xerophilic fungi. *International Journal of Food Microbiology.* 81: 11-19.

Government Gazettes No. 26849. 2009. Regulations governing the tolerance for fungus-produced toxins in foodstuffs in terms of the Foodstuffs, Cosmetics and Disinfectants Act 54 of 1972 (GN R1145 in GG 26849 of 8 October 2004).

Government Gazettes No. 32190. 2009. Regulations Relating to the Grading, Packing and Marking of Maize intended for sale in the Republic of South Africa, Act no. 119 of 1990 (GN R 473 published in GG 32190 of 08 May 2009).

Government Gazette No. 32935. 2010. Feed destined for animals are regulated by the Fertilizers, Farm Feeds, Agricultural Remedies and Stock Remedies Act no.36 of 1947 (GN R 70 published in GG 32935 of 12 February 2010).

Hassan, R.M., Mekuria, M. & Mwangi, W. 2001. Maize breeding research in Eastern and Southern Africa: current status and impacts of past investments made by the public and private sectors-1966-1997. CIMMYT.

Hayes, A.W. 1980. Mycotoxins: a review of biological effects and their role in human disease. *Clinical Toxicology.* 17 (**1**): 45-83.

Hendricks, K. 1999. Fumonisins and neural tube defects in South Texas. *Epidemiology.* 10 (**2**): 198-200.

Holzapfel, C.W., Steyn, P.S. & Purchase, I.F.H. 1966. Isolation and structure of aflatoxins M1 and M2. *Tetrahedron Letters*. 25:2799-2803.

Hoseney, R.C. & Faubion, J. M. 1992. Physical Properties of Cereal Grains. Chapter 1. Pg. 1. In Storage of Cereal Grains and their Products. 4th edition. Ed. D.B. Sauer. American Association of Cereal Chemists. 615 pp.

Huff, W.E., Doerr, J.A., Hamilton, P.B. & Vesonder, R.F. 1981. Acute toxicity of vomitoxin (deoxynivalenol) in broiler chickens. *Poultry Science.* 60: 1412-1414.

Hult, K., Plestina, R., Habazin-Novak, V., Radic, B. & Ceovic, S. 1982. Ochratoxin A in human blood and Balkan endemic nephropathy. *Archives of Toxicology.* 51: 313-321.

Humpf, H.U. & Kenneth, A.V. 2004. Review: Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins. *Molecular Nutritional Food Research.* 48: 255–269.

Javed, T., Richard, J.L., Bennet, G.A., Dombrink-Kurtzman, M.A., Bunte, R.M., Koelbeck, K.W., Cote, L.M., Leeper, R.W. & Buck, W.B. 1993. Embryopathic and embryocidal effects of purified Fumonisin B1 or *Fusarium proliferatum* culture material extract on chicken embryos. *Mycopathologia*. 123: 185-193.

Jeebhay, M.F. 2002. Occupational allergy and asthma among food-processing workers in South Africa. *African Newsletter on Occupational Health and Safety.* 12 (**3**): 59-62.

Jimenez, M., Manez, M. & Hernandez, E. 1996. Influence of water activity and temperature on the production of zearalenone in corn by three *Fusarium* species. *International Journal of Food Microbiology.* 29:417-421.

Jonsyn, F.E., Maxwell, S.M. & Hendrickse, R.G. 1995. Ochratoxin A and aflatoxins in breast milk samples from Sierra Leone. *Mycopathologia*. 131: 121-126.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). 2008. Safety evaluation of certain mycotoxins in food. WHO Food Additive Series 59. WHO Publications. 479 pp.

Jurado M, Vázquez C, Marín S, Sanchis V & González-Jaén MT. 2006. PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in maize. *Systematic and Applied Microbiology*.

Kendrick, B. 2000. The Fifth Kingdom. 3rd Edition. Focus publishing.

Kuehn, H.K. & Gunderson, M.F. 1962. Psychrophilic and mesophilic fungi in fruit-filled pastries. *Applied Microbiology.* 10: 354-356.

Kuehn, H.K. & Gunderson, M.F. 1963. Psychrophilic and mesophilic fungi in frozen food products. *Applied Microbiology*. 11: 352-356.

Kuiper-Goodman, T. 1995. Mycotoxins: risk assessment and legislation. *Toxicology Letters.* 82/83: 853-859.

Kuiper-Goodman, T. 1998. Food safety: mycotoxins and phycotoxins in perspective, In Miraglia M., van Egmond H., Brera C. and Gilbert J., *Mycotoxins and Phycotoxins – Developments in Chemistry, Toxicology and Safety. Proceedings of the IX IUPAC International Symposium*, Fort Collins, CO, Alalken Inc, 25-48.

Kumar, V., Basu, M.S. & Rajendran, T.P. 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. *Crop Protection.* 27: 891-905.

Lee, H.B. & Magan, N. 2000. Impact of environmental and interspecific interactions between spoilage fungi and *Aspergillus ochraceus* on growth and ochratoxin production in maize grain. *International Journal of Food Microbiology*. 61:11-16.

Lipp, P.E. & Deep, I.W. 1991. Influence of Tillage and crop rotation on yield, stalk rot and recovery of *Fusarium* and *Trichoderma* spp. from corn. *Plant Disease*. 75:828-833.

Magan, N. & Lacey, J. 1984. Effect of water activity, temperature and substrate on interactions between field and storage fungi. *Transactions of the British Mycological Society*. 82:83-93.

Maheshwari, R., Bharadwaj, G. & Bhat, M.K. 2000. Thermophilic fungi: their physiology and enzymes. *Microbiological and Molecular Biology Reviews.* 64 (**3**): 461-488.

Marasas, W.F.O., Gelderblom, W.C.A., Shephard, G.S. & Vismer, H.F. 2008. Mycotoxins: a global problem. Chapter 4. Pg. 29. In Mycotoxins: detection methods, management, public health and agricultural trade. Eds. Leslie, J.F., Bandyapadhyay, R. & Visconti, A. CAB International.

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

Marasas, W.F.O., Kellerman, T.S., Pienaar, J.G. & Naude, T.W. 1976. Leukoencephalomalacia: a mycotoxin of Equidae caused by *Fusarium moniliforme* Sheldon. *Onderstepoort Journal of Veterinary Research.* 43: 113-122.

Marasas, W.F.O., Kriek, N.P.J., van Rensburg, S.J., Steyn, M. & van Schalkwyk, G.C. 1977. Occurrence of Zearalenone and deoxynivalenol, Mycotoxins produced by *Fusarium graminearum* Schwabe, in maize in Southern Africa. *South African Journal of Science*. 73:346-349.

Marin, S., Sanchis, V., Arnau, F., Ramos, A.J. & Magan, N. 1998. Colonization and competitiveness of *Aspergillus* and *Penicillium* species on maize grain in the presence of *Fusarium moniliforme* and *Fusarium proliferatum*. *International Journal of Food Microbiology*. 45: 107-117.

Martin, P.M.D. 1974. Fungi associated with common crops and crop products and their significance. *South African Medical Journal*. 48: 2374.

Martins, M.L. & Martins, H.M. 2002. Influence of water activity, temperature and incubation time on the simultaneous production of deoxynivalenol and zearalenone in corn (*Zea mays*) by *Fusarium graminearum.* Food Chemistry. 79: 315-318.

Mclean, M. & Berjak, P. 1987. Maize grains and their associated mycoflora-a micro ecological consideration. *Seed Science Technology*. 15: 831-850.

Mejia, D. 2009. Maize post harvest operation. Chapter 23. In Compendium for Post Harvest Operations for FAO. Eds. Mejia, D. & Parrucci, E.

Merrill, A.H. Jr., Sullards, M.C., Wang, E., Voss, K.A. & Riley, R.T. 2001. Sphingolipid metabolism: roles in signal transduction and disruption by Fumonisins. *Environmental Health Perspectives.*109 (**2**): 283-289.

Miller, J.D. 1995. Fungi and Mycotoxins in Grain. Implications for stored product research. *Journal of Stored Products*. 31(**1**):1-16.

Mishra, H.N. & Das, C. 2003. A review on biological control and metabolism of aflatoxin. *Critical Reviews in Food Science and Nutrition.* 43 (**3**): 245-264.

Moss, M.O. 2000. Toxigenic fungi and mycotoxins. Chapter 53. Pg. 1490. In The microbiological safety and quality of food. Ed. Lund, B.M, Baird-Parker, T. C. & Gould, G. W. Springer. 2080 pp.

Mulé, G., Susca, A., Stea, G. & Moretti, A. 2004. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium*

verticillioides, *F. proliferatum* and *F. subglutinans*. *European Journal of Plant Pathology*. 110: 495-502.

Munkvold, G.P. & Desjardins, A.E. 1997. Fumonisins in Maize: Can we reduce their occurrence? *Plant Disease*. 81 (**6**): 556-565.

Murray, P.R. & American Society for Microbiology. 1995. Manual of Clinical Microbiology. 6th Ed. Ed. Murray, P.R. ASM Press. 1482 pp.

National Department of Agriculture (NDA). 16 April 2004. Maize profile. http://www.nda.agric.za/ docs/Maize/maize.htm

National Department of Agriculture (NDA). 28 April 2004. General Information on agriculture in South Africa. http://www.nda.agric.za/history/

Oancea, S. & Stoia, M. 2008. Mycotoxins: a review of toxicology, analytical methods and health risks. *Acta Universitatis Cibiniensis Series E: Food Technology.* 1 (**12**): 19-36.

Pfohl-Leszkowicz, A. & Manderville, R.A. 2007. Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. *Molecular Nutritional Food Research.* 51: 66-99.

Pitt, J.I. 1989. An introduction to Mycotoxins. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Pitt, J.I., Basilico, J.C., Abarca, M.L. & Lopez, C. 2000. Mycotoxins and toxigenic fungi. *Medical Mycology.* 38 (**1**): 41-46.

Pitt, J.I. & Christian, J.H.B. 1968. Water relations of xerophilic fungi isolated from prunes. *Applied Microbiology*. 16 (**12**): 1853-1858.

Pomeranz, Y. 1992. Biochemical, Functional and Nutritive changes during storage. Chapter 3. Pg. 55. In Storage of Cereal Grains and Their Products. 4th edition. Ed D. B. Sauer. American Association of Cereal Chemists. 615pp.

Prelusky, D.B., Trenholm, H.L., Hamilton, R.M.G. & Miller, J.D. 1987. Transmission of [^{14}C] deoxynivalenol to eggs following oral administration to laying hens. *Journal of Agricultural Food Chemistry.* 35: 182-186.

Rabie, C.J. & Marais, G.J. 2000. Toxigenic fungi and mycotoxins in South African foods and feeds. Report to the Department of Health. 31 March 2000. 113pp.

Rabie, C.J., Lübben, A., Schipper, M.A.A., van Heerden, F.R. & Fincham, J.E. 1985. Toxigenicity of *Rhizopus* species. *International Journal of Food Microbiology.* 1 (**5**): 263-270.

Richard, J.L. 2007. Some major mycotoxins and their mycotoxicoses-an overview. *International Journal of Food Microbiology.* 119: 3-10.

Richard, J.L., Meerdink, G., Maragos, C.M., Tumbleson, M., Bordson, G., Rice, L.G. & Ross, P.F. 1996. Absence of detectable fumonisins in the milk of cows fed *Fusarium proliferatum* (Matsushima) Nirenberg culture material. *Mycopathologia.* 133: 123-126.

Riley, R.T. 1998. Mechanistic interactions of mycotoxins: theoretical consideration. Chapter 7. Pg. 227. In Mycotoxins in agriculture and food safety. Eds. Sinha, K.K., & Bhatanagar, D. Volume 68 of Books in soils, plants and the environment. CRC Press. 511 pp.

Robinson, C.H. 2001. Cold adaptation in Arctic and Antarctic fungi. *New Phytologist.* 151 (**2**): 341-353.

Rotter, B.A., Prelusky, D.B. & Pestka, J.J. 1996. Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health*. 48: 1-34.

Salunkhe, D.K., Chavan, J.K. & Kadam, S.S. 1985. Post harvest Biotechnology of Cereals. CRC Press. 208 pp.

Sanchis, V. & Magan, N. 2004. Environmental conditions affecting mycotoxins. Chapter 8. pg. 174. in Mycotoxins in food: detection and control. Eds. Magan, N. & Olsen, M. CRC Woodhead Publishing Ltd.

Sauer, D.B., Meronuck, R.A. & Christensen, C.M. 1992. Microflora. Chapter 9. Pg. 313. In Storage of Cereals grains and their Products. 4th edition. Ed D.B. Sauer. American Association of Cereal Chemists.

Schindler, A.F., Palmer, J.G. & Eisenberg, W.V. 1967. Aflatoxin production by *Aspergillus flavus* as related to various temperature. *Applied and Environmental Microbiology.*15 (**5**): 1006-1009.

Sharma, H.S.S. 1989. Economic importance of thermophilous fungi. *Applied Microbiology Biotechnology.* 31: 1-10.

Sharma, R.P. 1993. Immunotoxicity of mycotoxins. *Journal of Dairy Science.* 76(**3**): 892-897.

Sherif, S.O., Salama, E.E. & Abdel-Wahhab, M.A. 2009. Mycotoxins and child health: the need for health risk assessment. *International Journal of Hygiene and Environmental Health.* 212: 347-368.

Siame, B.A. & Nawa, I.N. 2008. Mycotoxin contamination in food systems in eastern and southern Africa. Chapter 10. Pg. 117. In Mycotoxins: Detection methods, management, public health and agricultural trade. Eds. Leslie, J.F., Bandyopadhyay, R. & Visconti, A. CAB International.

South African Government. 2009a. Abstract of agricultural statistics. Department of Agriculture. 115pp.

South African Government. 2009b. Monthly Food Security Bulletin of South Africa: July 2009. Department of Agriculture, Forestry and Fisheries.

South African Government. 2009c. www.info.gov.za. Department of Agriculture and Land Affairs.

South African Government. 2009d. Maize industry situational analyses, market indicators and outlook for 2009 season. Department of Agriculture and Land Reform.

Speijers, G.J.A. & Speijers, M.H.M. 2004. Combined toxic effects of mycotoxins. *Toxicology Letters.* 153: 91-98.

Steyn, P.S., van der Merwe, K.J., Fourie, L., Scott, D.B. & Theron, J.J. 1965. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. *Nature.* 205: 1112-1113.

Sukprakarn, C. 31 March 2004. Insect Pests of stored Products. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

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

Sydenham, E.W., Shephard, G.S., Thiel, P.G., Marasas, W.FO. & Stockenstrom, S. 1991. Fumonisin contamination of commercial corn-based human foodstuffs. *Journal of Agricultural and Food Chemistry.* 39 (**11**): 2014-2018.

Sydenham, E.W., Thiel, P.G., Marasas, W.F.O., Shephard, G.S., van Schalkwyk, D.J. & Koch, K.R. 1990. Natural occurrence of some *Fusarium* mycotoxins in corn from low and high oesophageal cancer prevalence areas of the Transkei. *Journal of Agricultural and Food Chemistry.* 38: 1900-1903.

Tansey, M.R. 1972. Effect of temperature on growth rate and development of the Thermophilic fungus *Chaetomium thermophile. Mycologia.* 64 (**6**): 1290-1299.

Tipples, K.H. 1995. Quality and nutritional changes in stored grain, Chapter 10. In Stored Grain Ecosystems. Volume 39. Eds. Jayas, D.S., White, N.D.G. & Muir, W.E. CRC Press. 757 pp.

Tomkins, R.G. 1929. Studies of the growth of moulds. *Proceedings of the Royal Society of London.* 105 (**738**): 375-401.

Tongaat Hulett, 2010.Maize in products. 2pp. Tongaat Hulett.

Velluti, A., Marin, S., Bettuci, L., Ramos, A.J. & Sanchis, V. 2000. The effect of fungal competition of maize grains by *Fusarium moniliforme, Fusarium proliferatum, Fusarium graminearum* and Fumonisin B1 and zearalenone formation. *International Journal of Food Microbiology*. 59:59-66.

Wannop, C.C. 1961. The histopathology of Turkey "X" disease in Great Britain. *Avian Diseases.* 5(**4**): 371-381.

Wareing, P.W. 1997. Incidence and detection of Thermotolerant and Thermophilic fungi from maize with particular reference to *Thermoascus* species. *International Journal of Food Microbiology.* 35: 137-145.

Warfield, C.Y. & Gilchrist, D.G. 1999. Influence of kernel age on Fumonisins in B1 production by *Fusarium moniliforme. Applied and Environmental Microbiology*. 65(7): 1-17.

Warren, H.L. 1978. Comparison of normal and high lysine maize inbreds for resistance to kernel rot caused by *Fusarium moniliforme*. *Phytopathology.* 68: 1331-1335.

Weinstein, R.N, Plamer, M.E, Johnstone, K. & Wynn-Williamson, D.D. 1997. Ecological and physiological characterization of *Humicola marvinii*, a new psychrophilic fungus from Fellfield soils in maritime Antarctic.*Mycologia.* 89(**5**): 706-711.

White, T.J., Bruns, T., Lee, S. & Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pgs. 315-322. In PCR Protocols: A Guide to Methods and Applications. Ed. Innis, M.A.. Academic Press.

Wicklow, D.T., Weaver, D.K. & Throne, J.E. 1998. Fungal colonists of maize grain conditioned at constant temperatures and humidities. *Journal of Stored Product Research*. 34(4): 355-361.

Wijnands, L.M. & van Leusden, F.M. 2000. An overview of adverse health effects caused by mycotoxins and bioassays for their detection. National Institute of Public Health and the Environment. 99 pp.

Wild, C.P. & Gong, Y.Y. 2010. Mycotoxins and human disease: a largely ignored global health issue. *Carcinogenesis*. 31 (**1**): 71–82.

Wilson, D.M. & Abramson, D. 1992. Mycotoxins. Chapter 10. Pg. 341. In Storage of Cereal grains and their Products. 4th edition. Ed D.B. Sauer American Association of Cereal Chemists. 615 pp.

Table 1: Types and characteristics of the maize kernels, as well as their uses and cultivation distribution worldwide.

Type of Endosperm	Thickness of pericarp	Crown Appearance	Texture of Endosperm (Mature)	Uses and % of the total maize used in areas mentioned	Countries were cultivated
Pop	Very thick	Pointed/ Rounded	Hard	Confection (<1%)	USA, Eventually all regions
Flint	Thick to medium	Rounded	Mostly hard	General (14%)	Argentina, Southern Europe
Dent	Medium	Dented	Hard & Soft	Livestock feed, industrial process, mill ground meal (73%)	World wide
Floury	Stretched to thin	Slightly dented	Soft	Direct human consumption as flour, direct at milk stage, parched, beverages (12%)	Latin America and South West, South Africa
Sweet	Thick to medium	Wrinkled	Glassy	Direct at milk stage, with 70% moisture used in frozen foods, canned foods, dehydrated foods and beverage (~1%)	North America (USA & Canada)

Source: Updated version of Meija (2004)

Table 2: Fungus species associated with foods and feeds in southern Africa.

Fungus Name	Fungus Name
Acremonium spp.*	*Aspergillus rugulosum**
*Alternaria alternate**	*Aspergillus* spp.*
*Alternaria chartarum**	*Aspergillus sydowii*
Alternaria spp.*	*Aspergillus tamari**
Aspergillus alliaceus	*Aspergillus terreus**
*Aspergillus amstelodami**	*Aspergillus ustus**
*Aspergillus avenaceus**	*Aspergillus versicolor**
*Aspergillus auricomus**	*Aspergillus wentii**
*Aspergillus biplanes**	*Aureobasidium pullulans**
*Aspergillus candidus**	*Bipolaris sorokiniana**
*Aspergillus carneus**	*Bipolaris* spp.*
*Aspergillus chevalieri**	*Botryodiplodia theobromae**
*Aspergillus clavatus**	*Chaetomium* sp.*
*Aspergillus echinosporus**	*Cladosporium cladosporioides**
*Aspergillus flavipes**	*Cladosporium sphaerospermum**
*Aspergillus flavus**	*Candida albicans**
Aspergillus flavus var. *columnaris**	*Candida chodati**
*Aspergillus fumigatus**	*Candida krusei**
*Aspergillus giganteus**	*Candida macedoniensis**
*Aspergillus glaucus group**	*Curvularia lunata**
*Aspergillus granulosus**	*Curvularia* spp.*
*Aspergillus mangini**	*Diplodia macrospore**
Aspergillus melleus	*Diplodia maydis**
*Aspergillus nidulans**	*Drechslera campanulata**
*Aspergillus niger**	*Drechslera* spp.*
*Aspergillus niveus**	*Epicoccum purpurascens**
*Aspergillus ochraceus**	*Fusarium acuminatum**
*Aspergillus parasiticus**	*Fusarium anthophilum**
*Aspergillus repens**	*Fusarium aridum**
Aspergillus restrictus	*Fusarium avenaceum**
*Aspergillus ruber**	*Fusarium camptoceras**
*Aspergillus rubrum**	*Fusarium chlamydosporum**

* Species that have been found to be toxic to animals.

Source: Rabie & Marais (2000)

Table 2 continued: Fungus species associated with foods and feeds in southern Africa.

Fungus Name	Fungus Name
Fusarium chlamydosporum var. *fuscum**	*Neocosmospora vasinfecta**
*Fusarium compactum**	*Mucor circinelloides**
*Fusarium concolor**	*Mucor indicus**
*Fusarium crookwellense**	*Mucor* spp.*
*Fusarium culmorum**	*Nigrospora oryzae**
*Fusarium equiseti**	*Paecilomyces varioti**
*Fusarium fujikuroi**	*Paecilomyces* spp.*
*Fusarium fusarioides**	*Hansenula anomale**
*Fusarium globosum**	*Kluyveromyces marxianus*
*Fusarium graminearum**	*Monodictys* sp.
Fusarium lateritium	*Oidiodendron* sp.*
*Fusarium verticillioides**	*Penicillium variable**
Fusarium verticillioides var. *subglutinans*	*Penicillium aculeatum*
*Fusarium napiforme**	*Penicillium camemberti*
*Fusarium nygamai**	*Penicillium charisii**
*Fusarium oxysporum**	*Penicillium chrysogenum*
*Fusarium poae**	*Penicillium citrinum**
*Fusarium proliferatum**	*Penicillium corylophilum*
*Fusarium reticulatum**	*Penicillium crustosum**
*Fusarium roseum**	*Penicillium cyclopium**
Fusarium sacchari var. *subglutinans**	*Penicillium expansum**
*Fusarium sambucinum**	*Penicillium fellutanum*
*Fusarium scirpi**	*Penicillium frequentans**
*Fusarium semitectum**	*Penicillium funiculosum**
*Fusarium solani**	*Penicillium herqui*
*Fusarium sporotrichioides**	*Penicillium implicatum**
Fusarium spp.*	*Penicillium islandicum**
*Fusarium subglutinans**	*Penicillium italicum**
*Fusarium verticillioides**	*Penicillium janthinellum**
*Geotrichum candidum**	*Penicillium janczewskii**
*Gliocladium catenulatum**	*Penicillium jenseni**
*Gliocladium roseum**	*Penicillium lanosum**
*Macrophomina phaseolina**	*Penicillium meleagrinum**
*Myrothecium verrucaria**	*Penicillium multicolor*
*Lasiodiplodia theobromae**	*Penicillium nigricans*

* Species that have been found to be toxic to animals.

Source: Rabie & Marais (2000)

Table 2 continued: Fungus species associated with foods and feeds in southern Africa.

Fungus Name	Fungus Name
Penicillium notatum	*Pithomyces chartarum**
*Penicillium oxalicum**	*Pleospora herbarum**
*Penicillium piceum**	*Rhizoctonia* spp.*
*Penicillium pulvillorum**	*Rhizopus arrhizus**
*Penicillium purpurogenum**	*Rhizopus chinensis**
Penicillium purpurogenum var. *rubrisclerotium*	*Rhizopus microsporus**
*Penicillium raistrickii**	*Rhizopus microsporus* var. *chinensis**
*Penicillium roqueforti**	*Rhizopus nigricans**
*Penicllium rubrum**	*Pestalotia* spp.*
*Penicillium rugulosum**	*Phoma arachidicola**
Penicillium simplicissimum	*Rhizopus rhizopodiformis**
Penicillium steckii	*Rhizopus* spp.*
Penicillium spp.*	*Rhizopus stolonifer**
Penicillium thomii	*Saccharomycopsis fibuligera**
*Penicillium urticae**	*Saccharomycopsis* sp.*
*Rhizopus oryzae**	*Sclerotium rolfsii**
*Penicillium viridicatum**	*Scopulariopsis brevicaulis**
*Penicillium aurantiogriseum**	*Scopulariopsis* spp.
*Penicillium brevi-compactum**	*Stachybotrys chartarum**
*Phoma exigua**	*Streptomyces* spp.*
*Phoma herbarum**	*Stemphylium solani**
*Phoma jolyana**	*Stenocarpella maydis**
*Phoma sorghina**	*Syncephalastrum racemosum*
Phoma spp.*	*Trichoderma lignorum**
*Phomopsis leptostromiformis**	*Trichoderma* spp.*
Phomopsis spp.*	*Trichoderma viride**
Piptocephalis sp.	*Trichothecium roseum**

* Species that have been found to be toxic to animals.

Source: Rabie & Marais (2000)

Table 3: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
Allergic illness				
Alternaria	F	Tenuazonic acid, alternariol Irritation thought to be directly due to mechanical action of spores	Allergy resembling bronchitis (mainly humans).	Secondary lung changes, emphysema and fibrosis (human).
Aspergillus	S			
Candida	S			
Cladosporium	F			
Fusarium	F/S			
Helminthosporium	F			
Penicillium	F/S			
Verticillium	F			
Mycotoxicosis: I. Mouldy Corn Toxicosis				
Alternaria	F	Various acting synergistically	Toxicosis with multiple symptoms affecting skin of face, eyes,GIT, liver,viscera, blood system and nervous system (various animals)	Not Known
Aspergillus flavus & spp.	S			
Cladosporium	F			
Mucor	S			
Penicillium rugulosum & spp.	S			
Scopulariopsis brevicaulis	S			
Stachybotrys alternans	S			

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
Mycotoxicosis: II. Primarily involving liver or kidney				
a) Aflatoxicosis				
Aspergillus flavus	S	Aflatoxin	Centrilobular necrosis and proliferation of bile ducts and fibrosis in liver. Impairment of blood clotting system, hemorrhage, weakening of gastric motor function (animals and birds)	Hepatoma in rats, ducks and other animals. Strong circumstantial evidence for human hepatoma
A. niger	S			
A. ostianus	S			
A. parasiticus	S			
A. ruber	S			
A. wentii	S			
Penicillium citrinum	S			
P. frequentus	S			
P. puberulum	S			
P. variabile	S			
b) Sterigmatocystin toxicosis				
Aspergillus nidulans	S	Sterigmatocystin	Necrosis and peritonitis (rats)	Hepatoma, cholangio-sarcoma in experimental animals
A. rugulosum	S			
A. versicolor	S			
Bipolaris sorokiniana	F			
Eurotium repens	S			

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
c) Luteoskyrin toxicosis				
Penicillium islandicum	S	Luteoskyrin	Centrilobular necrosis and cirrhosis of liver in many experimental animals	Hepatoma in experimental rats only
d) Ochratoxicosis				
Aspergillus carbonarius	S	Ochratoxin A	Necrosis and fatty infiltration of liver.	Not known
A. niger	S			
A. ochraceus	S		Damage to renal tubules and fibrosis of kidney (liver)	
Penicillium viridicatum	S			
e) *Aspergillus fumigatus* toxicosis				
Aspergillus fumigatus	S	Fumagillin, fumigatin, fumitremorgin	Multiple internal damage including liver and kidney (animals)	Not known

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
f) Patulin (Clavacin) toxicosis				
Aspergillus clavatus	S	Patulin (clavacin)	Maltgerm intoxication of cattle. Necrosis and granulomata in liver and pancreas, degeneration of kidney, haemolysis of red cells	Sarcomas in experimental rats only.
A. giganteus	S			
A. terreus	S			
Byssochlamys nivea	S			
Penicillium claviforme	S			
P. expansum	S			
P. urticae	S			
g) Penicillic acidosis				
Aspergillus melleus		Penicillic acid	Liver damage and loss of co-ordination.	Tumours after injction in rats only
A. ochraceus	S			
A. quercinus	S			
A. sulphureus	S			
Penicillium baarnense	S			
P. cyclopium	S			
P. palitans	S			
P. puberulum	S			
P. stoloniferum	S			
P. suaveolens	S			
P. thomii	S			

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
h) Facial eczema (sheep)				
Pithomyces chartarum	F	Sporidesmin	Biliary obstruction leading to photosentisation of facial skin	None reported
Periconia minutissima	F			
i) Diplodiosis				
Diplodia zeae	F	*Diplodia zeae* toxin	Kidney degeneration, catarrhal enteritis, lung hyperaemia, inco-ordination	Not known
j) Rubratoxicosis (camels, birds)				
Penicillium purpurogenum	S		Liver engorgement, haemorrhagia	Not known
P. rubrum	S	Rubratoxin		
k) Polyuria (Sassoon Hospital syndrome)			Glomerulonephrosis	Not known
Absidia ramose	S	Toxin unnamed	Fatty infiltration of liver (humans)	
Aspergillus clavatus	S	Citrinin		
Penicillium citrinum	S	Citrinin		
Rhizopus stolonifer	F	Toxin unnamed		

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
I) Haematuria				
Chaetomium globosum	S	Chaetoein	Haemorrhagia in kidney (animals)	Not known
Gliocladium fimbriatum	S	Gliotoxin		
Trichoderma viridae	F	Gliotoxin		
Mycotoxicosis: II. Primarily involving organs other than liver or kidney				
a) Drunken Bread Syndrome				Not known
Fusarium graminearum	S	Zearelenone	Ataxia, diarrhea (humans)	
b) Hyperoestrogenism				Not known
Fusarium graminearum	S	Zearelenone	Abortion, necrosis and inflammation of genitalia. Increase in weight of uterus, GIT, blood and nervous system also affected (animals)	
c) Alimentary toxic aleukia (alimentary septic angina)				
F. sporotrichioides	S	Sporotrichin	Haemorrhage, necrosis, inflammation of memebranes, GIT disorders, disturbance of nervous system, blood system etc.	Not known
F. poae	S			

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
d) Leucoencephalomalacia				
F. verticillioides	F/S	Fumonisins	Fetal necrosis of brain matter (horses)	Not known
e) Fescuetoxicosis				
F. equiseti	F/S	T2 toxin (diacetoscirpenol)	Lameness and gangrene etc. (cattle)	Not known
F. nivale				
F. tricinctum				
f) Stachybotryotoxicosis		T2 Toxin		
Stachybotrys alternans	F	Stachybotrytoxin	Haemorrhage, inflammation of membranes, disturbance of nervous system (cattle)	Not known
g) Dendrodochiotoxicosis		Dendrodochiotoxin		
Dendrodochium toxicum	F		Paralysis, generalized haemorrhage (cattle)	Not known

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
h) Ergotism				
Claviceps purpurea	F	Ergotamine	Abortion, contractile effect on uterus and circulatory system, gangrene (cattle,humans)	Not known
i) Tremor convulsion				
Penicillium crustosum	S	Tremorgen (tremortin)	Tremors and convulsions (animals)	Not known
P. cyclopium	S			
P. granulatum	S			
P. palitans	S			
j) Haemorrhagia				
Alternaria tenuis	S	Cyclopiazonic acid	As above (animals)	Not known
	F	Alternarin, tenuazonic acid	Haemorrhage,multiple lesions	
Cladosporium epiphyllum	F	Not named		
C. fagi	F	Not named		

Source: Updated version of Martin (1974)

Table 3 continued: Disease syndromes associated with specific fungi.

Major disease category, type of illness and fungi	Field (F) or Storage (S)	Toxin	Acute Toxicity Symptoms	Chronic or Sublethal Effect
k) Ustilagotoxicosis				
Puccinia graminis	F	Not named	Epileptiform convulsions, salivation, other multiple effects (animals)	Not known
Tilletia laevis	F			
Ustilago avenae	F			
U. hordei	F			
U. zeae	F			

Source: Updated version of Martin (1974)

Table 4: Factors affecting mycotoxin formation in maize.

Factor	In Field	At Harvest and Drying	In Storage
Physical			
Moisture	+	+	+
rapidity of drying	-	+	+
rewetting	-	+	+
relative humidity	+	+	+
net evaporation	+	+	-
Temperature	+	+	+
Mechanical Damage	+	+	+
Blending of grain	-	+	+
Hot Spots	-	-	+
Time	+	+	+
Chemical			
CO_2	-	-	+
O_2	-	-	+
Nature of substrate	+	-	+
Mineral nutrition	+	-	+
Chemical Treatment	-	-	+
Biological			
Plant stress	+	-	+
Invertebrate vectors	+	-	+
Plant varietal differences	+	-	+
Fungal strain differences	+	-	+
Spore load	+	+	+
Microbiological ecosystem	+	-	+
Insect damage	+	+	+
Damage by plant disease	+	-	+

Source: Wilson & Abramson (1992)

Table 5: Main differences between field and storage fungi.

Field Fungi	Storage Fungi
1. Persist in the seed or fruit under dry conditions. Limited by excess moisture.	1. Persist under dry conditions but require moisture contents in excess of a specific level (13-14% for cereals) for development. These moisture levels are not usually present in pre-harvest conditions
2. Except in the case of destructive parasites (e.g. *Fusarium* scab), species do not attack the germ of the seed or contribute to rapid deterioration. Many may parasitise leaves, stems and inflorescences, and merely discolor seed.	2. This group includes a number of species specifically invading seeds only (e.g. *Aspergillus restrictus*), that bring about deterioration and death of seeds within a short time
3. No significant biochemical change in seeds or fruits.	3. Increase fat acidity. Increase reducing sugars. Decrease non-reducing sugars Decrease protein content
4. Do not cause heating.	4. May cause localized heating of grain if certain thermophilic species are present (*Penicillium cyclopium, Penicillium funiculosum*)
5. Representative examples: Saprophytes: *Alternaria alternata* *Cladosporium cladosporioides* *Cochliobolus geniculatus* (*Curvularia geniculata*) *Epicoccum purpurascens* *Fusarium graminearum* *Penicillium oxalicum* Parasites: *Drechslera rostrata* *Leptosphaerulina arachidicola* *Physalospora rhodina* (*Botryodiplodia theobromae*) *Trichometasphaeria turcica*	5. Representative examples: *Aspergillus candidus* *Aspergillus flavus* *Eurotium repens* *Corticium solani* *Gliocladium catenatulatum* *Macrophomina pahseolina* *Penicillium chrysogenum* *Penicillium viridicatum*

Source: Updated version of Martin (1974)

Table 6: Fungi associated with South African maize.

Fungus Name	Fungus Name
Acremonium spp.	*Fusarium compactum*
Aspergillus flavus	*Fusarium equiseti*
Stenocarpella maydis	*Fusarium oxysporum*
Fusarium graminearum	*Fusarium poae*
Fusarium verticillioides	*Fusarium semitectum*
Fusarium subglutinans	*Fusarium solani*
Lasiodiplodia theobromoae	*Geotrichum candidum*
Mucorales	*Macrophomina phaseolina*
Nigrospora spp.	*Neocosmospora* spp.
Penicillium spp.	*Neocosmospora vasinfecta*
Giberella fujikuroi	*Pestalotia* spp.
Fusarium moniliforme var. subglutinans	*Phomopsis* spp.
Fusarium roseum	*Rhizoctonia* spp.
Penicillium oxalicum	*Aspergillus parasiticus*
Diplodia macrospora	*Fusarium globusum*
Penicillium pulvillorum	*Fusarium proliferatum*
Aspergillus clavatus	*Eurotium* spp.
Aspergillus ochraceus	*Mucor* spp.
Aspergillus niger	*Rhizopus oryzae*
Phoma sorghina	*Rhizopus stolonifer*

Source: Rabie & Marais (2000)

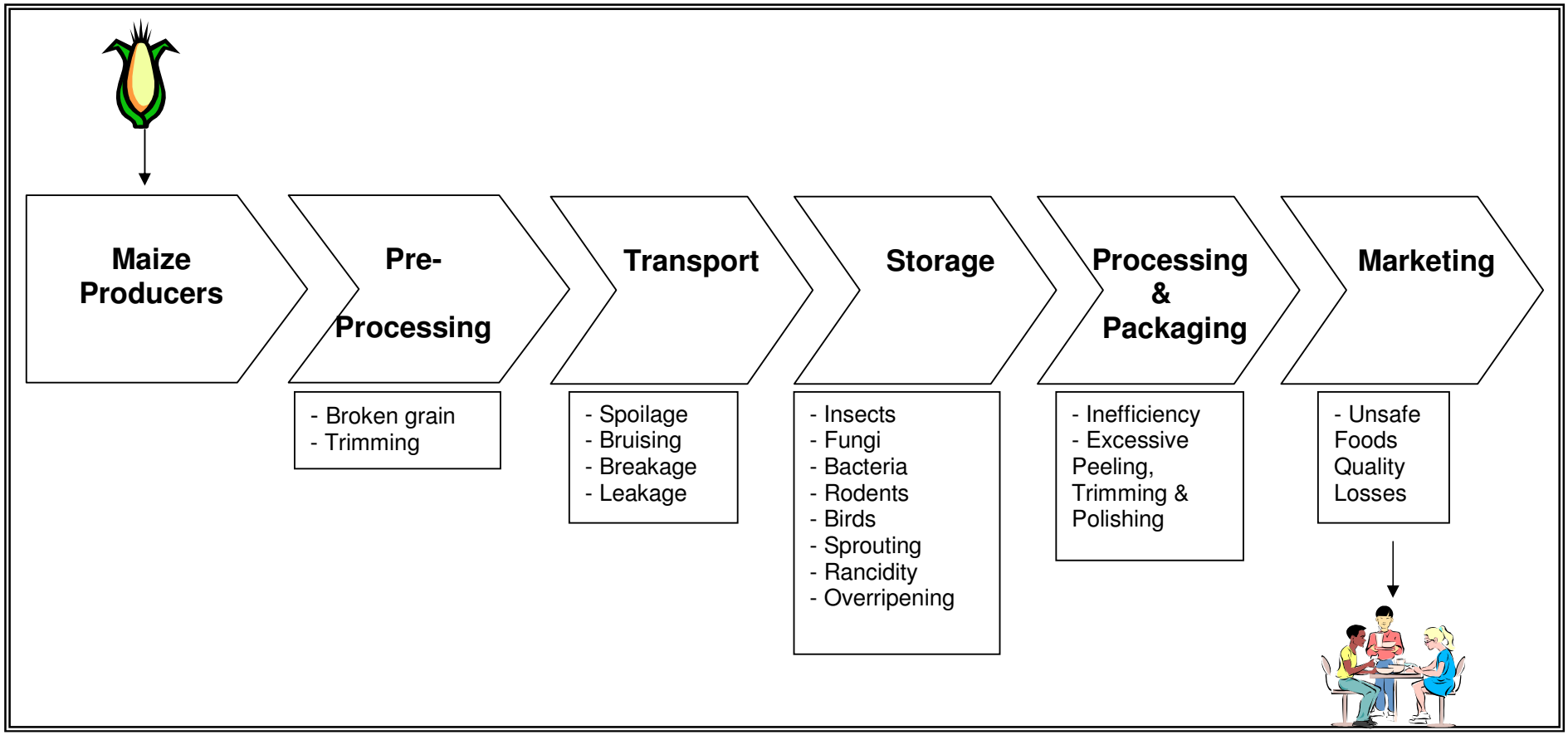

FIGURE 1: The Food Pipeline and the possible reasons for maize damage at the various stages.

Source: Updated version of Meija (2004)

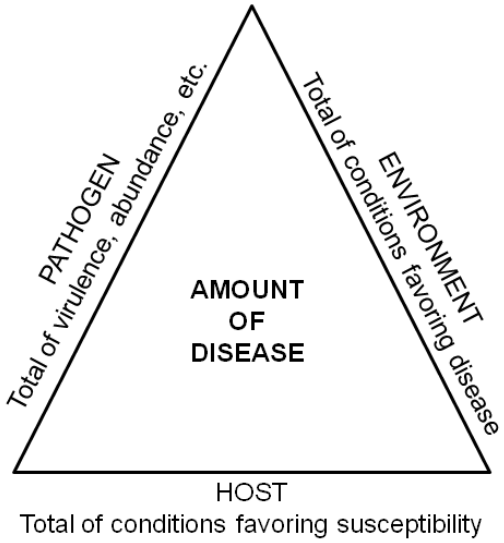

FIGURE 2: The disease triangle.

Source: Agrios (1997)

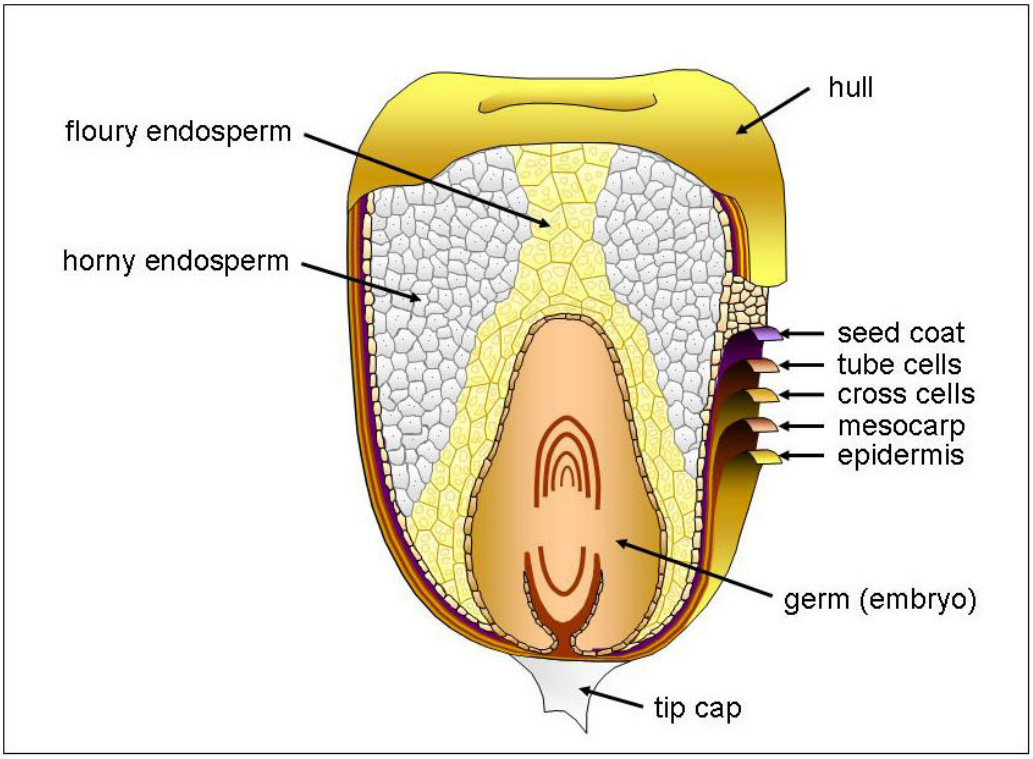

FIGURE 3: Composite parts of a maize kernel.

Source: Updated version of Meija D (2004)

Chapter 2:

Comparative study on the fungal infestation of 49 commercially produced maize cultivars

1. Summary

Maize is the staple food of people living in South Africa and is constantly exposed to mycotoxigenic fungi and their mycotoxins. It is estimated that at least 25% of grain crops, worldwide, are annually contaminated with mycotoxins. The situation in South Africa is most likely similar, with a considerable burden being placed on the health of animals and humans, particularly where the immune system is already compromised by malnutrition and HIV/AIDS.

In this study, 49 commercially produced maize cultivars from the South African National Cultivar Trials of the 2003/2004 planting season were cultivated in the same field. After harvesting, the cultivars were compared, based on the level of invasion of selected mycotoxigenic fungi in the kernels. The purpose of this study was to compare *in vivo* the ability of commercially available maize cultivars to withstand the natural invasion of these fungi. The levels of selected mycotoxins, such as fumonisins and zearalenone, were also determined.

Results showed that 6 of the 49 cultivars tested, indicated a lower infestation level. Cultivar DKC 80-10 showed the lowest infestation rate. It was evident that *Fusarium verticillioides* was the most prominent fungus in all cultivars tested. The reason for the significantly lower levels of other fungi could be explained by the relative domination of *F. verticillioides* rather than the resistance of the maize cultivars to fungal attack. The levels of mycotoxins did not necessarily correlate with the levels of the relevant fungi. This was likely due to the differences in the ability of individual fungal isolates to produce mycotoxins.

2. Introduction

Maize is the staple food of South Africa of which slightly more than half (approximately 4 million tons) per year is destined for human consumption and the rest (about 3.5 million tons) for animal feed (NDA, **2003**). The maize industry in South Africa is also the largest contributor to the total gross value of agriculture in this region. All South Africans consume maize in some form or another, with the majority consuming it as maize meal (NDA, **2004**). Many other products such as pharmaceuticals, confectionary, toothpaste, popcorn, and soups can include maize as an ingredient in various forms (Tongaat Hulett, **2010**). Maize can also indirectly reach the human food chain through the consumption of animal products such as meat, dairy products, cheeses and eggs (FAO, **1992**).

A wide variety of field- and storage fungi have previously been associated with maize in South Africa (Marasas *et al.*, **1981;** Rheeder *et al.*, **1995**). One of the more important fungi in South African maize is *Fusarium verticillioides*. This fungus is implicated in oesophageal cancer in humans and leucoencephalomalacia (LEM) in horses, mules and donkeys (Marasas *et al.*, **1984**) due to the production of mycotoxins such as fumonisins (Gelderblom *et al.*, **1988**). Other fungi that are regularly associated with maize include *Fusarium graminearum* that produces deoxynivalenol, nivalenol and zearalenone (Marasas *et al.*, **1979**), *Stenocarpella maydis* of which the mycotoxin is still unknown (Rheeder *et al.*, **1990**), and *Penicillium islandicum* that produces luteoskyrin (Bouchet *et al.*, **1976**).

The role of the environment, together with the ability of a plant to withstand fungal attack, are the main factors influencing the fungal populations associated with a specific crop. It was stated by Hassan *et al.* (**2001**) that the ecological adaptation of maize varieties, released by public breeding programs and private seed companies in eastern and southern Africa, reflects the characteristics of local production environments. It is, therefore, reasonable to believe that the fungal populations and their mycotoxins could

differ between cultivars and geographical areas. Based on a report to the Department of Health (Rabie & Marais, **2000**) approximately 190 toxigenic fungi are associated with food and feed commodities in South Africa.

The aim of this study was to quantitatively determine the level of contamination of 49 commercially produced maize cultivars by mycogenic fungi, taken from the South African National Cultivar Trial of the 2003/2004 season. One of the objectives was to determine whether infection levels of certain mycogenic fungi will differ between cultivars collected under similar geographical and environmental conditions.

3. Materials and Methods:

3.1. Maize production

Forty nine cultivars (see Table 1, pg. 86) were grown at the premises of the Agricultural Research Council (ARC) in the Potchefstroom area under natural conditions without any fungicide treatments. Of each cultivar, four rows of 20 meters each were planted and cultivated for approximately 6 months and then harvested. Between 5 and 10 kg of maize kernels were harvested for each cultivar from the two inner rows. Sub-samples were taken by randomly selecting 20 separate samples and mixing them together to form one sub-sample from each cultivar. The sub-samples were used to conduct fungal enumerations according to the method described by Rabie *et al.* (**1997**).

3.2. Fungal enumeration

Fungal enumeration was done by surface sterilizing maize kernels with 76% (v/v) ethanol, rinsing them three times with sterile distilled water, and plating them onto three different growth media including potato dextrose agar, pentachloro-nitrobenzene agar and malt salt agar. The fungi were identified based on the morphology of fruiting structures and the results were expressed as the percentage of kernels infested with a specific fungus. Results are summarized in Tables 2 to 11 (see pgs. 87-96). The growth media was prepared as described below:

3.2.1. Culture Media

3.2.1.1. Potato Dextrose Agar (PDA)

PDA (Merck, South Africa) was prepared according to manufacturers instructions. It was then poured into 90 mm petri dishes and allowed to settle.

3.2.1.2. Malt Salt Agar (MSA)

MSA was prepared by dissolving 90 g NaCl (Promark Chemicals, South Africa) in 360 ml distilled water in a 500 ml Schott bottle. 24 g of malt extract (Merck, South Africa) and 24 g of agar (Merck, South Africa) were added to 840 ml distilled water in a 1 L Schott bottle. The solutions were then autoclaved separately for 20 min at 121^{0}C. The solution containing the NaCl was then added aseptically the solution containing the malt extract and agar. The medium was then aseptically poured into 90 mm petri dishes and allowed to settle.

3.2.1.3. Pentachloronitrobenzene agar (PCNB)

PCNB was prepared by adding 30 g agar (Merck, South Africa), 22.5 g peptone (Merck, South Africa), 3 g pentachloronitrobenzene (Sigma Aldrich, South Africa), 1.5 g KH_2PO_4 (ACE, South Africa) and 0.75 g $MgSO_4.7H_20$ (ACE, South Africa) to 1500 ml distilled water with 250 mg of chloramphenicol. The solution was then autoclaved for 20 min at 121^{0}C, after which it was aseptically poured into 90 mm petri dishes and allowed to settle.

3.3. Surface sterilization

Ethanol (76% v/v) was prepared as disinfectant. A small sample of maize (approximately 500 kernels) was weighed out and shaken for 1 min in disinfectant. After draining the ethanol from the maize sample, it was rinsed twice in sterilized distilled water and padded dry in an autoclaved paper towel before plating.

3.4. Plating

After the maize kernels were dried subsequent to surface sterilization, they were plated by aseptically placing 5 kernels per petri dish (as shown in Figure 1) using forceps. In total, 50 kernels were used per cultivar on all three media respectively. The plates were incubated for at least 10 days at approximately 25^0C with 12-hour dark and mixed light cycles as described by Rabie *et al.,* **1997**.

FIGURE 1: Illustration indicating the placing of five maize kernels onto an agar plate.

3.5. Fungal identification

All fungal cultures were identified according to their morphological characteristics by using stereo and light microscopy. Identifications of most fungi were based on morphological characteristics. *Penicillium* species identification was done according to the method of Pitt (**1991**) that is based on seven-day growth studies, including morphological and physiological characteristics. In some cases, it was not possible to identify fungi to species level due to the lack of fruiting structures. In such cases the fungi were identified only to genus level where possible.

3.6. Mycotoxin analysis

Samples of 100 g were collected from each cultivar and were sent to the ARC Onderstepoort for analysis. About 50 kernels were milled to a fine powder by a hand mill (Sprong and Co, England). The samples were then prepared and

extracted according to the prescribed enzyme linked immunosorbent assay (ELISA) method for each mycotoxin (Neogen Veratox kits – Analytical Diagnostic Products, South Africa). The analytical tests were performed according to the manufacturer's instructions that are based on direct competitive ELISA. The optical densities were read in a Neogen micro well reader. The levels of total fumonisins and zearalenone are given in Table 14 (pg. 100).

4. Results:

Fungal populations were quantitatively determined for each maize cultivar by expressing the levels as the percentage of kernels infested with a specific fungus. The results are summarized in Tables 2 to 11 (pgs. 87-96). In total, 19 different fungal species and groups were found to be associated with the 49 maize cultivars used in this study (see Table 12, pg. 97). This represents a wide variety of both field and storage fungi.

All maize cultivars tested contained high levels of *Fusarium verticillioides* (see Figure 2, pg. 102). In most cultivars tested, 100% of the maize kernels were infested with this fungus, except in 6 of the cultivars. Cultivars 2 (DKC 80-10), 5 (SNK 2682), 8 (PAN 6146), 25 (PAN 6479), 38 (DKC 78-15).and 40 (PAN 6995 Bt) indicated an infestation level below 100%. Due to the dominance of this fungus, it was, therefore, used as an indicator of possible resistance.

The level of *Penicillium oxalicum* infestation in the 49 maize cultivars tested is summarized in Figure 3 (pg. 103). This fungus did not show the same pattern of infestation when compared with those cultivars that showed lower levels of *Fusarium verticillioides* (Figure. 2, pg. 102). For example, cultivar 5 (SNK 2682) had an infestation rate of 4%, 25 (PAN 6479) had 22%, 38 (DKC 78-15) had 6% and 40 (PAN 6995 Bt) had 4% infestation with *P. oxalicum*. Cultivars 2 (DKC 80-10) and 8 (PAN 6146) showed no infestation with this fungus Cultivar 30 (PHB 30D05) showed the highest level of infestation of 84%. The majority of the cultivars showed infestation levels of below 20%. Cultivars 2

(DKC 80-10), 7 (LS 8504), 8 (PAN 6146) and 9 (DKC 80-12 Y) also showed no infestation with this fungus.

Figure 4 (pg. 104), indicates the level of infestation of *Stenocarpella maydis*. Cultivar 25 (SNK 2682) showed the highest incidence of infestation of 10%. The majority of the cultivars showed a zero level of infestation. The cultivars with lower *F. verticillioides* levels including cultivar 8 (PAN 6146), cultivar 5 (SNK 2682), and 25 (PAN 6479) showed infestation levels with *S. maydis* of 2%, 6% and 10%, respectively. Cultivars 2 (DKC 80-10), 38 (DKC 78-15) and 40 (PAN 6995 Bt) showed no infestation with this fungus.

The presence of *Phoma sorghina* in the 49 maize cultivars is summarised in Figure 5 (pg.105). Results indicated that cultivar 8 (PAN 6146) had the highest level of infestation of 56%. The majority of the cultivars showed a zero level of infestation. When comparing the six cultivars with lower *F. verticillioides* levels it was found that cultivars 8 (PAN 6146), 38 (DKC 78-15) and 40 (PAN 6995 Bt) had infestation levels of 56%, 24% and 2%, respectively. Whereas cultivars 2 (DKC 80-10), 5 (SNK 2682) and 25 (PAN 6479) showed no infestation with *P. sorghina*.

A total of ten cultivars showed an infestation level of a 100% with *Mucor* species (Figure 6, pg.106). The majority of the cultivars had infestation levels of below 20% and eight cultivars showed no infestation with *Mucor* spp. Those cultivars with lower *F. verticillioides* levels contained diverse levels of these fungi. For example, cultivar 2 (DKC 80-10) had 18%, 5 (SNK 2682) had 14%, 8 (PAN 6146) had 58%, 25 (PAN 6479) had 2%, 38 (DKC 78-15) had 12 % and 40 (PAN 6995 Bt) had 16% of the kernels infested with *Mucor* species.

Other fungi found on the cultivars were summarized in Table 12 (pg. 97). This table represents the fungal diversity found on all the 49 cultivars used in this study. The majority of fungi found in the cultivars are field fungi e.g. *Acremonium* spp., *Cladosporium* spp., *Nigrospora* spp. This is an indication that ideal conditions were experienced for fungal contamination while the maize plants were still cultivated in the field just before or during harvesting.

A list of possible mycotoxins that could be present in the cultivar samples tested was compiled based on the fungal species and levels found (see Table 13, pg. 98). For the purpose of this study, and the available analytical methodology, the level of fumonisins and zearalenone were determined (see Table 14, pg. 100). Fumonisins were detected in a total of 41 cultivars with the highest level of 11,1 mg/kg fumonisins that was detected in cultivar 47 (ZM 421). In contrast, zearalenone was only detected in cultivars 5 (SNK 2682), 11 (Goldfinger), 14 (NS 5914) and 16 (SNK 2472) with cultivar 16 (SNK 2472) that indicated the highest level of zearalenone nl. 62,2 µg/kg. Comparisons between the levels of *Fusarium verticillioides* and the levels of fumonisins, and the levels of *Fusarium graminearum* and the levels of zearalenone, are shown in Figures 7 and 8 respectively (pgs. 107-108).

5. Discussion:

Based on the results obtained it was evident that a wide variety of fungi were associated with the maize cultivars tested (see Table 12). The most prominent fungi were members of *Fusarium* and *Penicillium*. It is also these two genera that represent quite a number of mycotoxigenic fungi that are detrimental to human and animal health. Most of the fungi identified in this study, are capable of producing a number of known mycotoxins. However, some fungi such as *Phoma sorghina* and *Stenocarpella maydis* produce unknown mycotoxins that are not detectable by any existing analytical methods, molecular or otherwise. In such cases, risk assessment will always rely on the determination of fungal populations, either by means of morphological or genetically based identifications.

By quantitatively determining the fungal populations in the 49 maize cultivars (see Tables 2-11) that were grown under the same conditions and exposure, a comparative overview could be obtained of the possible resistance against mycotoxigenic fungi in these cultivars. This should only be regarded as an indication of resistance, as no confirmation could be given that the level of

exposure to mycotoxigenic fungi was the same in all cultivars during cultivation. Nevertheless, all cultivars were subjected to the same environmental conditions such as exposure to the sun, wind, temperature, rainfall, insect damage, nutrition, and soil type. As such, the relative risk of fungal attack was likely similar in all cultivars tested.

The results of this study indicate that the kernels of South African maize cultivars are quite susceptible to the infestation of *Fusarium verticillioides*. Extremely high levels in most of the cultivars tested in this study are evidence of this phenomenon (Figure 2). Although the levels of *F. verticillioides* were still comparatively high in cultivars 2 (DKC 80-10), 5 (SNK 2682), 8 (PAN 6146), 25 (PAN 6479), 38 (DKC 78-15) and 40 (PAN 6995 Bt), the possibility does exist that these cultivars could have a mechanism that slow down fungal attack compared to the rest of the cultivars tested.. However, it still has to be confirmed whether this reflects true resistance.

Penicillium oxalicum was found in high numbers in many of the cultivars (Figure 3) even though it is a fungus that is usually associated with poor storage conditions rather than in the field. Comparative lower infestation rates with *P. oxalicum* were found in the same cultivars that demonstrated lower infestation with *F. verticillioides.* This strengthens the notion that a possible resistance mechanism could be in action in these cultivars. There were, however, contradictory findings where the levels of *P. oxalicum* were relatively low in certain cultivars while the levels of *F. verticillioides* were extremely high. This could possibly be due the dominance of *F. verticillioides,* influencing the competitiveness of *P. oxalicum*.

Stenocarpella maydis occurred in very low levels in all the cultivars (Figure 4) even though it is regarded as one of the more important mycotoxigenic fungi of maize in South Africa. Possible reasons for the lower level of contamination are that *F. verticillioides* is likely a more aggressive colonizer than *S. maydis* or the risk of exposure of the maize cultivars to *S. maydis* was likely lower than *F. verticillioides*. It could also be that the dominance of *F. verticillioides* and *P. oxalicum* could have limited the infestation of this fungus.

The level of infestation of *Phoma sorghina* (Figure 5) is relatively low compared to *F. verticillioides* and *P. oxalicum*. This is similar to the lower levels of *S. maydis*, indicating that *P. sorghina* is likely a less aggressive colonizer of maize than the other field fungi. If this is the case, it should be seen that the results obtained here is not a true reflection of the resistance of the maize cultivars to *P. sorghina*, but rather the ability of this fungus to compete with other field fungi for the same food source such as maize. Results also showed no resemblance between the levels of *F. verticillioides* and *P. sorghina*.

Although certain cultivars indicated high levels of infestation with *Mucor* species (Figure 6), no pattern could be found that was similar to either *F. verticillioides* or *P. oxalicum*. *Mucor* species are known to act as both field and storage fungi (Pitt & Hocking, **2009**), which give these fungi the advantage to invade the maize during field conditions and propagate during storage. The fact that a number of cultivars indicated extremely high levels of this fungus, emphasizes the ability of these fungi to compete with the more dominant fungi in maize for the same nutrient.

Although only the presence of mycotoxins such as fumonisins and zearalenone were tested in this study, other mycotoxins such as secalonic acid D and tenuazonic acid could also be present. In addition, unknown mycotoxins of *Mucor* species, *Stenocarpella maydis*, *Penicillium* species and *Phoma sorghina* could also be present in certain samples. Due to the limited availability of analytical methods, the presence of the latter mycotoxins was not tested.

The majority of cultivars tested in this study contained fumonsins. This was expected as all cultivars contained high levels of *Fusarium verticillioides*. Twenty five of the 49 cultivars showed fumonisin levels above 1 mg/kg, which is below the 2ppm international standard recommended by the FDA (**2001**). This finding further substantiates the high risk of fumonisins to be present in South African maize.

When comparing the level of infestation of *F. verticillioides* and the level of fumonisins in the maize cultivars it became evident that fungal infestation does not necessarily correlate with levels of these mycotoxins (Figure 7). In general, low levels of fungal infestation normally correlate with relatively low levels of fumonisins. It is only when high fungal infestation is experienced, that the levels of fumonisins can range from absent to levels of up to 11,1 mg/kg. By implication, higher levels of fungal infestation could thus pose a higher risk for fumonisins to be present at levels in food not acceptable for human and animal consumption.

Zearalenone was only detected in 4 of the maize cultivars tested (Figure 8). The highest level of zearalenone that was detected was 62,2 µg/kg found in cultivar 16 (SNK 2472). The cultivars that indicated a low or zero level of infestation of *Fusarium graminearum* tended to have the highest level of zearalenone produced. In Figure 7, the histogram highlights that even though the fungus might be present on various cultivars, it is not a certainty that zearalenone will be present.

6. Conclusion:

Based on the results obtained in this study it is evident that cultivars 2, 5, 8, 25, 38 and 40 might indicate a resistance to at least two fungi, namely *Fusarium verticillioide*s and *Penicillium oxalicum*. This observation of possible resistance nevertheless needs to be confirmed.

The level of infestation of fungi such as *F. verticillioides* does not always correlate with the levels of mycotoxins present (Figure 7), but it indicates the risk involved. Low levels of the fungus normally indicate a relatively low level of the mycotoxin, but high fungal infestation only increases the risk of the mycotoxin to be present, whether at low or high levels.

This study also emphasizes the importance of mycotoxigenic fungi in commercial maize cultivars in South Africa. The presence of *F. verticillioides* in the staple food of the majority of South Africans is a serious threat, both for humans and animals. Ways of lowering the presence of this fungus and its mycotoxins is of utmost importance and the use of resistant cultivars could prove to be worthwhile to investigate.

7. References:

Bouchet, J., Chuong, P.P.V., Toma, F., Kirszenbaum, M. & Fromageot P. 1976. Isolation and characterization of luteoskyrin and rugulosin, two hepatotoxic anthraquinonoids from *Penicillium islandicum* Sopp. and *Penicillium rugulosum* Thom. *Journal of Food Chemistry.* 24(**5**)**:** 964-972.

Food and Drug Administration (FDA). 2001. Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds. Background Paper in Support of Fumonisin Levels in Corn and Corn Products Intended for Human consumption.

Food and Agricultural Organisation (FAO). 1992. Maize in Human Nutrition. FAO Food and Nutrition Series, No. 25. FAO. 112pp.

Gelderblom, W.C.A., Jaskiewicz, K., Marasas, W.F.O., Thiel, P.G., Horak, R.M., Vleggaar, R. & Kriek, P.J. 1988. Fumonisins – Novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Applied and Environmental Microbiology.* 54(**7**)**:** 1806-1811.

Hassan, R. M., Mekuria, M. & Mwangi, W. 2001. Maize breeding research in eastern and southern Africa current status and impacts of post harvest investments made by the public and private sectors-1966-1997. CIMMYT.

Marasas, W.F.O., Nelson, P.E. & Toussoun, T.A. 1984. Toxigenic *Fusarium* species, identity and mycotoxicology. The Pennsylvania State University Press. University Park and London. 328 pp.

Marasas, W.F.O., van Rensburg, S.J. & Mirocha, C.J. 1979. Incidence of *Fusarium* species and the mycotoxins, dexynivalenol and zearalenone, in corn produced in esophageal cancer areas in Transkei. *Journal of Agricultural Food Chemistry.* 27(**5**)**:** 1108-1112.

Marasas, W.F.O., Wehner, F.C., van Rensburg, S.J. & van Schalkwyk, D.J. 1981. Mycoflora of corn produced in human esophageal cancer areas in Transkei, Southern Africa. *Postharvest Pathology and Mycotoxins.* **71:** 792-796.

National Department of Agriculture (NDA). 2003. Food Pricing Monitoring Committee: Final report. December 2003. pp310. http://www.nda.agric.za/docs/fpmc/default.htm

National Department of Agriculture (NDA). 2004. General information on agriculture in South Africa. http://www.nda.agric.za/history/

Pitt, J.I. & Hocking, A.D. 2009. Fungi and food spoilage. Springer. 540pp.

Pitt, J.I. 1991. A laboratory guide to common *Penicillium* species. Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, 187 pp.

Rabie, C.J. & Marais, G.J. 2000. Toxigenic fungi and mycotoxins in South African foods and feeds. Report to the Department of Health. 31 March 2000. 113pp.

Rabie, C. J., Lübben, A., Marais, G. J. & Jansen van Vuuren, H. 1997. Enumeration of fungi in barley. *International Journal of Food Microbiology*. 35:117-127.

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

Rheeder, J.P., Sydenham, E.W., Marasas, W.F.O., Thiel, P.G., Shephard, G.S., Schlechter, M., Stockenström, S., Cronje, D.W. & Viljoen, J.H. 1995. Fungal infestation and mycotoxin contamination of Southern African

commercial maize harvested in 1989 and 1990. *South African Journal of Science.* **91:** 127-131.

Tongaat Hulett, 2010.Maize in products. 2pp. Tongaat Hulett.

Table 1: Maize cultivars used in the study.

Cultivar Name	Cultivar Name
1. PAN 6734	26. SNK2551
2. DKC 80-10	27. Saffier
3. PHB 3442	28. PAN 6043
4. PAN 6844	29. SC 405
5. SNK 2682	30. PHB 30d05
6. Synercus	31. PAN 6633
7. LS 8504	32. LS 8509
8. PAN 6164	33. CRN 3549
9. DKC 80-12 Y	34. PAN 6611
10. AFG 4410	35. SC 407
11. Goldfinger	36. CAP 341-NG
12. PAN 6966	37. PAN 6615
13. DKC 66-22	38. DKC 78-15
14. NS 5914	39. Caracal
15. PHB 30H22	40. PAN 6995 Bt
16. SNK 2472	41. LS 8507
17. AFG 4512	42. PAN 6927
18. DKC 63-20	43. Panthera
19. CRN 3760	44. PAN 6537
20. MRI 514	45. SNK 2969
21. PAN 6053	46. PAN 6071
22. CRN 3505	47. ZM 521
23. SC 621	48. PAN 6335
24. MRI 624	49. PHB 30N35
25. PAN 6479	

Table 2: Fungal enumeration of cultivars 1-5, expressed as percentage of kernels infested.

Cultivar No:	1 (PAN 6734)			2 (DKC 80-10)			3 (PHB 3442)			4 (PAN 6844)			5 (SNK 2682)		
Sample No:	22040172			20040173			20040174			20040175			20040176		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Acremonium spp.	2			10									16		
Basidiomycetes	8														
Cladosporium spp.															2
Fusarium graminearum							2		2				2	2	
Fusarium oxysporum	2												22	14	8
Fusarium verticillioides	74	100	76	30	38	28	100	100	100	100	100	100	42	34	48
Geotrichum spp.	4														
Mucor spp.	40		36	18		2	6			18			14		4
Nigrospora spp.	4					2							2		
Penicillium islandicum	4			2						4					
Penicillium oxalicum	26		30				14		16	6			4		
Penicillium spp.	2			16		14				4		6	10		12
Stenocarpella maydis													2		

Table 3: Fungal enumeration of cultivars 6-10, expressed as percentage of kernels infested.

Cultivar No:	**6 (Synercus)**			**7 (LS 8504)**			**8 (PAN 6146)**			**9 (DKC 80-12 Y)**			**10 (AFG 4410)**		
Sample No:	**20040177**			**20040178**			**20040179**			**20040180**			**20040181**		
	PDA	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**
Cladosporium spp.									2	2		4			
Fusarium graminearum						2				2					
Fusarium oxysporum	14							12							
Fusarium verticillioides	100	100	100	100	100	100	56	98	96	100	100	100	100	100	100
Mucor spp.	4			100			58		30				100		100
Penicillium islandicum	8														
Penicillium oxalicum	12												46		64
Penicillium spp.	2		8	54		70	18		26	12		12			
Phoma sorghina							18		56			4			
Stenocarpella maydis							6								
Bacteria				100			100								

Table 4: Fungal enumeration of cultivars 11-15, expressed as percentage of kernels infested.

Cultivar No:	11 (Goldfinger)			12 (PAN 6966)			13 (DKC 66-22)			14 (NS 5914)			15 (PHB 30H22)		
Sample No:	20040182			20040183			20040184			20040185			20040186		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Fusarium oxysporum		94			18						58				
Fusarium poae		2													
Fusarium verticillioides	100	100	100	100	100	90	100	100	100	100	100	100	100	100	100
Mucor spp.	100			100			100		100	68		46	100		32
Penicillium islandicum			50							2			12		
Penicillium oxalicum	76			66		68			80			4	66		66
Penicillium spp.							64								26
Stenocarpella maydis							8								

Table 5: Fungal enumeration of cultivars 16-20, expressed as percentage of kernels infested.

Cultivar No:	16 (SNK 2472)			17 (AFG 4512)			18 (DKC 63-20)			19 (CRN 3760)			20 (MRI 514)		
Sample No:	20040187			20040188			20040189			20040190			20040191		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Fusarium graminearum									4			4			
Fusarium oxysporum		24													
Fusarium verticillioides	100	92	100	100	100	100	100	100	100	100	100	100	100	100	100
Mucor spp.	4			6			70			66			24		
Nigrospora spp.															6
Penicillium islandicum				4											
Penicillium oxalicum	16		16	14		10	64		42	4		8	6		2
Penicillium spp.				6					8						
Phoma sorghina							28		10						
Stenocarpella maydis	4						2								
Bacteria										100			100		

Table 6: Fungal enumeration of cultivars 21-25, expressed as percentage of kernels infested.

Cultivar No:	21 (PAN 6053)			22 (CRN 3505)			23 (SC 621)			24 (MRI 624)			25* (PAN 6479)		
Sample No:	20040192			20040193			20040194			20040195			20040196		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Acremonium spp.															6
Fusarium verticillioides	100	100	100	100	100	100	100	100	88	100	100	100	48	84	26
Mucor spp.	6		2	24			14			6		2			2
Nigrospora spp.	8		8			6	4			2		4	14		
Penicillium islandicum	12		6			2	10		4	2			2		2
Penicillium oxalicum	16		16	18		32	24		24	20		14	22		18
Penicillium spp.										4					
Stenocarpella maydis	4									4			10		

*Cultivar no. 25: Some seeds had no fungal growth.

Table 7: Fungal enumeration of cultivars 26-30, expressed as percentage of kernels infested.

Cultivar No:	26 (SNK 2551)			27 (Saffier)			28 (PAN 6043)			29 (SC 405)			30 (PHB 30D05)		
Sample No:	20040197			20040198			20040199			20040200			20040201		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Fusarium verticillioides	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Mucor spp.	12		10	12		8	50						34		8
Nigrospora spp.			22												
Penicillium islandicum	30			32		6	38		16	12			18		
Penicillium oxalicum	38		44	42		72	42		36	26		68	48		84
Penicillium spp.				28									10		
Phoma sorghina			2						2						
Stenocarpella maydis				2						2			2		
Bacteria	100									100			100		

Table 8: Fungal enumeration of cultivars 31-35, expressed as percentage of kernels infested.

Cultivar No:	31 (PAN 6633)			32 (LS 8509)			33 (CRN 3549)			34 (PAN 6611)			35 (SC 407)		
Sample No:	20040202			20040203			20040204			20040205			20040206		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Aspergillus flavus							2								
Eurotium rubrum									2						
Fusarium verticillioides	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Mucor spp.				12			94		100				2		
Nigrospora spp.				40		54			4	2		8			2
Penicillium islandicum				26			2		4	4			4		2
Penicillium oxalicum	46		60	72		46			14	2		22	12		20
Penicillium spp.													6		6
Phoma sorghina													6		2
Stenocarpella maydis							4								
Bacteria	100			100			100			100			100		

Table 9: Fungal enumeration of cultivars 36-40, expressed as percentage of kernels infested.

Cultivar No:	36 (CAP 341-NG)			37 (PAN 6615)			38 (DKC 78-15)			39 (Caracal)			40 (PAN 6995Bt)		
Sample No:	**20040207**			**20040208**			**20040209**			**20040210**			**20040211**		
	PDA	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**	**PDA**	**PCNB**	**MSA**
Alternaria spp.							2								
Cladosporium spp.									36						72
Eurotium repens			2												
Fusarium graminearum													2		
Fusarium verticillioides	100	100	100	100	100	100	42	66	38	100	100	100	50	58	8
Mucor spp.	10						12		4	100		100	16		4
Nigrospora spp.							24		22				36		16
Penicillium islandicum			2			2									
Penicillium oxalicum	6		12	8		12	6		6			18	2		4
Penicillium spp.	2		2				2		2	8					
Phoma sorghina	6		4	4			24		10	6		2			2
Bacteria										100					

Table 10: Fungal enumeration of cultivars 41-45, expressed as percentage of kernels infested.

Cultivar No:	41 (LS 8507)			42 (PAN 6927)			43 (Panthera)			44 (PAN 6537)			45 (SNK 2969)		
Sample No:	20040212			20040213			20040214			20040215			20040216		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Alternaria spp.															2
Cladosporium spp.			24												
Fusarium graminearum			4												
Fusarium verticillioides	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Mucor spp.	100			26		24	100			2					
Nigrospora spp.						18									
Penicillium islandicum							14			2					
Penicillium oxalicum	4		32	26		32	4			2			6		
Penicillium spp.									26			10			2
Phoma sorghina	10			4											
Bacteria	100			100			100			100			100		

Table 11: Fungal enumeration of cultivars 46-49, expressed as percentage of kernels infested.

Cultivar No:	46 (PAN 6071)			47 (ZM 521)			48 (PAN 6335)			49 (PHB 30N35)		
Sample No:	20040217			20040218			20040219			20040220		
	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA	PDA	PCNB	MSA
Alternaria spp.			2									
Aspergillus flavus				2								
Fusarium verticillioides	100	100	100	100	100	100	100	100	100	100	100	100
Mucor spp.							6			28		
Penicillium islandicum	2			4								
Penicillium oxalicum	8			4			20		14	34		58
Penicillium spp.			26			16						
Phoma sorghina							8			32		
Stenocarpella maydis							2					
Bacteria	100			100			100			100		

Table 12: Fungal species found to be associated with the 49 maize cultivars in the study.

Fungus Name	*Fungus Name*
Acremonium spp. (F)	*Fusarium verticillioides* (F)
Alternaria spp. (F)	*Geotrichum* spp. (F/S)
Aspergillus flavus (S)	*Mucor* spp. (F/S)
Basidiomycetes (F)	*Nigrospora* spp. (F)
Cladosporium spp. (F)	*Penicillium islandicum* (S)
Eurotium rubrum (S)	*Penicillium oxalicum* (S)
Fusarium graminearum (F)	*Penicillium* spp. (S)
Fusarium oxysporum (F)	*Phoma sorghina* (F)
Fusarium poae (F)	*Stenocarpella maydis* (F)
Fusarium spp. (F)	

F/S indicates whether the fungus is regarded as a field or storage fungus.
Source: Martin (1974).

Table 13: Possible mycotoxins present in cultivars produced in Potchefstroom, based on the presence of fungi during the fungal enumeration test.

Cultivar no.	Cultivar description	Possible mycotoxins present
1	PAN 6734	Fumonisins, secalonic acid D
2	DKC 80-10	Fumonisins
3	PHB 3442	Fumonisins
4	PAN 6844	Fumonisins
5	SNK 2662	Fumonisins, moniliformin, zearalenone
6	Syncerus	Fumonisins, toxins from *Mucor* spp.
7	LS 8504	Fumonisins, toxins from *Penicillium* spp.
8	PAN 6146	Fumonisins, tenuazonic acid, toxins from *Mucor* spp.
9	DKC 80-12 Y	Fumonisins
10	AFG 4410	Fumonisins
11	Goldfinger	Fumonisins, moniliformin, zearalenone, luteoskyrin, secalonic acid D
12	PAN 6966	Fumonisins, secalonic acid D, toxins from *Mucor* spp.
13	DKC 66-22	Fumonisins, secalonic acid D, toxins from *Mucor* and *Penicillium* spp.
14	NS 5914	Fumonisins, moniliformin, zearalenone, toxins from *Mucor* spp.
15	PHB 30H22	Fumonisins, secalonic acid D, toxins from *Mucor* spp.
16	SNK 2472	Fumonisins, moniliformin, zearalenone
17	AFG 4512	Fumonisins
18	DKC 63-20	Fumonisins, secalonic acid D, tenuazonic acid, toxins from *Mucor* spp.
19	CRN 3760	Fumonisins, toxins from *Mucor* spp.
20	MRI 514	Fumonisins
21	PAN 6053	Fumonisins
22	CRN 3505	Fumonisins, secalonic acid D
23	SC 621	Fumonisins
24	MRI 624	Fumonisins
25	PAN 6479	Fumonisins
26	SNK 2551	Fumonisins, luteoskyrin, secalonic acid D
27	Saffier	Fumonisins, luteoskyrin, secalonic acid D
28	PAN 6043	Fumonisins, luteoskyrin, secalonic acid D, toxins from *Mucor* spp.
29	SC 405	Fumonisins, secalonic acid D
30	PHB 30D05	Fumonisins, secalonic acid D, toxins from *Mucor* spp.

Table 13 continued: Possible mycotoxins present in cultivars produced in Potchefstroom, based on the presence of fungi during the fungal enumeration test.

31	PAN 6633	Fumonisins, secalonic acid D
32	LS 8509	Fumonisins, secalonic acid D
33	CRN 3549	Fumonisins, toxins from *Mucor* spp.
34	PAN 6611	Fumonisins
35	SC 407	Fumonisins
36	CAP 341 NG	Fumonisins
37	PAN 6615	Fumonisins
38	DKC 78-15	Fumonisins
39	Caracal	Fumonisins, toxins from *Mucor* spp.
40	PAN 6995 Bt	Fumonisins
41	LS 8507	Fumonisins, secalonic acid D, toxins from *Mucor* spp.
42	PAN 6927	Fumonisins, secalonic acid D
43	Panthera	Fumonisins, toxins from *Mucor* spp.
44	PAN 6537	Fumonisins
45	SNK 2969	Fumonisins
46	PAN 6017	Fumonisins
47	SM 521	Fumonisins
48	PAN 6335	Fumonisins
49	PHB 30N35	Fumonisins, secalonic acid D, tenuazonic acid

Table 14: Levels of fumonisins and zearalenone in the 49 maize cultivars tested based on the Neogen Veratox kits.

Cultivar no.	Cultivar description	Total fumonisins (ppm)	Zearalenone (ppb)
1	PAN 6734	0.7	ND
2	DKC 80-10	1.8	ND
3	PHB 3442	8.6	ND
4	PAN 6844	0.8	ND
5	SNK 2662	0.1	46.3
6	Syncerus	2.6	ND
7	LS 8504	1.5	ND
8	PAN 6146	0.4	ND
9	DKC 80-12 Y	0.3	ND
10	AFG 4410	3.6	ND
11	Goldfinger	8.5	26.7
12	PAN 6966	5.9	ND
13	DKC 66-22	6	ND
14	NS 5914	0.1	6.8
15	PHB 30H22	7.6	ND
16	SNK 2472	4.7	62.2
17	AFG 4512	5.2	ND
18	DKC 63-20	3.1	ND
19	CRN 3760	6.5	ND
20	MRI 514	5	ND
21	PAN 6053	0.2	ND
22	CRN 3505	1.4	ND
23	SC 621	ND	ND
24	MRI 624	1.1	ND
25	PAN 6479	3.7	ND
26	SNK 2551	ND	ND
27	Saffier	1.9	ND
28	PAN 6043	0.1	ND
29	SC 405	4.1	ND
30	PHB 30D05	2.1	ND
31	PAN 6633	0.4	ND
32	LS 8509	0.4	ND
33	CRN 3549	0.2	ND
34	PAN 6611	ND	ND
35	SC 407	8.3	ND
36	CAP 341 NG	9.9	ND
37	PAN 6615	0.1	ND
38	DKC 78-15	ND	ND

Table 14 continued: Levels of fumonisins and zearalenone in the 49 maize cultivars tested based on the Neogen Veratox kits.

39	Caracal	ND	ND
40	PAN 6995 Bt	ND	ND
41	LS 8507	8.8	ND
42	PAN 6927	ND	ND
43	Panthera	5.1	ND
44	PAN 6537	ND	ND
45	SNK 2969	10.2	ND
46	PAN 6017	0.5	ND
47	SM 521	11.1	ND
48	PAN 6335	0.1	ND
49	PHB 30N35	0.8	ND

*ND = not detected, ppm = parts per million (mg/kg), ppb = parts per billion (µg/kg)

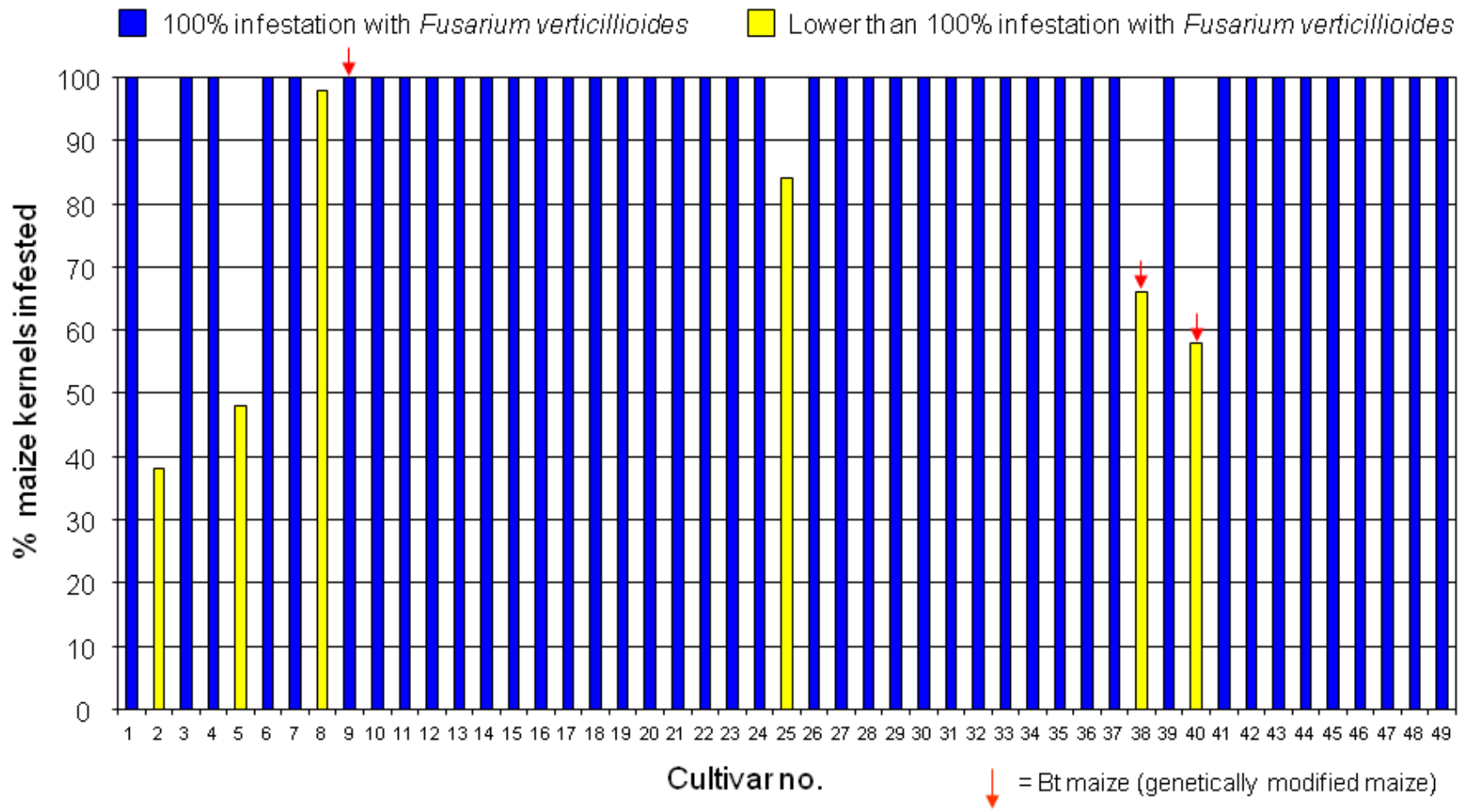

FIGURE 2: Presence of *Fusarium verticillioides* in 49 maize cultivars.

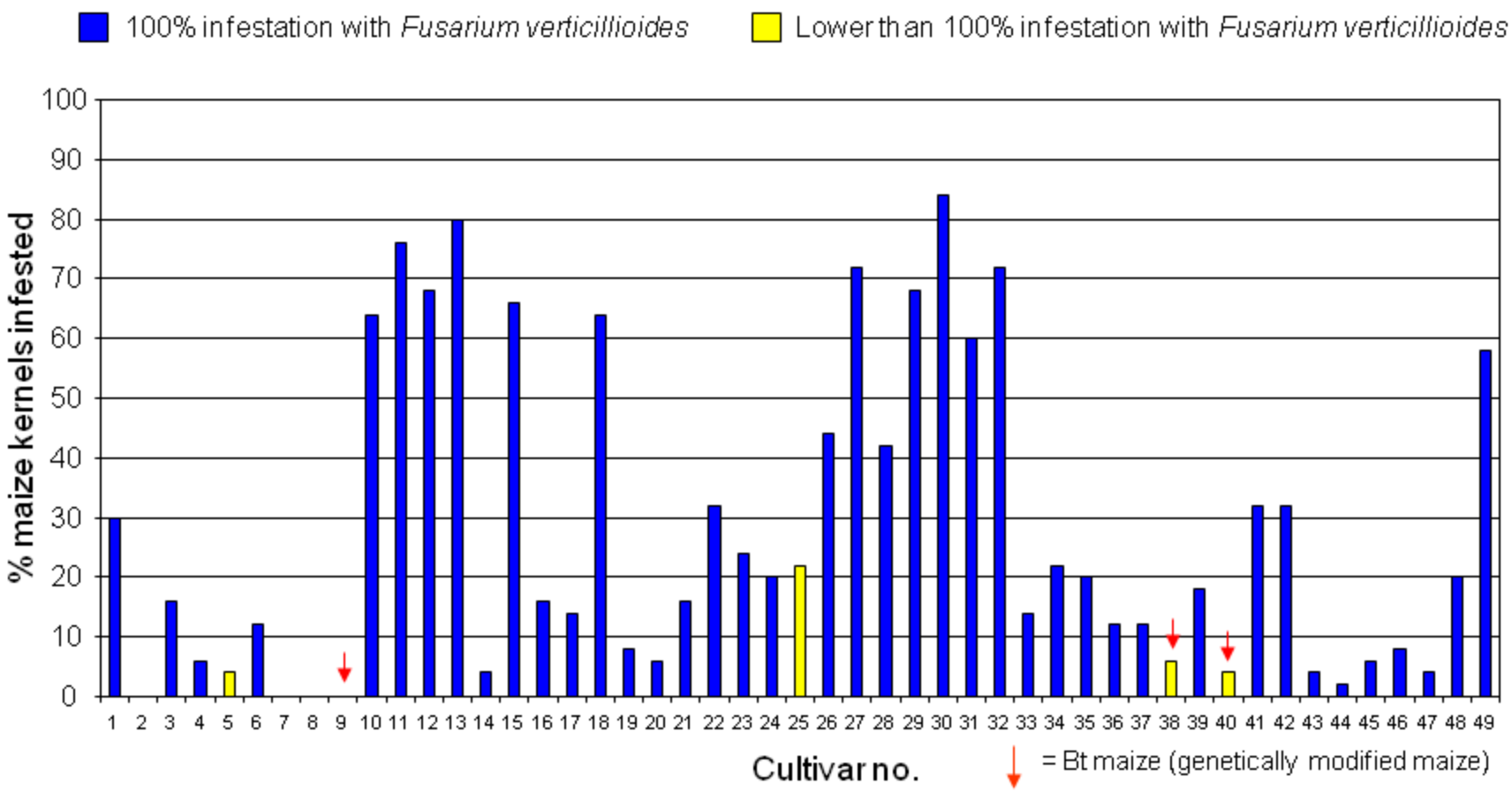

FIGURE 3: Presence of *Penicillium oxalicum* in 49 maize cultivars.

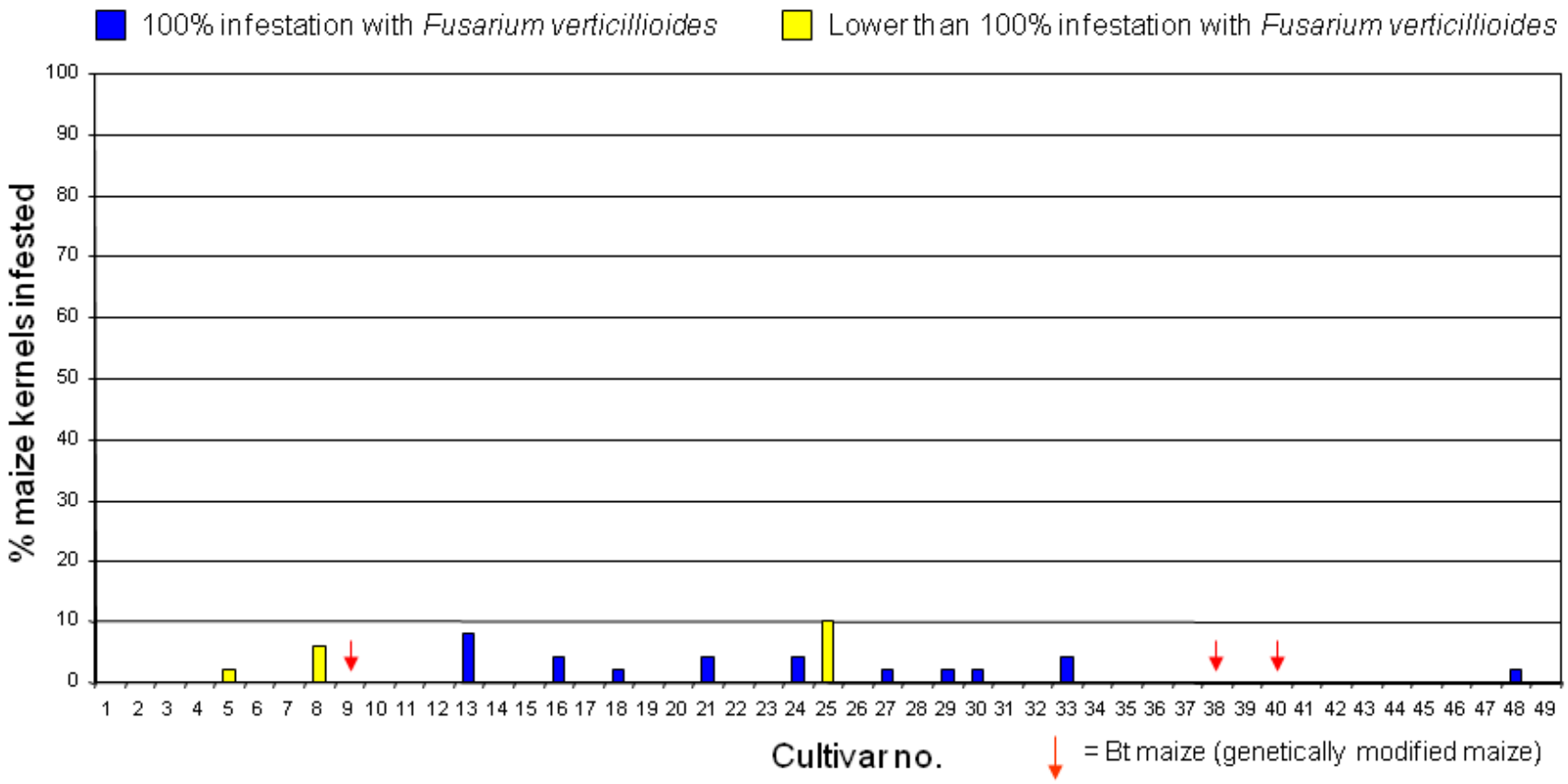

FIGURE 4: Presence of *Stenocarpella maydis* in 49 maize cultivars.

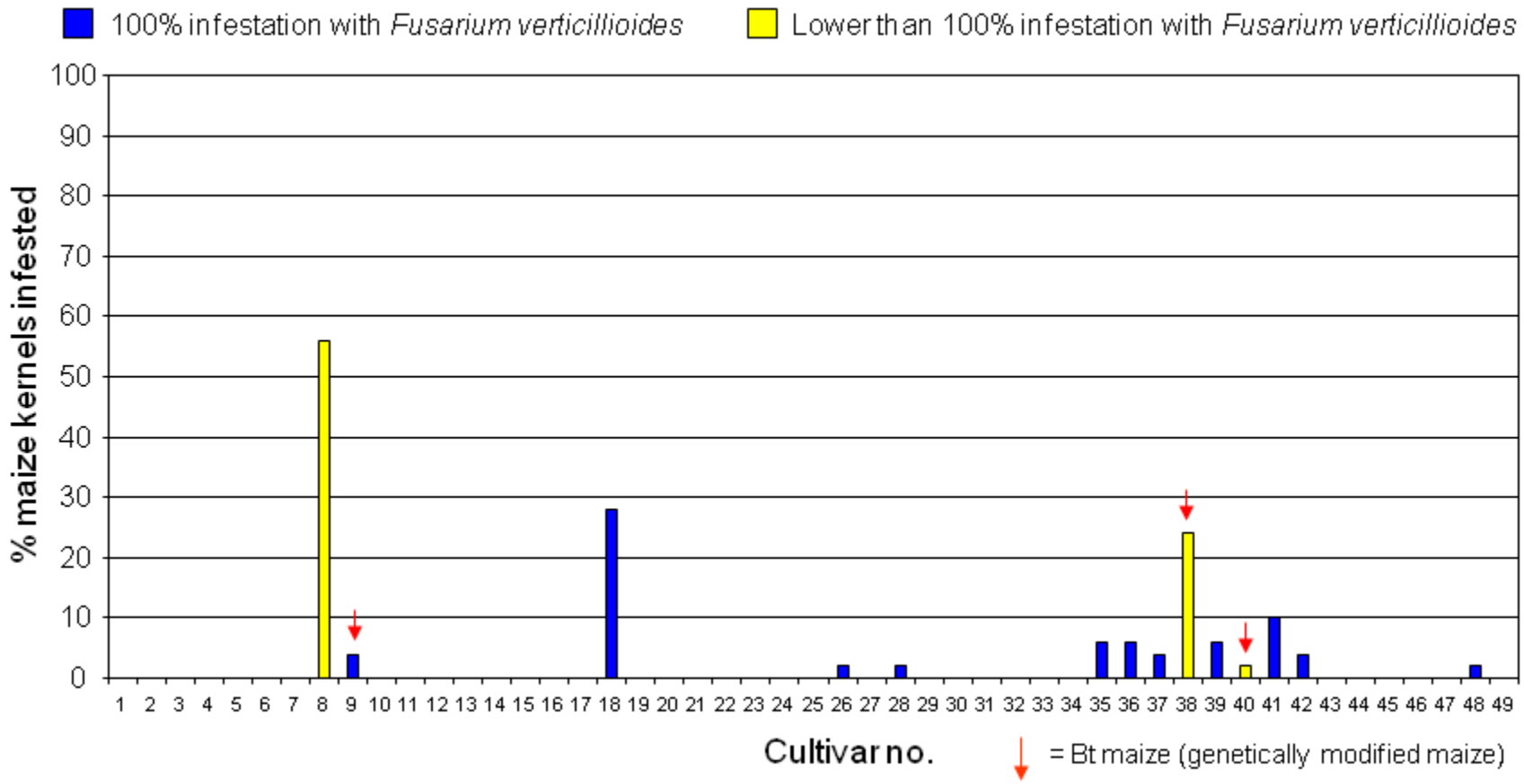

FIGURE 5: Presence of *Phoma sorghina* in 49 maize cultivars.

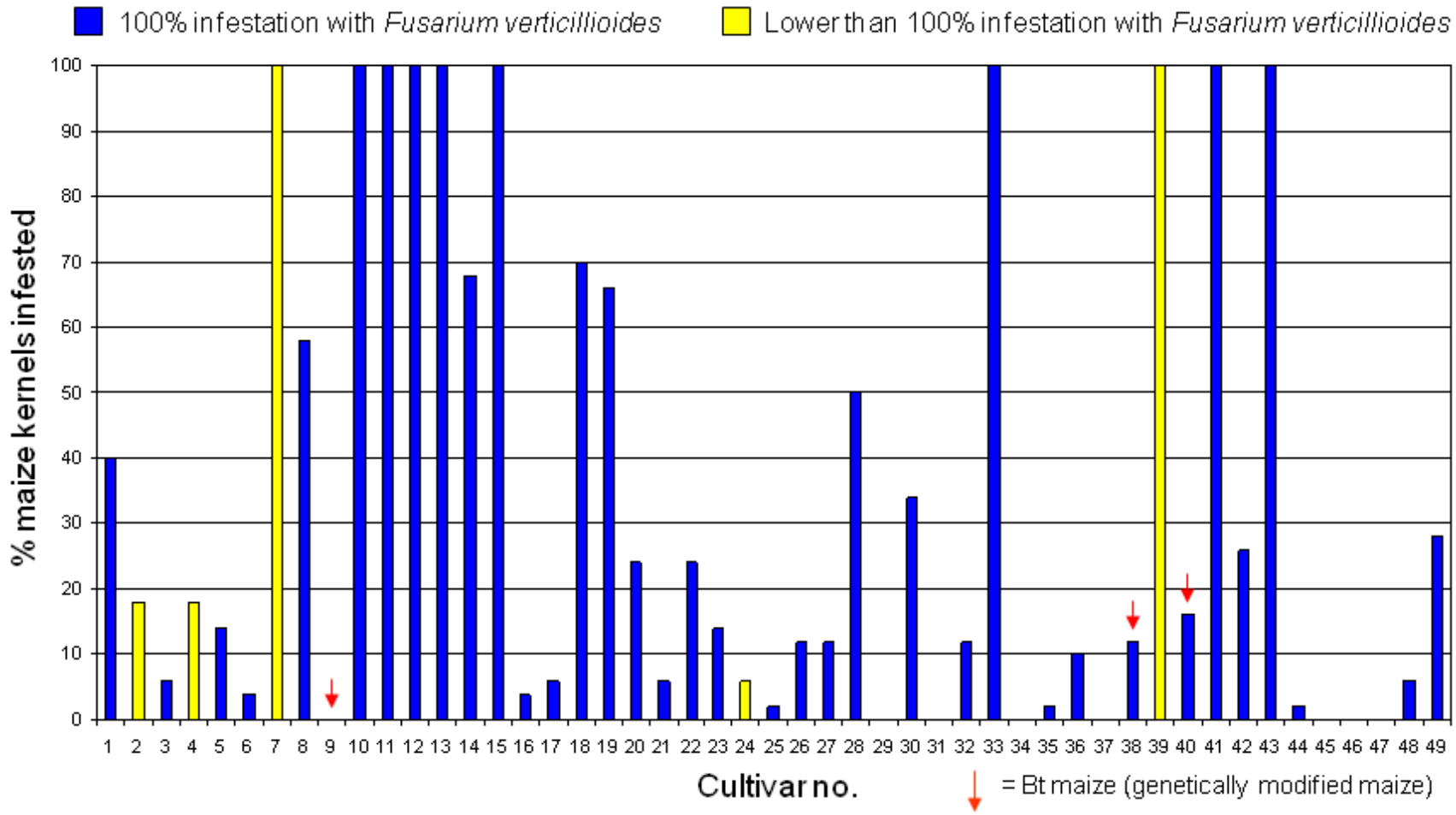

FIGURE 6: Presence of *Mucor* species in 49 maize cultivars.

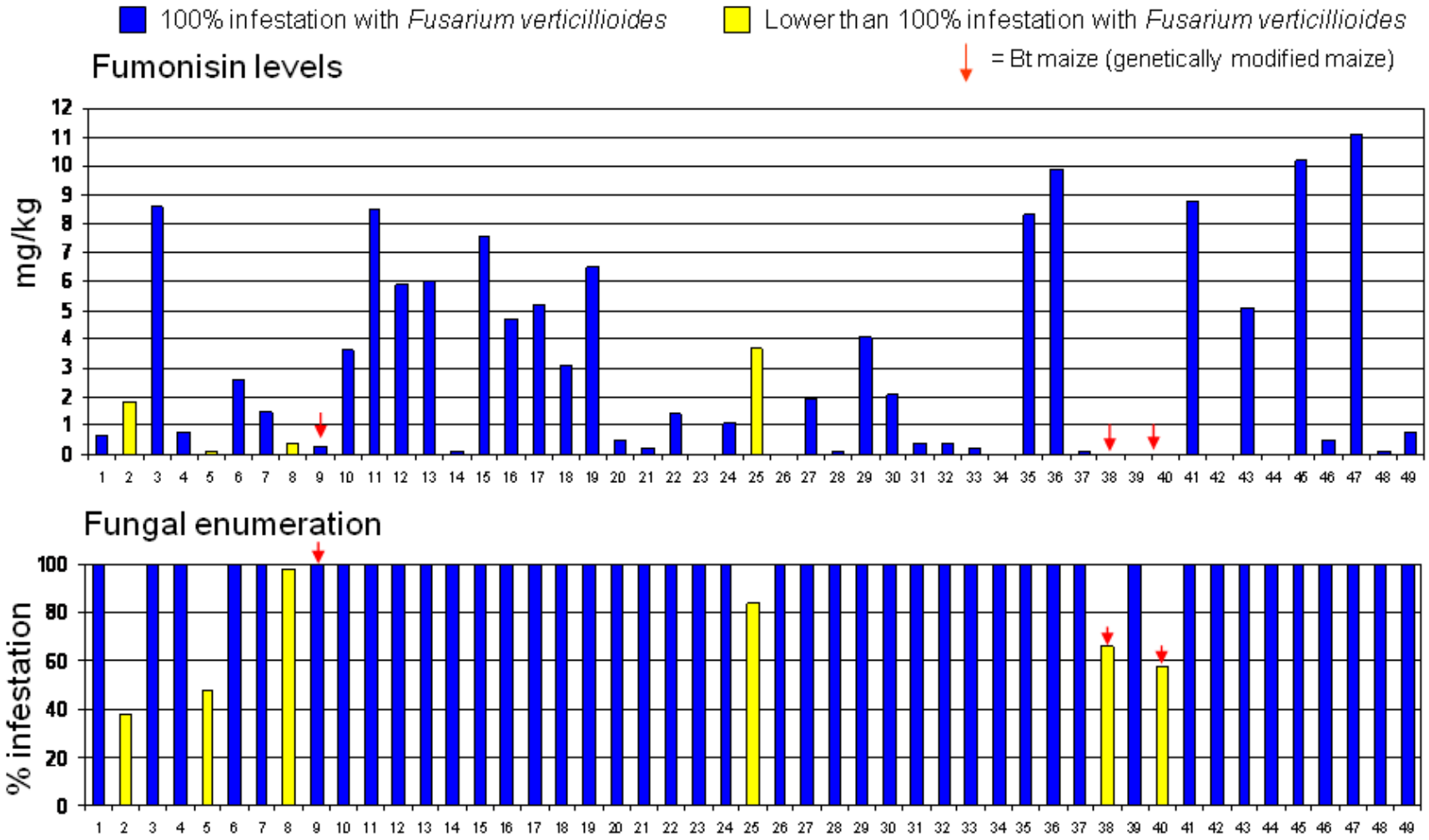

FIGURE 7: Fumonisin levels using the Neogen Veratox kit compared to levels of *Fusarium verticillioides* in maize cultivars.

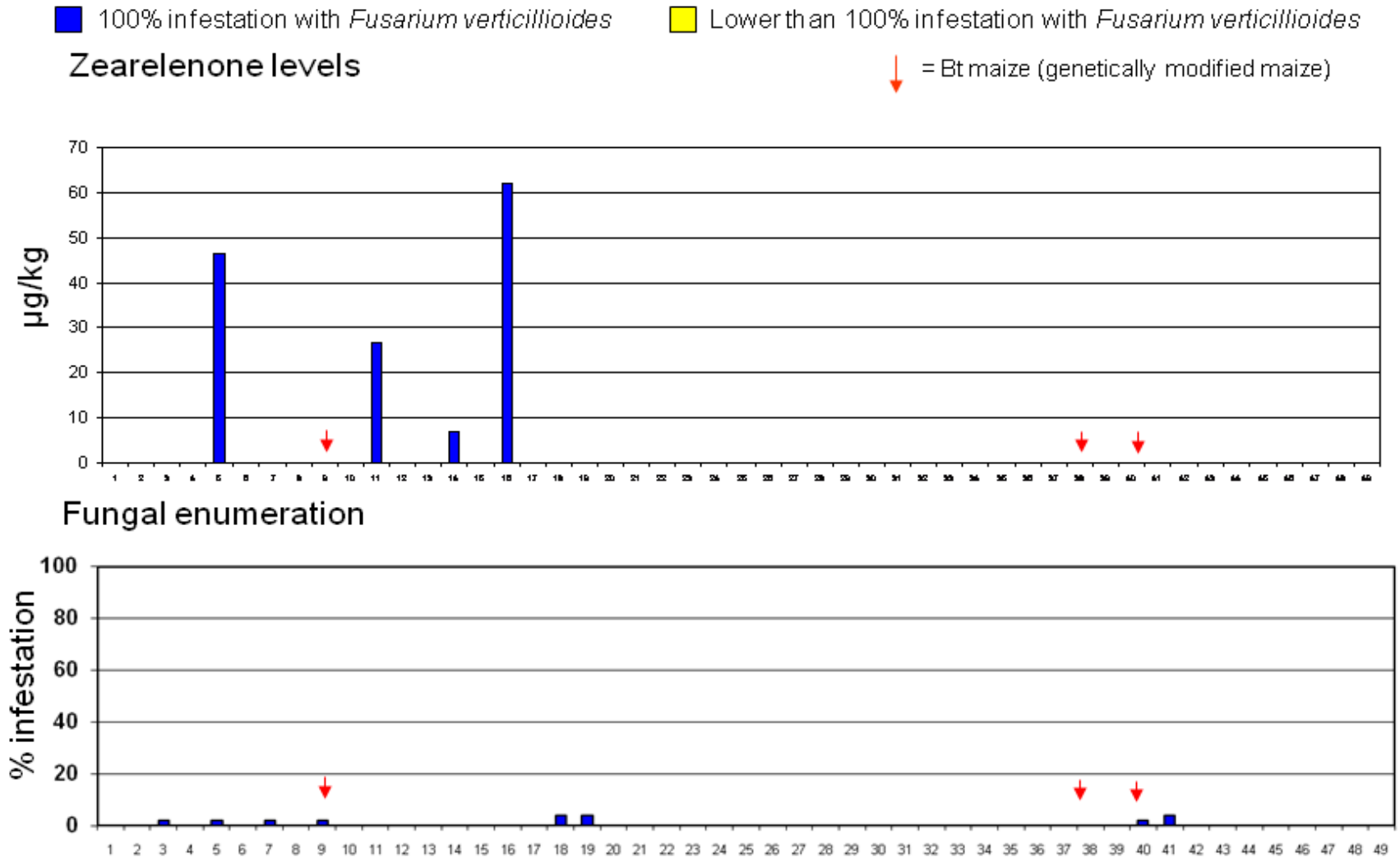

FIGURE 8: Zearelenone levels using the Neogen Veratox kit compared to levels of *Fusarium graminearum* in maize cultivars.

Chapter 3:

The effect of irradiation on mycotoxigenic fungi in commercially produced maize cultivars

1. Summary

Maize is the staple food of the majority of people living in South Africa. It is, therefore, of great importance to ensure that the quality of this food source does not pose a threat to the health of humans and animals. Maize is known to be contaminated by a wide range of both storage and field fungi that are responsible for 25% of grain crops worldwide being contaminated with mycotoxins on a yearly basis.

In an attempt to lower the levels of viable fungi in maize, a study was conducted to determine the effect of irradiation against the viability of mycotoxigenic fungi in 49 commercially produced maize cultivars. Two cultivars were initially exposed to various intervals of gamma-irradiation including 2, 4, 6, 8 and 12 kGy respectively.

Results showed that exposure to 8 kGy had a dramatic decrease in the levels of viable fungi without substantially affecting the germination of the maize kernels. All 49 maize cultivars were exposed to 8 kGy of gamma-irradiation that showed similar results.

The levels of *Fusarium verticillioides* were extremely high in the initial samples and were almost non-existent after irradiation. Gamma-irradiation proved to successfully decrease both field and storage fungi to almost a non-viable level, thus also lowering the potential of further formation of mycotoxins during storage of the maize.

2. Introduction

South Africa produces about 8 million tons of maize per annum, making it the country with the largest maize industry in Africa (NDA, **2003**). Just less than half of the crop is destined for human consumption as a staple food for the majority of people, while the rest is used for animal feed. All South Africans consume maize in some form or another, with the majority eating it in the form of maize meal (Viljoen, **2003**). Nevertheless, South Africa produces a surplus of maize in some years that is destined for export purposes to other countries, such as Zimbabwe, Japan, Zambia, Malawi, Mauritius, Kenya and Mozambique (NDA, **2003**).

One of the main concerns in South African maize is the occurrence of a wide variety of both field and storage fungi, some of which are known mycotoxin producers (Rabie *et al*., **1982**). Mycotoxins are mainly secondary metabolites that are produced by fungi and have the capacity to impair human and animal health (mycotoxicoses) (D'Mello *et al.,* **1997**). Fungal toxins are not necessarily detected by antigens and hence in certain cases produce no obvious symptoms (Fung *et al.,* **2004**). The estimation is that 25% of grain crops worldwide are contaminated with mycotoxins, making it a serious quality and safety issue on a global scale (Pitt, **1989**).

One possible way of controlling the development of fungi and their mycotoxins in maize is the use of gamma irradiation. This entails the use of emissions given off by various radioactive substances such as Cobalt 60 or Cesium 137 (Steele, **2000**). These substances give off high energy photons called gamma rays, which have the ability to penetrate up to a depth of several feet (Steele, **2000**; Shea, **2000**). Such rays do not make the product radioactive because no photons are given off. Some of the benefits that are indicated for the use of gamma irradiation include a high level of process reliability, total penetration potential, production of consistent results, and having broad product services

applications which are safe and environmentally friendly (Käferstein, **1993**; Kilcast, **1995**; Diehl, **2002**).

Gamma irradiation has been studied previously with different degrees of success on barley seeds at 4 kGy (Ramakrishna *et al*., **1991**), fruits at 5 kGy (Aziz *et al*., **2001**), black cumin at 6 kGy (EL-Bazza Zeinab *et al*., **2001**), walnuts at 2 kGy (Al-Bachir, **2002**), rice and pulses at levels above 4 kGy (Maity *et al.,* **2004**), and maize (Pfahler, **1970**; Adem *et al*., **1981**; Cuero *et al*., **1986;**). In most cases, a decrease in fungal levels and mycotoxin production was experienced.

The purpose of this study was to investigate the possibility of lowering the fungal infestation in maize through the use of gamma-irradiation, but without substantially influencing the germination of the kernels. Accordingly, one of the objectives was to obtain maize cultivars that were relatively free of viable fungi in order to conduct resistance trials during storage (see Chapter 4). These cultivars were then spiked with known mycotoxigenic fungi and the tempo of infestation was subsequently monitored in each cultivar. The *rationale* was that, if the ability of the maize kernel to germinate stayed intact after irradiation, then the resistance mechanism would also likely be uninfluenced.

3. Materials and Methods

3.1. Maize

Two out of 49 maize cultivars, each representative of yellow (PHB 3442 / Cultivar no. 3) and white maize (LS 8507 / Cultivar no. 41), were selected to conduct a series of irradiation levels. This was to determine the optimum irradiation level to eliminate, or drastically lower the viability of fungi and still obtain good germination of the kernels. All 49 cultivars were then exposed to this selected optimum irradiation level.

3.2. Irradiation

Subsamples of each of the 49 cultivars were taken by weighing out 180 g of maize into a container. These where then sent to Isotron (Pty) Ltd (Isando, Johannesburg), where the containers were exposed to various levels of gamma irradiation. These included levels of 2, 4, 6, 8 and 12 kGy. The populations and levels of the survival rate of the various fungi at various irradiation levels were determined by the method described by Rabie *et al.* (**1997**). The results were compared to determine the optimum level of irradiation for maximum effect on the viability of the fungi and minimum influence on the germinability of the maize kernels. Non-irradiated maize of both cultivars 3 and 41 was used as control.

3.3. Fungal enumeration

Enumeration was done by surface sterilizing the irradiated and non-irradiated maize kernels and plating them onto two different media, namely potato dextrose agar (PDA) and malt salt agar (MSA). This was done to determine the viability of both field- and storage fungi. The fungi were identified based on morphological characteristics and the results were expressed as the percentage of kernels infested with a specific fungus. Results are summarized in Tables 1 and 2, indicating the fungal infestation of the different irradiation levels in the two maize cultivars tested. This level of irradiation (8 kGy) was then used to treat all 49 cultivars, after which fungal enumeration was done (see Tables 3 to 12, pgs. 125-129). The detail on the composition of the media used is given below.

3.3.1. Culture Media

3.3.1.1. Potato Dextrose Agar (PDA)

PDA (Merck, South Africa) was prepared according to manufacturers instructions. It was then poured into 90 mm petri dishes and allowed to settle. Plates were stored at 4°C until used.

3.2.1.2. Malt Salt Agar (MSA)

MSA was prepared by dissolving 90 g NaCl (Promark Chemicals, South Africa) in 360 ml distilled water. Separately, 24 g of malt extract (Merck, South Africa) and 24 g of agar (Merck, South Africa) were added to 840 ml distilled water in a 1.2 L container. These were then separately autoclaved for 20 min at 121°C and allowed to cool down to approximately 55°C. The sterilized NaCl was then added aseptically to the malt extract and agar. The medium was then aseptically added to 90 mm petri dishes and allowed to settle. The plates were then stored at 4°C until it was used.

3.4. Surface sterilization

In order to remove all contaminants on the surface of the maize kernels, ethanol (approximately 76% v/v) was used as disinfectant. This was prepared by adding 200 ml distilled water to 800 ml 96% ethanol (Radchem Laboratory Supplies, South Africa). A small sample of maize (approximately 300 kernels) was rinsed for 1 min in disinfectant. After draining the ethanol from the maize sample, it was rinsed twice in sterile distilled water by shaking the submerged maize for 1 minute respectively and decanting the water. The sample was then dried on a sterile paper towel before plating.

3.5. Plating procedures

After the maize kernels were dried subsequent to surface sterilization, individual kernels were randomly selected and plated onto growth media. This was done by aseptically placing 5 kernels onto one 90 mm diam. petri dish and spreading them evenly by using forceps. In total, 100 kernels of each cultivar were placed on each medium respectively. The plates were incubated for at least 10 days at approximately 25^0C with 12-hour dark and light cycles, until fruiting structures emerged from the kernels.

3.6. Fungal identification

All fungal cultures were identified based on their morphological characteristics by using stereo and light microscopy. Identifications of most fungi were based on reference books and publications (Domsch, **1980**), as well as with the assistance of known mycologists in the field of grain mycology. *Penicillium* species were identified according to the method of Pitt (**1991**). In some cases, it was not possible to identify fungi to species level due to the lack of fruiting structures. In such cases the fungi were identified only to genus level.

4. Results

Results showed that different levels of irradiation can have a substantially diverse effect on the viability of the fungi and the germination of the maize kernels. Due to the fact that *Fusarium verticillioides* was the most dominant fungus in the samples (see Chapter 2, Figure 2, pg. 102), the levels of this fungus were used as an indicator to determine the effective loss of viability among the fungi present. It was apparent that viable levels of *F. verticillioides* in cultivar 3 (Table 1, pg. 123) were not drastically influenced, up to an irradiation level of 4 kGy. At a level of 6 kGy, however, the level of *F. verticillioides* was dramatically lowered but still at 18% infestation. At this level of radiation the germination of the kernels was only slightly influenced (lowered from 84% to 78%). At an exposure level of 8 kGy, contamination with *F. verticillioides* was almost eradicated. In addition, while the germinability of the maize kernels was affected, the majority of kernels

nevertheless demonstrated the ability to germinate, but with reduced fecundity (66% compared to an initial 94% before irradiation).

A similar trend was experienced with cultivar 41 (Table 2, pg. 124). A drastic decline in fungal viability was experienced at 6 kGy with no decline of germinability of the maize kernels. At 8 kGy the germinability only declined with approximately 10% and the fungal counts were almost non-existent.

Based on these results, it was decided to irradiate all 49 maize cultivars at a level of 8 kGy (Tables 3 – 12, pgs. 125-129). Results showed that different cultivars reacted differently to the germination of the kernels. For example cultivar 4 (Table 3, pg. 125) indicated only a slight decline in germination (from 92% to 90%) whereas cultivar 18 (Table 6, pg. 126) indicated a loss of 90% in germinability.

All cultivars showed a dramatic decline in the levels of the viability of fungi. The highest level of fungal viability was found in cultivar 11 (Table 5, pg. 126) containing a level of 12% for *F. verticillioides* after irradiation at 8 kGy, which is dramatically lower than in the pre-irradiated maize. In many cases the level of viable fungi were decreased to zero.

Figure 1 (pg. 130) and Figure 2 (pg. 131) respectively show levels of viable fungi after irradiation at 8 kGy and corresponding germination levels of the maize kernels, for the 49 cultivars examined.

5. Discussion

Results in this study indicated that irradiation could be a powerful tool to dramatically lower the levels of viable fungi, without significantly influencing the germinability of maize kernels. What also became apparent is that different maize cultivars could react differently to irradiation both with respect to lowered fungal viability as well as intrinsic fecundity of the kernels themselves. It should also be kept in mind, that other factors such as moisture content, the type of fungi present, as well as the area and conditions in which the cultivars were grown, could influence the outcome of the irradiation. It was beyond the scope of this dissertation to further investigate this combination of factors, or the levels of mycotoxins present after irradiation.

Although irradiation could have benefits in lowering the viability of maize-infesting fungi, previous studies have also shown that irradiated material could also be susceptible to post-irradiation formation of mycotoxins. Specifically, this has been shown to occur where pure cultures of mycotoxigenic fungi have been introduced after the irradiation process (El-Aal & Aziz, **1995**). Possible reasons for this, is that the substrate is void of any viable organisms, making it easy for a newly introduced fungus to propagate and develop without substantial competition from other organisms. One example to illustrate this phenomenon is from a study conducted by Paster & Bullerman (**1988**) on wheat. These authors showed that once *Aspergillus ochraceus* was exposed to low levels of irradiation, it actually increased the production of mycotoxins (ochratoxin A). However, once the irradiation levels continued to increase, the fungus lost its viability. Although the viability of fungi can be drastically influenced by gamma-irradiation, mycotoxins are normally not effected. This was proven by Krska *et al.* (**2003**) in a study where all *Fusarium* species present in maize were killed without decreasing the mycotoxin levels.

Gamma-irradiation can also be utilized in combination with other physical and chemical methods, such as modified atmosphere and fungistats, to decrease fungal development and subsequently decrease mycotoxin levels in a commodity (Paster & Bullerman, **1988**). Each physical means for controlling mycotoxin formation can interact differently for each mycotoxin production. By utilizing a combination of methods, synergistically, at lower levels of exposure, can control the development of fungal growth in a commodity (Paster *et al.,* **1992**). These authors have shown that the use of a combination of 0.2% propionic acid, in combination with 2 kGy irradiation and 40-60% carbon dioxide (CO_2) in a single treatment, reduced the spoilage effects significantly compared to any single method used independently.

6. Conclusion

While the use of gamma-irradiation was found to lower the fecundity of maize kernels, viability of fungi were shown to be radically reduced at a level of 8 kGy.

As such, the objective of this study, namely to substantially reduce the viable levels of fungi without dramatically influencing the viability of the maize kernels was successfully achieved. Nevertheless, it should be kept in mind that gamma-irradiation could have a negative impact on the subsequent formation of mycotoxins, especially in cases where the irradiated material is destined for food or feed production (Shea, **2000**; Ferreira-Castro *et al.,* **2007**) and that the material will be exposed to possible further contamination from the environment. In South Africa, irradiation is used to treat meat products and is regarded as safe (de Bruyn, **2000**). In such cases this method should be used with the understanding that post-irradiated material could be exposed to the re-introduction of spoilage organisms, including mycotoxigenic fungi.

7. References

Adem, E., Uribe, R.M., Watters, F.L. & Bourges, H. 1981. Present status of corn grain disinfestations by irradiation in Mexico. *Radiation Physics and Chemistry.* 18: 555-567.

Al-Bachir, M. 2002. Effect of gamma irradiation on fungal load, chemical and sensory characteristics of walnuts (*Juglans regia* L.). *Journal of Stored Products Research*. 40: 355-362.

Aziz, N.H. & Moussa, L.A.A. 2002. Influence of gamma-radiation on mycotoxin producing moulds and mycotoxins in fruits. *Food Control*. 13: 281-288.

Cuero, R.G., Smith, J.E. & Lacey, J. 1986. The influence of gamma irradiation and sodium hypochlorite sterilization on maize seed microflora and germination. *Food Microbiology*. 3: 107-113.

de Bruyn, I.N. 2000. The application of high dose food irradiation in South Africa. *Radiation Physics and Chemistry*. 57: 223-225.

Diehl, J.F. 2002. Food irradiation-past, present and future. *Radiation Physics and Chemistry.* 63: 211-215.

Domsch, K.H. 1980. Compendium of soil fungi. Volume 1. Academic Press. 900pp.

D'Mello, J.P.F. & Macdonald, A.M.C. 1997. Mycotoxins. *Animal Feed Science Technology.* 69: 155-166.

El-Aal, A.S.S. & Aziz, N.H. 1995. Influence of competitive microbial flora and Innoculum size of *Penicillium purpurogenum* on the production of rubratoxin-B in corn medium before and after exposure to gamma radiation. *Food Research International.* 28 (**6**): 521-524.

EL-Bazza, Z.E.M., Hala, A.F., EL-Fouly, M.E.D.Z. & EL-Tablawy, S.Y.M. 2001. Inhibitory effects of gamma irradiation and *Nigella sativa* seeds oil on growth, spore germination and toxin production of fungi. *Radiation Physics and Chemistry.* 60: 181-189.

Ferreira-Castro, F.L., Aquino, S., Greiner, R., Ribeiro D.H.B., Reis, T.A. & Corrêa, B. 2007. Effects of gamma-irradiation on maize samples contaminated with *Fusarium verticillioides*. *Applied Radiation and Isotopes.* 65 (**8**): 927-933.

Fung, F. & Clark, R.F. 2004. Health effects of mycotoxins: A toxicological overview. *Clinical Toxicology.* 42 (**2**): 217-234.

Käferstein, F.K. & Moy, G.G. 1993. Public health aspects of food irradiation. *Journal of Public Health Policy.* 14 (**2**): 149-163.

Kilcast, D. 1995. Food irradiation: Current problems and future potential. *International Biodeterioration and Biodegradation.* 279-296.

Krska, R., Pettersson, H., Josephs, R.D., Lemmens, M., Mac Donald, S. & Welzig, E. 2003. Zearalenone in maize: stability testing and matrix characterization of a certified reference material. *Food Additives and Contaminant.* 20 (**12**): 1141-1152.

Maity, J.P., Chakraborty, A., Saha, A., Santra, S.C. & Chanda, S. 2004. Radiation-induced effects on some common storage edible seeds in India infested with surface microflora. *Radiation Physics and Chemistry.* 71: 1065-1072.

National Department of Agriculture (NDA). 2003. Food Pricing Monitoring Committee: Final report. December 2003. pp310. http://www.nda.agric.za/docs/fpmc/default.htm

Paster, N. & Bullerman, L.B. 1988. Mould spoilage and mycotoxin formation in grains as controlled by physical means. *International Journal of Food Microbiology.* 7: 257-265.

Paster, N., Menasherov, M., Lacey, J. & Fanelli, C. 1992. Synergism between methods for inhibiting the spoilage of damp maize during storage. *Postharvest Biology and Technology.* 2: 163-170.

Pfahler, P.L. 1970. Reproductive characteristics of *Zea mays* L. plants produced from gamma-irradiated kernels. *Radiation Botany.* 10(**4**): 329-335.

Pitt, J.I. 1989. An introduction to Mycotoxins. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Pitt, J.I. 1991. A laboratory guide to common *Penicillium* species. Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, 187 pp.

Rabie, C.J., Marasas, W.F.O., Thiel, P.G., Lübben, A. & Vleggaar, R. 1982. Moniliformin production and toxicity of different *Fusarium* species from Southern Africa. *Applied and Environmental Microbiology.* 43 (**3**): 517-521.

Rabie, C.J., Lübben, A., Marais, G.J. & Jansen van Vuuren, H. 1997. Enumeration of fungi in barley. *International Journal of Food Microbiology.* 35: 117-127.

Ramakrishna, N., Lacey, J. & Smith, J.E. 1991. Effect of sterilization, fumigation and gamma irradiation on microflora and germination of barley seeds. *International Journal of Food Microbiology*. 13: 47-54.

Shea, K.M. 2000. Technical report: Irradiation of food. *Pediatrics.* 106: 1505-1510.

Steele, J.H. 2000. Food irradiation: A public health opportunity. *International Journal of Infectious Diseases.* 4 (**2**), Editorial.

Viljoen, J.H. 2003. Mycotoxins in grain and grain products in South Africa and proposals for their regulation. PhD Thesis, University of Pretoria, pp. 305.

Table 1: Fungal enumeration and germination of maize (Cultivar no. 3), expressed as percentages, irradiated at different kGy's.

CULTIVAR NO. 3 (PHB 3442)	Control		2 kGy		4 kGy		6 kGy		8 kGy		12 kGy	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Acremonium spp.			2									
Aspergillus niger	50	48			12	10			2		6	
Eurotium rubrum		2										
Fusarium verticillioides	62	40	96	92	56	16	18	2	4		10	
Mucor spp.	2		22			2						
Penicillium spp.	36	38	10	6	18	14		10		2	8	
Phoma sorghina				4								
Germination (%)	84		60		20		78		66		16	

Table 2: Fungal enumeration and germination of maize (Cultivar no. 41), expressed as percentages, irradiated at different kGy's.

CULTIVAR NO. 41 (LS 8507)	Control		2 kGy		4 kGy		6 kGy		8 kGy		12 kGy	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Eurotium repens		12		2						2		
Eurotium rubrum		4						14		2		
Fusarium verticillioides	86	46	68	30	34	10	2	4	2		14	8
Mucor spp.	8											
Nigrospora spp.	6	2					4	2	4			
Penicillium spp.	8	20	12	6	8	14					16	24
Phoma sorghina	2				2							
Germination (%)	96		100		80		98		86		66	

Table 3: Fungal enumeration of irradiated maize cultivars 1-5, expressed as percentage of kernels infested.

Cultivar No:	1 (PAN 6734)		2 (DKC 80-10)		3 (PHB 3442)		4 (PAN 6844)		5 (SNK 2682)	
Sample No:	22040172		20040173		20040174		20040175		20040176	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Aspergillus niger								2		
Cladosporium spp.			2							
Coelomycetous fungi			2							
Fusarium oxysporum							2			
Penicillium spp.	2	2			2					
Germination (%)	38		56		26		90		44	

Table 4: Fungal enumeration of irradiated maize cultivars 6-10, expressed as the percentage of kernels infested.

Cultivar No:	6 (Synercus)		7 (LS 8504)		8 (PAN 6146)		9 (DKC 80-12 Y)		10 (AFG 4410)	
Sample No:	20040177		20040178		20040179		20040180		20040181	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Fusarium oxysporum			2							
Fusarium verticillioides	4							2		
Penicillium spp.					2		2			
Germination (%)	48		34		72		94		70	

Table 5: Fungal enumeration of irradiated maize cultivars 11-15, expressed as the percentage of kernels infested.

Cultivar No:	11 (Goldfinger)		12 (PAN 6966)		13 (DKC 66-22)		14 (NS 5914)		15 (PHB 30H22)	
Sample No:	20040182		20040183		20040184		20040185		20040186	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Fusarium verticillioides	12						2		2	
Germination (%)	68		90		80		64		66	

Table 6: Fungal enumeration of irradiated maize cultivars 16-20, expressed as the percentage of kernels infested.

Cultivar No:	16 (SNK 2472)		17 (AFG 4512)		18 (DKC 63-20)		19 (CRN 3760)		20 (MRI 514)	
Sample No:	20040187		20040188		20040189		20040190		20040191	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Coelomycetous fungi		2								
Fusarium oxysporum	2									
Penicillium spp.	2			2					2	
Germination (%)	58		78		8		72		36	

Table 7: Fungal enumeration of irradiated maize cultivars 21-25, expressed as the percentage of kernels infested.

Cultivar No:	21 (PAN 6053)		22 (CRN 3505)		23 (SC 621)		24 (MRI 624)		25 (PAN 6479)	
Sample No:	20040192		20040193		20040194		20040195		20040196	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Fusarium verticillioides					2					
Germination (%)	56		60		34		20		80	

Table 8: Fungal enumeration of irradiated maize cultivars 26-30, expressed as the percentage of kernels infested.

Cultivar No:	26 (SNK 2551)		27 (Saffier)		28 (PAN 6043)		29 (SC 405)		30 (PHB 30D05)	
Sample No:	20040197		20040198		20040199		20040200		20040201	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Aspergillus niger				2						
Cladosporium spp.		2								
Eurotium rubrum						4				
Penicillium spp.	4			6	4				2	
Germination (%)	36		26		34		18		94	

Table 9: Fungal enumeration of irradiated maize cultivars 31-35, expressed as the percentage of kernels infested.

Cultivar No:	31 (PAN 6633)		32 (LS 8509)		33 (CRN 3549)		34 (PAN 6611)		35 (SC 407)	
Sample No:	20040202		20040203		20040204		20040205		20040206	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Cladosporium spp.								2		
Fusarium verticillioides									2	
Mucor spp.					4					
Penicillium spp.							2			
Germination (%)	70		76		90		78		90	

Table 10: Fungal enumeration of irradiated maize cultivars 36-40, expressed as the percentage of kernels infested.

Cultivar No:	36 (CAP 341-NG)		37 (PAN 6615)		38 (DKC 78-15)		39 (Caracal)		40 (PAN 6995Bt)	
Sample No:	20040207		20040208		20040209		20040210		20040211	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Nigrospora spp.					10					
Phoma sorghina								2		
Germination (%)	66		74		80		46		58	

Table 11: Fungal enumeration of irradiated maize cultivars 41-45, expressed as the percentage of kernels infested.

Cultivar No:	41 (LS 8507)		42 (PAN 6927)		43 (Panthera)		44 (PAN 6537)		45 (SNK 2969)	
Sample No:	20040212		20040213		20040214		20040215		20040216	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Aspergillus niger							2			
Eurotium repens				2						
Penicillium spp.	2	6	2							
Germination (%)	72		76		28		80		34	

Table 12: Fungal enumeration of irradiated maize cultivars 46-49, expressed as the percentage of kernels infested.

Cultivar No:	46 (PAN 6071)		47 (ZM 521)		48 (PAN 6335)		49 (PHB 30N35)	
Sample No:	20040217		20040218		20040219		20040220	
	PDA	MSA	PDA	MSA	PDA	MSA	PDA	MSA
Fusarium verticillioides							2	
Penicillium spp.	2						2	
Germination (%)	64		36		50		66	

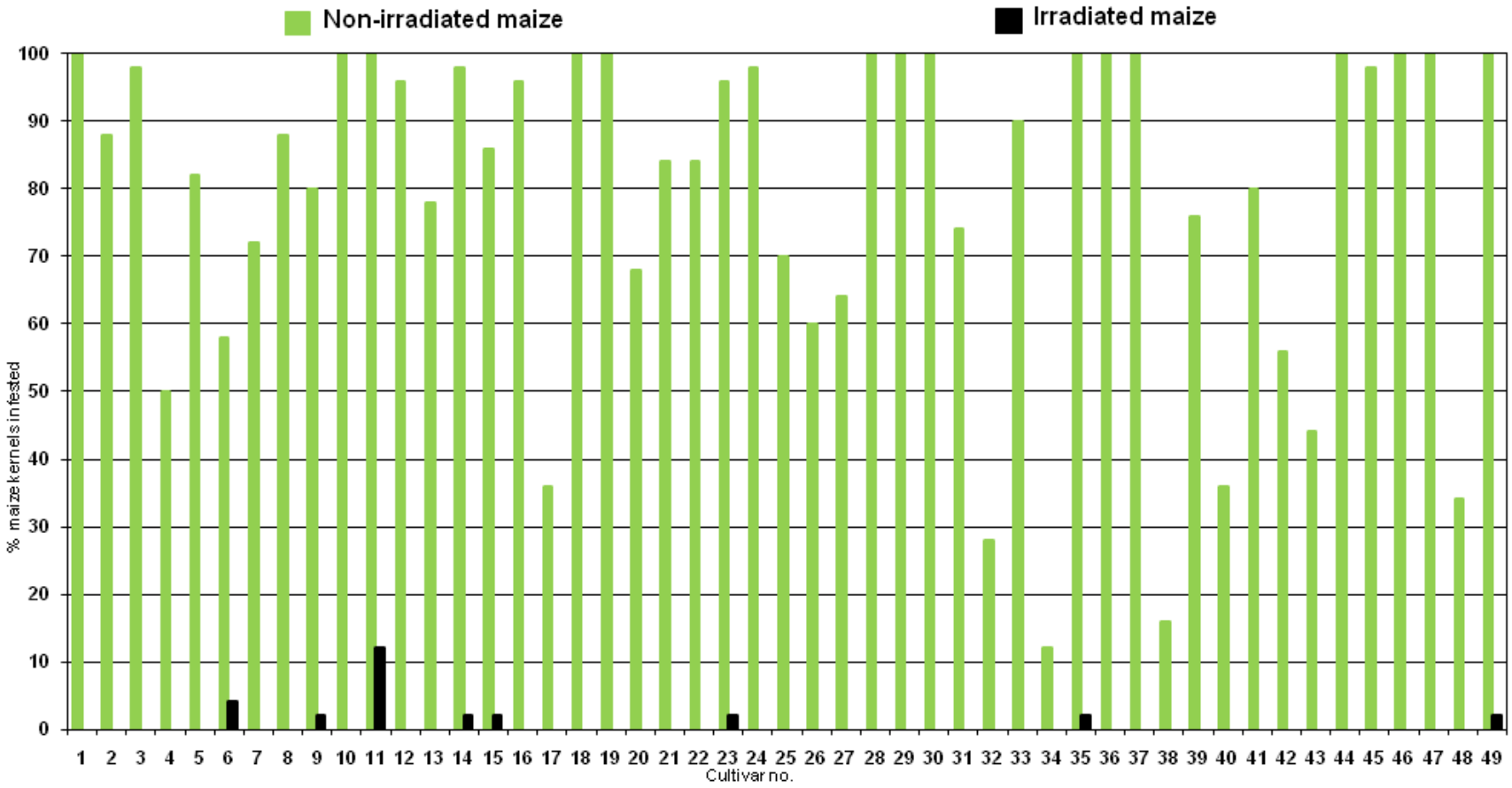

FIGURE 1: Levels of *Fusarium verticillioides* in 49 maize cultivars before and after irradiation.

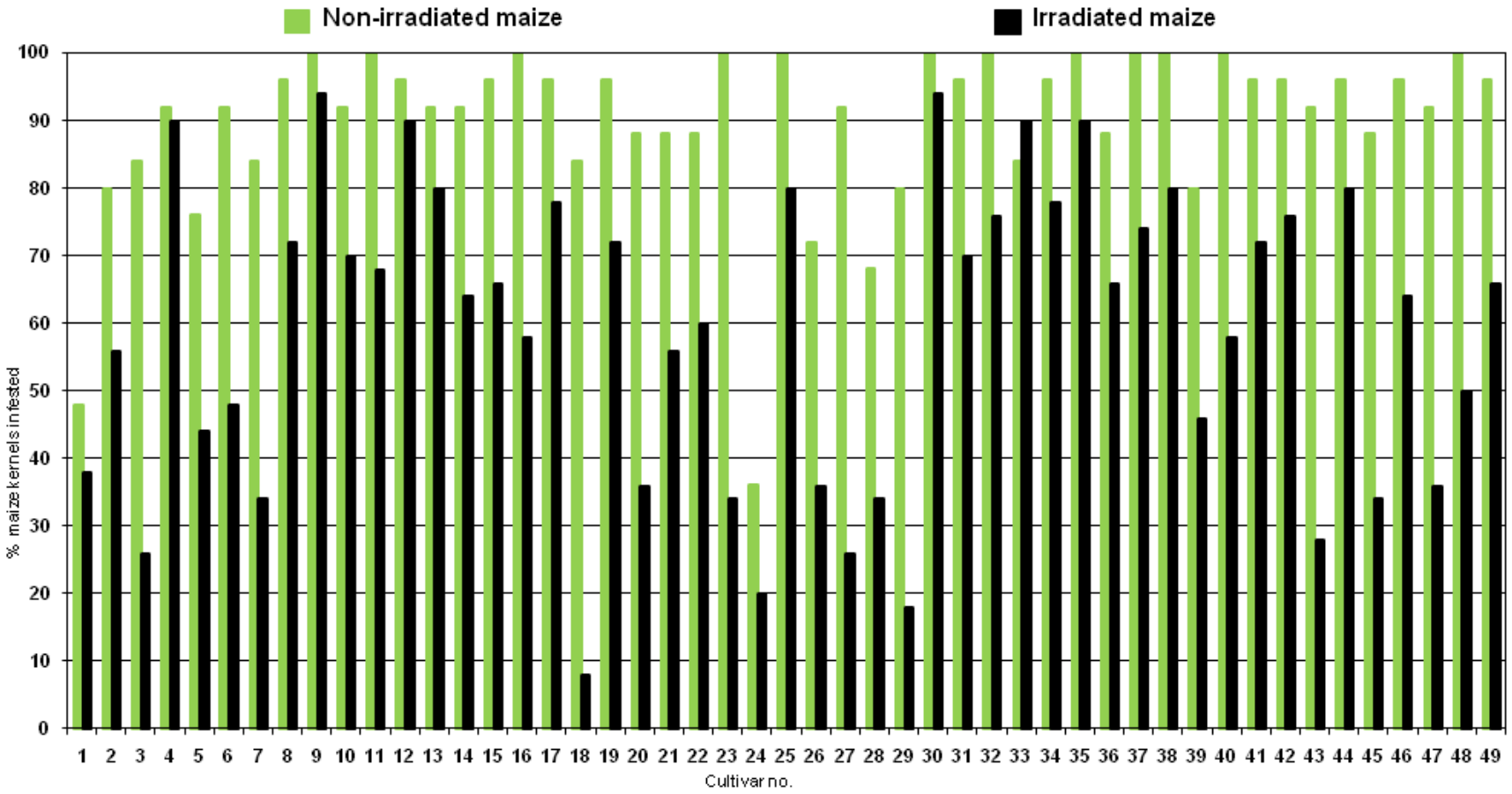

FIGURE 2: Kernel germination of 49 maize cultivars before and after irradiation.

Chapter 4:

Resistance of 49 commercially produced maize cultivars against the infestation of mycotoxigenic fungi during storage

1. Summary:

As maize comprises the largest area of farmland in South Africa, it is also the largest food crop consumed by a vast number of the South African population in some form or another. However, mycotoxin contamination occurs quite frequently in this crop that is mainly destined for human and animal consumption.

The purpose of this study was to establish what levels of resistance could be found in commercially produced South African maize cultivars against the infestation with selected mycotoxigenic fungi. The overall objective was to infect 49 maize cultivars with ten different mycotoxigenic fungi. Five of these fungi, which are known to contaminate maize in the field, included *Alternaria alternata, Fusarium graminearum, Fusarium verticillioides, Phoma sorghina* and *Stenocarpella maydis*. The other five species, which are known to contaminate maize during storage, included *Aspergillus flavus, Aspergillus ochraceus, Eurotium repens, Penicillium islandicum* and *Rhizopus oryzae*.

A unique inoculation technique was used by infecting kernels of each maize cultivar, serially, with each of the ten fungi in a container that simulated storage conditions.

Results showed that cultivars 2 (DKC 80-10), 8 (PAN 6164), 17 (AFG 4512) and 38 (DKC 78-15) indicate resistance towards the field fungi whereas cultivars 4 (PAN 6844), 8 (6164) & 34 (PAN 6611) showed resistance towards the storage fungi. Cultivar 8 (PAN 6164) showed overall resistance against both the field and storage fungi. Results also indicated the possibility that different resistance mechanisms could be present in maize when attacked by field and storage fungi respectively.

2. Introduction

South Africa is suitable for the cultivation of a wide variety of crops of which maize comprises the largest area of farmland. Hence, it plays an important role in the economy of South Africa (NDA, **2004**). In total, about 10 million tons of maize is produced annually in South Africa. Of these, approximately half is white maize, destined for human consumption, and the other yellow maize that is used for animal feed. The surplus maize is usually exported (NDA, **2003**).

While still in the field, mycotoxin contamination of forages and cereals frequently occurs after infection of plants with particular pathogenic fungi or with symbiotic endophytes. During processing and storage of harvested products and feeds, contamination may also occur whenever environmental conditions are ideal for spoilage fungi to develop (Bailey, **1992**). Mycotoxin contamination can occur in any of the stages from cultivating the crop in the field to storage and processing of the maize. Although one of these stages could play a more important role in mycotoxin contamination it is almost never only associated with only one stage (Wilson & Abramson, **1992**).

Plant host-fungus and other biological interactions (e.g. insects) govern the invasion of fungi before harvest whereas crop (nutrients), physical (temperature, moisture) and biotic factors (insects, interference competition) factors govern the invasion of fungi post harvest (Miller, **1995**). Therefore, the original source of fungi in storage is from the field, although contamination can also occur during storage or processing. Resistance of maize to fungal attack has mainly been studied from a plant pathogenic point of view in the past (Agrios, **1997**). However, this study focuses on the resistance of maize cultivars against fungal infestation during storage.

The main objective of this study is to evaluate the resistance of 49 commercially available maize cultivars against ten selected mycotoxigenic fungi, consisting of five field fungi (*Alternaria alternata Fusarium graminearum,*

Fusarium verticillioides, Phoma sorghina and *Stenocarpella maydis*) and five storage fungi (*Aspergillus ochraceus, Aspergillus flavus, Eurotium repens, Penicillium islandicum* and *Rhizopus oryzae*) during storage.

3. Materials and Methods:

3.1. Fungal Cultures

In total, 10 fungi (Figure 1) representing the more important mycotoxigenic fungi in South Africa were selected. The 10 fungi were either obtained from the CSIR culture collection, or were freshly isolated from maize samples. Single spore isolations were made for each fungus to obtain pure cultures for the use in the resistance trial (see Table 1, pg. 155). These cultures were deposited in the CAMS culture collection.

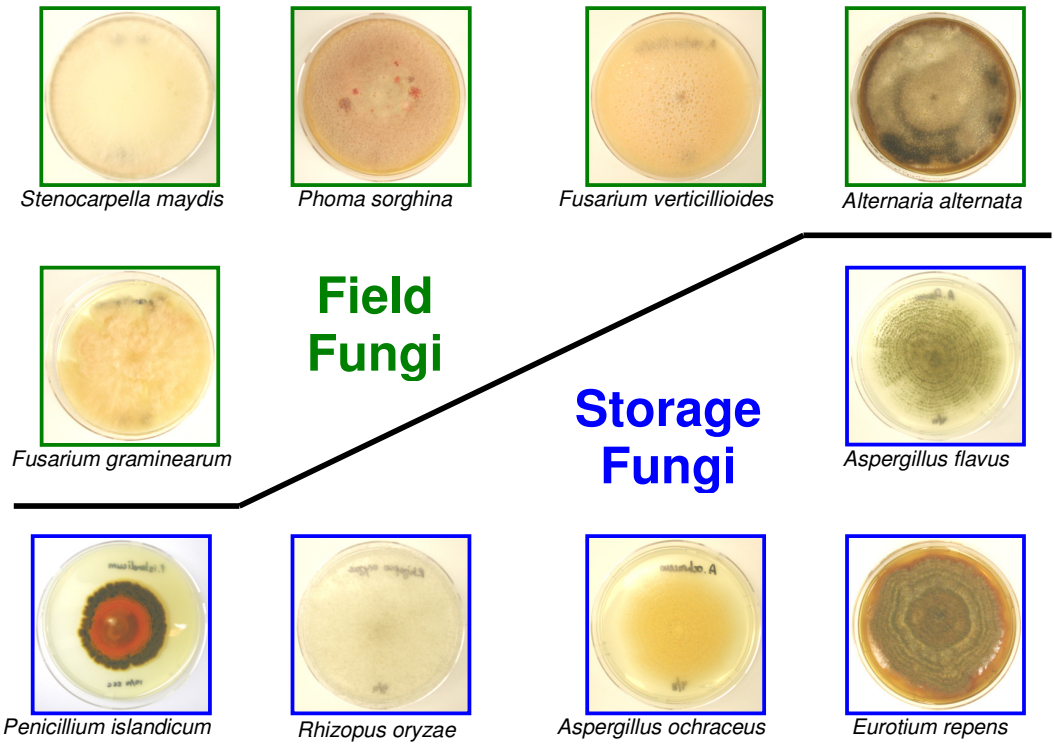

FIGURE 1: Field and storage fungi used in the resistance trial

3.1.1. Fungal isolates selected

Ten fungi were selected for the resistance trial. The reasons for selecting these fungi and their importance are given below:

3.1.1.1. *Alternaria alternata*

Alternaria spp. is a genus that can be found worldwide and in high quantities in the atmosphere, soil, seeds and in agricultural commodities. *Alternaria alternata* is the most common species to be found in most plants, throughout seasons and in geographic regions. It is a field fungus that generally invades crops in the field. This fungus is associated with a variety of grains, such as wheat, barley and oats in which it is more prominent. It can cause a disease called 'black point' which, due to the high mycelial and conidial masses in the seed and germ, causes a loss of colour in the kernels (Logrieco *et al.*, **2003**). *Alternaria alternata* produces the mycotoxins alternariol, alternariol methyl ether, and tenuazonic acid. This fungus was included in this study to represent this genus and to determine the ability of the fungus to propagate under storage conditions.

3.1.1.2. *Fusarium graminearum*

Fusarium graminearum is a field fungus that is generally associated with cereals grown in warmer areas. This fungus is predominantly associated with head blight of wheat but also causes 'red ear rot' of maize (Logrieco *et al.,* **2003**). *Fusarium graminearum* produces the mycotoxins, trichothecenes and zearalenone. These mycotoxins cause alimentary toxic aleukia and Akabitoxicosis illness in humans; digestive disorders, haemorrhagic syndrome in internal organs and skin inflammation in animals (Goswami & Kistler, **2004**).

3.1.1.3. *Fusarium verticillioides*

Fusarium verticillioides is the most prominent fungus in South African maize. This fungus is a plant pathogen that causes stalk rot of maize in which the fungus is able to spread throughout the plant via systemic infection from infected seeds. *Fusarium verticillioides* is also capable of penetrating roots and stalks directly and may also enter the plant through wounds. Unlike *Fusarium graminearum, Fusarium verticillioides* causes 'red ear rot' of maize

(Logrieco *et al.,* **2003**). The isolation of this fungus has also occurred from symptomless plants. This fungus is so prominent in South Africa, that due to its infection of home grown maize, it is associated with esophageal cancer in South Africa (Marasas *et al.,* **1984**), due to the fact that it produces the mycotoxins called fumonisins. It also causes leucoencephalomalacia (LEM) in horses, mules and donkeys.

3.1.1.4. *Phoma sorghina*

Phoma sorghina can be isolated from soil and air. It is a fungus that can be found everywhere in both tropical and subtropical regions. *Phoma sorghina* is not host specific. It is generally transmitted via contaminated seeds and accordingly generally causes loss of seedlings (Punithalingham, **1983**). *Phoma sorghina* is a field fungus that is associated with maize, to a much lesser extent, than that of sorghum and millet in Southern Africa. This pathogen causes root rot and stem rot in crops. This fungus was included, as it might be responsible for the disease, onyalai, in humans. Once this fungus is consumed by means of contaminated foods, it leads to the lowering of blood platelet counts and causes patients to become bleeders. These related symptoms were also observed in animals consuming contaminated feed (Rabie *et al.,* **1975**). *Phoma sorghina* was included to represent the genus, *Phoma,* in this study.

3.1.1.5. *Stenocarpella maydis*

Stenocarpella maydis, in addition to *Fusarium verticillioides,* is also one of the more important fungi associated with maize in South Africa. This fungus is able to survive in residue of maize stalks, cobs and fallen kernels. *Stenocarpella maydis* causes stalk rot, white ear rot and seedling blight of maize (EPPO Quarantine Pest, **2004**). It is able to affect the yield of the commodity, if it infects at the early stages of development of the plant. Due to this fact, it is therefore also able to affect the feeding and milling qualities of the grain (Vincelli, **1997**). Currently, the mycotoxins of this fungus are unknown, but it does cause a disease called diplodiosis among cattle and sheep, as well as deaths in poultry. Due to the different disease symptoms in different animals, it is believed that there could be more than one mycotoxin

responsible. *Stenocarpella maydis* is usually associated with poor conditions in the field. It was included in this study to determine its ability to propagate under storage conditions.

3.1.1.6. *Aspergillus ochraceus*

Aspergillus ochraceus is a known storage fungus. It is associated to a much lesser extent with maize although it is found on many other foods and feed commodities. A few strains of *A. ochraceus* are able to infect living plants. This fungus produces the mycotoxin ochratoxin A that causes fatal kidney disease in humans and ochratoxicosis in animals (chronic nephropathy and carcinogenicity) (Logrieco *et al.*, **2003**). Due to the fact that the spores are airborne and, if ideal conditions for growth are experienced, the fungus would be able to germinate and colonize the commodity.

3.1.1.7. *Aspergillus flavus*

In other countries outside South Africa, *Aspergillus flavus* is known to be a field fungus, but likely due to environmental conditions in South Africa, this fungus is also known to be a storage fungus. It produces spores that easily infests conditioning bins of maize mills and is able to contaminate the end product. This fungus is able to produce mycotoxins called aflatoxins, that are potent hepatotoxins and carcinogens that cause aflatoxicosis in humans and animals.

3.1.1.8. *Eurotium repens*

Eurotium spp. is very common. These fungi are able to develop on substrates that are low in moisture (xerophilic). *Eurotium repens* is very common in stored seeds and is normally found in a commodity when poor storage conditions are experienced and with very low moisture content. It produces the mycotoxin, sterigmatocystin, which is a precursor of aflatoxins. The presence of this fungus can cause vomiting and ill thrift among humans and animals.

3.1.1.9. *Penicillium islandicum*

Penicillium is the most complex genus due to its numbers of species that colonize a wide range of habitats. A few species are among the most common and destructive agents of post harvest maize kernels, but causing spoilage of grain during harvesting, storage or transit (Logrieco *et al.*, **2003**). This fungus also serves as a food source for mites in storage. *Penicillium islandicum* causes *Penicillium* ear rot of maize that causes the loss of color of the embryo or 'blue eyes', once it invades kernels stored at high moisture levels. The infection generally occurs on maize ears that have been injured by mechanical means or by insects (University of Hawaii, Botany Faculty, **2007**). Due to this fact, over the past few years, this fungus has become more prominent during harvesting, indicating that the fungus has probably infected the maize commodity while in the field. It produces the mycotoxin, luteoskyrin, which is carcinogenic to humans and animals (Ghosh, **1978**).

3.1.1.10. *Rhizopus oryzae*

Rhizopus oryzae is a member of the phylum, Zygomycota. From the rest of the fungi included in this study, the fungi belonging to this phylum differ considerably, based on their physiology and morphology. *Rhizopus oryzae* is a fungus that can be found in soil, decaying fruit and vegetables, animal faeces and old bread. It is regularly associated with maize and can propagate either in the field, or under storage conditions. *R. oryzae* produces the mycotoxin, ergot alkaloid, agroclavine that is toxic to man, sheep and cattle.

3.2. Preparation of inoculum

The ten test fungi were grown on 90 mm petri dishes containing either PDA or MSA, depending on the ability of the fungus to sporulate on the media. The plates were incubated at approximately 25^{o}C for about two weeks to allow fungal coverage of the plates and adequate sporulation. A spore suspension was prepared by suspending the spores in five milliliters of sterile distilled water and gently scratching the surface with a blunt pinsette. A sterile syringe was used to remove the spore suspension from the plate and to inject the suspension onto sterile earbuds (flow Figure 2**C**, pg. 140). Earbuds were bought at a Pick 'n Pay supermarket, cut in halve and autoclaved at 121^{0}C for

20 min. Once the spore suspension was added to 60 sterile earbuds, 40 ml Czapek dox broth was added. The inoculated earbuds were then incubated at 25^{0}C until the heads were covered with fungal growth (Figure 2**D**).

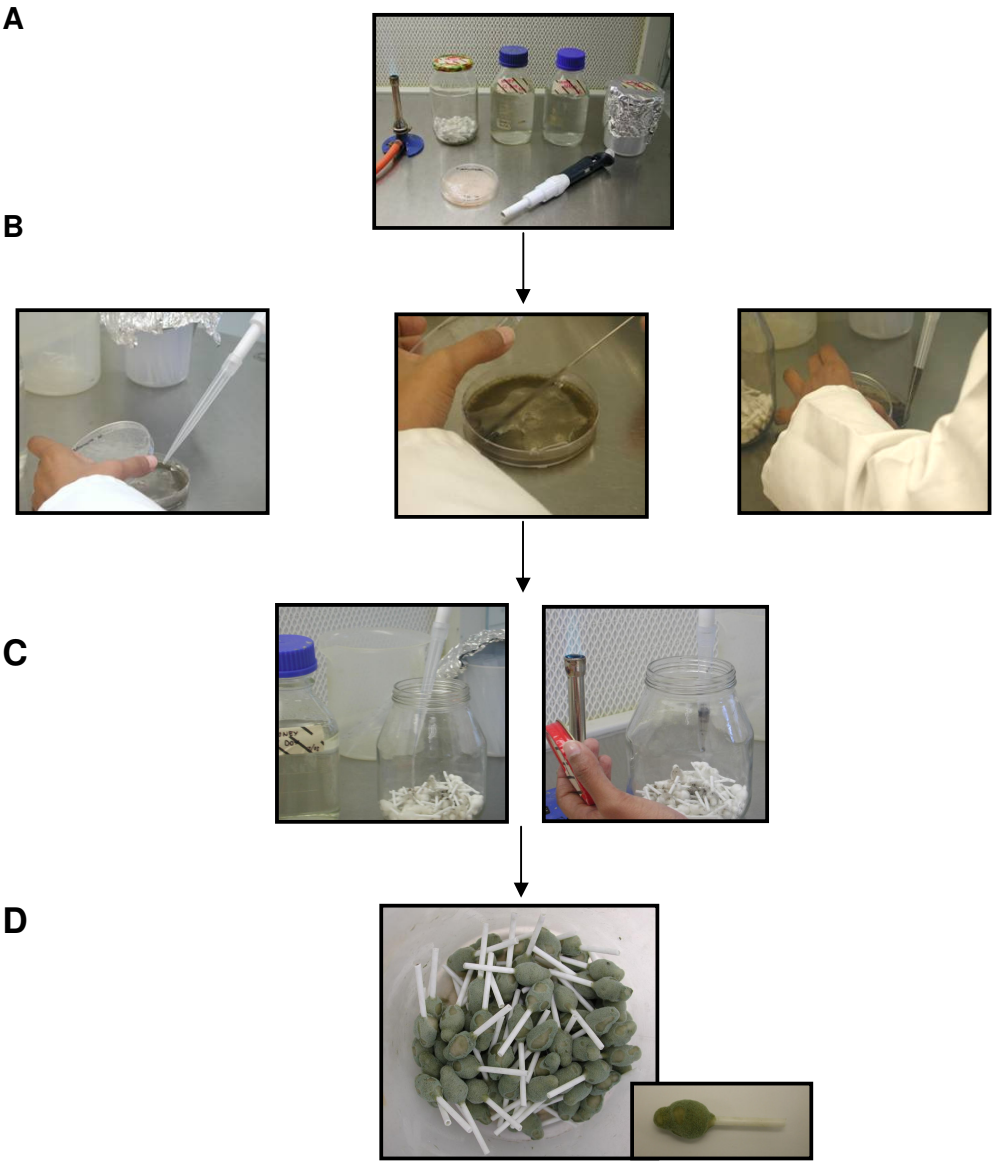

FIGURE 2: Preparation of inoculum

Apparatus in (A) comprising a bunsen burner, sterile earbuds, sterile distilled water, Czapek dox broth, pipette and fungus was subsequently used to plate out the test fungi (B), after which a fungal suspension was injected onto sterile earbuds (C). D shows the growth of the fungus on the earbuds after a 2 week incubation period.

3.2.1. Adaptation of protocol

This protocol had to be adapted as *Rhizopus oryzae* did not perform well on the broth. A spore suspension was made as described previously and added to potato dextrose agar that was cooled down to 55^0C. The medium was gently shaken to allow even dispersal of spores. Each sterile earbud was dipped into the inoculated medium, allowed to solidify and placed onto empty 90mm petridishes. The inoculated earbuds were incubated at 25^0C until the heads were covered with fungal growth.

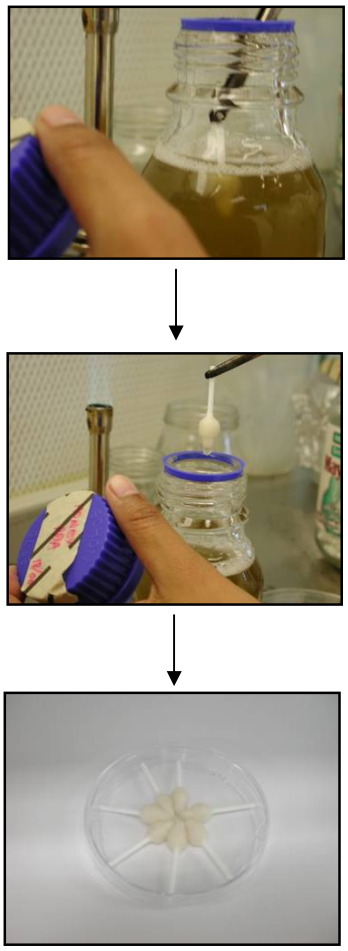

FIGURE 3:

Inoculation of sterile earbuds containing PDA with fungal spores.

3.3. Moisture content

In total, 180 g of each maize cultivar was weighed out into a 250 ml plastic container with a screw cap. Each cultivar was weighed out into 10 such containers, resulting in a total of 1,8 kg kernels tested per maize cultivar. The

moisture content of each maize cultivar was pre-determined at approximately 10%. Each fungus is known for their ability to grow at specific moisture contents and, therefore, moisture contents for individual cultivars were adjusted according to the support needed for each fungus to grow (see Table 2, pg. 156).

3.4. Inoculation of 250 ml containers

Each container, with 180 g of maize, was inoculated by placing a colonised ear bud in the middle of the container. Each fungal carrying ear bud was inserted approximately 3.5 cm deep in the centre of each bottle, as shown in Figure 4. The inoculated bottles were incubated at 25^0C with 12-hour light and dark cycles. All containers were observed daily for visible fungal growth.

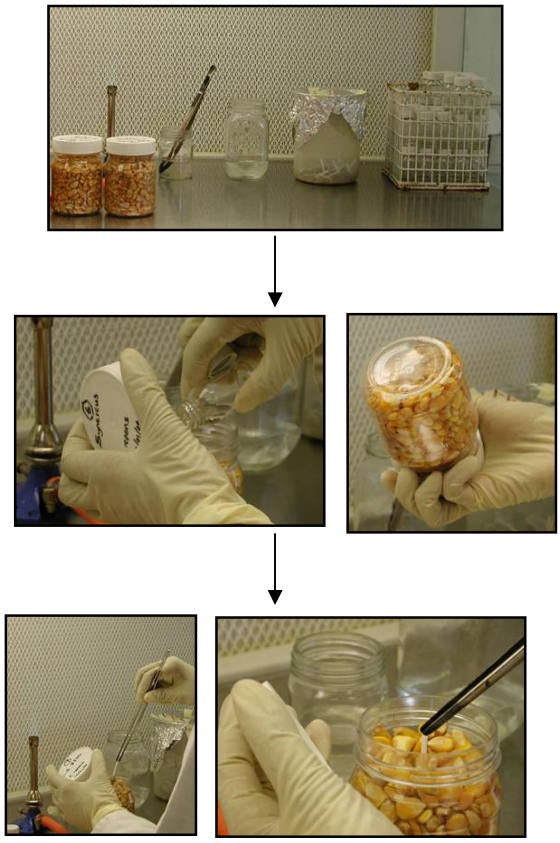

FIGURE 4:

Inoculation of maize kernels with earbuds colonised by fungi.

3.5. Fungal identification and enumeration

Once fungal growth started to be visibly observed in any of the containers, all the containers were removed from the incubator. The populations and levels of the various fungi were determined by the method described by Rabie *et al.* (**1997**) (see Chapter 2, pg. 73). Either potato dextrose agar (PDA) or malt salt agar (MSA) was used, depending whether the fungus is regarded as either a field fungus or a storage fungus.

3.5.1. Culture media

3.5.1.1. Potato Dextrose Agar (PDA)

PDA (Merck, South Africa) was prepared according to manufacturers instructions. Approximately 20 ml was then poured into 90 mm petri dishes and allowed to settle.

3.5.1.2. Malt Salt Extract (MSA)

MSA was prepared by dissolving 90 g NaCl (Promark Chemicals, South Africa) in 360 ml distilled water in a 500 ml Schott bottle. 24 g of malt extract (Merck, South Africa) and 24 g of agar (Merck, South Africa) were added to 840 ml distilled water in a 1 L Schott bottle. The solutions were then autoclaved separately for 20 min at 121^{0}C and cooled down to approximately 50^{o}C. The solution containing the NaCl was then added aseptically to the solution containing the malt extract and agar. The medium was then mixed and approximately 20 ml was aseptically added to 90 mm petri dishes and allowed to settle.

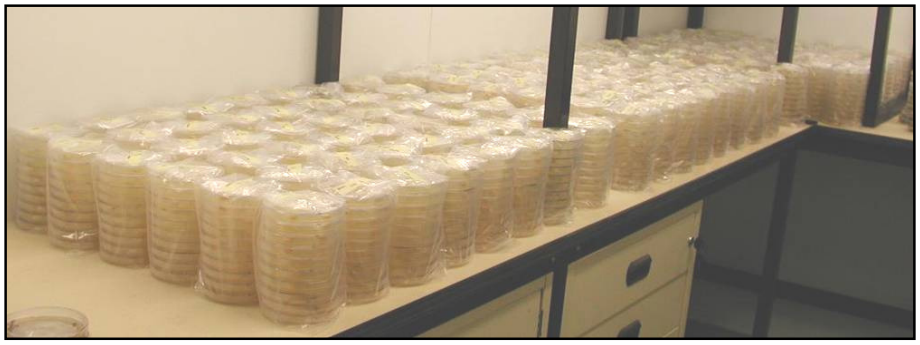

FIGURE 5: Malt extract being added to agar upon which infected maize kernels were allowed to germinate.

4. Results:

Results indicated that nine mycotoxigenic fungi tested against the 49 cultivars, under storage conditions, were successful in infecting maize. Table 3 (pg. 157) shows a summary of the infestation levels of each fungus on each cultivar. Figures 6 to 15 (pg. 161-170) compare the level of infestation for each of the individual test fungi in the 49 maize cultivars.

Initially, the rate of infestation of each individual fungus on the 49 cultivars was determined (Figures 6-15). The results of the various field fungi were then grouped together and those of the storage fungi grouped separately. This was done to determine whether any differences could be observed in the resistance of the maize kernels between the field and the storage fungi. The field fungi included (*Fusarium graminearum, Fusarium verticillioides, Phoma sorghina* and *Stenocarpella maydis* (see Figure 16, pg. 171)*;* and the storage fungi included *Aspergillus ochraceus, Aspergillus flavus, Eurotium repens* and *Rhizopus oryzae* (see Figure 17, pg. 172). *Alternaria alternata* (field fungus) and *Penicillium islandicum* (storage fungus) were excluded when analyzing the data as *Alternaria alternata* showed no growth in the bottles and *Penicillium islandicum*, showed overwhelming aggressiveness on the maize kernels. Inclusion of these results into the calculations would have distorted the true results.

Alternaria alternata (Figure 6, pg. 161) was the only fungus found unable to infest the maize cultivars under storage conditions.

Fusarium graminearum was able to infest 29 out of the 49 cultivars tested under storage conditions (Figure 7, pg. 162). Cultivar 30 (PHB 30D05) seemed to be the most susceptible cultivar with an infestation level of 78%. Thirty-six cultivars showed no infestation or infestation levels below 10% with this fungus. In total, 13 cultivars showed infestation levels that ranged between 12 and 78%.

Fusarium verticillioides infested 14 cultivars out of 49 (Figure 8, pg. 163). Nine cultivars showed an infestation level of lower than 10%; whereas 5 cultivars showed an infestation level of 10% and higher. Cultivar 40 (PAN 6995 Bt) showed a considerable infestation of *Fusarium verticillioides*. Cultivars 12 (PAN 6966), and 33 (CRN 3549) were also highly infested with this fungus during storage; with 30 (PHB 30D05) having the highest rate of infestation at 90%.

In total, 25 out of 49 cultivars were infested with *Phoma sorghina* (Figure 9, pg. 164). Cultivar 33 (CRN 3549) was identified as the more susceptible cultivar with the highest infestation level of 48%. Twenty-three of the cultivars showed infestation levels of less than 10% whereas 24 cultivars showed no infestation at all.

Stenocarpella maydis was able to infest 39 cultivars out of 49 (Figure 10, pg. 165). Cultivars 12 (PAN 6966), 33 (CRN 3549) and 47 (ZM 521) indicated 100% infestation by this fungus. Seven cultivars indicated infestation levels between 60-80% and eighteen cultivars had infestation levels between 10-40%. Eleven cultivars indicated levels below 10% and only 10 cultivars had zero infestation by this fungus

Cultivars 6 (Synercus), 12 (PAN 6966), 30 (PHB 30D05), 33 (CRN 3549) and 46 (PAN 6071) indicated a 100% infestation level by *Aspergillus ochraceus* (Figure 11, pg. 166). Ten cultivars were infested at levels between 80-100%

and twenty cultivars at levels between 20-80%. A total of seven cultivars indicated infestation levels below 20%, whereas no cultivars had a zero infestation level by this fungus.

All test cultivars showed infestation by *Aspergillus flavus* (Figure 12, pg. 167). In total, 5 cultivars i.e. 18 (DKC 63-20), 23 (SC 621), 27 (Saffier), 38 (DKC 78-15) and 48 (PAN 6335) showed a 90-100% infestation level. Twenty-seven cultivars showed infestation levels higher than 10% but less than 80%, and seventeen cultivars showed infestation levels of 10% and less.

Eurotium repens (Figure 13, pg. 168) was able to infect all cultivars except cultivar 34 (PAN 6611). Eleven cultivars showed infestation levels between 80-100% with 5 cultivars showing a 100% infestation level i.e. cultivar 12 (PAN 6966), 20 (MRI 514), 30 (PHB 30D05), 33 (CRN 3549) and 45 (SNK 2969). Twenty-six cultivars showed infestation levels between 30-80% with the majority above 35%. Only 12 cultivars showed infestation levels below 30%.

In Figure 14, pg. 169, *Penicillium islandicum* was very aggressive in the infestation of the 49 test cultivars. It was able to infect all 49 cultivars with infestation levels of 60% and above. The majority of the cultivars showed 100% infestation level.

Rhizopus oryzae (Figure 15, pg. 170) was able to infest all 49 cultivars except cultivar 35 (SC 407). A 100% infestation level was seen in 14 cultivars. Twenty-four cultivars showed infestation levels between 20-90% with the majority being above 50%. Eleven cultivars showed infestation levels below 20%.

For complete analysis of all the results, to determine the reactions of the cultivars against all the fungi, the averages of the fungal infestation, for each cultivar, were determined by calculating all levels of infestation for each cultivar divided by the amount of fungi used. For analysis of the results for the field fungi, the infestation levels of *Fusarium graminearum, Fusarium*

verticillioides, Phoma sorghina and *Stenocarpella maydis* were calculated together and the averages determined (Table 4, pg.159). Since *Alternaria alternata* showed no infestation during the trial, it was excluded from the calculations so as to not distort the true results. For the storage fungi, the averages were determined in the same manner by using *Eurotium repens, Aspergillus ochraceus, Aspergillus flavus* and *Rhizopus oryzae. Penicillium islandicum* was excluded, due to its overwhelming aggressiveness in the trial (Table 5, pg. 160).

Figure 16 (pg. 171) shows the graphical presentation of the averages of all the field fungi screened in this study. The majority of the cultivars showed infestation levels of below 10% (32 cultivars). Cultivar 33 (CRN 3549) showed the highest average infestation level of 62%. Overall, the results indicated that the cultivars that seem to prolong the invasion of field fungi under storage conditions are cultivars 2 (DKC 80-10), 8 (PAN 6164), 17 (AFG 4512) and 38 (DKC 78-15 Bt). The more easily infested cultivars were identified as being cultivars 12 (PAN 6966), 30 (PHB 30D05), 33 (CRN 3549) and 40 (PAN 6995 Bt).

Figure 17 (pg. 172) represents the averages of all the storage fungi. Cultivar 4 (PAN 6844) showed the lowest average infestation level of storage fungi with a 14% infestation. The highest average level was found in cultivar 33 (CRN 3549) with a 90.5% infestation level. Cultivars that showed slower infestation rates of storage fungi were cultivars 4 (PAN 6844), 8 (PAN 6164) and 34 (PAN 6611). The cultivars that were more easily infested were cultivars 23 (SC 621), 27 (Saffier), 33 (CRN 3549) and 45 (SNK 2969).

5. Discussion:

Fungi are one of the most important causes of deterioration in stored maize. Fungi may be divided into two groups, mostly based upon the moisture content of food commodities needed for fungal growth (Christensen *et al.*; **1974**) i.e. field fungi and storage fungi. One of the characteristics of field fungi

is that they require high moisture content in order to grow (22 to 25% wet weight basis or 30-33% on dry weight basis). Field fungi generally infect a commodity, while still in the field and, in addition, most are plant pathogenic.

Storage fungi can grow at much lower moisture content than that of the field fungi, even on occasion, when no free water is present. This is a special biological niche to which, over the long period of evolution, they have become adapted to and in which they thrive with no competition from other fungi (Sauer *et al.,* **1992**). Due to their ability to produce vast amounts of spores, it is much easier to infect crops during storage.

Alternaria alternata was able to develop quite effectively on the ear buds but was unable to colonize any of the maize cultivars during the trial. This could be due to the fact that *Alternaria* can survive as a latent infection in seeds and has the ability to attack the seedling once it begins to germinate (Thomma, **2003**). In **1990**, a survey done by Logrieco *et al*., showed that maize was uninfected by *Alternaria alternata* under storage conditions. A study done by Torres *et al.* (**2003**), on the competition of growth between *Aspergillus ochraceus, Alternaria alternata* and *Fusarium verticillioides* on maize kernels at different water activities and temperatures showed *Alternaria alternata* to have the lowest growth rate when inoculated singularly on the substrate. Their study further showed that, when inoculated on maize kernels with *Aspergillus ochraceus* and *Fusarium verticillioides,* respectively*, Alternaria alternata* actually showed a two-fold increase in growth rate. This could have been due to competition between the fungi for the substrate, and possibly also the reason why *Alternaria alternata* in this study showed no growth in the inoculated bottles.

Based on the findings in this study, it is thus unlikely that *Alternaria alternata* (Figure 6) has the capability to cause spoilage of maize during storage. The presence of this fungus in maize would thus rather be an indication of spoilage before or during harvesting, rather than during storage and processing of maize.

Although *Fusarium graminearum* is a field fungus, it was included in this study to observe and determine whether it would propagate during storage conditions. *Fusarium graminearum* (Figure 7) was able to infest 29 out of the 49 cultivars during storage.

Fusarium verticillioides was included in this study to determine its aggressiveness on the maize kernels during storage. *Fusarium verticillioides* (Figure 8) was able to invade 14 of the maize cultivars during storage; this was in great contrast of what was experienced during field conditions where it had a 100% infestation level in almost all the cultivars (see Chapter 2, Figure 2, pg. 102). Torres *et al.* (**2003**), showed that *Fusarium verticillioides*, when inoculated with other fungi i.e. *Alternaria alternata*, showed faster rates of growth. The authors also showed that, when inoculated with *Aspergillus ochraceus*, *Fusarium verticillioides* was inhibited by the spores of *A. ochraceus*. In this study, it became apparent that additional methods should be sought to reduce the competitive growth of *Fusarium verticillioides* in natural conditions, so as to subsequently decrease mycotoxin production in a commodity.

Phoma sorghina was able to invade 25 of the maize cultivars that were screened in the trial (Figure 9). The ability of this particular fungal species to infest the cultivars, while under storage conditions, was relatively slow, compared to the other field and storage fungi included in this study. This fungus is known to be a plant pathogen and could therefore be easily dominated by the other storage fungi during storage conditions.

Stenocarpella maydis seemed to be relatively well adapted to propagate under storage conditions (Figure 10). The resistance of the maize cultivars against the attack by *S. maydis*, under storage conditions, varied greatly among the cultivars tested in the trial. This fungus could potentially cause serious economic losses in South Africa, under ideal moisture conditions, even though it is not present in high density.

Aspergillus ochraceus was able to infest all the cultivars under storage conditions (Figure 11). *Aspergillus flavus* was included in this study to determine its ability to invade the 49 maize cultivars under storage conditions (Figure 12). When, in this study, the levels of infestation of *A. flavus* were compared to that of *A. ochraceus*, it was noted that *A. flavus* was able to infect all the cultivars, but at much lower levels than that of *A. ochraceus*. According to the study conducted by Lee & Magan (**2000**), they showed that when *A. flavus* was grown on the same substrate as *A. ochraceus, A. flavus* was able to dominate at higher temperatures. Marin *et al.* (**1998**) showed that when *A. flavus* is grown on the same substrate as *Fusarium verticillioides, F. verticillioides* is able to inhibit the growth of *A. flavus*.

Eurotium repens (Figure 13) was able to infect all cultivars except cultivar 34 (PAN 6611).

Based on the results in this study, it is evident that *Penicillium islandicum* (Figure 14) can aggressively invade maize during storage. Results also indicated that all cultivars were highly susceptible to be colonised by this fungus. It is, therefore, not possible to identify any maize cultivars in this study that could be targeted for future resistance studies against this fungus.

Infestation of *Rhizopus oryzae* (Figure 15) was quite diverse between the various cultivars, with only cultivar 35 that had no infestation. This fungus is known to grow well under both field and storage conditions and therefore was expected to be dominant among the 49 cultivars during storage.

When the invasion of field fungi and storage fungi are compared during storage (see Figures 16 and 17), it is evident that those fungi that are known to cause spoilage during storage are more prominent.

6. Conclusion:

By looking at the overall average infestation of both field and storage fungi combined, cultivar 8 (PAN 6164) indicated a slower infestation of both field and storage fungi. Cultivar 33 (CRN 3549), on the other hand, showed susceptibility to both groups of fungi. These results indicated that there is likely a difference in the mechanism how field and storage fungi infest maize, or even how maize cultivars resist attack from these two groups. Moreno-Martinez *et al.* (**1971**), and Cantone *et al*. (**1983**) show that there are differences in maize types in susceptibility to damage from invasion by storage fungi.

7. References:

Agrios, G.N. 1997. Plant Pathology. 4th Ed. Academic press. 635 pp.

Bailey, J.E. 1992. Whole grain storage, Chapter 5, Pg. 157. In Storage of Cereals Grains and their Products. 4th edition. Ed D.B Sauer. American Association of Cereal Chemists. 615 pp.

Cantone, F.A., Tuite, I., Barman, L.F. and Stroshine, R. 1983. Genotype differences in reaction of stored corn kernels to attack by selected *Aspergillus* and *Penicillium* species. *Phytopathology.* 73: 1250-1255.

Christensen, C.M. & Kaufmann, H.H. 1974. Microflora in Storage of Cereal grains and their Products. Ed D. B. Sauer.

EPPO Quarantine Pest. 2004. *Stenocarpella macrospora* and *Stenocarpella maydis.*

Ghosh, A.C., Manmade, A, Kobbe, B, Townsend, J.M. & Demain, A.L. 1978. Production of luteoskyrin and isolation of a new metabolite, Pibasterol, from *Penicillium islandicum* Sopp. *Applied and Environmental Microbiology.* 3: 563-566.

Goswami, R.S. & Kistler, H.C. 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. *Molecular Plant Pathology.* 5 (**6**): 515-525.

Lee, H.B. & Magan, N. 2000. Impact of environment and interspecific interactions between spoilage fungi and *Aspergillus ochraceus* on growth and ochratoxin production in maize. *International Journal of Food Microbiology.* 1: 11-16.

Logrieco, A., Battalico, A., Solfrizzo, M. & Mulé, G. 1990. Incidence of Alternaria species in grains from Mediterranean countries and their ability to produce mycotoxins. *Mycologia.* 82(**4**): 501-505.

Logrieco, A., Battalico, A., Mulé, G., Moretti, A. & Perrone, G. 2003. Epidemiology of toxigenic fungi and their assocaitted mycotoxins for some Mediterranean crops. *European Journal of Plant Pathology.* 109: 645-667.

Marasas, W.F.O., Nelson, P.E. & Toussoun, T.A.. 1984. Toxigenic *Fusarium* species, identity and mycotoxicology. The Pennsylvania State University Press, University Park and London, pp328.

Marín, S., Sanchos, V., Arnau, F., Ramos, A.J. & Magan, N. 1998. Colonisation and competitiveness of *Aspergillus* and *Penicillium* species on maize grain in the presence of *Fusarium moniliforme* and *Fusarium proliferatum. International Journal of Food Microbiology.* 45: 107-117.

Miller, J.D. 1995. Fungi and Mycotoxins in Grain. Implications for stored product research. *Journal of Stored Products*. 31(**1**): 1-16.

Moreno-Martinez, E. & Christensen, C. M. 1971. Differences among line varieties of maize in susceptibility to damage by storage fungi. *Phytopathology*. 61: 1498-1500.

National Department of Agriculture (NDA). 2003. Food Pricing Monitoring Committee: Final report, December 2003, pp310. http://www.nda.agric.za/docs/fpmc/default.htm

National Department of Agriculture (NDA). 2004. General Information on agriculture in South Africa. http://www.nda.agric.za/history/

Punithalingham, E. 1983. *Phoma sorghina.* C.M.I Desriptions of Pathogenic Fungi and bacteria No.825.

Rabie, C.J., Van Rensburg, S.J., Van Der Watt, J.J. & Lübben, A. 1975. Onyalai--the possible involvement of a mycotoxin produced by *Phoma sorghina* in the aetiology. *South African Medical Journal.* 49 (40): 1647-1650.

Rabie, C. J., Lübben, A., Marais, G. J. and Jansen van Vuuren, H. 1997. Enumeration of fungi in barley. *International Journal of Food Microbiology*. 35: 117-127.

Sauer, D. B., Meronuck, R. A. & Christensen, C. M. 1992. Microflora Chapter 9. Pg. 313. In Storage of Cereals grains and their Products. 4th edition. Ed DB Sauer. American Association of Cereal Chemists.

Thomma, P.H.J.B. 2003. *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology*. 4 (**4**): 225-236.

Torres, M.R., Ramos, A.J., Soler, J., Sanchos, V. & Marín, S. 2003. SEM study of water activity and temperature effects on the initial growth of *Aspergillus ochraceus, Alternaria alternata* and *Fusarium verticillioides* on maize grain. *International Journal of Food Microbiology.* 81: 185-193.

University of Hawaii, Botany Faculty, 2007. Mycotoxins.
http://www.botany.hawaii. edu/faculty/wong/bot135/lect11.htm

Vincelli, P. 1997. Ear rot of corn caused *Stenocarpella maydis (=Diplodia maydis)*. Cooperative extension servise. University of Kentucky. College of agriculture.

Wilson, D.M. & Abramson, D. 1992. Mycotoxins. Chapter 10. Pg. 341. In Storage of Cereal grains and their Products. 4th edition. Ed D.B. Sauer American Association of Cereal Chemists. 615 pp.

Table 1: Five field- and five storage fungi identified to be used in the resistance trial.

CAMS no.	Fungal name
Field fungi	
CAMS 1255	*Alternaria alternata*
CAMS 1256	*Fusarium graminearum*
CAMS 1257	*Fusarium verticillioides*
CAMS 1258	*Phoma sorghina*
CAMS 1259	*Stenocarpella maydis*
Storage fungi	
CAMS 1260	*Aspergillus ochraceus*
CAMS 1261	*Aspergillus flavus*
CAMS 1262	*Eurotium repens*
CAMS 1263	*Penicillium islandicum*
CAMS 1264	*Rhizopus oryzae*

Table 2: Moisture contents used to support fungal growth in the resistance trial.

CAMS no.	Fungal name	Moisture content (%)
Field fungi		
CAMS 1255	*Alternaria alternata*	30
CAMS 1256	*Fusarium graminearum*	25
CAMS 1257	*Fusarium verticillioides*	30
CAMS 1258	*Phoma sorghina*	25
CAMS 1259	*Stenocarpella maydis*	25
Storage fungi		
CAMS 1260	*Aspergillus ochraceus*	20
CAMS 1261	*Aspergillus flavus*	20
CAMS 1262	*Eurotium repens*	20
CAMS 1263	*Penicillium islandicum*	20
CAMS 1264	*Rhizopus oryzae*	30

Table 3: Resistant trial results of 10 mycotoxigenic fungi against 49 maize cultivars.

Cultivar No:	*Alternaria alternata*	*Fusarium graminearum*	*Fusarium verticillioides*	*Phoma sorghina*	*Stenocarpella maydis*	*Aspergillus ochraceus*	*Aspergillus flavus*	*Eurotium repens*	*Penicillium islandicum*	*Rhizopus oryzae*
1				6	10	62	52	48	68	100
2				2		52	16	30	100	100
3					18	68	10	28	100	100
4				6		14	2	30	98	10
5			8		10	30	28	46	100	10
6		4		2	6	100	10	40	100	20
7		12			20	36	10	46	100	80
8				2		30	4	14	94	20
9		4	18		20	32	6	74	100	100
10		6	14			52	12	66	100	80
11					28	20	44	18	100	82
12		8	66	8	100	100	60	100	62	50
13					8	16	4	38	100	100
14		42			30	38	8	40	96	20
15		4	10	2	70	94	30	54	72	22
16		4		4	2	54	8	40	100	100
17				2		56	32	40	100	80
18		2			80	20	100	96	100	70
19		6			22	86	10	50	100	90
20		24		2	34	82	8	100	100	10
21		2		4	30	80	6	50	100	50
22			6			28	2	6	100	100
23		22	8	6	30	46	100	88	100	100
24					10	12	30	74	100	30
25		6			20	86	14	54	100	20
26		2			30	76	66	80	100	80
27		12	24	8	20	74	100	68	96	100
28					10	54	66	56	100	80
29					16	50	28	66	100	80

Table 3 continued: Resistant trial results of 10 mycotoxigenic fungi against 49 maize cultivars.

30		78	90	16	30	100	76	100	100	20
31		6			8	76	24	54	100	60
32		16		2	10	44	28	16	100	90
33		24	76	48	100	100	62	100	98	100
34			2		10	40	8		100	10
35		6		2	10	96	6	80	100	
36				6		50	16	10	100	30
37		6			18	70	16	84	100	50
38				2		40	94	38	80	60
39				2	78	80	6	88	100	100
40		28	68		80	92	30	70	94	90
41		10			20	74	24	66	100	100
42			12	2	18	44	22	56	98	100
43		4		4	60	54	28	50	100	30
44		12				56	24	66	96	90
45		34		2	40	94	52	100	100	100
46				2	60	100	18	30	100	10
47			16		100	6	10	22	100	80
48		12				20	100	28	98	30
49		16		4	80	98	18	32	100	30

Results expressed as the percentage of maize kernels infested with a specific fungus.

Table 4: Averages of the % infestation of all field fungi combined, except *Alternaria alternata*, during storage.

No:	*Fusarium graminearum*	*Stenocarpella maydis*	*Phoma sorghina*	*Fusarium verticillioides*	Average
1	-	10	6	-	**4**
2	-	-	2	-	**0.5**
3	-	18	-	-	**4.5**
4	-	-	6	-	**1.5**
5	-	10	-	8	**4.5**
6	4	6	2	-	**3**
7	12	20	-	-	**8**
8	-	-	2	-	**0.5**
9	4	20	-	18	**10.5**
10	6	-	-	14	**5**
11	-	28	-	-	**7**
12	8	100	8	66	**45.5**
13	-	8	-	-	**2**
14	42	30	-	-	**18**
15	4	70	2	10	**21.5**
16	4	2	4	-	**2.5**
17	-	-	2	-	**0.5**
18	2	80	-	-	**20.5**
19	6	22	-	-	**7**
20	24	34	2	-	**15**
21	2	30	4	-	**9**
22	-	-	-	6	**1.5**
23	22	30	6	8	**16.5**
24	-	10	-	-	**2.5**
25	6	20	-	-	**6.5**
26	2	30	-	-	**8**
27	12	20	8	24	**16**
28	-	10	-	-	**2.5**
29	-	16	-	-	**4**
30	78	30	16	90	**53.5**
31	6	8	-	-	**3.5**
32	16	10	2	-	**7**
33	24	100	48	76	**62**
34	-	10	-	2	**3**
35	6	10	2	-	**9**
36	-	-	6	-	**1.5**
37	6	18	-	-	**6**
38	-	-	2	-	**0.5**
39	-	78	2	-	**20**
40	28	80	-	68	**44**
41	10	20	-	-	**7.5**
42	-	18	2	12	**8**
43	4	60	4	-	**17**
44	12	-	-	-	**3**
45	34	40	2	-	**19**
46	-	60	2	-	**15.5**
47	-	100	-	16	**29**
48	12	-	-	-	**3**
49	16	80	4	-	**20**

Results expressed as the percentage of kernels infested with a specific fungus.

Table 5: Averages of the % infestation of all storage fungi combined, except *Penicillium islandicum,* during storage.

No:	*Eurotium repens*	*Aspergillus flavus*	*Aspergillus ochraceus*	*Rhizopus oryzae*	Average
1	48	52	62	100	**65.5**
2	30	16	52	100	**49.5**
3	28	10	68	100	**51.5**
4	30	2	14	10	**14**
5	46	28	30	10	**28.5**
6	40	10	100	20	**42.5**
7	46	10	36	80	**43**
8	14	4	30	20	**17**
9	74	6	32	100	**53**
10	66	12	52	80	**52.5**
11	18	44	20	82	**41**
12	100	60	100	50	**77.5**
13	38	4	16	100	**39.5**
14	40	8	38	20	**26.5**
15	54	30	94	22	**59.5**
16	40	8	54	100	**50.5**
17	40	32	56	80	**52**
18	96	100	20	70	**71.5**
19	50	10	86	90	**59**
20	100	8	82	10	**50**
21	50	6	80	50	**46.5**
22	6	2	28	100	**34**
23	88	100	46	100	**83.5**
24	74	30	12	30	**36.5**
25	54	14	86	20	**43.5**
26	80	66	76	80	**75.5**
27	68	100	74	100	**85.5**
28	56	66	54	80	**64**
29	66	28	50	80	**56**
30	100	76	100	20	**74**
31	54	24	76	60	**53.5**
32	16	28	44	90	**44.5**
33	100	62	100	100	**90.5**
34	-	8	40	10	**14.5**
35	80	6	96	-	**45.5**
36	10	16	50	30	**26.5**
37	84	16	70	50	**55**
38	38	94	40	60	**58**
39	88	6	80	100	**68.5**
40	70	30	92	90	**70.5**
41	66	24	74	100	**66**
42	56	22	44	100	**55.5**
43	50	28	54	30	**40.5**
44	66	24	56	90	**59**
45	100	52	94	100	**86.5**
46	30	18	100	10	**39.5**
47	22	10	6	80	**29.5**
48	28	100	20	30	**44.5**
49	32	18	98	30	**44.5**

Results expressed as the percentage of kernels infested with a specific fungus.

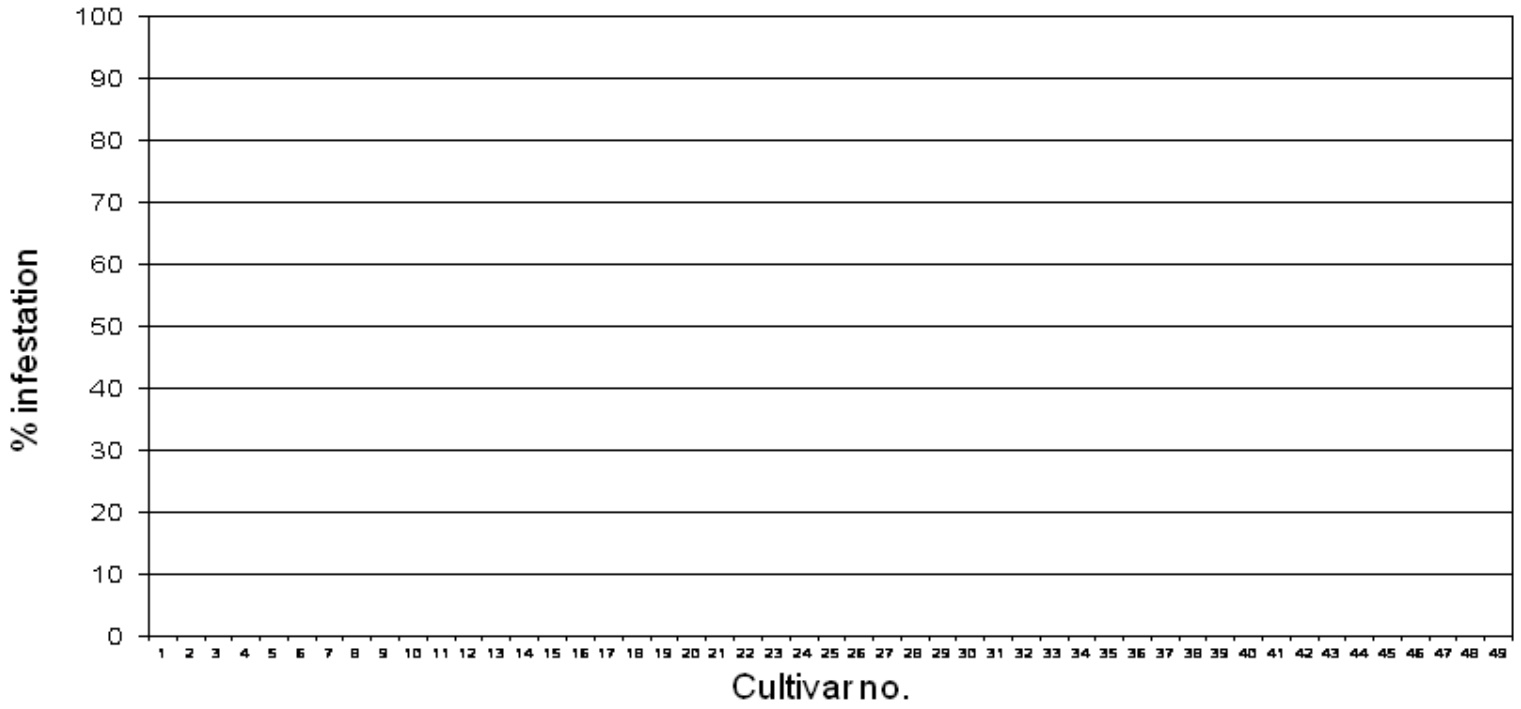

FIGURE 6: Comparison of 49 cultivars against the infestation of *Alternaria alternata* during storage.

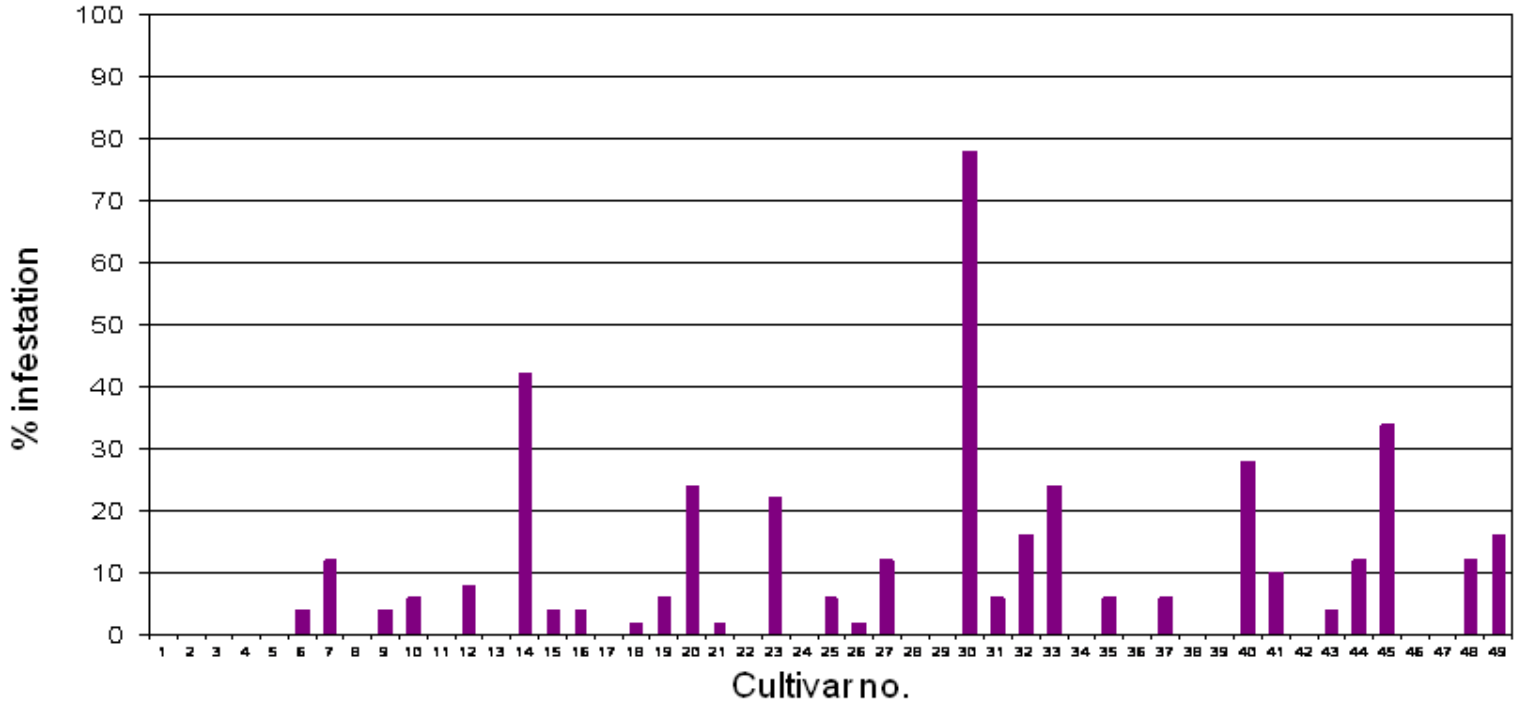

FIGURE 7: Comparison of 49 cultivars against the infestation of *Fusarium graminearum* during storage.

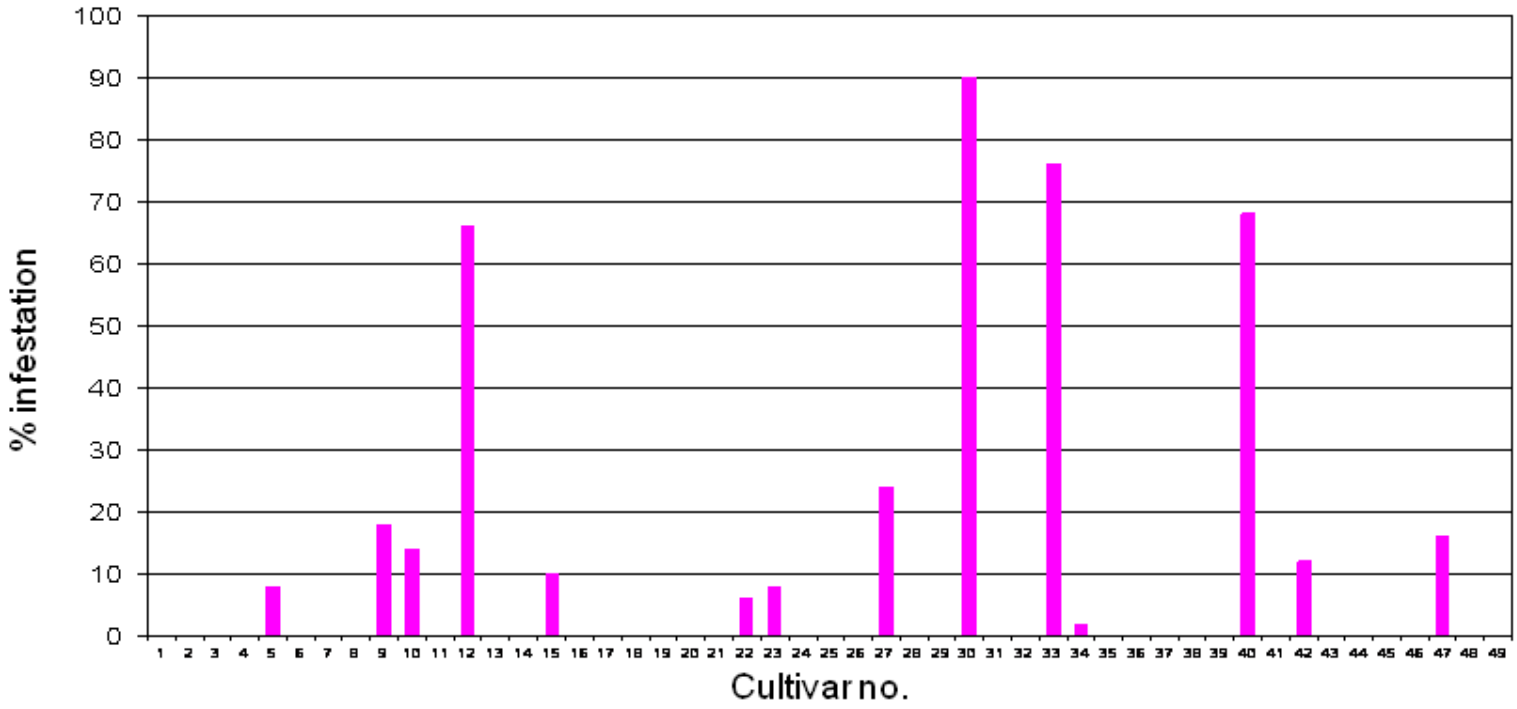

FIGURE 8: Comparison of 49 cultivars against the infestation of *Fusarium verticillioides* during storage.

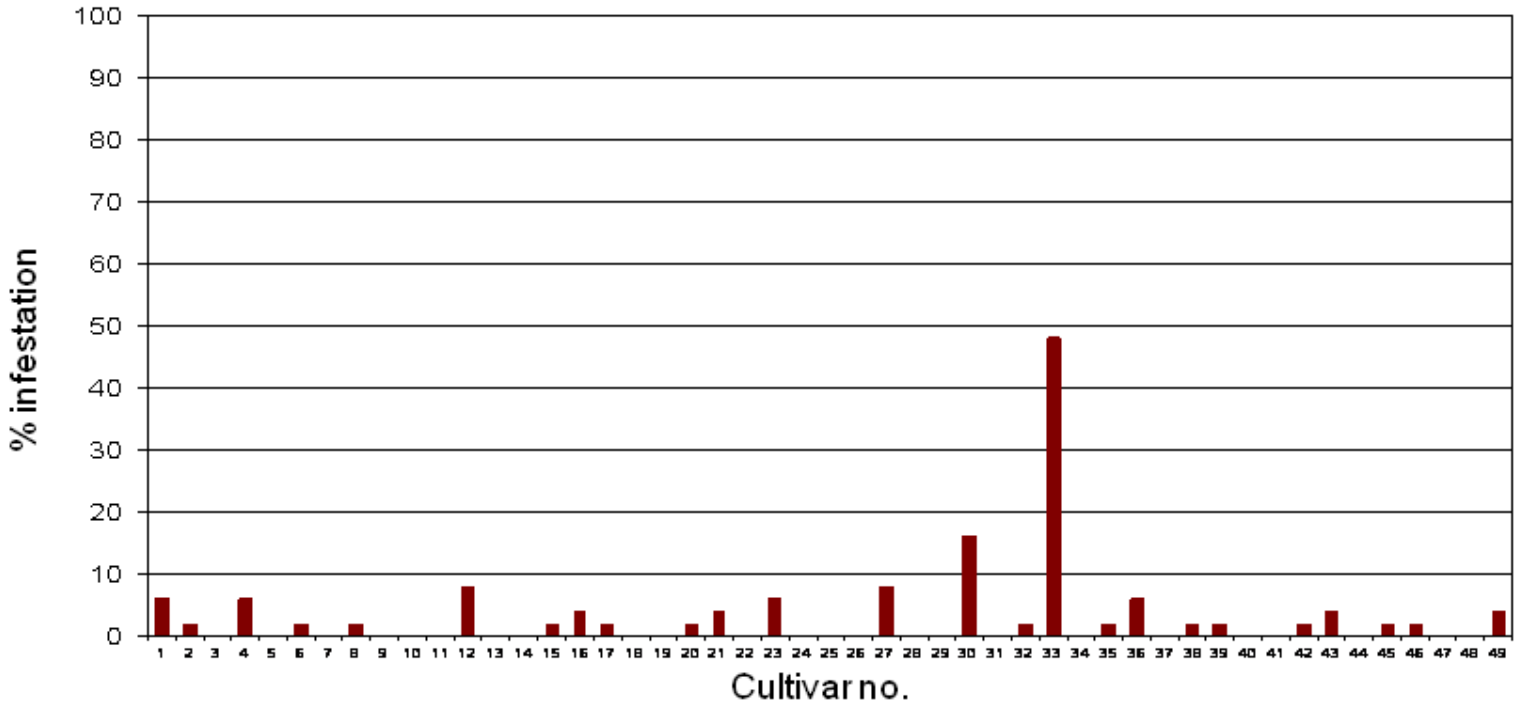

FIGURE 9: Comparison of 49 cultivars against the infestation of *Phoma sorghina* during storage.

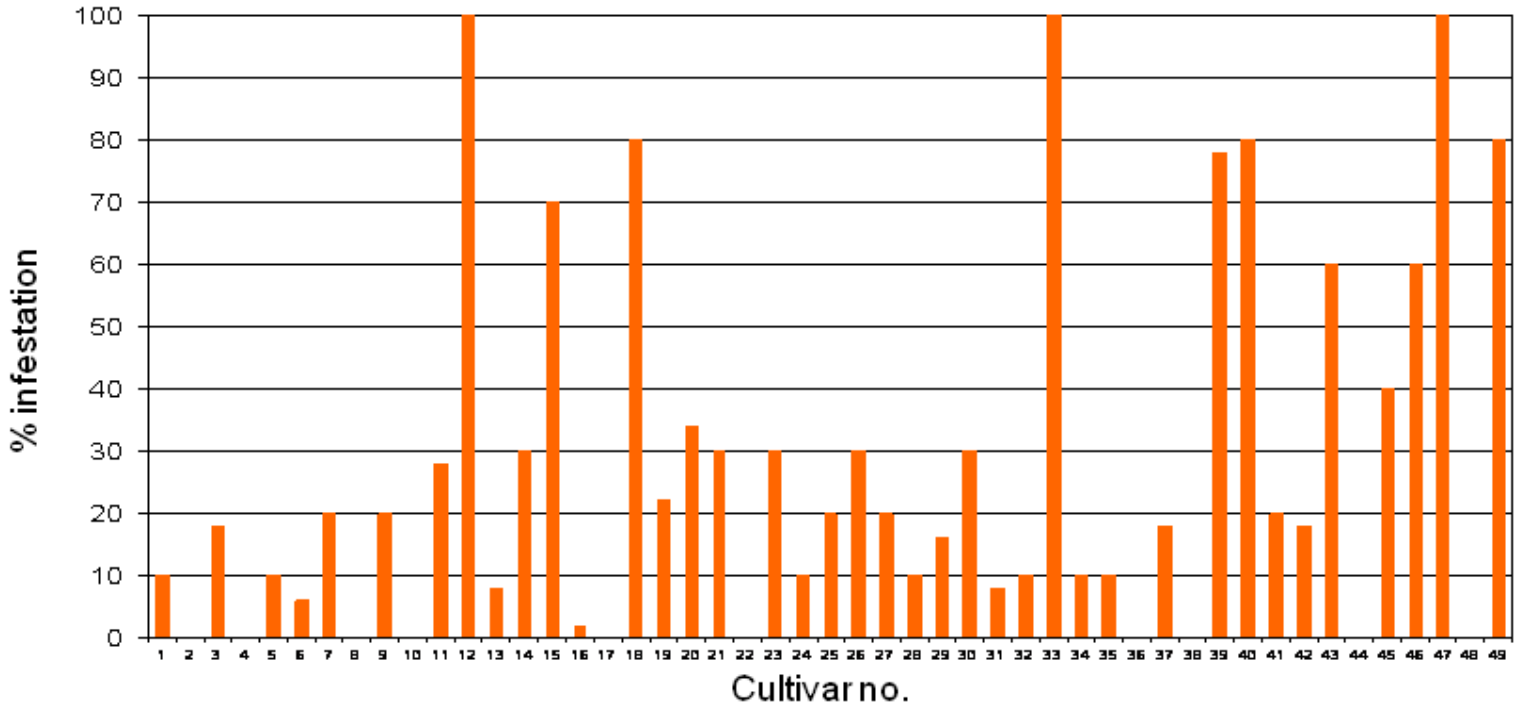

FIGURE 10: Comparison of 49 cultivars against the infestation of *Stenocarpella maydis* during storage.

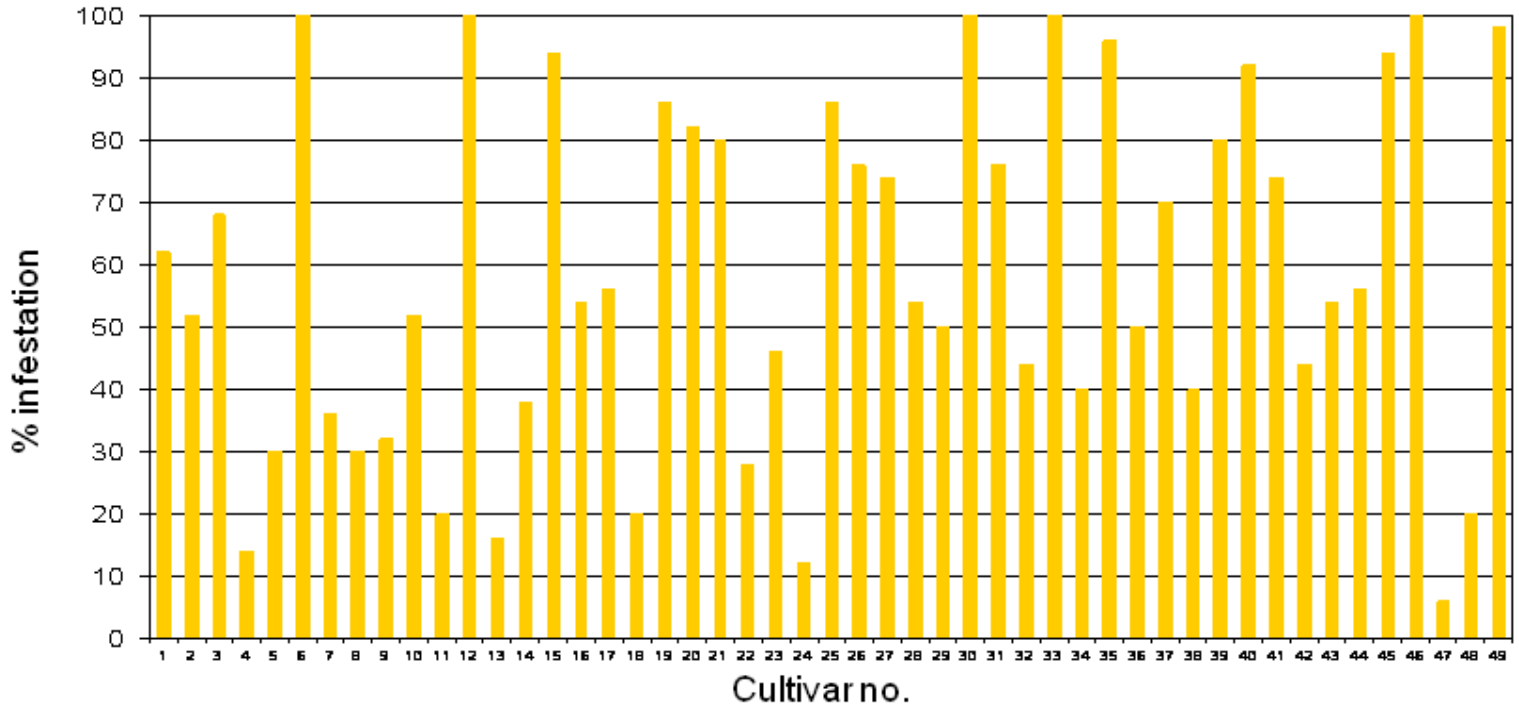

FIGURE 11: Comparison of 49 cultivars against the infestation of *Aspergillus ochraceus* during storage.

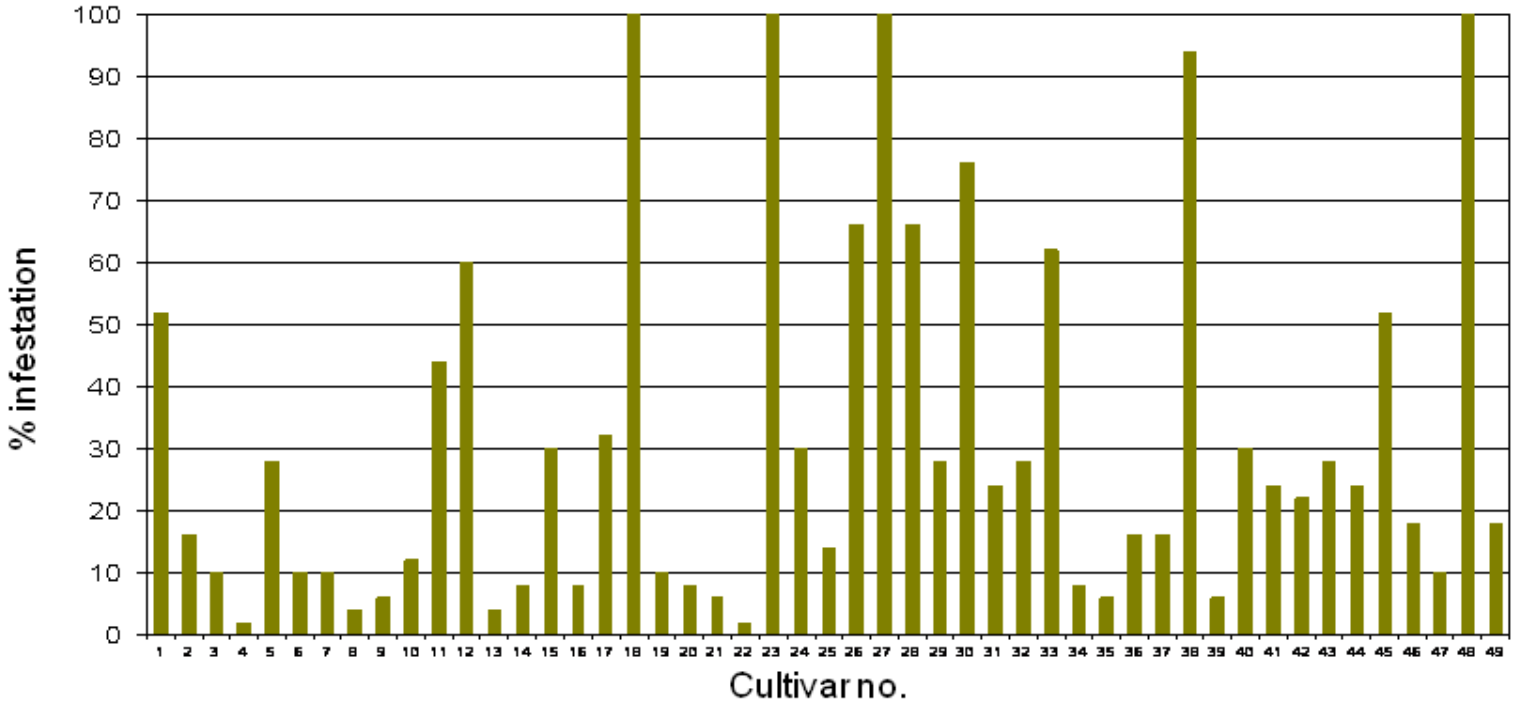

FIGURE 12: Comparison of 49 cultivars against the infestation of *Aspergillus flavus* during storage.

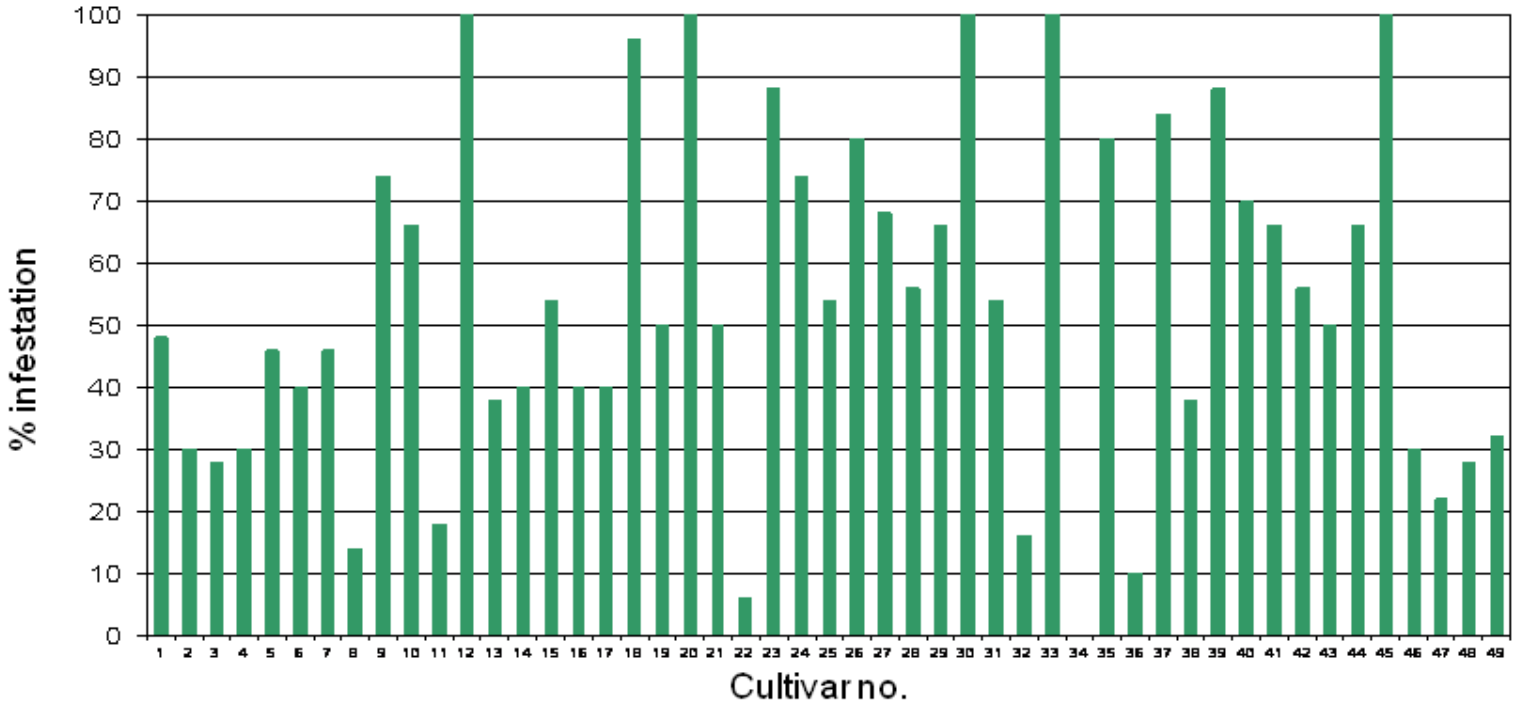

FIGURE 13: Comparison of 49 cultivars against the infestation of *Eurotium repens* during storage.

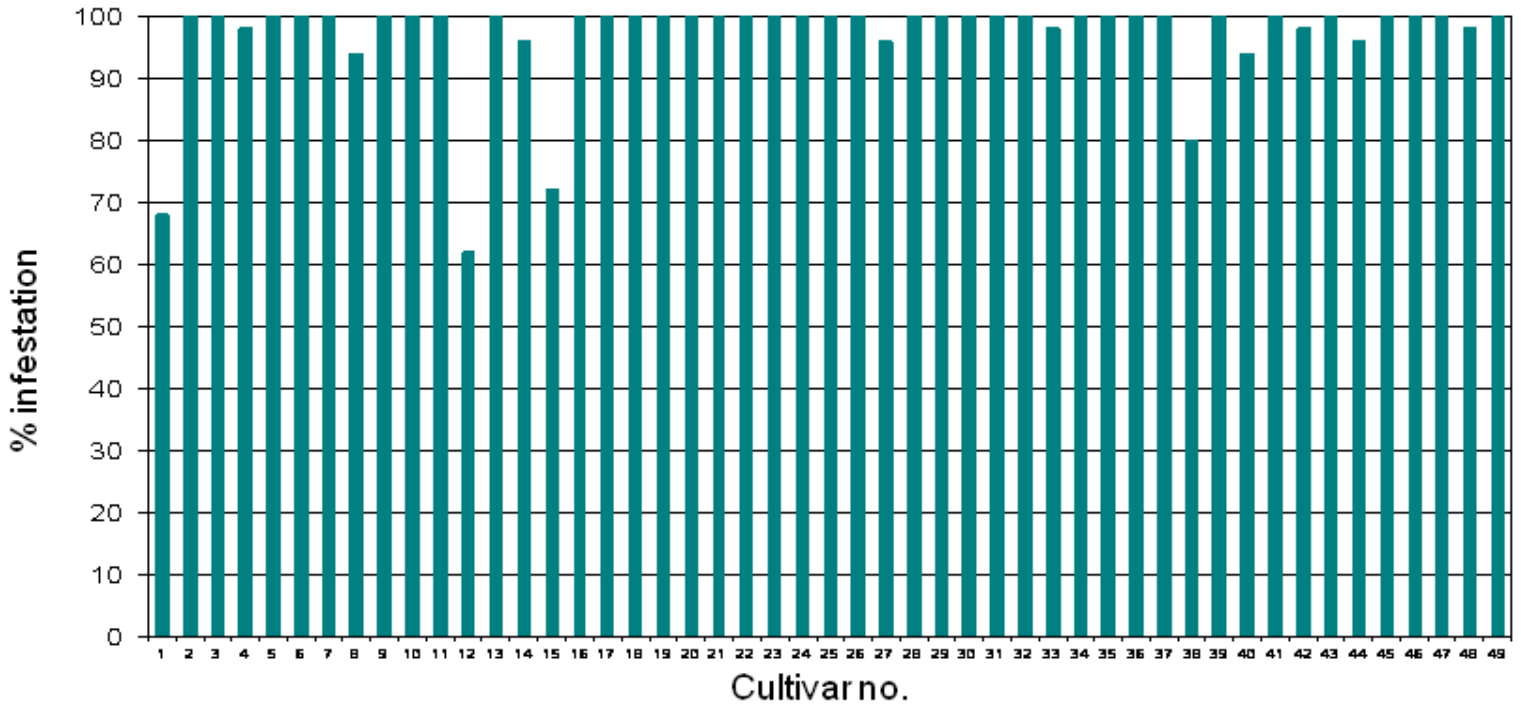

FIGURE 14: Comparison of 49 cultivars against the infestation of *Penicillium islandicum* during storage.

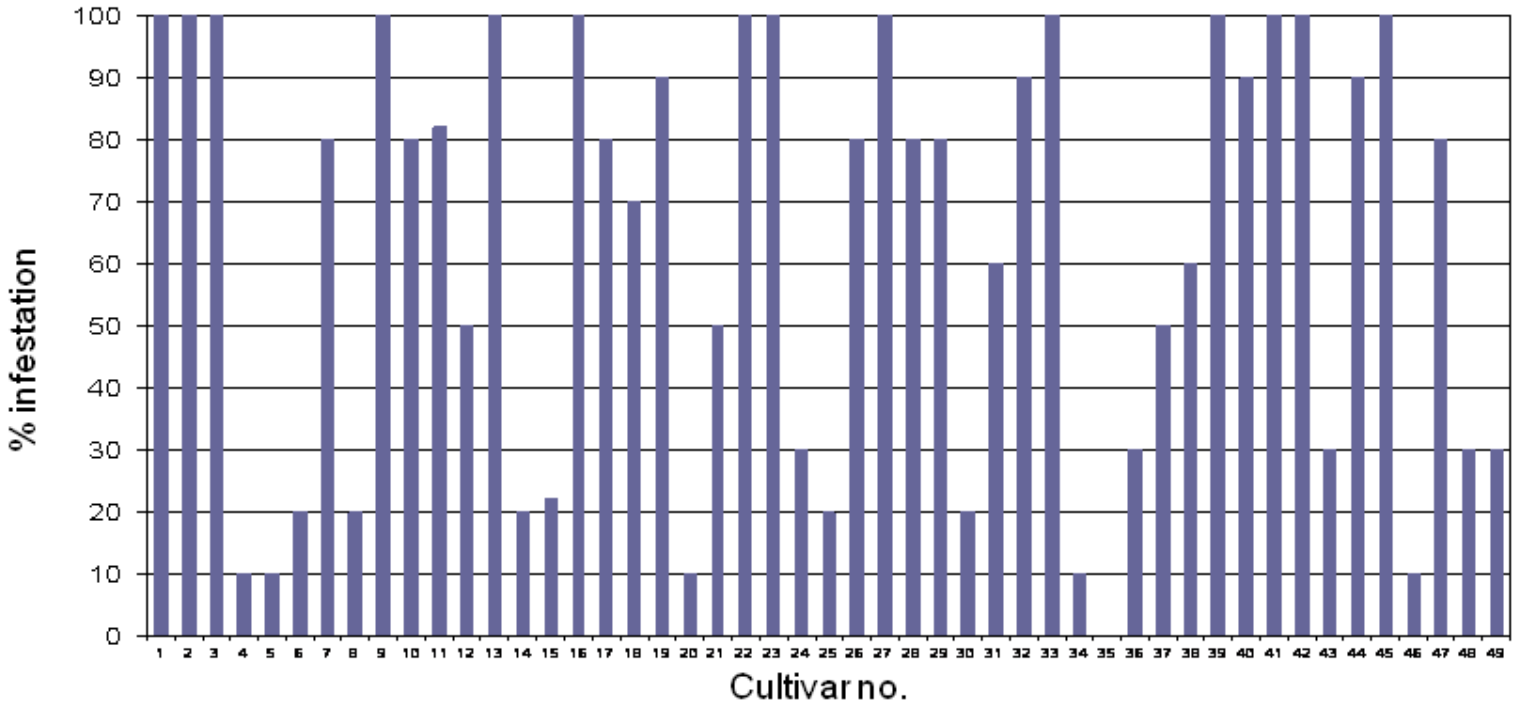

FIGURE 15: Comparison of 49 cultivars against the infestation of *Rhizopus oryzae* during storage.

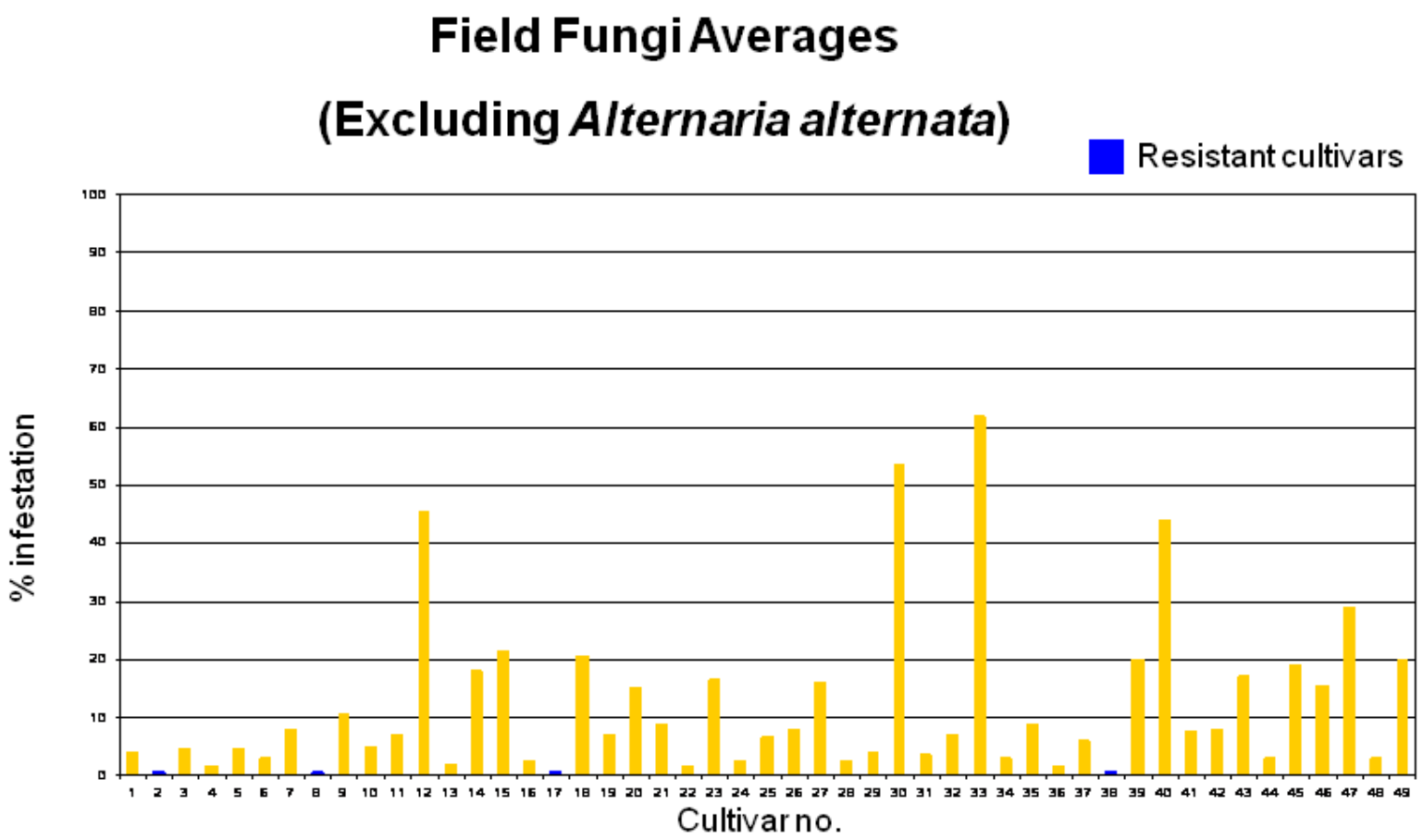

FIGURE 16: Comparison of 49 cultivars against the average infestation of all field fungi namely *Fusarium graminearum, Fusarium verticillioides, Phoma sorghina* and *Stenocarpella maydis* during storage.

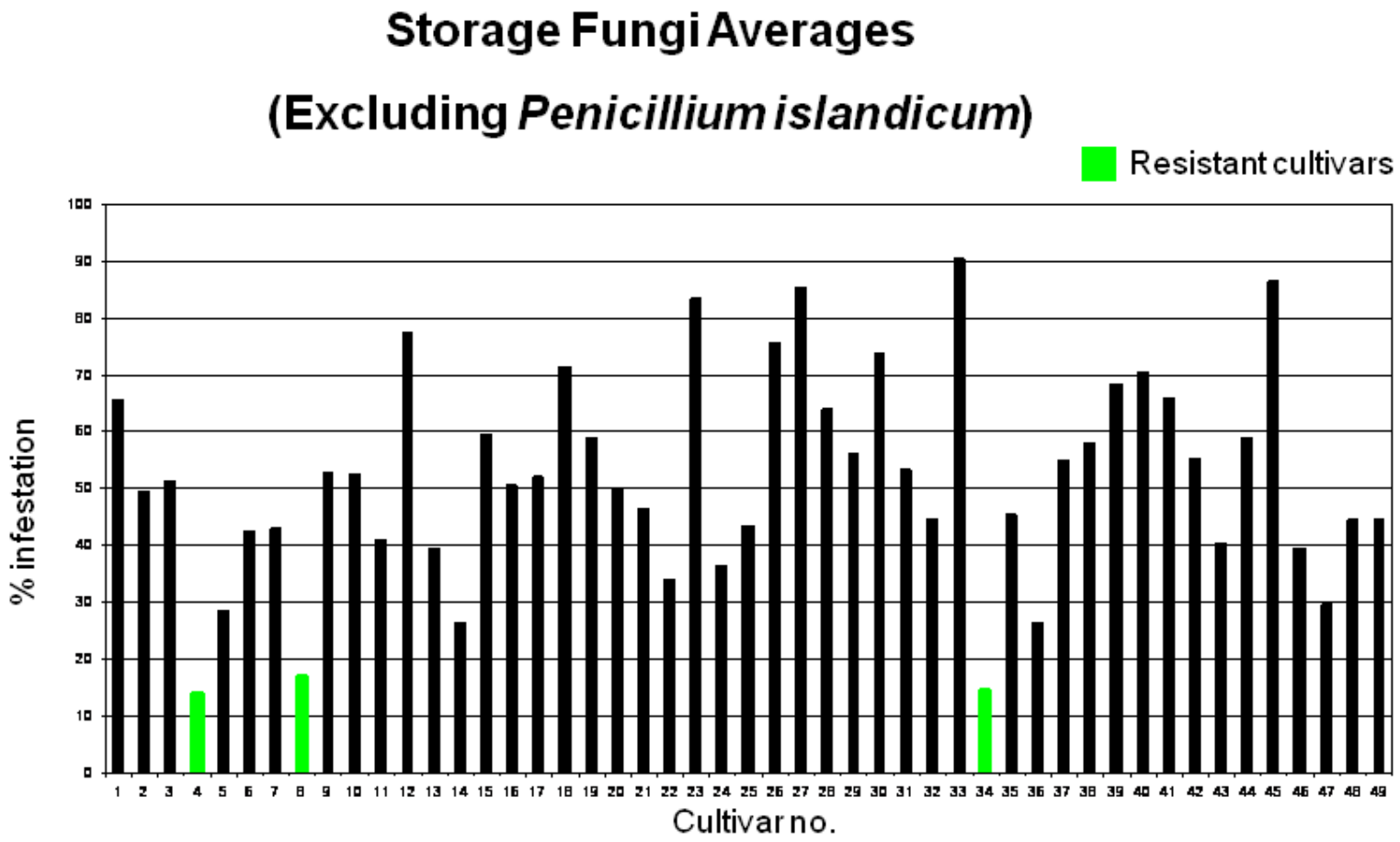

FIGURE 17: Comparison of 49 cultivars against the average infestation of all storage fungi namely *Aspergillus ochraceus, Aspergillus flavus, Eurotium repens,* and *Rhizopus oryzae* during storage.

Chapter 5:

The use of PCR-based technology to identify mycotoxigenic fungi in maize

1. Summary:

A wide variety of field and storage fungi are associated with maize, which is regarded as the staple food of South Africa. Previous studies have shown that in South Africa, food and feed commodities are associated with at least 190 mycotoxigenic fungi making the risk of humans and animals that can be exposed to mycotoxins a reality.

The aim of this study was to develop a rapid and precise method for detection of the ten relevant mycotoxigenic fungi in South African maize. The fungi included in the study were *Alternaria alternata, Aspergillus flavus*, *Aspergillus ochraceus*, *Eurotium repens*, *Fusarium graminearum, Fusarium verticillioides*, *Penicillium islandicum*, *Phoma sorghina*, *Rhizopus oryzae* and *Stenocarpella maydis*. To develop a system of detection, the fungi and maize were analyzed by exploiting the sequence variations of the internal transcribed spacer (ITS) and D1/D2 regions of the fungal rRNA gene.

The results obtained showed that ITS regions can be used to identify contamination from some of these fungi by applying the variability of internal transcribed spacer (ITS) regions. The use of this application is to provide the maize industry with a tool to determine mycotoxigenic risks in maize through a rapid and more cost effective method.

2. Introduction

Maize is the largest locally produced field crop and the most important source of carbohydrates in the South African Custom Union (SACU) for animal and human consumption (NDA, **2003**). The majority of South Africans consume maize in some form or another, mostly as maize meal. Various field and storage fungi are associated with maize, and previous studies have shown that at least 190 toxigenic fungi are associated with food and feed commodities in South Africa (Rabie & Marais, **2000**).

Mycotoxin contamination is seen as a serious quality and safety problem worldwide (FAO, **2004**). Fungal toxins are low molecular weight chemical compounds, which are not detected by antigens and hence sometimes produce no obvious symptoms (Pitt, **1989**). Mycotoxins can cause acute or chronic toxicity in humans and animals that eat infected crops or foods. This depends on the quantities of mycotoxins produced and consumed. In addition, the high incidence of HIV/AIDS in Southern Africa, combined with the consumption of maize infected with mycotoxins, could negatively affect the immune system of infected persons. This emphasizes the importance of preventing the intake of food contaminated with mycotoxins, especially on the African continent (Sharma, **1993**).

Due to the fact that the presence of mycotoxins are not visible to the naked eye, and do not affect the taste of foods and feeds, their detection and elimination from the food chain is not always seen as a priority. It is, therefore, preferable to minimize possible risks through prevention as the majority of these mycotoxins are chemically stable and resistant to temperature, storage and processing conditions (Starkl, **2004**). One of the measures that can be taken to ensure the use of low risk commodities in the human and animal food chain is the use of molecular based technology to evaluate the level of fungal contamination (Konietzny & Greiner, **2003**). Microbiological methods have shown to be very time consuming and sometimes inaccurate as the morphological characteristics of the fungi could

be similar among genetically diverse organisms (Geiser *et al.*, **2000**). In addition, morphological identification is dependent on the development of fruiting structures that could take weeks in cases where vegetative growth is mainly experienced. According to Rabie & Marais (**2000**) the majority of fungi isolated from maize in South Africa have shown to be toxigenic (see Table 2 in Chapter 1, pg. 47). The use of molecular methods to identify these fungi is becoming more relevant, especially in cases where the mycotoxins are still unknown and rapid identification of the fungi is needed to conduct risk assessments.

To date a variety of molecular methods have been used for the identification and differentiation between fungal pathogens. These include restriction length polymorphisms (RFLPs) that were used for the identification of brown rot and white rot fungi in wood decay (Jasalavich *et al.*, **2000**), Southern blots have been used to examine the molecular nature of resistant genes in wild maize varieties specifically to a fungal pathogen, *Cochliobolus carbonum* (Multani *et al.*, **1998**) and PCR-enzyme immunoassays to identify dimorphic and yeast like fungal pathogens (Lindsley *et al.*, **2001**). According to Bruns *et al.* (**1991**), most molecular studies on fungi have focused on rRNA genes due to the presence of universally DNA conserved regions that serve as ideal primer sites. In **1990**, White *et al.* designed a set of primers from most plant and fungal kingdoms that allowed the amplification of the internal transcribed spacer (ITS) regions. ITS regions are stretches of DNA between the 18S, 5.8S and 28S rRNA regions of the fungal rRNA gene. These regions have been used in many different applications including the ability to diagnose ear rot disease in maize (Möller *et al.*, **1999**), detection of mycotoxigenic fungi in food (Konietzny & Greiner, **2003**), rapid identification of *Fusarium* cultures (Abd-Elsalam *et al.*, **2003**; Mulé *et al.*, **2004**) and to detect mycotoxigenic *Fusarium* species in maize (Jurado *et al.*, **2006**).

Another region that has been used is the D1/D2 26S rDNA region. Evaluation by Sugita & Nishikawa (**2003**) of the ITS method used by Japanese Pharmocopeia, and the D1/D2 region used by the Pharmaceutical Society of Japan, showed the D1/D2 method to be superior to the ITS regions. The

authors stated that the D1/D2 region is defined based on the species concept and can further allow phylogenetic analysis. The D1/D2 region has previously been used to differentiate between *Fusarium* species (Edel *et al.*, **1996**), the identification of basidiomycetous yeasts (Fell *et al.*, **2000**), differentiation between *Saccharomyces* species (Ramos *et al.*, **2001**), and the identification of a novel ascomycetous yeast (Lu *et al.*, **2004**).

In this study, two different methods, based on DNA sequence analysis, were evaluated for the identification of the ten more important mycotoxigenic fungi in contaminated maize. It is foreseen that if this technique can be used to detect fungal contamination in maize, it could contribute to the rapid evaluation of samples and give maize producers and consumers a tool to make timely decisions as to whether a commodity is fit for consumption.

3. Materials and Methods:

3.1. Fungal inoculation of maize kernels

Spore suspensions were prepared for each of the five selected field fungi namely *Alternaria alternata*, *Fusarium graminearum, F. verticillioides*, *Phoma sorghina, Stenocarpella maydis* and five storage fungi namely *Aspergillus flavus*, *A. ochraceus*, *Eurotium repens, Penicillium islandicum*, and *Rhizopus oryzae*. This was done by adding five milliliters of sterile distilled water to each of the 90 mm petri dishes that was fully covered with growth of each of the selected fungi. The surface of the petri dish was then gently scratched with a blunt pincette. In total, 50 ml of spore suspension was added to non-solidified potato dextrose agar (PDA) at 50^0C. The PDA was prepared as per manufacturer's instructions (Merck, South Africa). The suspension was then mixed to allow even distribution of spores throughout the medium and poured onto 90mm petri dishes to solidify. The inoculated petri dishes were then incubated at 25^0C for 3 days. Twenty-five kernels were plated onto the petri dishes by the method described by Rabie *et al.* (**1997**) (also see Chapter 2, pg. 68). The plates were incubated at 25^0C for 7 days after which kernels, infested with each fungus, were harvested. Table 1 shows the maize cultivars used in this study that previously showed to be either rapidly or more slowly

colonized by the selected fungi (see Chapter 4). This was done to evaluate the two extraction methods to obtain plant and fungal DNA.

Table 1: Selected cultivars that indicated slower and faster rates of infestation to 4 field fungi and 4 storage fungi.

Field fungi (*S. maydis, P.sorghina, F. verticillioides* and *F. graminearum*)	Cultivars with slower rate of infestation	Cultivars with faster rate of infestation
	2 (DKC 80-10)	12 (PAN 6966)
	8 (PAN 6146)	30 (PHB 30D05)
	17 (AFG 4512)	33 (CRN 3549)
	38 (DKC 78-15)	40 (PAN 6995 Bt)

Storage fungi (*A. flavus, A. ochraceus, E. repens* and *R. oryzae*)	Cultivars with slower rate of infestation	Cultivars with faster rate of infestation
	4 (PAN 6844)	23 (SC 621)
	8 (PAN 6146)	27 (Saffier)
	34 (PAN 6611)	33 (CRN 3549)
		45 (SNK 2969)

3.2. DNA extraction

The CTAB method is generally used for plant DNA extractions and DEB method for fungal DNA extractions. However both methods were used to extract DNA from the artificially inoculated maize kernels in order to compare the yield of both plant and fungal DNA in the artificially inoculated maize.

3.2.1 CTAB method

Genomic DNA was extracted from pre-inoculated maize kernels as described by Murray & Thompson (**1980**). The CTAB extraction buffer contained 5% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA pH8, 0.1 M Tris-HCl pH8, and 1% (w/v) PVP; which was made up to 500 ml. Just before use, 0.2% (v/v) 2-Mercapto-ethanol was added to the extraction buffer (i.e. 180 µl of 2-

Mercapto-ethanol per 1 ml of CTAB buffer). Two maize kernels were ground with a mortar and pestle into a fine powder using liquid nitrogen. An amount of 1 ml of CTAB extraction buffer was added to the contents of the mortar and transferred to a 2 ml eppendorf tube. The samples were incubated for at least 30 mins at 65^0C in a water bath (tubes inverted every 10 mins). The samples were then centrifuged for 7 mins at 10621 g. The supernatant (600 µl) was transferred to an empty 1.5 ml tube and 600 µl of 24:1 chloroform: iso-amyl alcohol was added and the tubes kept on ice for 30 mins and inverted every 10 mins. The samples were then centrifuged for 15 mins at 17949 g. The supernatant (500 µl) was transferred to an empty 1.5 ml tube. An amount of 500 µl of isopropanol was added and the solution incubated at 20^0C for 15 mins, after which it was centrifuged for 15 mins at 17949 g. The supernatant was discarded, 500 µl of 70% ethanol added into the same tube and inverted 5 times gently. The samples were centrifuged for 5 mins at 17949 g and washed once again with 70% ethanol as mentioned above. The supernatant was discarded. An amount of 300 µl of 95% ethanol was added to the samples and centrifuged for 1 min at 17949 g. The supernatant was removed and the pellet was allowed to air dry. The pellet was then re-suspended with 50 µl of sterile distilled sabax water. This method was used for the DNA extractions of all the pre-inoculated maize kernels.

3.2.2. DEB method

Fungal DNA was extracted by using the DEB method (Raeder & Broda, **1985**). The DNA extraction buffer (DEB) contained 200mM Tris-HCl pH8, 150 mM NaCl, 25 mM EDTA pH8, 0.5% SDS and 1% PVP. Just before use 0.2% (v/v) 2-Mercapto-ethanol was added to the extraction buffer (i.e. 160 µl of 2-Mercapto-ethanol per 500 µl DEB). Fungal mycelium was gently scraped off the culture and placed into 2 ml screw cap eppendorf tubes along with metal yellow balls (3 mm, tungsten carbide beads) and 500 µl of DEB. The samples were put into a Fastprep machine at speed 4 for 5 seconds. The metal balls were removed and the contents transferred to a 2 ml tube to which 50 µl of 1 M Tris-HCl pH8.0, 100 µl of phenol and 170 µl of chloroform were added and the tubes incubated on ice for 5 mins. The suspension was then centrifuged for 15 mins at 20817 g. The aqueous phase was transferred to a new tube

and, once again, cleaned with 50 µl of 1 M Tris-HCl pH8.0, 100 µl phenol and 170 µl chloroform as mentioned previously until the interface was clean. In total, 1 X volume of chloroform was added and samples centrifuged for 15 mins at 10621 g. The aqueous phase was transferred to an empty tube and 2.5 X volume of ethanol and 0.5M ammonium acetate were added. The solution was allowed to precipitate overnight at -20^{0}C and then centrifuged for 10 mins at 10 000 rpm, supernatant discarded and pellet washed with 2.5 X volume of 70% ethanol. It was then centrifuged for 10 mins at 10621 g. The supernatant was discarded and the pellet was allowed to air dry. The pellet was re-suspended with 50 µl of sterile distilled sabax water. This method was used to extract DNA from the ten selected fungi. In addition this method was also used to extract DNA from the artificially inoculated fungal maize.

3.3. PCR amplification

Prior to PCR amplification all extracted samples were column-purified using the QIAquick PCR Purification Kit (QIAGEN GmbH) (Southern Cross Biotechnology) as per manufacturer's instructions. DNA quantification was done by using the Nanodrop IS 500 system.

3.3.1. D1/D2 Region

Amplification of the D1/D2 region was performed in 25 µl volumes containing 2.5 mM of each dNTP, 10 µM of each oligonucleotide primer, NL1 (5'-GCATATCAATAAGCGGAGGAAAA-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), 8 ng of fungal DNA template and 1 Unit of *Taq* DNA polymerase (Inqaba Biotech, South Africa), 0.75 µl of 50mM $MgCl_2$ and 1 X PCR reaction buffer. The conditions for PCR amplification included an initial denaturation at 94^{0}C for 5 mins, followed by 35 cycles for 1 min at 94^{0}C, 1 min at 55^{0}C and 2 mins at 72^{0}C, and a final extension of 72^{0}C for 5 mins.

3.3.2. ITS Regions

Amplification reactions of the ITS regions were carried out in 25 µl volumes containing 0.4 mM of each dNTP, 0.4 µM of each oligonucleotide primer, ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and 0.4 µM of primer ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'), 8 ng DNA template and 1 Unit of Taq polymerase (Inqaba Biotech, South Africa), 1 µl of 50 mM $MgCl_2$ and 1 X PCR reaction buffer. The PCR amplification was conducted with an initial denaturation step at 94^0C for 5 mins, followed by 35 cycles for 45 secs at 94^0C, 1 min at 50^0C and 1 min 30 secs at 72^0C, and a final extension of 72^0C for 5 mins.

3.4. Electrophoresis

3.4.1. TBE (Tris-borate EDTA)

Amplified products were resolved on 1.1% agarose gels using 1 X TBE (Tris-borate -EDTA) buffer. To allow for separation of bands, these gels were run for about 15 mins at 120 V on a 20 cm gel. The bands were then detected by ethidium bromide fluorescence on a UV transilluminator.

3.4.2. TAE (Tris-acrylamide-EDTA)

Amplified products were resolved on 1.5% agarose gels using 1 x TAE (Tris-acrylamide-EDTA) buffer. The gels were run for about 160 mins at 120 V on a 20 cm gel to ensure separation of bands, and then detected by ethidium bromide fluorescence on a UV transilluminator.

PCR products of the ITS regions for post-inoculated cultivar no. 38 where run on TBE and TAE gels to determine which buffer would give a clearer band definition.

4. Results

4.1. DNA extraction

The CTAB method is normally used for rapid isolation of high molecular weight plant DNA. This method was used for DNA extraction of the pre-inoculated maize kernels and for the extraction of both maize DNA and fungal DNA from the post-inoculated maize kernels. The DEB method is generally used for isolation of DNA from filamentous fungi. Although good yields of fungal DNA were obtained using this method, higher DNA yields were obtained using the CTAB method on the post-inoculated maize kernels as shown in Table 2.

Table 2: Comparison of DNA concentrations extracted from post-inoculated maize kernels using two DNA extraction methods, DEB and CTAB.

DEB Method	
Cultivar no.	DNA concentration (ng/µl)
4	226.26
8	159.81
34	131.63
23	152.68
27	182.70
33	143.87
45	55.67

CTAB Method	
Cultivar no.	DNA concentration (ng/µl)
4	543.35
8	471.36
34	492.18
23	525.93
27	1034.53
33	565.90
45	943.98

DNA extractions of the post-inoculated kernels of *A. flavus* were done by both the CTAB and DEB methods. The CTAB method (Figure 1 and Table 2) yielded a reasonable amount of both plant and fungal DNA when used for DNA extraction of the post-inoculated maize kernels.

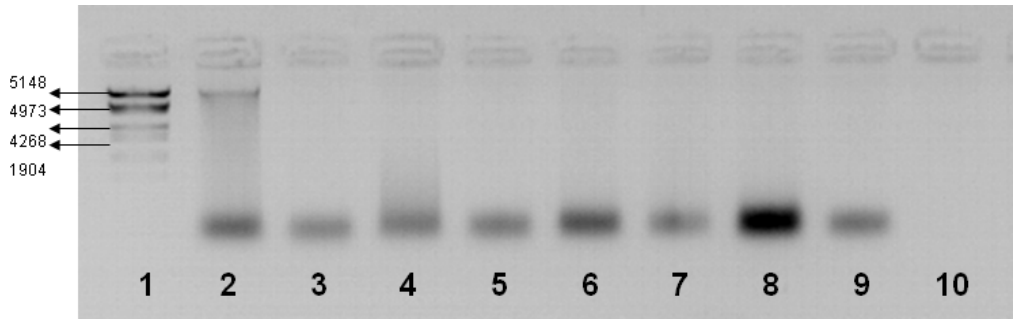

FIGURE 1: DNA extraction of post-inoculated maize kernels (*A. flavus*) using the CTAB extraction method. Lane 1: DNA marker, *Eco RI* and *HIND III*; Lane 2: cultivar no. 4; Lane 3: cultivar no. 8; Lane 4: cultivar no. 34; Lane 5: cultivar no. 23; Lane 6: cultivar no. 27; Lane 7: cultivar no. 33; Lane 8: cultivar no. 45; Lane 9: cultivar no. 33 and Lane 10: negative control (no DNA).

DNA extractions of the post-inoculated kernels of *Aspergillus flavus* using the DEB method (Figure 2 and Table 2) yielded very little DNA (faint bands visible in lanes 2-6) and no DNA (no visible bands in lanes 7-10).

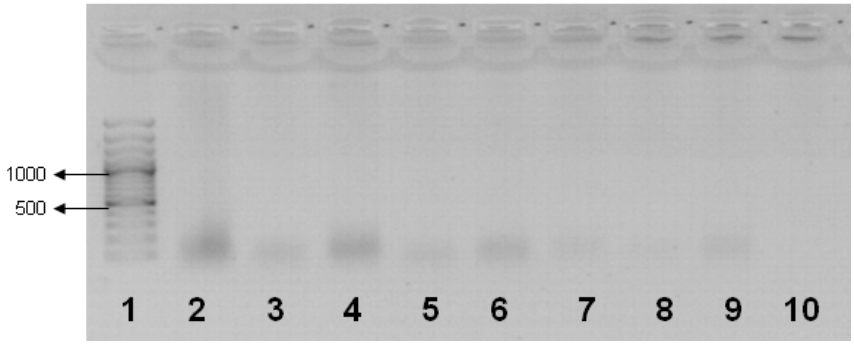

FIGURE 2: DNA extraction of post-inoculated kernels (*A. flavus*) using the DEB extraction method. Lane 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: cultivar no. 4; Lane 3: cultivar no. 8; Lane 4: cultivar no. 34; Lane 5: cultivar no. 23; Lane 6: cultivar no. 27; Lane 7: cultivar no. 33; Lane 8: cultivar no. 45; Lane 9: cultivar no. 45 and Lane 10: negative control (no DNA).

4.2. PCR amplification

For the optimization of the PCR for post-inoculated maize kernels (Figure 3), different concentrations of DNA from post-inoculated cultivars were used to determine the optimum amount needed to obtain a PCR product in the reaction. The optimization of the PCR reaction showed that 2 µl of DNA sample was sufficient to obtain a PCR product for all the post-inoculated maize kernels.

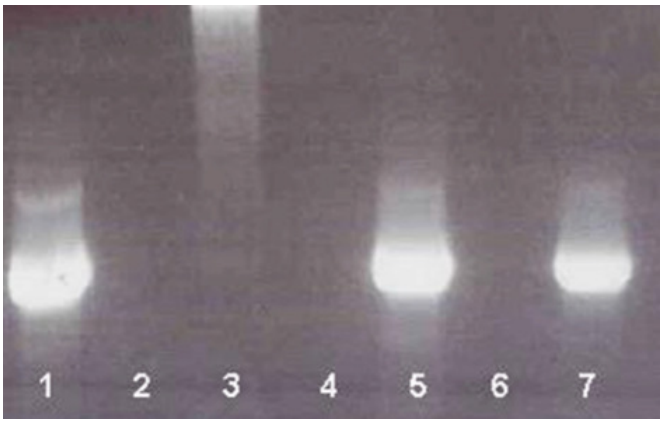

FIGURE 3: Optimization of PCR amplification of ITS regions run on a TAE gel. Lane 1: 2µl of DNA from cultivar no. 4 Lane 2: 4µl of DNA from cultivar no. 8; Lane 3: 2µl of DNA from cultivar no. 34; Lane 4: 8µl of DNA from cultivar no. 23; Lane 5: 2µl of DNA from cultivar no. 27; Lane 6: 8µl of DNA from cultivar no. 33; Lane 7: 2µl of DNA from cultivar no. 45.

Amplification was obtained for some of the fungi using the D1/D2 region. Of the 10 fungi, the D1/D2 region was able to amplify *A. alternata, S. maydis* and *F. graminearum* from the field fungi and for the storage fungi it was able to amplify *E. repens, R. oryzae* and *A. flavus.* This region did not give a good differentiation between the fungal bands (lanes 5-10) and for some fungi no amplification was observed (no bands visible in lanes 2-4 and 11).

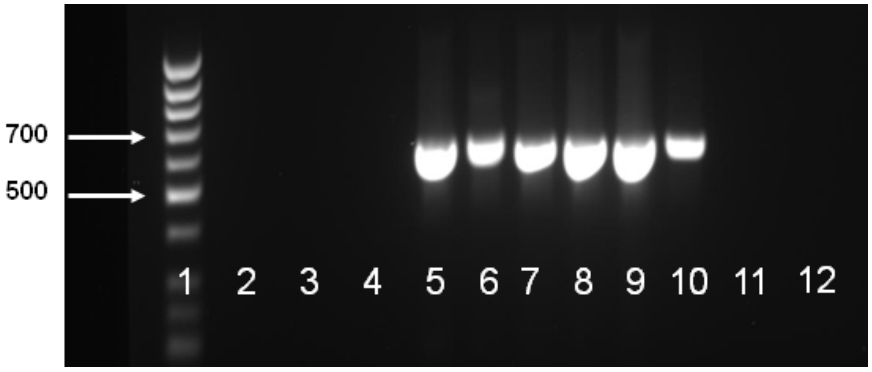

FIGURE 4: PCR amplification of the D1/D2 region in the 10 fungi run on a TAE gel. Lane 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: *F. verticillioides;* Lane 3: *A. ochraceus*; Lane 4: *P. sorghina;* Lane 5: *A. alternata;* Lane 6: *E. repens;* Lane 7: *R. oryzae;* Lane 8: *S. maydis;* Lane 9: *F. graminearum;* Lane 10: *A. flavus;* Lane 11: *P. islandicum;* Lane 12: negative control (no DNA).

PCR amplification of the 10 fungi was also carried out using the ITS regions (Figure 5). Unlike the D1/D2 region, the ITS regions showed better differentiation among the 10 fungi.

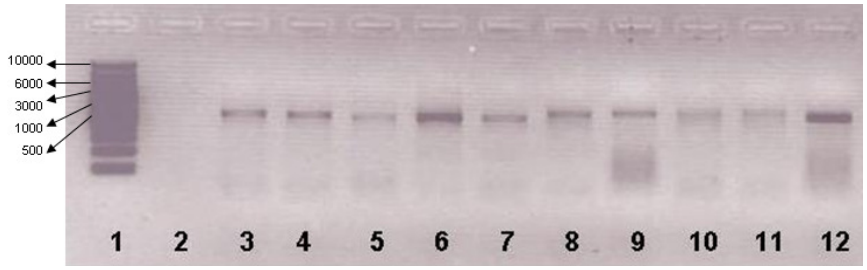

FIGURE 5: PCR amplification of the ITS regions of the 10 fungi run on a TAE gel. Lane 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: negative control (no DNA); Lane 3: *S. maydis;* Lane 4: *A. alternata;* Lane 5: *P. sorghina;* Lane 6: *F. verticillioides;* Lane 7: *F. graminearum;* Lane 8: *A. flavus;* Lane 9: *P. islandicum;* Lane 10: *A. ochraceus;* Lane 11: *R. oryzae;* Lane 12: *E. repens.*

PCR amplification of pre-inoculated maize kernels from four different cultivars and the 10 fungi with the ITS regions (Figure 6) showed a different banding pattern between the maize and the fungi. The difference observed in the bands size between the fungi and the maize was further investigated for cultivar 38 that was inoculated with 4 different fungi. Pre- and post-inoculated cultivar 38 and the 4 fungi were PCR amplified with the ITS regions and the amplification products separated using two different gel buffer systems, TBE (Figure 7) and TAE (Figure 8).

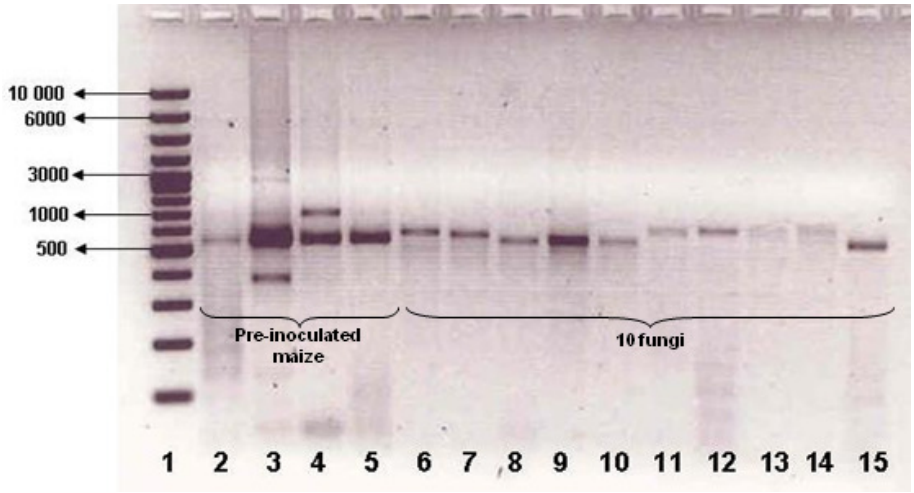

FIGURE 6: PCR amplification, optimization of the ITS regions, showing different DNA amplification products between the pre-inoculated maize and the 10 fungi run on a TAE gel. Lane 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: cultivar no. 17; Lane 3: cultivar no. 30; Lane 4: cultivar no. 34; Lane 5: cultivar no. 45; Lane 6: *S. maydis;* Lane 7: *A. alternata;* Lane 8: *P. sorghina;* Lane 9: *F. graminearum;* Lane 10: *F. verticillioides;* Lane 11: *A. flavus;* Lane 12: *P. islandicum;* Lane 13: *A. ochraceus;* Lane 14: *R. oryzae;* Lane 15: *E. repens.*

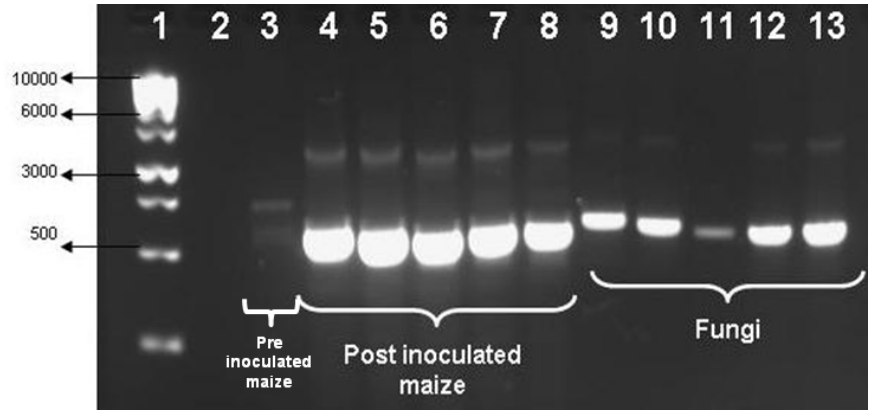

FIGURE 7: PCR amplification of the ITS regions, of pre and post-inoculated maize of cultivar no. 38 with five field fungi run on a TBE gel. Lane 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: negative control (no DNA); Lane 3: pre-inoculated maize of cultivar no. 38; Lanes 4-8: post-inoculated maize of cultivar no. 38; Lane 4: *S. maydis*; Lane 5: *A. alternata*; Lane 6: *P. sorghina*; Lane 7: *F. verticillioides* and Lane 8: *F. graminearum*. Lanes 9-13 amplification of the field fungi only, Lane 9: *S. maydis*; Lane 10: *A. alternata*; Lane 11: *P. sorghina*; Lane 12: *F. verticillioides* and Lane 13: *F. graminearum*.

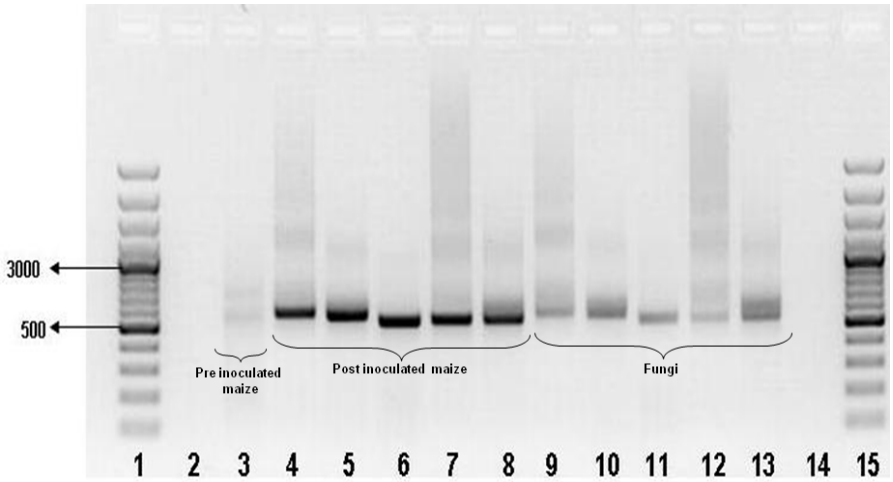

FIGURE 8: PCR amplification of the ITS regions, of pre and post-inoculated maize of cultivar no. 38 with five field fungi run on a TAE gel. Lane 1 and 15: Generuler 1kb DNA ladder (Fermentas); Lanes 2 and 14: negative control (no DNA); Lane 3: pre-inoculated maize of cultivar no. 38; Lanes 4-8: post-inoculated maize of cultivar no. 38; Lane 4: *S. maydis*; Lane 5: *A. alternata*; Lane 6: *P. sorghina*; Lane 7: *F. verticillioides* and Lane 8: *F. graminearum*. Lanes 9-13: amplification of the field fungi only, Lane 9: *S. maydis*; Lane 10: *A. alternata*; Lane 11: *P. sorghina*; Lane 12: *F. verticillioides* and Lane 13: *F. graminearum*.

The parameters evaluated for the detection and identification of field and storage fungi in post-inoculated maize kernels included two DNA extraction methods, two regions of the rRNA complex, ITS and D1/D2 and two gel buffer systems. Two different maize cultivars were used representing two different rates of infestation to field fungi and storage fungi. Cultivar no. 17 represented maize that had a faster rate of infestation towards field fungi such as *S. maydis, A. alternata, P. sorghina, F. verticillioides* and *F. graminearum*, while cultivar no. 30 represented maize with a slow rate of infestation. Cultivar no. 34 represented maize cultivars which had a slower rate of infestation towards storage fungi such as *A. flavus, A. ochraceus, R. oryzae* and *E. repens*, except *P. islandicum* and cultivar no. 45 represented cultivars with a faster rate of infestation. The differences in the amplification products are shown in Figures 9 and 10. For the field fungi (Fig. 9), the pre- (pink blocks) and post- inoculated maize kernels (green blocks) have a distinct 900 bp band. The fungal amplifications did not show a band of this size, indicating it to be a maize band. The 5 field fungi had bands of slightly different sizes: *S. maydis* is ~550 bp, *A. alternata* is ~525 bp, *P. sorghina* is ~400 bp, *F. verticillioides* is ~520 bp and *F. graminearum* is ~530 bp. Lane 2, corresponding to pre-inoculated maize cultivar 17, showed the same size band as the post inoculated maize, lanes 3 and 4, indicating the presence of maize. Lanes 3 and 4 show two bands where the lower band represents the maize band and the bands above corresponds with the fungal bands of *S. maydis* and *A. alternata* in lanes 8 and 9, respectively, indicating the presence of the fungi in the post-inoculated maize kernels.

In the post-inoculated maize kernels for cultivar no. 30, lane 13 shows a band corresponding to the pre-inoculated maize cultivar no. 30 and lanes 14 and 15 show the same maize band in the lanes for the post-inoculated maize kernels along with the corresponding fungal bands to those of lanes 8 and 9 indicating the presence of *S. maydis* and *A. alternata*, respectively.

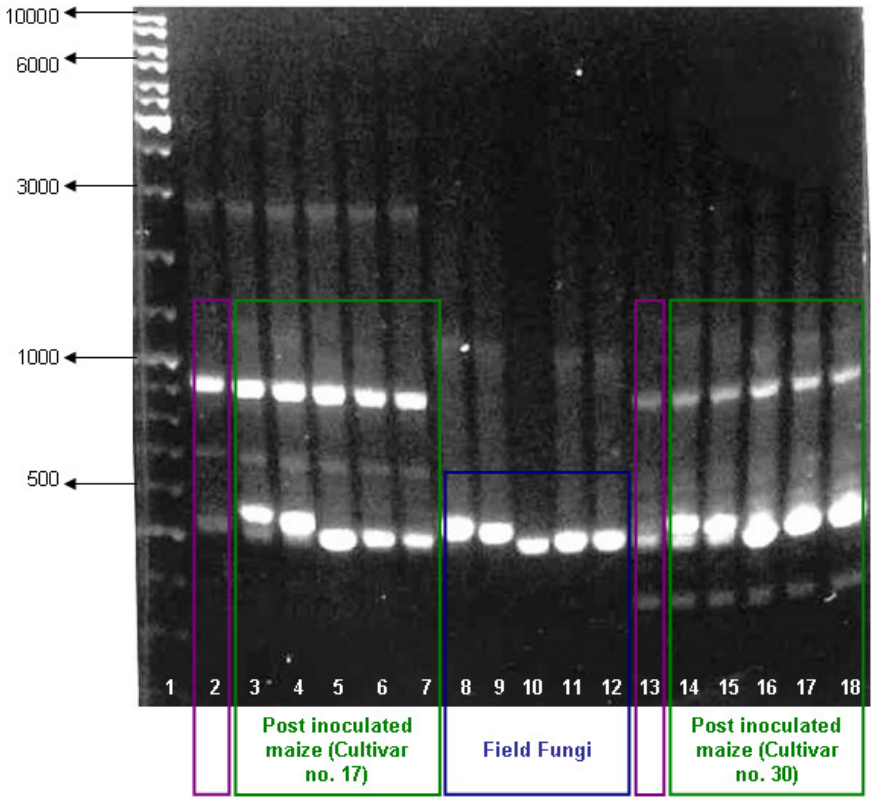

FIGURE 9: PCR amplification of the ITS regions, of post-inoculated maize cultivar no. 17 and cultivar no. 30 with field fungi (*S. maydis, A. alternata, P. sorghina, F. verticillioides* and *F. graminearum*) run on a TAE gel. Lanes 1: Generuler 1kb DNA ladder (Fermentas); Lane 2: pre-inoculated maize, cultivar no. 17; Lanes 3-7: post-inoculated maize of cultivar no. 17; Lane 3: *S. maydis*; Lane 4: *A. alternata*; Lane 5: *P. sorghina*, Lane 6: *F. verticillioides* and Lane 7: *F. graminearum*. Lanes 8-12: amplification of the field fungi only, Lane 8: *S. maydis*; Lane 9: *A. alternata*; Lane 10: *P. sorghina*; Lane 11: *F. verticillioides* and Lane 12: *F. graminearum*; Lane 13: pre-inoculated maize, cultivar no. 30; Lanes14-18: post-inoculated maize of cultivar no. 30; Lane 14: *S. maydis*; Lane 15: *A. alternata*; Lane 16: *P. sorghina*; Lane 17: *F. verticillioides* and Lane 18: *F. graminearum*.

For the storage fungi, (Figure 10) lanes 4-7, post-inoculated maize (green block), shows a band corresponding to maize cultivar no. 34, lane 3 (pre inoculated maize kernels) (pink block). Lanes 4-7, also show the fungal bands from the corresponding fungi in lanes 8-11 of *A. flavus, A. ochraceus, R. oryzae* and *E. repens*, respectively (orange block), showing the presence of the fungi in the post-inoculated maize samples. The band size for *A. flavus*

is ~600 bp, *A. ochraceus* is ~625 bp, *R. oryzae* is ~690 bp and *E. repens* is ~580 bp. For cultivar no. 45 (green block) the maize band corresponding to the pre-inoculated maize kernels can be seen in lane 16 as well as in lanes 12-15, corresponding to the same maize bands in post-inoculated maize samples from cultivar no. 45. The fungal bands present in lanes 8-11 corresponding to *A. flavus, A. ochraceus, R. oryzae* and *E. repens*, respectively, also correspond to those shown in lanes 12-15 for post-inoculated maize from cultivar no. 45, once again indicating the presence of the fungi in the post-inoculated maize samples. The isolate of *P. islandicum,* used in this project was unable to amplify with ITS regions and therefore was not used.

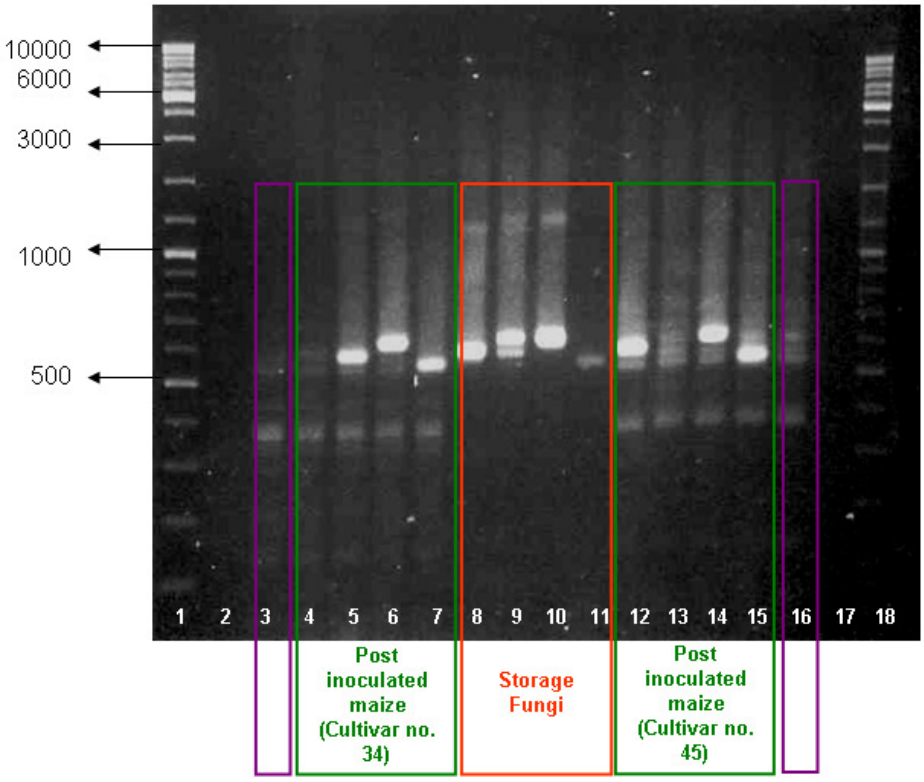

FIGURE 10: PCR amplification of the ITS regions, of post inoculated maize cultivar no. 34 and cultivar no. 45 with storage fungi (*A. flavus, A. ochraceus, R. oryzae* and *E. repens*) run on a TAE gel. Lanes 1 and 18: Generuler 1kb DNA ladder (Fermentas); Lanes 2 and 17: negative control (no DNA); Lane 3: pre-inoculated maize, cultivar no. 34; Lanes 4-7: post-inoculated maize of cultivar no. 34; Lane 4: *A. flavus*; Lane 5: *A. ochraceus*; Lane 6: *R. oryzae*, and Lane 7: *E. repens;* Lanes 8-11

amplification of the storage fungi only; Lane 8: *A. flavus*; Lane 9: *A. ochraceus*; Lane 10: *R. oryzae*; and Lane 11: *E. repens*. Lanes 12-15: post-inoculated maize of cultivar no. 45; Lane 12: *A. flavus*; Lane 13: *A. ochraceus*; Lane 14: *R. oryzae*, and Lane 15: *E. repens*. Lane 16: pre-inoculated maize of cultivar no. 45.

5. Discussion:

Food security is of primary concern in Africa. Subsistence farming is common in South Africa where homegrown crops are the only source of food, irrespective of quality considerations. The subsistence farmer's livelihood depends mostly upon having good quality maize for market trading and for feeding of people and animals. However, due to the fungal infestation of maize and subsequent mycotoxin contamination, the maize is, in some cases unsuitable for human and animal consumption. The effect that even low quantities of mycotoxins can have on the immune system, an additional burden is placed on immune compromised people including those suffering from HIV/AIDS if they were to consume food that carries high mycotoxin levels.

It is generally accepted that the greater the degree of seed infestation by fungi, the greater the likelihood of toxin formation by the fungus concerned. The use of microbiological methods to identify mycotoxigenic fungi in maize can be time consuming due to the long period it takes for certain fungi to produce fruiting structures. In addition, fungal identification based on morphology is not always reliable. The use of molecular methods can considerably speed up the identification process and is likely to be more reliable. Taylor *et al.* (**2000**) were the first to compare the contrast of the morphological, biological and phylogenetic species concepts. The authors demonstrated that using molecular methods (e.g. ITS regions) to identify fungi was more reliable and efficient than the use of morphological methods (e.g. the identification of fruiting structures of fungi).

In this study the use of two different methods to extract DNA from maize inoculated with fungi were evaluated. The CTAB method, normally used for rapid isolation of high molecular weight plant DNA, was used for the DNA extractions of the post-inoculated maize kernels to obtain both maize and fungal DNA. The ability of CTAB to form complex polysaccharides with the starch in maize allowed the removal of more superfluous substances resulting in higher yields of DNA. The DEB method, which is primarily used for isolation of DNA from filamentous fungi, was used for the extraction of fungal DNA. Although the two methods yielded high quantities of DNA the CTAB method proved to be a better extraction method for the post-inoculated maize kernels (Figure 1 and 2).

Different DNA concentrations were used to optimize the PCR amplifications of the ITS and D1/D2 regions. Optimization of PCR showed that 2 µl (50 ng/µl in 50 µl) of DNA sample in each PCR reaction was sufficient for good visualization of the amplified products (Figure 3) and this DNA concentration was used for all PCR amplifications throughout this study. The amplification of different DNA regions using the ITS and D1/D2 region was evaluated in the detection and identification of fungal presence in maize cultivars. The ITS regions showed better differentiation among the 10 fungi. An advantage of using the ITS regions is that it covers a larger region of the rRNA gene than the D1/D2 26S rDNA region.

When the pre-inoculated maize samples were run on the same gel along with the 10 fungi, differences in the band migration were observed between the maize and the fungi (Figure 6). Different buffers were evaluated to determine which buffer shows higher resolution when running amplified PCR products on the gel. The agarose gels prepared with TBE buffer did not allow good separation of the bands (Figures 7). In contrast gels prepared with TAE buffer allowed better separation of both maize and fungal bands (Figure 8). Brody & Kern (**2004**) have reviewed different media for DNA electrophoresis including the use of TAE and TBE buffers. In this review the authors showed that TAE buffer is better suited for separating DNA on gels although the fundamental reason of TAE working better is not suitably documented.

The ITS regions made it possible to differentiate between the maize bands at ~450 bp and the fungal bands of the five different field fungi (Figure 9). Differentiation between the two cultivars could also be seen by amplification of the ITS regions where cultivar no. 17 has 3 maize bands and cultivar no. 30 has 2 maize bands indicating a difference in the maize cultivars. The resulting banding pattern of the maize and the fungi in the post-inoculated maize samples allowed the identification of two field fungi, *A. alternata* and *S. maydis.* Owing to the close molecular weight of the maize bands and of the fungal bands of the other three fungi, *P. sorghina, F. verticillioides* and *F. graminearum*, the identification of these fungi in the post-inoculated samples was not possible. Another methodology, like quantitative real time PCR will need to be evaluated for the identification of these fungi in post-inoculated maize samples. As reviewed by Edwards *et al.* (**2002**), real time PCR assays can be used to differentiate between mycotoxigenic genera and species, especially for closely related mycotoxigenic species in genera such as *Fusarium, Penicillium* and *Aspergillus* that could not be differentiated by normal PCR. Real time PCR is more rapid, convenient and has the potential to monitor the expression of certain genes under certain environmental conditions in different commodities (Konietzny & Greiner, **2003**). Möller *et al.,* (**1999**) and Jurado *et al.,* (**2006**), demonstrated the potential of ITS regions for the detection of the *Fusarium* spp. but not to the identification at species level. Harrow *et al.,* (**2010**), reported the translation elongation factor 1 α (αEF) and beta tubulin (β-TUB) to be much better genes to identify species of *Fusarium*. Geiser *et al.,* (**2004**) have used the translation elongation factor 1 α (EF1 α) which has shown to give good definition between species and be able to populate the FUSARIUM-ID sequence database for effective identification of *Fusarium* spp. to species level.

PCR amplification of the ITS regions for the detection and identification of the 4 storage fungi was successful (Figure 10). The ITS regions in the storage fungi gave a higher, better banding pattern than that of the field fungi which made it easier to distinguish from the lower banding pattern of the maize.

The amplification of the ITS region could be used in industry for the identification of mycotoxigenic fungi causing seedling blight in maize. This disease can cause a maize plant not to grow and produce fruit to its maximum potential; they remain stunted throughout their life cycle which leads to great decrease in yield. Detection of fungi in maize pre-planting by use of this method, i.e. by obtaining a random sample of maize from a bag of seed; doing basic DNA extraction, PCR amplification of the ITS regions and identifying the fungi present in the sample; could possibly save industry massive losses in yield in the field and prevent potential outbreaks of some plant pathogenic diseases. For the fungi that could not be identified, *F. graminearum, F. verticillioides* and *P. sorghina,* further optimization is needed and more fungal isolates should be included in future studies.

6. Conclusion:

This study showed that the ITS regions can be used as a tool to detect and identify the presence of *S. maydis, A. alternata* (field fungi), as well as *A. flavus, A. ochraceus, R. oryzae* and *E. repens* (storage fungi) in maize that had been inoculated with these fungi. Application of the ITS regions for the field and storage fungi have shown to provide better detection and identification for the storage fungi. The final confirmation of these results would need the collection of maize samples that were naturally infested with fungi and to carry out detection and identification of these fungi. Future prospects would be to optimize this method using a wider variety of common storage fungi that would allow their detection and identification in maize. Fungi could be quickly detected by analyzing the maize cultivars for fungal contamination therefore decreasing the consumption of “contaminated” food and planting of infected material by small-scale farmers. Further studies would have to be conducted to determine the cost effectiveness of this method if it would be applied routinely.

7. References:

Abd-Elsalam, K.A., Aly, I.N., Abdel-Satar, M.A., Khalil, M.S. & Verreet, J. A. 2003. PCR identification of *Fusarium* genus based on nuclear ribosomal-DNA sequence data. *African Journal of Biotechnology.* 2(**4**): 82-85.

Brody, J.R. & Kern, S.E. 2004. History and principles of conductive media for standard DNA electrophoresis. *Analytical Biochemistry.* 333: 1-13.

Bruns, T.D., White, T.J. & Taylor, J.W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Systematics.* 22: 525-564.

Edel, V., Steinberg, C., Gautheron, N. & Alabouvette, C. 1996. Evaluation of restriction analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA for the identification of *Fusarium* species. *Mycological Research.* 101(**2**):179-187.

Edwards, G.S., O'Callaghan, J. & Dobson, A.D.W. 2002. Review: PCR-based detection and quantification of mycotoxigenic fungi. *Mycological Research.* 106(**9**): 1005-1025.

Food and Agricultural Organisation (FAO). 2004. Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition paper. 163 pp.

Fell, J.W., Boekhout, T., Fonseca, A., Scorzetti, G. & Statzell-Tallman, A. 2000. Biodiversity and systematics of Basidiomycetous yeasts as determined by large-subunit rDNA D1/D2 domain sequence analysis. *International Journal of Systematic and Evolutionary Microbiology.* 50: 1351-1371.

Geiser, D.M., Dorner, J.W., Horn, B.W. & Taylor, J.W. 2000. The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae. Fungal Genetics and Biology.* 31: 169-179.

Geiser, D.M., a del Mar Jime´nez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, T.J., Zhang, N., Kuldau, G.A & O'Donnell, K. 2004. FUSARIUM-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. *European Journal of Plant Pathology.* 110: 473-479.

Harrow, S.A., Farrokhi-Nejad, R., Pitman, A.R., Scott, I.A.W., Bentley, A., Hide, C. & Cromey, M.G. 2010. Characterisation of New Zealand *Fusarium* populations using a polyphasic approach differentiates the *F. avenaceum/F. acuminatum/F. tricinctum* species complex in cereal and grassland systems. *Fungal Biolog.* 1-19.

Jasalavich, C.A., Ostrofsky, A. & Jellison, J. 2000. Detection and identification of decay fungi in spruce wood by restriction fragment length polymorphism analysis of amplified genes encoding rRNA. *Applied and Environmental Microbiology*. 66 (**11**): 4725-4734.

Jurado, M., Vázquez, C., Marín, S., Sanchis, V. & González-Jaén, M.T. 2006. PCR-based strategy to detect contamination with mycotoxigenic *Fusarium* species in maize. *Systematic and Applied Microbiology*.

Konietzny, U. & Greiner, R. 2003. The application of PCR in the detection of mycotoxigenic fungi in foods. *Brazilian Journal of Microbiology*. 34: 283-300.

Lindsley, M.D., Hurst, S.F., Iqbal, N.J. & Morrison, C.J. 2001. Rapid identification of dimorphic and yeast-like fungal pathogens using specific DNA probes. *Journal of Clinical Microbiology*. 39(**10**): 3505-3511.

Lu, H.Z., Cai, Y., Wu, Z.W., Jia, J.H. & Bai, F.Y. 2004. *Kazachstania aerobia* sp. nov., an ascomycetous yeast species from aerobically deteriorating corn silage. *International Journal of Systematic and Evolutionary Microbiology*. 54: 2431-2435.

Möller, E.M., Chelkowski, J. & Geiger, H.H. 1999. Species-specific PCR assays for the fungal pathogens *Fusarium moniliforme* and *Fusarium subglutinans* and their applications to diagnose maize ear rot disease. *Journal of Phytopathology*. 147: 497-508.

Mulé, G., Susca, A., Stea, G. & Moretta, A. 2004. A species-specific PCR assay based on the calmodulin partial gene for identification of *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans*. *European Journal of Plant Pathology*. 110: 495-502.

Multani, D.S., Meeley, R.B., Paterson, A.H., Gray, J., Briggs, S.P. & Johal, G.S. 1998. Plant-pathogen microevolution: Molecular basis for the origin of a fungal disease in maize. *Proceedings of the National Academy of Sciences of the United States of America*. 95: 1686-1691.

Murray, M.G. & Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*. 8(**19**): 4321-4326.

National Department of Agriculture (NDA). 2003. Food Pricing Monitoring Committee: Final report, December 2003, 310 pp. http://www.nda.agric.za/docs/fpmc/default.htm

Pitt, J.I. 1989. An introduction to Mycotoxins. In Mycotoxin prevention and control in foodgrains. Eds. Semple, R.L., Frio, A.S., Hicks, P.A. & Lozare, J.V. http://www.fao.org/docrep

Rabie, C.J. & Marais, G.J. 2000. Toxigenic fungi and mycotoxins in South African foods and feeds. Report to the Department of Health. 31 March 2000. 113pp.

Rabie, C.J., Lübben, A., Marais, G.J. & Jansen van Vuuren, H. 1997. Enumeration of fungi in barley. *International Journal of Food Microbiology*. 35: 117-127.

Raeder, U. & Broda, P. 1985. Rapid preparation of DNA from filamentous fungi. *Letters in Applied Microbiology*. 1: 17-20.

Ramos, J.P., Valente, P., de Souza, R.A., Rosa, C.A. & Leocini, O. 2001. Heteroduplex mobility assay of the D1/D2 region of the 26S rDNA for differentiation of *Saccharomyces* species. *Letters in Applied Microbiology*. 33(**3**): 206.

Sharma, R.P. 1993. Immunotoxicity of mycotoxins. *Journal of Dairy Science.* **76:** 892-897.

Starkl, V. 31 March 2004. Mycotoxins in feeds-Prevention and control. http://www.afma.co.za/AFMA_Template/feedpaper14.html

Sugita, T. & Nishikawa, A. 2003. Fungal identification method based on DNA sequence analysis: Reassessment of the methods of the Pharmaceutical Society of Japan and the Japanese Pharmacopoeia. *Journal of Health Science*. 49(**6**): 531-533.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., & Fisher, M.C. 2000. Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology*. 31: 21-32.

White, T.J., Bruns, T., Lee, S. & Taylor, J. 1990.Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: A Guide to Methods and Applications.

Chapter 6:

Summary and conclusions

1. Summary:

The purpose of this chapter is to give an overview of the work conducted in this thesis, and to put the rationale for conducting each chapter in perspective. This chapter also gives an overview of how the various chapters are relevant to the overall questions that are addressed by this study.

Chapter 2 provides an overview of the fungal diversity on the 49 maize cultivars that were used throughout this study. The cultivars were initially grown in the Potchefstroom area, after which a fungal enumeration was done to determine the type and levels of fungi in each of the cultivars. In addition, this chapter also gives valuable information on the ability of each cultivar to withstand or prolong fungal infestation under natural conditions in the field. The purpose was also to use this opportunity to produce enough material of each of the maize cultivars for further study such as resistance trials, analyses and molecular work. As a result of this initial evaluation of the cultivars and subsequent pilot resistant trials, it was noted that the content of one of the maize pathogens, namely *Fusarium verticillioides*, was extremely high in basically all the maize cultivars (Chapter 2, Figure 2, pg. 102). It was realised at this stage that a way needs to be found to lower the viability of this fungus. This was necessary because the resistance trials in chapter 4 are based on the spiking of the cultivars with specific fungi and following the tempo of colonization of the maize kernels. This would not be possible in the presence of high levels of *Fusarium verticillioides*, as this fungus will overwhelm the other fungi and distort the results to a level that is not accurate.

In chapter 3 it was decided to investigate irradiation technology as a possible means to lower the levels of *F. verticillioides* and other fungi in the original maize cultivar samples. This method has shown to be highly effective, not only in reducing the fungal load in the cultivars (Chapter 3, Figure 1, pg. 130), but still allowing subsequent germination of the kernels (Chapter 3, Figure 2, pg. 131). The germination of the kernels was used as an indicator that the kernel is still viable and, therefore, likely that the resistance mechanisms

against fungal attack, if present, would also still be intact. In most cases, the levels of viable fungi in the cultivars were lowered by more than 90%.

The successful irradiation of the maize cultivars to eliminate viable fungi allowed that the resistance trials could be conducted in chapter 4. Due to the fact that the resistance of maize kernels against fungi during storage have not been evaluated before, various attempts were made to set up a resistance trial. Finally a decision was made to use 250 ml screw cap containers for the resistance trials. Specifically, the containers were able to maintain the integrity of the sample conditions such as temperature, moisture and preventing cross contamination.

Maize cultivars that have shown to be either easily infested or more resistant to fungal infestation during storage were selected from the resistance trial. The infested maize cultivars were then used to develop a molecular based method to identify each of the ten mycotoxigenic fungi used in the resistance trial (Chapter 5). This allowed that the molecular method used could be evaluated and fine tuned to distinguish between fungal and maize DNA.

The initial fungal evaluation of the 49 maize cultivars in chapter 2 indicated that 6 cultivars i.e. cultivar 2 (DKC 80-10), cultivar 5 (SNK 2682), cultivar 8 (PAN 6164), cultivar 25 (PAN 6479), cultivar 38 (DKC 78-15) and cultivar 40 (PAN 6995 Bt) could possibly be considered as candidates for resistance against mycotoxigenic fungi. In chapter 4, cultivars 2 (DKC 80-10) and 8 (PAN 6164) showed a slower rate of infestation when it comes to field fungi. Based on these findings a resistant trial was set up and mycotoxin analyses were conducted.

The evaluation of the type and level of individual fungi also gave an indication of possible mycotoxins that could be associated with the 49 maize cultivars. The identification of mycotoxigenic fungi, based on morphological characteristics, could sometimes be difficult due to the lack of fruiting structures. The purpose of chapter 5 was to develop a molecular method to distinguish between the most common fungi in maize, as well as to confirm

that the fungal identifications done in chapter 2 were accurate. The results of chapter 5 thus provide an alternative tool for determining the presence of mycotoxigenic fungi, especially in cases where morphological characteristics are not adequately visible.

2. Conclusions

The main purpose of this study was to evaluate commercially produced maize cultivars in South Africa for their ability to withstand fungal infestation during storage. Results indicated that at least 2 maize cultivars could possibly have resistance mechanisms against mycotoxigenic fungi. It also seems that different mechanisms could be in place for field and storage fungi respectively. This, however, is based on the comparison of the tempo of fungal infestation between the 49 cultivars screened in this study. Therefore, the repeatability and confirmation of resistance at molecular level needs to be confirmed.

Regulations in South Africa on mycotoxins do not necessarily address all relevant mycotoxins. For example, fumonisins are regularly associated with South African maize, although this group of mycotoxins are not yet regulated by law. There is thus a significant risk that South Africans could be exposed to fumonisins, especially those that are dependant on maize as a staple food. However, regulations on fumonisins could have a dramatic impact on the availability of maize if levels laid down in South Africa are too strict. On the other hand, if legislation is not enforced, the health of South Africans could be at risk if the maize industry does not take proactive steps to eliminate contaminated food from the human food chain. Therefore, a balance should be reached between the availability of maize for human and animal consumption, and the safety of using contaminated maize. This is not an easy task, especially in a country where the availability of grains, such as maize, is constantly under threat due to market trends and unfavourable environmental conditions.

It was shown in chapter 5 that it is possible to distinguish between the more prominent fungi in maize in South Africa at molecular level. This could prove in future to be of importance to the maize industry to conduct risk assessments of the possible presence of mycotoxins in food. In some cases, such as *Stenocarpella maydis*, the mycotoxins of certain fungi are unknown and this method could be of significance to conduct a complete health risk analysis on such commodities. In addition, if resistant genes can be identified in those maize cultivars that showed resistance to fungal infestation, these genes can ultimately be used to combat mycotoxin contamination in the commercial cultivars that are planted in South Africa. This could show to be a possible way of lowering the exposure of South Africans to mycotoxins, such as fumonisins, and making it possible to lay down legislation that can be enforced, without compromising the food security in South Africa.

The resistance trials conducted in chapter 4, provides an indication that there are commercially available “wild type” maize cultivars, containing intrinsic resistance mechanisms that seem to slow down the infestation of mycotoxigenic fungi in certain cultivars e.g. cultivars 2 (DKC 80-10) and 8 (PAN 6146) during storage. Such cultivars could be of use in future to lower the levels of fungi and their mycotoxins, especially in rural areas where the use of pesticides and fungicides are not an option due to cost implications. In the long term, this could lead to the gradual decline of fungal levels in the environment, and ultimately lower the risk of mycotoxins in the staple food of South Africa.