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STORAGE FUNGI AND MYCOTOXINS ASSOCIATED WITH COWPEA

BY

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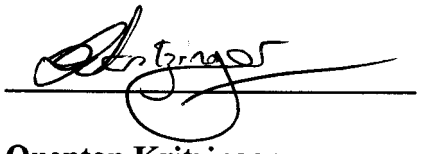
DECLARATION

These studies have not been submitted in any form to another University and, except where acknowledged in the text, are results of my own work.



Quenton Kritzinger

SUMMARY

Cowpeas (*Vigna unguiculata* (L.) Walp) is an important African indigenous legume crop for the livelihoods of many relatively poor people residing in less developed countries of the tropics. Rural families derive a nutritious food, animal feed and income from the production of this crop. Storage of seed is certainly the most important post-harvest operation but the losses incurred are great. These losses, due to an inability to effectively control physical and biological factors, result in problems with storage insects, moisture and associated fungi. Seeds are particularly susceptible to fungal contamination when stored at high ambient temperatures and relative humidities. To determine the storage fungi associated with cowpea seeds, surface-sterilised cowpea seeds (200 seeds from each of nine cultivars) were plated out on malt extract agar. After 5-7 days incubation at 25 °C, the most dominant and common fungi recorded were *Alternaria* spp. followed by *Penicillium* spp., *Aspergillus flavus* and *A. niger*. The influence of a three-year cold storage period at ± 5 °C on the fungi associated with the seeds was also investigated. *Alternaria, Aspergillus* and *Penicillium* spp. appeared to dominate. Some fungal species recorded prior to cold storage were not recorded thereafter. Certain storage fungi are known to produce mycotoxins, which are secondary fungal metabolites that are toxic to both farm animals and humans, under poor storage conditions. The presence of the fusarial mycotoxins, fumonisin B_1, B_2 and B_3 in four cowpea cultivars (Bechwana White, Glenda, Iron Grey, Rhino) was investigated. The samples were extracted with methanol/water (70:30 v/v) and cleaned-up on strong anion exchange solid phase extraction cartridges. High performance liquid chromatography with pre-column derivatisation using *o*-phthaldialdehyde (OPA) was used for the detection and quantification of fumonisin B_1, B_2 and B_3. All samples were contaminated with FB_1, with levels

ranging from 81 - 1002 ng g^{-1}. Fumonisin B_2 and B_3 were not detected in any samples. This is believed to be the first report of fumonisin B_1 in cowpea seeds. Since the known fumonisin-producing *Fusarium* species were not found in the six different *Fusarium* species isolated from these four cultivars, further investigations are required to determine which fungal species are responsible for the FB_1 production.

An alternative approach to the prevention and control of fungal contamination and mycotoxin production of seeds by treating cowpea seed with essential plant oils was tested. The inhibitory activity of five essential oils (thyme, clove, peppermint, soybean and peanut) was investigated, *in vitro* and *in vivo*, on five fungal species (*A. flavus, A. niger, Penicillium chrysogenum, Fusarium oxysporum* and *F. equiseti*) commonly associated with cowpea seeds and on two cowpea cultivars. Thyme and clove oil significantly inhibited the growth of all five fungal species *in vitro* at 500 and 1000 ppm, while peppermint oil was successful at 2000 ppm. Peanut and soybean oil did not show any significant inhibition of fungal growth. The *in vivo* effect of thyme, clove and peppermint oils on naturally infected seed revealed that only thyme at 1000 ppm reduced fungal growth of storage fungi in the PAN 325 cultivar. In the PAN 311 cultivar, thyme and clove oils at 1000 ppm and peppermint oil at 2000 ppm significantly reduced growth of storage fungi. In artificially infected seed, all three oils significantly inhibited the growth of *P. chrysogenum*. Thyme reduced the growth of *F. oxysporum* and *F. equiseti*, whilst peppermint oil inhibited only *F. oxysporum*. These oils did not seem to adversely affect the germination nor emergence of cowpea seed.

The storage fungi significantly reduced percentage germination and emergence of the white (IT 93K452-1) seed but had little or no effect on the brown (CH 14) seed. Furthermore, all three oils significantly inhibited the storage fungi on the white seed, possibly increasing the percentage germination and emergence.

Keywords: mycotoxins, cowpea, *Fusarium*, fumonisin, storage fungi, essential oils, post-harvest storage

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LIST OF ABBREVIATIONS

AFB_1	-	Aflatoxin B_1
AFB_2	-	Aflatoxin B_2
AFROX	-	African Oxygen Ltd.
ARC	-	Agricultural Research Council
ATA	-	alimentary toxic aleukia
AOAC	-	Official Association of Analytical Chemists
CCD	-	charge-coupled device
CTA	-	Technical Centre for Agricultural and Rural Co-operation ACP-EU
CZE	-	capillary zone electrophoresis
DAS	-	diacetoxyscirpenol
DON	-	deoxynivalenol
ELISA	-	enzyme-linked immunosorbent assays
FAO	-	Food and Agricultural Organisation of the United Nations
FB_1	-	fumonisin B_1
FB_2	-	fumonisin B_2
FB_3	-	fumonisin B_3
GASGA	-	Group for Assistance on Systems Relating to Grain after Harvest
GC	-	gas chromatography
GC/MS	-	gas chromatography / mass spectrometry
GLC	-	gas liquid chromatography
HPLC	-	high performance liquid chromatography

HPTLC	-	high performance thin-layer chromatography
IARC	-	International Agency for Research on Cancer
IITA	-	International Institute of Tropical Agriculture
INPhO	-	Information Network on Post-harvest Operations
ISTA	-	International Seed Testing Association
JIRCAS	-	Japan International Research Center for Agricultural Sciences
LC	-	liquid chromatography
LEM	-	leukoencephalomalacia
MEA	-	malt extract agar
MRC	-	Medical Research Council
MS/MS	-	mass spectrometry / mass spectrometry
NIV	-	nivalenol
OA	-	ochratoxin A
OPA	-	*o*-phthaldialdehyde
PDA	-	potato dextrose agar
PES	-	pulmonary edema syndrome
PROMEC	-	Programme on Mycotoxins and Experimental Carcinogenesis
R_F	-	relative mobility
SAX	-	strong anion exchange
SFC	-	supercritical fluid chromatography
SGI	-	scabby grain intoxication
SIM	-	selected ion monitoring
SPE	-	solid-phase extraction

TLC	-	thin layer chromatography
USA	-	United States of America
UV-Vis	-	ultraviolet - visible
VOPI	-	Vegetable and Ornamental Plant Institute
ZEA	-	zearalenone

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CHAPTER 1

GENERAL INTRODUCTION

Grains and their by-products are important sources of energy and protein for farm livestock and humans. This study deals with the indigenous African legume, cowpea (*Vigna unguiculata* (L.) Walp). This is an important crop for subsistence farmers and rural communities living in tropical and sub-tropical countries in Africa, largely due to the nutritional value of cowpeas. It provides the communities with a good source of proteins, vitamins, carbohydrates and other minerals (Singh *et al.*, 1997).

Despite their potential in upgrading diets of the poor people of the world, there are numerous constraints to the optimal utilisation of cowpea as food. Many of these constraints are due to the postharvest storage of the cowpea seeds (Uzogara and Ofuya, 1992). It is well known that cowpea seeds and other post-harvest commodities can be contaminated by fungi when the seeds are not properly stored after harvesting. They are especially susceptible to fungi if stored at high moisture levels and warm temperatures. Storage fungi not only play a role in the decrease of germination of seed, they are also responsible for the spoilage of the seed, often rendering it unfit for human and animal consumption. It is important to determine what storage fungi are associated with cowpea seeds (Chapter 3 and 4). Since the longevity of fungi is dependent on storage conditions of the seed and since temperature plays an important role in the spread and establishment of fungi, the mycoflora present in cowpea seeds before and after a three-year period of cold storage was determined (Chapter 3).

It is known that when grains and animal feed are colonised by fungi, the presence of certain fungi capable of producing toxic mycotoxins pose a health risk and can cause various diseases in both farm animals and humans (Yagen and Joffe, 1976; Marasas, 1994). Furthermore, economic losses can also be incurred due to condemned foods and decreased animal productivity. Little work has been done on mycotoxins associated with cowpea seeds. Thus far, it has been reported that cowpea seeds are susceptible to *Aspergillus* infection and subsequent aflatoxin production (Seenappa *et al.*, 1983). The possible presence of the *Fusarium* toxins, fumonisin B_1, B_2 and B_3 on cowpea seeds was determined during this study (Chapter 4). Fumonisins are the most recently described mycotoxins that have a major impact on the health of farm animals and humans (Moss, 1996).

Since fungi are the most important causative agents of crop deterioration during storage (Mishra and Dubey, 1994), certain control measures and preventative strategies must be carried out on newly harvested seed so that losses both in quality and quantity are avoided. This can be achieved by controlling the environmental conditions during storage. This, however, is sometimes not economically feasible for subsistence farmers.

Chemical treatment of seeds, through the use of synthetic fungicides, has been widely exploited but due to the harmful side effects on the community and environment, an alternative approach should be looked at. It has been well recorded that a large number of naturally occurring compounds, such as essential oils from plants, possess antifungal activity and inhibit growth of mycotoxigenic fungi (Mishra and Dubey, 1994; Yin and Cheng, 1998). There are, however, only a few reports on cowpea seeds treated with essential oils to inhibit storage fungi and mycotoxins (Adegoke and Odesola, 1996). Certain essential oils, for example, the essential oil of lemon grass (*Cymbopogon citratus* (DC. ex Nees) Stapf.) (Adegoke and Odesola, 1996), have been recorded to inhibit the growth of fungi associated with cowpea seeds. Essential plant

oils appear not to affect the germination of seeds, including maize (*Zea mays* L.) (Montes-Belmont and Carvajal, 1998) and chick-peas (*Cicer arietinum* L.) (Pacheco *et al.*, 1995). It has been reported that no significant reduction in germination was seen when cowpea seeds were treated with soybean (*Glycine max* (L.) Merrill) oil (Cruz and Cardona, 1981). The antifungal activity of five essential plant oils namely, thyme (*Thymus vulgaris* L.), clove (*Syzygium aromaticum* (L.) Merr. and Perry), peppermint (*Mentha piperita*), soybean and peanut (*Arachis hypogaea* L.) were tested *in vitro* and *in vivo* on five fungal species that could be possible mycotoxigenic species and on the storage fungi associated with cowpea cultivars (Chapter 5). Since storage fungi are one of the causes of a decrease in germination of seeds (Neergaard, 1977), the control of these fungi and the effect the essential oils of clove, thyme and peppermint have on cowpea seed germination was investigated (Chapter 5).

The aim of this study is to: 1) Provide a record of the storage fungi present on cowpea seeds; 2) Determine the effect of cold storage in these fungi; 3) Determine the presence of fumonisins in fungal contaminated seed; 4) Test the efficacy of essential plants oils in controlling storage fungi of cowpea seed and; 5) Investigate the effect of the control of the storage fungi by these plant oils on the germination and emergence of cowpea seed.

Structure of thesis

Some of the chapters presented in this thesis have been prepared for publication in international scientific journals. In Chapter 4, the manuscript entitled "Detection of fumonisin B_1 in cowpea (*Vigna unguiculata* (L.) Walp) seeds", has been written in the format for submission to the *Journal of Food and Agricultural Chemistry*. In Chapter 5, the manuscript titled "Effect of essential plant oils on storage fungi, germination and emergence of cowpea seeds", has been

prepared for *Seed Science and Technology*. Thus, full nomenclature for each fungus mentioned for the first time in each of these chapters is used. The reader will thus note some inconsistencies within the contents page and format of chapters. Author citation and literature citation in Chapters 1, 2, 3 and 6 are according to the *Seed Science and Technology*.

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CHAPTER 2

LITERATURE REVIEW

2.1. Importance of cowpea

Cowpea, belonging to the family Fabaceae, is an important indigenous African legume crop for many subsistence farmers and millions of relatively poor people living in less developed countries of the tropics. Cowpeas are heat- and drought-tolerant annual plants that are also efficient in nitrogen fixation (Bittenbender, 1992). Cowpeas can be produced for human food, animal feed, soil cover and as a source of income through the trading of seeds (Johnson and Raymond, 1964; Singh *et al.*, 1997). Furthermore, because the seed is widely traded outside of the major production areas, it provides a cheap and nutritious food for many poor urban communities of developing countries (Singh *et al.*, 1997). The young leaves, immature pods and peas are used as vegetables, while numerous snacks and main meal dishes are prepared from the seed. All the plant parts that are used for food are nutritious, providing a good source of proteins, carbohydrates, vitamins and minerals (Johnson and Raymond, 1964; Singh *et al.*, 1997). Cowpea seed contains on average 23-25% protein and 50-67% starch (Singh *et al.*, 1997). Moreover, cowpea fodder makes an important contribution to feed supplies for large and small animals (Singh *et al.*, 1997).

It is well known that cereal and leguminous seeds in storage support an extensive array of fungal flora, which may be active or dormant, depending on storage conditions (van Warmelo, 1967; Ushamalini *et al.*, 1998). These storage fungi invade the seed during storage starting on the farm, and have the ability to grow at moisture contents in equilibrium with relative humidities of

70-90%. As a result, deterioration and spoilage of seed generally occurs. It is under such conditions that these fungi may form secondary metabolites (mycotoxins) which could be toxic to animals and humans (van Warmelo, 1967).

2.2. Storage fungi and mycotoxins associated with legume seeds

The growth of toxigenic fungi and mycotoxin contamination have been found associated with various legume seeds, including chickpea (Ahmad and Singh, 1991), different types of beans (El-Kady *et al.*, 1991; Saber, 1992; Pitt *et al.*, 1994; Tseng and Tu, 1997), lupine (*Lupinus* spp. L.) (Abdel-Hafez, 1984) and pea (*Pisum sativum* L.) (Abdel-Hafez, 1984; Saber *et al.*, 1998). Ahmad and Singh (1991) reported that *Aspergillus flavus* Link ex. Fries, *A. niger* van Tieghem, *A. nidulans* (Eidam) Winter, *A. ochraceus* Wilhelm and *Penicillium* spp. grew vigorously and initiated grain spoilage and aflatoxin elaboration in chickpea. More recently, Saber *et al.* (1998) compared the mycoflora associated with three types of pea (dry, frozen and fresh) seeds and determined the natural occurrence of mycotoxins in the three types of seeds. *A. niger*, *A. flavus*, *A. fumigatus* Fres., *Penicillium chrysogenum* Thom, *P. aurantiogriseum* Dierckx, *P. citrinum* Thom, *Cladosporium cladosporiodes* (Fres.) de Vries, *C. herbarum* (Pers.: Fr.) Link, *C. spaerospermum* Penzig, *Alternaria alternata* (Fr.:Fr.) Keissler, *Fusarium oxysporum* Schlecht. emend. Snyd. and Hans and *F. verticillioides* (Sacc.) Nirenberg were detected in all three types of pea seeds, exhibiting variable frequencies and counts. Thin layer chromatography (TLC) analyses showed that some samples were contaminated with aflatoxin B_1 and B_2 and T-2 toxin (Saber *et al.*, 1998). Most samples (different cultivars) of cowpea seeds in Western Nigeria were found to harbour *A. flavus*, *A. niger*, *F. verticillioides*, *F. solani* (Mart.) Appel and Wollenw. emend. Snyd. and Hans., *Penicillium digitatum* Sacc. and *Rhizopus arrhizus* Fischer (Esuruoso, 1975). Other

fungi included *Chaetomium*, *Cladosporium*, *Curvularia* and *Macrophomina* species. Seenappa *et al.* (1983) investigated *Aspergillus parasiticus* Speare infection and aflatoxin production in some cowpea lines in Tanzania and found that all the cowpea samples were susceptible to *A. parasiticus* infection and subsequent aflatoxin production. In spite of several studies on aflatoxins in different legume seeds, there is little information on the production of aflatoxins on cowpea seeds (Hitokoko *et al.*, 1981).

The invasion of storage fungi of the embryos of seeds is one of the several causes of decreased germination (Neergaard, 1977). According to Agarwal and Sinclair (1987), if a decrease in seed germination is due to storage fungi, the effect on germination is influenced by the moisture content of the seeds, period of storage, storage temperature and other factors. Christensen (1973) reported that samples of seeds, including that of barley (*Hordeum vulgare* L.), maize, peas, sorghum (*Sorghum bicolor* (L.) Moench.), soybean and wheat (*Triticum aestivum* L.), stored at moisture contents and temperatures favourable for the growth of storage fungi, but free of storage fungi, retained a 95-100% germination percentage for some months. Similar samples inoculated with storage fungi, however, showed germinability reduced to near zero or zero. Furthermore, peas that were kept at 85% relative humidity and 30 °C lost germination capacity within six months when invaded by *Aspergillus* spp. Uninfected seed maintained a germination percentage of 95% (Fields and King, 1962).

2.3. Mycotoxins

Mycotoxins can be defined as low molecular weight fungal metabolites that are toxic to vertebrates. They have dramatic adverse effects on the health of farm animals and humans that eat contaminated agricultural products (Desjardins and Hohn, 1997). They can occur in a variety

of grains and the ingestion of mycotoxins can produce both acute and chronic toxicities ranging from chronic interferences with the functioning of the central nervous, cardiovascular and pulmonary systems and of the alimentary tract, to death. Mycotoxins can be carcinogenic, mutagenic, teratogenic and immunosuppressive (Coker, 1994). The poisoning of animals and humans by feed and food products contaminated by toxin-producing fungi is called mycotoxicosis (Hawksworth *et al.*, 1995). Mycotoxins have attracted worldwide attention over the past three decades because of their perceived impact on human health, the economic losses occurring from condemned foods and feeds and decreased animal productivity (Coker, 1994).

2.3.1. Aflatoxins

The aflatoxin-producing moulds, *Aspergillus flavus* and *A. parasiticus*, occur widely on inadequately dried food and feed grains in sub-tropical and tropical climates throughout the world. The aflatoxins may occur both before and after harvest, on virtually any food or feed which supports fungal growth, including cereals, oilseeds, edible nuts, cottonseed (*Gossypium* spp.), copra (*Cocos nucifera* L.) (Coker, 1994) and chickpea (Ahmad and Singh, 1991). The most common aflatoxins are divided into the B and G groups, based on their blue or green fluorescence under ultraviolet (UV) light illumination on TLC. Aflatoxin B_1 (AFB_1) is considered the most important aflatoxin since it is acutely toxic to humans and is responsible for liver necrosis following chronic exposure, and may be involved in the epidemiology of human liver cancer in some parts of the world, perhaps synergistically with the hepatitis B virus (Moss, 1996). Furthermore, aflatoxin B_1 has been confirmed as a highly potent animal and human liver carcinogen (Desjardins and Hohn, 1997). The ingestion of aflatoxin B_1–contaminated animal feed by dairy cattle can result in the presence of aflatoxin M_1, a metabolite of aflatoxin B_1, in milk

(Moss 1996). The acute toxicity of the aflatoxins has been demonstrated in both animals and man. The outbreak of 'Turkey X' disease in Britain, in the early 1960s, resulting in the death of thousands of turkeys, ducklings and other domestic animals, is thought to have arisen as a result of an aflatoxin-contaminated groundnut diet meal (Coker, 1994).

2.3.2. Trichothecenes

The trichothecenes comprise a large group of mycotoxins, produced by a variety of *Fusarium* moulds (Coker, 1994) and other fungi such as *Myrothecium, Stachybotrys, Trichothecium, Acremonium* and *Cylindrocarpon* (Ueno, 1983). They are sesquiterpene epoxides that inhibit eukaryotic protein synthesis (Desjardins and Hohn, 1997). Some trichothecenes attack at the initiation of protein synthesis whilst others inhibit elongation or termination, resulting in differences in toxicity and symptoms (Hocking, 1998). Among the large family, four occur naturally in cereal grains and cause mycotoxicoses in animals and possibly humans: type A trichothecenes, T-2 toxin and diacetoxyscirpenol (DAS), and the type B trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) (Marasas, 1994).

The most important producer of T-2 toxin is *Fusarium sporotrichioides* Sherb. The highest T-2 producing strains of *F. sporotrichioides* are reportedly those isolates from overwintered cereals implicated in the aetiology of the human haemorrhagic disease, alimentary toxic aleukia (ATA) in Russia (Yagen and Joffe, 1976; Joffe, 1978). Thousands of people, who had been forced to eat grain that had overwintered in the field were affected and entire villages were eliminated. The symptoms of ATA include fever, nausea, vomiting, bloody diarrhoea, dermatitis, acute inflammation of the alimentary tract, anaemia, circulatory failure and convulsions (Joffe, 1978; Marasas, 1994). T-2 toxin has been implicated in haemorrhagic toxicoses in farm animals.

Oral lesions, severe oedema of the body cavity, neurotoxic effects and finally death have been reported in poultry after the ingestion of feed contaminated with T-2 (Coker, 1994). Furthermore, Marasas *et al.* (1969) reported that T-2 toxin, from *Fusarium tricinctum* (Corda.) Sacc. Emend Snyd. and Hans *pro parte*, is acutely toxic to rats and rainbow trout. The most significant effect of T-2 toxin and other trichothecenes may be their immunosuppressive activity. There is limited evidence that T-2 toxin may be carcinogenic in animals (Coker, 1994).

Deoxynivalenol (DON) is the most frequently occurring trichothecene and is produced by species such as *Fusarium graminearum* Schwabe and *Fusarium culmorum* (W.G. Smith) Sacc. (Marasas, 1994). Despite the low acute toxicity of DON, also known as vomitoxin, it is a potent appetite suppressant and induces a vomiting response in pigs at very low concentrations (Moss, 1996). In Japan, scabby barley and wheat infected with *F. graminearum* have been implicated in scabby grain intoxication (SGI) (Marasas, 1994). Diacetoxyscirpenol (DAS) is produced mainly by *Fusarium equiseti* (Corda) Sacc. and *Fusarium poae* (Peck) Wollenw (Marasas, 1994). These fungi also occur on overwintered cereals and are implicated in the aetiology of ATA in Russia (Marasas, 1994). Many of the characteristic features of ATA, including skin erythema with burning sensations, neurological disorders and haematological abnormalities have also been recorded in human cancer patients treated with DAS as a chemotherapeutic agent (Goodwin *et al.*, 1978). Nivalenol, also produced by *F. graminearum*, can be linked to the sporadic epiphytotics of "akakabi-byo" (red mould disease) in Japan. This is due to the common occurrence of *F. graminearum* on the wheat, barley, oats (*Avena sativa* L.), rye (*Secale cereale* L.) and rice (*Oryza sativa* L.). These symptoms include anorexia, nausea, vomiting, headaches, abdominal pain, diarrhoea, chills, giddiness and convulsions (Yoshizawa, 1983).

2.3.3. Zearalenone

Fusarium graminearum is the most important producer of zearalenone (ZEA), a widely occurring mycotoxin that is responsible for many outbreaks of oestrogenic syndromes amongst farm animals (Marasas, 1991, 1994). Pigs seem to be especially sensitive to zearalenone and it was first discovered as the agent causing vulvovaginitis in sows. Symptoms include hyperaemia and oedematous swelling of the vulva in prepubertal gilts, or in more severe cases, prolapse of the vagina and rectum. Reproductive disorders in sows include infertility, foetal resorption or mummification, abortions, reduced litter size and small piglets. In male pigs, atrophy of testes, decreased libido and hypertrophy of the mammary glands following ZEA ingestion are well documented (Marasas *et al.*, 1984).

2.3.4. Fumonisins

The fumonisins are a group of mycotoxins produced by several species of the genus, *Fusarium*, of which *Fusarium verticillioides* and *Fusarium proliferatum* (Matsushima) Nirenberg are the most important since they occur world-wide and are the most prevalent fungi associated with maize (Gelderblom *et al.*, 1988; Coker, 1994). They are amino polyalcohols and are similar in structure to the long-chain base backbones of sphingolipids. Fumonisins inhibit the activity of sphingosine N-acetyltransferase which leads to the accumulation of toxic sphingoid bases (Desjardins and Hohn, 1997). There are numerous derivatives of fumonisins known, including A, B, C and P analogues (Musser, 1996). The most important analogues found in contaminated maize and for which analytical methods have been developed are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Shephard *et al.*, 1996). Only FB_1 and FB_2, however, appear to

be toxicologically significant. The occurrence FB_1 in cereals, primarily maize, has been associated with serious outbreaks of leukoencephalomalacia (LEM) in horses and pulmonary oedema and hydrothorax in pigs (Harrison *et al.*, 1990; Coker, 1994). LEM is characterised by liquefactive necrotic lesions of the white matter of the cerebral hemispheres and has been reported in many countries, including the USA, Egypt and South Africa (Marasas, 1994). FB_1 is also toxic to the central nervous system, liver, pancreas, kidney and lung in numerous animal species (Coker, 1994). Furthermore, it is also a cancer promoter and initiator in rat liver, is hepatotoxic in horses, pigs, rats and vervet monkeys and phytotoxic to several plants (Marasas, 1995, 1996). The incidence of *F. verticillioides* in domestically produced maize has been statistically linked to the incidence of human oesophageal cancer rates in the Transkei, southern Africa and in China (Rheeder *et al.*, 1992; Marasas, 1996). The International Agency for Research on Cancer (IARC) has also stated that toxins produced by *F. verticillioides* are possibly carcinogenic to humans and they have characterised them as class 2B carcinogens (Vainio *et al.*, 1993).

2.3.5. Ochratoxins

Ochratoxins are also produced by species of *Aspergillus*, including *Aspergillus ochraceus* which may be responsible for the presence of ochratoxin in commodities such as coffee (*Coffea arabica* L.) and cocoa (*Theobroma cacao* L.) in warmer parts of the world. In temperate climates, the most important species is *Penicillium verrucosum* Dierckx which may occur on cereals such as barley (Moss, 1996) and on other commodities including rice, peas, beans (*Phaseolus vulgaris* L.) and cowpeas (Coker, 1994). Ochratoxins are potent nephrotoxins (Miller, 1995) and ochratoxin A (OA) produces renal toxicity, nephropathy and immunosuppression in several

animal species (Coker, 1994). Although there is currently inadequate evidence for the carcinogenicity of OA in humans, there is sufficient evidence in experimental animals (Coker, 1994; Miller, 1995). Ochratoxin A has been found in significant quantities in pig meat, as a result of its transfer from animal feed (Marquardt and Frolich, 1992; Coker, 1994). A correlation exists between human exposure to OA and Balkan endemic nephropathy, a fatal chronic renal disease occurring in limited areas of Bulgaria, the former Yugoslavia and Romania (Coker, 1994).

2.4. Common storage and mycotoxigenic fungi

2.4.1. *Fusarium*

Fusarium, one of the most important genera of plant pathogenic fungi, occurs mostly in plants and cultivated soils and has a record of causing devastating diseases in many economically important plants (Marasas, 1994; Hocking, 1998). The importance of *Fusarium* in the current context is, however, that infection may occur in developing seeds, especially cereals. Unlike most *Aspergillus* and *Penicillium* species, fusaria grow in crops before harvest and consequently mycotoxins are usually produced before or immediately after harvest (Hocking, 1998). The mycotoxins produced by *Fusarium* spp. have also traditionally been associated with temperate cereals, since these fungi require lower temperatures for growth and mycotoxin production (Placinta *et al.*, 1999). Marasas *et al.*, (1984) studied more than 200 toxigenic *Fusarium* isolates and provided accurate information on species identification and the corresponding toxins produced. At least 20 *Fusarium* spp. belonging to 10 sections of the genus have been reported in the literature to be toxigenic (Nelson *et al.*, 1983, Marasas *et al.*, 1984). *F. sporotrichioides*, *F. graminearum*, *F. verticillioides* are the three most important toxigenic *Fusarium* species with

respect to the production of mycotoxins in cereal grains that are known to cause field outbreaks of mycotoxicoses in animals and most probably are associated with foodborne diseases of humans (Marasas *et al.*, 1984; Marasas and Nelson, 1987; Marasas, 1991).

Fusarium sporotrichioides, not a commonly occurring species, is found mainly on cereal crops in temperate regions, but it has also been isolated from groundnuts (Pitt and Hocking, 1985). In addition to producing T-2 toxin, *F. sporotrichioides* are known to produce butenolide, fusarenon-X, neosolaniol and nivalenol (Marasas *et al.*, 1984). *F. graminearum* is distributed world wide as a serious plant pathogen that causes root, crown, stem and ear rot and head blight of cereals and other hosts. The principal toxins produced by *F. graminearum* are DON, NIV and ZEA (Marasas *et al.*, 1984). *F. verticillioides* is more common in the tropics than in temperate zones and its most common source in foods is maize, under both field and storage conditions (Pitt and Hocking, 1985). The most important toxins produced by this species are the fumonisin B mycotoxins (Hocking, 1998). *F. globosum* Rheeder, Marasas et Nelson, a recently described species, was originally isolated from maize kernels harvested in the Transkei region of South Africa (Sydenham *et al.*, 1997). Morphologically, *F. globosum* is closely related to the other fungal contaminants of maize, including *F. verticillioides*. *F. globosum* isolates produces FB_1, FB_2 and FB_3 (Sydenham *et al.*, 1997). *F. equiseti* has a distribution extending from Alaska to the tropics (Domsch *et al.*, 1993) and has been reported on a variety of grains especially maize and barley. *F. equiseti* produces NIV, fusarenon X, T-2, DAS, butenolide, ZEA and several others that are less well characterised (Marasas *et al.*, 1984).

2.4.2. *Aspergillus*

Aspergillus species are common contaminants in soils and decaying vegetation. A number of species are closely associated with human foods, mainly cereals and nuts (Pitt and Hocking, 1985). Many of the species are xerophilic and are capable of spoiling foods only just above safe storage moisture limits. The most important mycotoxigenic species are *A. flavus*, *A. parasiticus*, *A. ochraceus* and *A. versicolor* (Vuill.) Tiraboschi (Pitt,1998).

Aspergillus flavus is common in soil, especially in cultivated soil in which crop residues are incorporated. It is also common in decaying vegetation and in grains and seeds with moisture contents high enough to permit it to grow (Christensen and Kaufmann, 1969). Peanuts, maize and cottonseed are the three most important crops invaded by this fungus (Cole *et al.*, 1982). The invasion of peanuts occurs as a result of drought stress and related factors (Cole *et al.*, 1982). Furthermore, Pitt *et al.* (1994) reported that *A. flavus* was the major fungi found in soybeans and sorghum in a study investigating the mycoflora of beans and small grain in Thailand. *A. parasiticus* is a less common species but, as with *A. flavus*, it has a particular affinity for nuts and oilseeds (Cole *et al.*, 1982). Both *A. flavus* and *A. parasiticus* produce aflatoxins, which are both acutely and chronically toxic to animals and humans. *A. ochraceus* is found on drying or decaying vegetation, seeds, nuts and fruits. It is widely distributed in dried foods including various kinds of beans, nuts, cereals, dried fruit, biltong (jerky) and salt fish (Pitt and Hocking, 1985). The major toxin that *A. ochraceus* produces is OA but it also produces penicillin acid, a mycotoxin of lower toxicity (Pitt, 1998). *A. versicolor* has been reported from a wide variety of foods. It occurs at harvest but is more commonly found in stored commodities like wheat, rice and maize. This fungus has also been found in a variety of nuts, fermented and cured meats, biltong (jerky), cheese and spices (Pitt and Hocking, 1985). *A. versicolor* is the most important

fungus producing the mycotoxin sterigmatocystin. This toxin is a precursor of aflatoxins. The acute oral toxicity is low since it is very insoluble in water and gastric juices. Sterigmatocystin is thus unlikely to be responsible for acute poisoning in animals and man (Terao, 1983).

2.4.3. *Penicillium*

Penicillium is a large genus, with 150 recognised species, but the majority of important toxigenic and food spoilage species are found in the subgenus *Penicillium*. The most important toxigenic species in foods are *P. citreonigrum* Dierckx, *P. citrinum*, *P. islandicum* Sopp and *P. verrucosum* (Pitt, 1998).

Penicillium citreonigrum is widely distributed (Pitt and Hocking, 1985) and favours lower temperatures and shorter hours of daylight. *P. citreonigrum* produces citreoviridin which causes acute cardiac beriberi (Pitt, 1998). *P. citrinum* is commonly found on milled grains and flour and whole cereals, especially rice, wheat and maize (Pitt and Hocking, 1985). The toxin produced is citrinin which is a significant renal toxin to monogastric domestic animals and birds (Pitt, 1998). *P. islandicum* occurs infrequently in nature (Pitt and Hocking, 1985) and produces at least four unique mycotoxins, namely, cyclochlorotine, islanditoxin, luteoskyrin and erythoskyrin. The two latter toxins are liver and kidney toxins (Pitt, 1998). Luteoskyrin is also carcinogenic (Pitt, 1998). *P. verrucosum* has been reported exclusively from the temperate zones and appears to be uncommon elsewhere (Pitt and Hocking, 1985). *P. verrucosum* is the principal producer of the nephratoxin, OA (Pitt, 1998).

2.5. Detection and analysis of mycotoxins

A great deal of research has been directed toward the development of analytical methodologies to identify and quantitate mycotoxins in food and feed. Mycotoxins are a large group of chemically unrelated compounds which are linked only by their production by fungi and some toxic effect in man or animals. Thus, the isolation, identification and quantitation of each mycotoxin presents its own set of problems (Plattner, 1986).

2.5.1. Sampling and sample preparation

Sampling is the most important step, since if the sample taken for analysis is not representative of the bulk, the analytical results are meaningless (Semple *et al*,. 1998). The sample size should increase with increasing particle size. For example, samples of whole groundnuts, maize and rice should be in the order of 20, 10 and 5 kg, respectively (Coker, 1994). In uniform sampling, a sample is taken so as to represent the average of the entire population. The samples are taken in a small quantity from each section of the population. Sampling in this case, however, needs to be evenly reduced. This procedure is called dividing, performed by quartering, dividing or using a divider (Semple *et al.*, 1998). Selective sampling is carried out when products are disposed according to the lowest quality and sampling is made from sections with particularly poor quality. Random sampling is applied in cases when several samples are taken from a product when they do not have the same quality. In this case, individual samples, the quantity of material sampled amount of sampling and sampling period are not fixed before sampling. The subjects of sampling are chosen by the use of dice, lottery or a random table (Hongsuwong, 1998). Sample preparation involves grinding and sample division to obtain a representative analytical sample. Usually, 1 kg

of ground material is blended at high speed with an appropriate amount of water to give a homogeneous paste (slurry) from which 100 g aliquots are taken for analysis (Semple *et al.*, 1998).

2.5.2. Extraction

Chloroform, acetonitrile, methanol and acetone are the commonly used organic solvents for extraction of mycotoxins. These solvents aid the breaking of weak electrostatic bonds that bind some mycotoxins to other substrate molecules (Semple *et al.*, 1998). The ground sample, or aqueous slurry, is either shaken with the extraction solvent for 30-45 min or blended at high speed for about 3 min. An explosion-proof blender is recommended for use with flammable solvents such as acetone and methanol (Semple *et al.*, 1998).

2.5.3. Common clean-up techniques

Since mycotoxins are such a diverse group of chemical compounds, it is difficult to find a simple procedure which specifically removes non-mycotoxin "interfering" compounds whilst leaving the mycotoxins in the extract. It is difficult to find a good method for screening a wide-range of mycotoxins simultaneously. Procedures have, however, been devised which remove interfering non-mycotoxin compounds from the extract of a particular commodity and leave a particular mycotoxin or group of mycotoxins in the extract (Semple *et al.*, 1998). Various clean-up techniques, such as, defatting which is done prior to the toxin extraction step, have been used to remove interfering non-mycotoxin compounds (Semple *et al.*, 1998). Petroleum ether or hexane is used to extract lipids from the sample using a Soxhlet extractor (Semple *et al.*, 1998). Column

chromatography is used in numerous regulatory or officially approved methods. A glass column is packed with adsorbent materials and the crude extract is added to the top of the column. This column is then eluted with a series of solvents or solvent mixtures which are designed to first wash off interfering compounds and then elute the desired mycotoxins. A miniature column (mini-column) is used in rapid aflatoxin assay methods to remove interfering compounds and to qualitatively detect aflatoxin down to a few parts per billion (Semple *et al.*, 1998).

Precipitation is another useful technique in which various chemicals (cupric carbonate, ammonium sulphate, lead acetate and ferric gel) are added to the crude extract to adsorb certain pigments, proteins and other interfering compounds onto their surface. The complex formed then precipitates out of solution and can be filtered off (Semple *et al.*, 1998).

Liquid-liquid partitioning can also be used, often in conjunction with one of the other clean-up procedures. This technique provides additional clean-up and it can also transfer toxins from one solvent system to another whilst concentrating the toxins. The partition is carried out in a separating funnel which contains the two immiscible solvents. The funnel is shaken for a few minutes allowing the dissolved compounds, including mycotoxins, to partition between the two phases. Care must be taken in the choice of solvents in order to minimise the risk of emulsion formation (Semple *et al.* 1998). Although TLC is used for the detection and quantification of toxins, it is also useful for separating the mycotoxins from interfering compounds in the extract (Semple *et al.* 1998). Immunoaffinity columns are also widely used for cleanup and isolation of mycotoxins particularly aflatoxins, OA and fumonisins. They are prepared by binding antibodies specific for a given mycotoxin to a specially activated solid-phase support and packing this support, suspended in aqueous buffer solution, into a cartridge. The mycotoxin in the extract then binds to the antibody, the impurities are removed with water and then the mycotoxin is desorbed with a miscible solvent (methanol) (Scott and Trucksess, 1997).

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2.5.4. Work-up

According to Semple *et al.* (1998), work-up is carried out in order to prepare the extract for the detection and/or quantification step. Following clean-up, the extract is often dissolved in a large volume of aqueous solvent, so it must be concentrated into a small volume (10-50 ml) of a volatile solvent, such as chloroform. This chloroform solution is dried by passing it through a bed of anhydrous sodium sulphate. The solvent is evaporated to near dryness. To avoid possible mycotoxins breakdown at this stage, evaporation is best carried out using a steam bath (preferably under a stream of nitrogen). A great deal of care should be taken to ensure that flasks are removed from the steam bath prior drying and that the vials are removed at the point of dryness (Semple *et al.*, 1998).

2.5.5. Chromatographic methods for the detection and quantification of mycotoxins

In recent years, chromatography has thus far been widely accepted for the analysis of mycotoxins since there always seems to be a need to separate some primary and other secondary fungal metabolites simultaneously produced with mycotoxins (Lin *et al.*, 1998). Numerous chromatographic techniques, including liquid chromatography (LC), high performance liquid chromatography (HPLC), gas chromatography (GC), TLC, supercritical fluid chromatography (SFC) and capillary zone electrophoresis (CZE) have played a role in the analysis of mycotoxins (Holcomb *et al.*, 1992). Although many techniques have been applied in the determination of certain varieties of mycotoxins, TLC is the most popular technique because of its congruous features such as line principle, higher sample throughput, lower operating costs, convenience in identification by comparison of spot colour, R_F values and UV-Vis spectrum (Lin *et al.*, 1998).

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Placinta *et al.* (1999), however, reported that in global terms, HPLC and gas chromatography/mass spectrometry (GC/MS) have emerged as methods of choice and are largely replacing earlier technologies based on TLC.

2.5.5.1. Thin layer chromatography

As required for most analytical methods, sample preparation and procedures for extraction and clean-up are also carried out during TLC. A silica gel layer on a glass plate or plastic sheet is mainly used in normal phase development (Yu and Chu, 1991) but numerous other layers have also been employed as alternative methods to analyse mycotoxins (Lin *et al.*, 1998). Solvents like chloroform, ethyl acetate, methanol, acetone, toluene, acetic acid, ether and water have often been employed , at varying percentages, in the initial systems (Lin *et al.*, 1998). For the separation of mycotoxins, tank development and instrumental TLC have been increasingly used in mycotoxin analysis (Lin *et al.*, 1998). Two kinds of techniques are frequently applied for visualising the mycotoxin spots on thin layer plates. These are the examination under UV light of long or short wavelength for naturally fluorescent mycotoxins like aflatoxins, citrinin, OA, and the spraying of plates with a chemical reagent that reacts with the mycotoxins to produce a coloured or fluorescent product (Lin *et al.*, 1998). The quantification of mycotoxins has been dominated in the past by *in situ* evaluation with fluorescence densitometry, fluorescence quenching and absorbency densitometry as methods of choice. Recently, however, a scientifically operated charge-coupled device (CCD) detector has been developed for the quantitative analysis of aflatoxins (Lin *et al.*, 1998).

An important feature of TLC is its off-line operating principle and in practice, TLC is more suitable for crude extract analysis. Furthermore, both physical and chemical, pre- and post-chromatographic visualisation techniques are used for the TLC detection and confirmation of mycotoxins. Since mycotoxins occurring in a matrix are usually in very low quantity levels, the analytical method used needs to be sensitive enough even though an enriching procedure may be performed before measurement. TLC is capable of lowering the operating detection limits by applying bigger volumes of sample solutions. Thus, a detection range for a number of mycotoxins is reduced to ng per spot level without concentrating the samples of interest in the application solution. Therefore, owing to its coherent features, TLC is a well developed, quick, cost effective and suitable method for the analysis of a wide range of mycotoxins (Lin *et al.*, 1998).

2.5.5.2. Other chromatography techniques

HPLC has been used for the analysis of a wide range of mycotoxins including the aflatoxins, OA, ZEA, DON and the fumonisins (Sydenham *et al.*, 1992; Coker, 1994). High performance thin layer chromatography (HPTLC) has been applied mainly to the aflatoxins whereas gas liquid chromatography (GLC) has been used for the quantification of DON, T-2 toxin and ZEA (Coker, 1994). Enzyme-linked immunosorbent assays (ELISA) have also been applied to a range of mycotoxins including aflatoxins, OA, DON, T-2 toxin and zearalenone (Richard *et al.*, 1993; Coker, 1994). Solid phase ELISA kits have also been developed for aflatoxins, OA, ZEA and T-2 toxin (Coker, 1994). The ELISA format for aflatoxins contains three specific reagents: the mono-polyclonal antibodies which recognise and bind with a specific aflatoxin, an aflatoxin-enzyme conjugate and an enzyme substrate. The bonded enzyme catalyses the oxidation of a substrate to

form a coloured complex for further qualitative and quantitative evaluation (Park *et al.*, 1989; Trucksess *et al.*, 1989). Mass spectrometry/mass spectrometry (MS/MS) is a useful, sensitive and highly selective technique for the identification and quantitation of mycotoxins at low levels directly from crude extracts with minimal chemical or chromatographic clean-up. In this method, the mycotoxin is identified by selecting its molecular ion with a first mass filter and producing daughter ions from the collision of that selected ion with a neutral target gas. The daughters are mass analysed by a second mass filter and the resulting spectrum can be used to identify the mycotoxin and determine the amount present in the sample (Plattner, 1986). In comparison to TLC, GC requires that compounds be volatile enough and relatively polar. Thus, those mycotoxins which are not sufficiently volatile enough require reaction to obtain a volatile derivative and such steps add complexity to the procedure (Lin *et al.*, 1998).

Since fumonisins do not process any chromophores and do not adsorb UV light or visible light or fluoresce, the development of analytical methods for the quantification of this toxin has been difficult (Sydenham *et al.*, 1992). Between 1988 and 1991, analytical methods for the detection and quantification of FB_1 and FB_2 in maize were developed and used for the first time (Marasas, 1996). These were the analysis by HPLC of the maleyl derivative of FB_1 with ultraviolet detection and of the fluorescamine derivative with fluorescence detection (Marasas, 1996); the successful development of liquid chromatographic methods based on solid-phase extraction (SPE) of solvent extracts (Shephard, 1998); analysis by HPLC with pre-column derivatisation with *o*-phthaldialdehyde and fluoresence detection (Sydenham *et al.*, 1992; Marasas, 1996; Shephard, 1998) and analysis by hydrolysis and GC/MS with sensitivity:low ppm (Marasas, 1996). Walker and Meier (1998) developed a rapid and sensitive method, liquid chromatography with diode array detection and GC with electron-capture detection, for the simultaneous detection of NIV and DON in wheat flour. In this method samples were extracted

with acetonitrile and water and the extract filtered and purified by a column containing charcoal, celite and other adsorbents. After evaporation, the dried residue was analysed by reversed-phase liquid chromatography with diode ray detection. An improved fluorometric and chromatographic method for the quantification of FB_1, FB_2 and FB_3 was reported by Duncan *et al.* (1998). In this chromatographic process, the first step involves the extraction of fumonisins from a sample and isolation on an immunoaffinity column and in the second step, the fumonisins are converted to fluorescent derivatives and quantified through HPLC or by a fluorometer. Onji *et al.* (1998) developed an improved method for the qualitative and quantitative analysis of fusarium toxins by GC/MS using cold on-column injection. The toxins were subjected to GC/MS without chemical derivatisation by means of the on-column injection technique. This technique is more reliable than conventional methods for fusarium mycotoxins since it is simple, there is rapid purification without chemical derivatisation and the identification of toxins is carried out by inspecting their native mass spectrometer readings (Onji *et al.*, 1998).

The trichothecenes are difficult to analyse by TLC or HPLC since they do not exhibit UV fluorescence. Thus, GC combined with MS using selected ion monitoring (SIM) is the recommended technique for their analysis (Steyn *et al.*, 1991). The method of choice used by the Medical Research Council (MRC) to detect type A trichothecenes, T-2 and DAS is by capillary GC and for type B trichothecenes, DON and NIV, electron-capture GC (Marasas, 1994). According to the Official Association of Analytical Chemists (AOAC), the procedure for the determination of zealarenones in maize is based on TLC with visual detection under UV light, which is a rapid screening method (Horwitz ,1980). The method of choice for the analysis of feeds and food for zearalenones is, however, the HPLC method with fluorescence detection (Bagneris *et al.*, 1986).

2.6. Prevention and control of storage fungi and mycotoxins in stored grain and seed

In order to design strategies for the reduction or elimination of mycotoxins in stored seed, knowledge about their fungal sources is needed. Many factors are involved in enhancing the formation of mycotoxins. These factors include the plant's susceptibility to fungal infestation, the suitability of fungal substrate, temperature, climate, moisture content, relative humidity and physical damage of seeds due to insects and pests (Noomhorm and Cardona, 1998; Suttajit, 1998).

According to Suttajit (1998), prevention of the contamination of fungi and their mycotoxins can be divided into three levels, namely, primary, secondary and tertiary prevention. Primary prevention is the most important and effective plan for reducing fungal growth and mycotoxin production. This can be achieved by the development of fungal resistant plant varieties, lowering of moisture content of grain after harvesting and during storage, storing grain at low temperatures and by the use of fungicides and insecticides against fungi and insects, respectively. Secondary prevention is required when fungal invasion begins in the grain at an early phase. The measures suggested include re-drying of the products, removal of contaminated seeds, detoxification of mycotoxins and the protection of stored products from any conditions which favour fungal growth. When grain is heavily infected by toxic fungi, tertiary prevention is required. Two practices are recommended, namely, complete destruction of the contaminated products or the detoxification or destruction of mycotoxins to the minimal level (Suttajit, 1998).

The most effective means to control and prevent aflatoxin contamination of maize and groundnuts and mycotoxin contamination of any foodstuff, is the ability to control the environment so as to prevent mould growth (Mislivec, 1998). Since all mould species are obligate aerobes and cannot grow in the absence of free oxygen, a way to prevent mycotoxin

contamination of maize and groundnuts and small grains, is to store them under anaerobic conditions (carbon dioxide and nitrogen). This is, however, not economically feasible for the small-scale farmer since large air-tight silos need to be used (Mislivec, 1998).

Mislivec (1998) found that in Thailand, virtually all of the mycotoxin-producing species grow poorly, if at all, at 10 ^{0}C. Another way, therefore, to control or prevent mycotoxin production is a storage temperature of 10 ^{0}C or less. Low temperature facilities are, however, virtually non-existent for small-scale farmers. Moisture control is, therefore, probably the best and most economical means to prevent mould growth and mycotoxin production. (Mislivec, 1998). Drying is the process of moisture removal from, in this case, the grain. In the sun-drying process, grain is heated by solar radiation, thus creating a higher vapour pressure in grain than the surrounding air (Naewbanij and Thepent, 1998). On sunny days, the drying process, which is the cheapest process, will take two to three days depending upon the spreading density, prevailing atmospheric conditions and seed type (Noomhorm and Cardona, 1998). The critical water content for safe storage corresponds to a water activity (a_w) of about 0.7. Maintenance of commodities below 0.7 a_w is an effective technique used throughout the world for controlling fungal spoilage and mycotoxin production in foods (GASGA and CTA 1997). More conventional types of drying include mechanical driers and solar drying (Noomhorm and Cardona, 1998). Even after acceptable drying, the corn must be handled by the farmer, buyer and export in a manner that will not cause a moisture increase.

Contaminated mycotoxins in foods and feeds should be removed, inactivated or detoxified by physical, chemical and biological means (Suttajit, 1998). Physically, fungi-contaminated seeds can be removed by handpicking or by photoelectric detection machines. These methods are, however, time- and labour-consuming and expensive (Suttajit, 1998). Jackson *et al.* (1997) investigated the effect of baking and frying on FB_1 of corn-based foods and their study suggested

that fumonisins are heat stable compounds that survive under most conditions used during baking and frying. Furthermore, Feuell (1966) investigated the detoxification of aflatoxins in groundnuts by exposure to radiation, heat and reactive gases and found that no treatment was completely successful.

Chemical treatment has been used as the most effective means for the removal of mycotoxins from contaminated commodities (Suttajit, 1998). Ammonia, as an anhydrous vapour and an aqueous solution, has attracted the widest interest and has been exploited commercially by the feed industry for the destruction of aflatoxin (Bauer, 1994; Coker, 1994). On-farm procedures involve spraying with aqueous ammonia, followed by storage at ambient temperature, for about two weeks, in large silage bags (Coker, 1994). Other chemicals used as treatments for the detoxification of mycotoxins include ozone gas (Dwarakanath *et al.*, 1968), formaldehyde (Codifier *et al.*, 1976), calcium hydroxide monomethylamine (Bauer, 1994), malonic acid (Megella and Hafez, 1982), benzoic acid (Chipley and Uraih, 1980; Uraih and Offonry, 1981), lactic and citric acid (Reiss, 1979), benzoic derivatives, potassium sulfite and potassium fluoride (Davis and Diener, 1967). Hasan (1998) has reported that *Aspergillus flavus, A. niger, Penicillium chrysogenum, P. corylophilum* Dierckx and *Rhizopus stolonifer* (Ehrenberg: Fries) were significantly inhibited in seeds (30% moisture content) treated with NaCl (salt).

The chemical recommended as a grain protectant is malathion, which prevents moulds, bacterial and insect activity (Noomhorn and Cordona, 1998). BP Chemicals of England has developed chemicals which when applied to moist grains prevent moulds and bacterial activity. An example is Propcorn, which prevents deterioration, loss of dry matter, nutrition loss and removes associated health hazards. Furthermore, Propcorn adds to energy value of the grain (Noomhorn and Cordona, 1998). Kemin Industries in the United States has also produced a mould inhibitor, Myro Curb. This inhibitor is non-toxic and non-volatile (Noomhorn and

Cordona, 1998). Since insects can be carriers of spoilage fungi to feeds and food, an appropriate measure to inhibit insect infestation is to apply fumigants. Common fumigants used include methyl bromide, carbon disulfide, carbon tetrachloride and phosphine (Noomhorn and Cordona, 1998).

A large number of naturally occurring compounds, such as essential oils from plants, possess antifungal activity. Various reports have shown that many of these oils are potent inhibitors of fungal growth (Thompson and Cannon, 1986; Chatterjee, 1990). Sharma *et al.* (1979) reported the inhibition of aflatoxin-producing fungi by onion (*Allium cepa* L.) extracts, and Moore and Atkins (1977) investigated the fungicidal and fungistatic effects of an aqueous garlic (*Allium sativum* L.) extract on medical yeast-like fungi. Furthermore, it has been reported that spice oil inhibited the growth and aflatoxin B_2 (AFB_2) production by *Aspergillus parasiticus* (Tiwari *et al.*, 1983). Forty essential oils were screened for antifungal activity on the mycelial growth of twenty species of *Rhizopus, Mucor* and *Aspergillus* (Thompson and Cannon, 1986). Seven oils, bay (*Gordonia lasianthus* (L.) Ellis), cinnamon (*Cinnamomum zeylanicum* Blume) (leaf and bark), clove, pimenta (*Capsicum annuum* L.) (berries and leaves) and thyme, were found to be most effective against fungi. *A. flavus* and *A. parasiticus* were successfully inhibited by these above-mentioned essential oils at concentrations of 500 and 1000 ppm. These results revealed that the susceptibility of the fungi to the seven essential oils may have potential application for the control of these fungi (Thompson and Cannon, 1986). Mishra and Dubey (1994) also found that the essential oil of lemon grass exhibited a broad antifungal spectrum and it was superior to synthetic fungicides like copper oxychloride and dithane. Adegoke and Odesola (1996) reported that samples of maize and cowpea treated with lemon grass powder and essential oil showed no physical deterioration of the seed. The essential oil of lemon grass also inhibited the growth of moulds like *A. flavus, A. fumigatus, Macrophomina phaseoli* (Tassi.) Goid. and *P. chrysogenum*.

The phytochemical components like alkaloids, tannins and cardiac glycosides found in the powder are believed to be associated with the preservative and anti-microbial effects of lemon grass (Adegoke and Odesola, 1996). Neem (*Azadirachta indica* A. Juss.) has also demonstrated antifungal activity (National Research Council, 1992). Neem oil protected the seeds of chickpeas against serious fungal diseases (*Rhizoctonia solani* Kühn, *Sclerotium rolfsii* Sacc. and *Sclerotinia sclerotiorum* (Lib.) de Bary) and it slowed the growth of *Fusarium oxysporum*, but did not kill it. Neem leaf extract failed to kill *A. flavus* but it completely stopped the fungus from producing aflatoxin (National Research Council, 1992). Hasan (1996) reported that coffee and tea (*Camellia sinensis* (L.) Kuntze) powder extract had an anti-toxigenic activity on toxin production by *A. parasiticus var. globosus*. Other biological treatments with natural products from plants or herbs include star anise (*Illicium verum* Hook.f.) seeds (Hitokoto *et al.*, 1978) and white and black pepper (*Piper nigrum* L.) (Madhyastha and Bhat, 1984).

It has been reported by Montes-Belmont and Carvajal (1998) that essential oils of clove, cinnamon, peppermint, basil (*Ocimun basilicum* L.) and thyme exhibited no inhibitory effect on the germination of maize seeds. Furthermore, the growth of the seedlings seemed to be unaffected by the treatments, with the fresh and dry weights being similar to those of the controls (Montes-Belmont and Carvajal, 1998). There was no adverse effect observed on the germination of chick pea seeds when treated with soybean and castor (*Ricinus communis* L.) oil (Pacheco *et al.*, 1995). Moreover, Cruz and Cardona (1981) found that no reduction in germination of cowpea seeds after being treated with soybean oil. Extracts of oils including a type of hyssop (*Origanum syriacum* L.) and lemon grass did, however, strongly inhibit germination of several species including wheat, when applied at concentrations of 20-80 ppm. (Dudai *et al.*, 1999).

Chitinase and β-1.3-glucanase, which are antifungal enzymes, found in a number of plant seeds, also act as defence against pathogenic fungi (Suttajit, 1998). Chitin and glucan, the major

polymeric components of many fungal cell walls, can be enzymically hydrolysed into smaller products resulting in the damage or killing of fungal mycelia or spores (Suttajit, 1998). Seeds rich in such antifungal enzymes are thus likely to resist fungal infestation (Nelson *et al.*, 1969; Roberts and Selitrennikoff, 1986).

It has been reported by D'Mello and MacDonald (1998) that a selection of cultivars of cereals resistant to fusarium pathogens is currently viewed as a sustainable and viable option for reducing mycotoxin contamination of grain. Studies have indicated that the exploitation of genetic resistance to fusarium head blight in wheat has been successfully used to restrict kernel contamination with DON (D'Mello and MacDonald, 1988).

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CHAPTER 3

STORAGE FUNGI OF COWPEA SEED STORED UNDER OPTIMAL CONDITIONS

3.1. Introduction

Cowpea is a notably important nutritious legume crop for many subsistence farmers and people living in less developed countries of tropical and sub-tropical Africa. Rural families can derive income, animal fodder and most importantly food from the production of this crop (Singh *et al.*, 1997; Ushamalini *et al.*, 1998). Cowpea, as food, can be used in the form of young leaves, immature pods, peas and seed (Singh *et al.*, 1997). This crop is nutritionally important to the rural and urban communities since it supplies the people with a good source of proteins, vitamins and carbohydrates (Singh *et al.*, 1997).

It is known that cowpea seeds are susceptible to fungal contamination, especially when stored under inadequate and poor storage conditions (Esuruoso, 1975; Hitokoko *et al.*, 1981; Shama *et al.*, 1988; Seenappa, *et al.* 1983; Ushamalini *et al.*, 1998), and as a result, deterioration and spoilage of the seed occurs. It is under these conditions, together with high moisture activities and high temperatures, that certain fungi tend to produce secondary fungal metabolites (mycotoxins) (Moss, 1996). The ingestion of mycotoxins can produce acute and chronic toxicities ranging from chronic interferences with the functioning of various systems of the body to the death of farm animals and humans (Coker, 1994). Mycotoxins have attracted worldwide attention over the past few decades because of their impact on human health and the economic

losses occurring from condemned foods and feeds and decreased animal productivity (Coker, 1994).

This has lead to a great deal of research pertaining to the control and prevention of storage fungi on seeds and similarly, mycotoxins. According to Mislivec (1998), the most effective way to control and prevent mycotoxin contamination of seeds is the ability to control the environment, i.e. atmosphere, temperature and moisture. Low temperature storage has been recommended as a practice to deter fungal growth (Suttajit, 1998). Goyal (1998) suggested that natural cooling is an effective method to preserve seed, since low temperatures do not allow microflora to grow as most of these fungi are thermophillic. Furthermore, Mislivic (1998) reported that a storage temperature of 10 °C or less was an environmental way to control the growth of mycotoxin producing species detected in Thailand.

The purpose of this investigation was to determine the mycoflora of cowpea seeds before and after a 3-year period of cold storage.

3.2. Materials and methods

3.2.1. Samples

Nine cultivars of recently harvested cowpea seeds were obtained from the Vegetable and Ornamental Plant Institute (VOPI), Roodeplaat, Pretoria during July 1996. Sample sizes ranged from 22–62g. The recently harvested samples were first analysed at the Department of Botany, University of Pretoria, Pretoria during July 1996 and thereafter stored in sealed aluminium foil packets in a refrigerator at ± 5 °C for a period of three years. Samples were then analysed during July 1999. During the first analysis four replicates of 100 seeds, picked randomly from each

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cultivar, were used. Due to an inadequate amount of seeds, only four replicates of 50 seeds from each sample were used during the second analysis.

3.2.2. Fungal analysis

In both analyses the seeds were surface sterilised with 1% sodium hypochlorite for 1 min and rinsed three times with sterile distilled water. The seeds from each sample were plated (five seeds per Petri dish, one in the plate centre and one in each quadrant) onto malt extract agar (MEA) containing 0.025% chloramphenicol. The Petri dishes were incubated at 25 °C for 5-7 days and then examined with the unaided eye and using a stereomicroscope. Fungi were identified to genus level, and where possible species level. Fungal identification was accomplished with the aid of various references (Samson *et al.*, 1981, Nelson *et al.*, 1983, Domsch *et al.*, 1993, Watanabe, 1994).

3.2.3. Statistical analysis

Two-way analysis of variance (ANOVA) was performed on the data and least significant differences ($P = 0.05$) were determined according to the student's *t* test.

3.3. Results

Fungi isolated from the nine cultivars of cowpea seeds analysed during both 1996 and 1999 are recorded in Table 1. The most common fungal genera found during both analyses were *Aspergillus, Penicillium* and *Alternaria.* The incidence of *Aspergillus* spp. showed a significant increase in 1999 in cultivars 1 and 4 but in cultivar 8, it was higher in 1996. *Alternaria* spp. increased significantly in cultivars 2 and 8 in 1999 whereas only *Penicillium* showed a significant

increase in cultivar 4 when compared with the percentage found in 1996. The incidence of *Penicillium* spp. in cultivar 3 in 1996 was much higher than in 1999. The percentage *Phoma* spp. was significantly higher during 1996 in cultivars 6 and 7 but were not isolated from these two samples in 1999. This same trend can be seen with *Chaetomium* in cultivar 1. Three species of *Aspergillus* were isolated from the cultivars, namely *A. flavus*, *A. niger* and *A. ochraceus*, with *A. niger* being the most common species. Other fungi isolated from the various samples were found either only during the 1996 or the 1999 analysis. For example, *Fusarium* spp. found in 1996 were recorded from samples 1 and 8 whereas *Fusarium* spp. in 1999 were recorded in samples 2, 6 and 8. Certain fungi, including *Gilmaniella, Cladosporium, Curvularia, Nigrospora, Pithomyces, Torula, Tripospermum* and *Verticillium* were only isolated from samples during the first analysis and not after cold storage (Table 1.).

3.4. Discussion

Many of the fungi isolated from the nine samples of cowpea seeds during the present investigation have been recorded previously on cowpea (Esuruoso, 1975; Shama *et al.*, 1988; Ushamalini *et al.*, 1998). To this end, Esuruoso (1975) recorded that *Aspergillus flavus, A. niger, Fusarium verticillioides, Fusarium solani, Penicillium digitatum* and *Rhizopus arrhizus* occurred on the 12 different cowpea varieties tested and on nearly all the 81 samples examined in Western Nigeria. Furthermore, *F. verticillioides, Fusarium oxysporum, A. niger, Colletotrichum gleosporiodes* (Penzig) Penzig and Saccardo and *Penicillium* spp. were the predominant fungi isolated from cowpea seed samples assayed for seed-borne fungi in India (Shama *et al.*, 1988), while Ushamalini *et al.* (1998) isolated *Alternaria alternata, A. flavus, A. niger, Penicillium* spp., *Macrophomina phaseolina* and *Rhizopus stolonifer*. During the present investigation, it was

found that in either/both analyses, *Aspergillus* and *Penicillium* spp. were present on all the samples tested whereas *Rhizopus* spp. was found on five samples. Certain fungi were isolated from the samples analysed prior to cold storage (*Gilmaniella, Cladosporium, Curvularia, Nigrospora, Pithomyces, Torula, Tripospermum* and *Verticillium* spp.) whereas others were isolated from both analyses. There are numerous reports on the longevity of fungi during storage. Dungan and Koehler (1944) recorded that *Fusarium graminearum* in maize seeds, died out completely in two years, while some of the seeds still harboured viable *F. verticillioides* after eight years. *F. oxysporum* in red clover seeds could still be isolated after six years of storage in air-tight containers (Narkiewicz-Jodko, 1974). *Alternaria* spp., which are commonly encountered in seed, are known to survive five to six years during seed storage (Neergaard, 1977). The longevity of fungi is, however, dependent on the storage conditions and the contradictory results on the longevity for each individual fungus species may occur due to the differing environmental conditions (Neergaard, 1977). Lutey and Christensen (1963) reported that *Fusarium* spp. and *Alternaria* spp. kept for a few months at 20 °C, died out and was reduced, respectively. In the present study *Aspergillus*, *Alternaria* and *Fusarium* spp. showed, although not significant in most cases, a general increase in infection after the three-year storage period. This would substantiate Esuruoso's (1975) opinion that seed-borne species of *Aspergillus* and *Penicillium* can be of considerable importance in the deterioration of stored cowpea seeds (Esuruoso, 1975).

The high incidence of *Aspergillus*, *Penicillium* spp. and the presence, although low, of *Fusarium* spp. recorded from the two analyses and especially from the second analysis where the seed had been stored in aluminium foil sachets under optimum storage conditions, is of great importance since these genera contain certain species that are known to be potentially mycotoxic (Coker, 1994; Moss, 1996), posing potential adverse health implications to both farm animals and humans.

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Table 1. Seed-borne fungi (percentage) present on cowpea seeds before and after cold storage (± 5 °C for 3 years).

	CULTIVARS																	
	1		2		3		4		5		6		7		8		9	
FUNGI	1996	1999	1996	1999	1996	1999	1996	1999	1996	1999	1996	1999	1996	1999	1996	1999	1996	1999
Acremonium	0.00^{A}	0.00^{A}	0.00^{A}	0.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	2.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Alternaria	8.33^{A*}	6.67^{A}	5.50^{A}	14.00^{B}	0.50^{A}	3.00^{A}	0.00^{A}	5.00^{A}	0.00^{A}	0.00^{A}	5.50^{A}	2.00^{A}	8.25^{A}	7.00^{A}	13.75^{A}	26.00^{B}	0.00^{A}	0.50^{A}
Aspergillus	0.33^{A}	3.33^{B}	2.00^{A}	5.00^{A}	1.50^{A}	1.25^{A}	0.75^{A}	17.00^{B}	0.50^{A}	1.50^{A}	0.25^{A}	0.50^{A}	0.25^{A}	3.00^{A}	6.0^{B}	1.0^{A}	0.25^{A}	5.00^{A}
Chaetomium	10.67^{B}	0.00^{A}	0.50^{A}	0.50^{A}	0.00^{A}	1.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.50^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	1.00^{A}	0.00^{A}	0.00^{A}
Cladosporium	0.00^{A}	0.00^{A}	0.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Curvularia	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Fusarium	0.33^{A}	0.00^{A}	0.00^{A}	6.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.50^{A}	0.00^{A}	0.00^{A}	2.25^{A}	3.00^{A}	0.00^{A}	0.00^{A}
Gilmaniella	0.00^{A}	0.00^{A}	1.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}
Nigrospora	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Penicillium	0.00^{A}	0.00^{A}	0.25^{A}	1.50^{A}	19.00^{B}	0.50^{A}	0.25^{A}	16.00^{B}	3.75^{A}	1.00^{A}	1.25^{A}	2.00^{A}	3.00^{A}	1.00^{A}	1.75^{A}	2.50^{A}	0.50^{A}	1.00^{A}
Phoma	0.00^{A}	0.00^{A}	0.00^{A}	5.50^{A}	0.25^{A}	0.00^{A}	1.00^{A}	1.00^{A}	0.00^{A}	1.00^{A}	14.50^{B}	0.00^{A}	3.50^{B}	0.00^{A}	1.00^{A}	5.00^{A}	0.00^{A}	0.00^{A}
Pithomyces	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Rhizoctonia	0.00^{A}	0.00^{A}	0.00^{A}	1.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	3.50^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Rhizopus	0.00^{A}	0.00^{A}	0.75^{A}	5.50^{A}	0.00^{A}	0.50^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	1.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.75^{A}	2.50^{A}	0.00^{A}	0.00^{A}
Torula	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	2.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Tripospermum	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Verticillium	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.25^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}	0.00^{A}
Unidentified	5.67^{B}	0.67^{A}	12.50^{A}	5.00^{A}	1.25^{A}	0.50^{A}	3.50^{A}	18.00^{A}	0.00^{A}	3.00^{A}	2.50^{A}	0.50^{A}	2.50^{B}	0.00^{A}	13.75^{B}	0.00^{A}	0.00^{A}	0.00^{A}

* Each value is a mean of 4 replicates. Values within two successive rows of each cultivar, 1996 and 1999, not followed by the same letter are significantly different ($P = 0.05$) according to the student's t test.

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CHAPTER 4

Detection of fumonisins in cowpea (*Vigna unguiculata* (L.) Walp) seeds

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ABSTRACT

Cowpeas (*Vigna unguiculata* (L.) Walp) are important nutritious legume crops for many subsistence farmers and rural communities. In tropical and sub-tropical Africa, cowpeas are often stored at high relative humidities and high ambient temperatures and are susceptible to fungal contamination. Some of these fungi produce mycotoxins, which can have adverse effects on the health of both farm animals and humans. Eight cowpea seed samples from four different cultivars were analysed for the *Fusarium* mycotoxins, fumonisins B_1, B_2 and B_3. Samples were extracted with methanol/water (70:30) and cleaned-up on strong anion exchange solid phase extraction cartridges. High-performance liquid chromatography with pre-column derivatisation using o-phthaldialdehyde was used for the detection and quantification of fumonisins B_1, B_2 and B_3. The analyses revealed that all eight samples were contaminated with fumonisin B_1 at levels ranging between 81 and 1002 ng g^{-1}, whereas no fumonisins B_2 and B_3 were detected. It is believed that this is the first report of the natural occurrence of fumonisin B_1 in cowpea seeds. Since none of the known fumonisin-producing fungi were isolated from the cowpea seeds, it is necessary to identify which species are responsible for toxin production.

Keywords: cowpeas, health risk, fumonisin B_1, *Fusarium*, mycotoxins, *Vigna unguiculata*.

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INTRODUCTION

Cowpeas (*Vigna unguiculata* (L.) Walp.) are regarded as popular and important indigenous African legume crops by many rural communities living in less developed countries of tropical and sub-tropical Africa. They are grown as a pulse, vegetable, fodder and as a cover crop (Ushamalini et al., 1998). Cowpeas are mainly consumed as a favourite foodstuff in the form of dried seeds, either as flour or split (Johnson and Raymond, 1964; van Wyk and Gericke, 2000). They are a good source of carbohydrates, vitamins and protein, providing more than half of plant protein in human diets in some areas of the semi-arid tropics (Singh et al., 1997; Tuan and Phillips, 1992).

It is well known, however, that cowpea seeds are susceptible to fungal contamination when poorly stored at high relative humidities and high ambient temperatures (Esuruoso, 1975, Hitokoto et al., 1981; Seenappa et al., 1983). It is also under these conditions that certain fungi may produce toxic secondary metabolites namely, mycotoxins (van Warmelo, 1967). The ingestion of mycotoxins in contaminated agricultural products can lead to detrimental health problems for humans and farm animals (Desjardins and Hohn, 1997; Moss, 1996). Mycotoxins exhibit properties of acute, sub-acute and chronic toxicities, leading to interferences with the functioning of various body systems (Coker, 1994; Saber et al., 1998). Furthermore, they are capable of causing mutations and deformities in developing embryos (Saber et al., 1998).

Fumonisins are the most recently characterised mycotoxins that have major significance in human health (Moss, 1996). They are primarily produced by *Fusarium verticillioides* (Sacc.) Nirenberg, *Fusarium proliferatum* (Matsushima) Nirenberg and *Fusarium nygamai* Burgess and Trimboli (Coker, 1994; Marasas, 1994). Fumonisins are acutely toxic to the liver and kidney (Desjardins and Hohn, 1997). They are amino polyalcohols that inhibit the activity of sphingosine N-acetyltransferase that leads to the accumulation of toxic sphingoid bases (Desjardins and Hohn,

1997). Various fumonisins have been isolated and characterised (Musser 1996), of which fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are the most important analogues found in contaminated maize (Shephard et al., 1996). FB_1 and FB_2 are known to be toxicologically significant. FB_1 has been known to cause leukoencephalomacia (LEM), a fatal brain disease in horses (Coker, 1994; Desjardins and Hohn, 1997; Marasas, 1996) and pulmonary edema syndrome (PES) in pigs (Marasas, 1996). FB_1 is also toxic to the central nervous system, liver, pancreas, kidney and lung in numerous animal species (Coker, 1994). Furthermore, it is a cancer promoter and initiator in rat liver, hepatotoxic to horses, pigs, rats and vervet monkeys and phytotoxic to several plants (Marasas, 1995; 1996). Lastly, FB_1 has been statistically linked to the incidence of human oesophageal cancer rates in Transkei, South Africa and China (Marasas, 1996). FB_1 has been classified as a group 2B carcinogen by the International Agency for Research on Cancer (IARC) who considers it to be possibly carcinogenic to humans (Vainio et al., 1993).

There are various reports concerning mycotoxins associated with legume seeds, including chickpea (*Cicer arietinum* L.) (Ahmad and Singh, 1991), lupine (*Lupinus* spp. L.) (Abdel-Hafez, 1984), pea (Saber et al., 1998) and various types of beans (El-Kady et al., 1991; Saber, 1992; Tseng and Tu, 1997). There is, however, little literature regarding cowpea seeds and mycotoxins. Seenappa et al., (1983) found cowpea samples to be susceptible to *Aspergillus parasiticus* Speare infection, and in subsequent aflatoxin contamination. There is no report, however, concerning the presence of fumonisins in cowpea seeds.

This paper deals with the detection and quantification of the *Fusarium* toxins, specifically FB_1, FB_2 and FB_3 in cowpea seeds.

MATERIALS AND METHODS

Apparatus.

a) Liquid chromatography – Waters 6000 A pump (Waters Corp., Milford, MA 01757) and Rheodyne injector.

b) Fluorescence detector – Waters Fluorescence 474 set at 335 nm (excitation) and 440 nm (emission) (Waters Corp., Milford, MA 01757).

c) Column - Phenomenex Ultracarb 5 ODS (20) (150 x 4.6 mm id.).

d) Integrator - Borwin Chromatography Software 1.22 (JMBS Developments, France).

e) Solid-phase extraction (SPE) columns - Chromabond® Strong anion exchange (SAX) cartridges, 6 ml capacity, containing 500 mg SiOH (Machery-Nagel, Dűren, D-52313).

f) SPE manifold - 12-place vacuum manifold (Lida)

g) Reacti-Therm™ Heating module (Pierce, Rockford, IL 61105)

h) Reacti-Vap™ Evaporator (Pierce, Rockford, IL 61105)

Reagents. Fumonisin B_1, B_2 and B_3 standards were obtained from PROMEC, Medical Research Council, Tygerberg, South Africa. All other reagents and solvents were obtained from Merck (Darmstadt, D-64271).

Seed samples. Four cultivars (Bechwana White, Glenda, Iron Grey and Rhino) approximately 100 g each of cowpea seeds were received from A. Haasbroek from the Oilseed Institute in Potchefstroom, South Africa.

Determination of seed-borne fungi. One hundred seeds were randomly chosen from each sample. Prior to plating out, 50 seeds from each sample were surface-sterilised in 1% sodium hypochlorite for 1 min. The remaining 50 seeds from each sample were not surface-sterilised. The seeds were plated out on malt extract agar (MEA) consisting of 15 g malt extract (diastase free), 17 g Bacto agar, 1000 ml distilled H_20 and 0,125 g novobiocin. The plates were incubated

at 25 °C for 5-7 days. The fungi were isolated, identified with the aid of various references (Samson et al., 1981; Nelson et al., 1983; Watanabe, 1994) and recorded. The *Fusarium* spp. were identified by Dr. J. P. Rheeder of PROMEC, Medical Research Council, Tygerberg, South Africa.

Sample preparation, extraction and clean-up. Cowpea seeds of the four different cultivars were used as samples. The sample extraction and clean-up was based on the method described by Sydenham et al. (1992) and was carried out at the Department of Botany, University of Pretoria, Pretoria, South Africa. Approximately 50 g of seeds from each sample were ground using a coffee grinder and 20 g of the grinded seeds weighed. After adding 100 ml 70% (v/v) methanol, the ground samples were homogenised for 3 min at 5000 rpm using a hand-held mixer. The samples were then centrifuged for 10 min at 4000 rpm and filtered through Whatman No. 4 filter paper. The pH of the filtrate was measured and adjusted with 0.1 M NaOH to between ph 5.8-6.5. Clean-up and extraction of the filtrate was carried out on strong anion exchange (SAX) cartridges attached to a solid phase extraction (SPE) manifold. Prior to adding 10 ml of the filtrate, the SAX cartridges were conditioned by washing successively with 5 ml 100% methanol followed by 5 ml 70% (v/v) methanol, whilst maintaining a flow rate of 1 ml min^{-1}. Cartridges were then washed with 5 ml 70% (v/v) methanol and 3 ml 100% methanol. This was followed by elution with 10 ml 1% (v/v) methanolic acetic acid at a flow rate of 1 ml min^{-1} and the eluate collected in vials. Eluates then were evaporated to dryness in vials on a Reacti-Therm heating module and evaporator at 50 °C under a slight stream of nitrogen (AFROX). The collection vials were washed with methanol and the additional methanol was evaporated until a dry residue formed. The dry residues were maintained at 4 °C until used for high performance liquid chromatography.

High Performance Liquid Chromatography (HPLC). The HPLC analyses were undertaken at PROMEC, Medical Research Council, Tygerberg, South Africa. A derivatisation

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agent, *o*-phthaldialdehyde (OPA), was added to both the standards and samples prior to HPLC. This is necessary since fumonisins are unable to absorb either UV or visible light and are unable to fluoresce. OPA derives the fluorescent products from the fumonisins (Sydenham et al. 1992). OPA (225 µl) was added to 25 µl of the standard and 10 µl was injected into the HPLC, whilst 150 µl OPA was added to 100 µl of the sample (which had been redissolved in 200 µl CH_3CN: H_2O) and 50 µl was injected into the HPLC (Sydenham et al., 1992).

RESULTS AND DISCUSSION

The percentage of fungi isolated from each sample was higher in the untreated seeds than in the surface-sterilised seeds (Table 1.). The most fungi was isolated from Iron Grey (98% infection) followed by Rhino, Bechwana White and then Glenda with 94%, 92%, and 88% infection, respectively. In the surface-sterilised seeds, the most fungi were isolated from Rhino (68% infection) followed by Iron Grey (52% infection). Glenda and Bechwana White had low counts of fungal colonies (8 and 4% infection, respectively). The most common fungi found included members of the genera, *Aspergillus* and *Phoma*, present in both surface-sterilised and untreated seeds in all four samples. *Aspergillus glaucus* Link ex Gray was the most predominant species, present in three samples, followed by both *Aspergillus flavus* Link ex. Fries and *Aspergillus niger* van Tieghem. Seenappa et al., (1983) reported that all cowpea samples analysed were susceptible to *Aspergillus* infection and subsequent aflatoxin production.

Six *Fusarium* species were isolated of which *Fusarium equiseti* (Corda) Sacc. appeared to be the most dominant. Four of these *Fusarium* species were present in the Rhino seeds, two in the Bechwana White sample and one species in the Glenda sample. An interesting occurence can be noted here. While the most important fumonisin-producing species are *F. verticillioides* and *F.*

proliferatum (Coker, 1994; Marasas, 1994), neither of these two species were isolated from the samples. However, Esuruoso (1995) recorded *F. verticillioides* on nearly all cowpea samples (81) examined. Other *Fusarium* species known to produce high concentrations of other mycotoxins but not fumonisins, including *F. equiseti, F. sambucinum* Fuckel and *F. subglutinans* (Wollenw. and Reink.) Nelson, Toussoun, and Marasas were isolated. Further research is required to identify the fungal species present on cowpea seed responsible for the fumonisin production. Other fungal genera isolated from the samples included *Chaetomium, Cladosporium, Penicillium* and *Trichothecium* spp.. *Penicillium* spp. are also known to produce mycotoxins including ochratoxins (Moss, 1996) and citrinin (Pitt, 1998).

From the eight samples analysed for *Fusarium* toxins, specifically FB_1, FB_2 and FB_3, FB_1 was found to be present in all the samples (Table 2.), while FB_2 and FB_3 were not detected. The highest concentration of FB_1 was found in the Rhino A cultivar (1002 ng g^{-1}), followed by Rhino B (213 ng g^{-1}), Bechwana White A (178 ng g^{-1}), Glenda A and B (161 ng g^{-1}) and Iron Grey A (127 ng g^{-1}). Levels below 100 ng g^{-1}were detected in Bechwana White B and Iron Grey B. This is the first report of the natural occurrence of FB_1 on cowpea seeds.

Since large quantities of cowpea seeds are produced and consumed in tropical and sub-tropical countries (Seenappa et al., 1983) and in light of the various toxicological consequences as a result of fungal mycotoxin contamination, a potential health risk exists for both humans and animals. It is thus essential that care is taken when seeds are stored such that fungal infestation and subsequent mycotoxin production can be effectively controlled and prevented. There are various reports concerning the antifungal activity of essential plant oils (Adegoke and Odesola, 1996; National Research Council, 1992) which can be used as an alternative approach to controlling and preventing fungal contamination of cowpea seeds.

Table 1. Percentages of fungi isolated from four cultivars of cowpea seeds

Fungi	Cultivar							
	Glenda		Bechwana White		Rhino		Iron Grey	
	+[a]	-[b]	+	-	+	-	+	-
Aspergillus flavus	4	10		26				2
A. glaucus		4			8	8	40	68
A. niger		18		14			4	2
Chaetomium sp.	2	2					2	2
Cladosporium sp.		18		14			2	
Diplodia sp.		4						
Fusarium chlamydosporum				2				
F. equiseti		2			2	10		
F. graminearum						2		
F. sambucinum						2		
F. scirpi					6			
F. subglutinans				2				
Penicillium sp.		4				32		16
Phoma sp.	2	14	4	28	52	36	2	
Trichothecium roseum		2		2				2
Other		10		4		4	2	6
Total % infection	8	88	4	92	68	94	52	98

[a] surface-sterilised seeds, [b] untreated seeds

Table 2. Fumonisin concentrations in cowpea seed cultivars

Cultivar	Fumonisin concentration (ng g^{-1})		
	FB_1	FB_2	FB_3
Bechwana White A	178	0	0
Bechwana White B	81	0	0
Glenda A	161	0	0
Glenda B	161	0	0
Iron Grey A	127	0	0
Iron Grey B	99	0	0
Rhino A	1002	0	0
Rhino B	213	0	0

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CHAPTER 5

EFFECT OF ESSENTIAL PLANT OILS ON STORAGE FUNGI, GERMINATION AND EMERGENCE OF COWPEA SEEDS

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Summary

The production of cowpea (*Vigna unguiculata* (L.) Walp) provides many subsistence farmers and rural communities of tropical and sub-tropical Africa with a nutritious food, animal feed and a source of income. Fungal invasion of the seeds inhibits the optimal utilisation of the seeds. Some storage fungi are known to produce mycotoxins that can adversely affect the health of farm animals and humans. Chemical control of storage fungi can have serious harmful effects on the community and environment. The antifungal activity of plant essential oils is a widely exploited alternative approach. The antifungal activity of the essential oils of thyme, clove, peppermint, soybean and peanut were tested against *Aspergillus flavus, A. niger, Fusarium oxysporum, F.*

equiseti and Penicillium chrysogenum in vitro. Thyme and clove significantly inhibited growth of all five fungi at 500 and 1000 ppm. Peppermint oil successfully inhibited growth at 2000 ppm. Soybean and peanut oil did not appear to exhibit any antifungal properties. Soybean oil stimulated growth of *F. equiseti*. Thyme, clove and peppermint were tested *in vivo* against storage fungi on naturally infected (cultivars PAN 325 and PAN 311) and artificially infected (cultivar CH 14) cowpea seed. Only thyme oil (1000 ppm) showed antifungal activity against storage fungi associated with PAN 325 cowpea seeds. In the PAN 311 cultivar, clove and thyme at 1000 ppm and peppermint at 2000 ppm exhibited antifungal activity against the storage fungi. In cultivar CH 14, thyme, clove and peppermint significantly reduced growth of *P. chrysogenum* whilst thyme and peppermint inhibited growth of *F. oxysporum*. Only thyme had an antifungal effect on *F. equiseti*. No treatment showed antifungal activity against *A. flavus* and *A. niger*. None of the oils showed harmful effects on the germination and emergence of cowpea seeds. The storage fungi significantly reduced percentage germination and emergence of the white (IT 93K452-1) seed but had little or no effect on the brown (CH 14) seed. Furthermore, all three oils significantly inhibited the storage fungi on the white seed thereby increasing the percentage germination and emergence.

Introduction

Cowpea seeds (*Vigna unguiculata* (L.) Walp) are susceptible to fungal contamination especially when stored under poor conditions together with high relative humidities and high temperatures (Esurouso, 1975; Seenappa *et al.*, 1983). It is well known that storage fungi are one of the several causes leading to a decrease in germination of seeds (Neergaard 1977). Fields and King (1962) reported that pea seed kept at 85% relative humidity and 30 °C lost germination capacity within six months when invaded by *Aspergillus* spp. Uninfected pea seed maintained a germination percentage of 95%.

Control measures to prevent fungal contamination and post-harvest losses include the control of environmental factors (temperature and moisture) (Mislivic, 1998) and chemical control (Bauer, 1994; Codifier *et al.*, 1976; Hasan, 1998; Megella and Hafez, 1982) including ammonia, which has been exploited commercially (Bauer, 1994; Coker, 1994). Since chemicals have potential harmful effects on the environment and the community, investigation into the antifungal properties of essential plant oils has attracted wide interest. There have been many reports regarding the antifungal properties of plant essential oils. Some of these oils include thyme (*Thymus vulgaris* L.) (Thompson and Cannon, 1986; Zambonelli *et al.*, 1996); cinnamon (*Cinnamomum zeylanicum* Blume) (Thompson and Cannon, 1986), clove (*Syzygium aromaticum* (L.) Merr. and Perry) (Thompson and Cannon, 1986; Chatterjee, 1990); pimenta (*Capsicum anuum* L.) (Thompson and Cannon, 1986), basil (*Ocimum basilicum* L.) (Chatterjee, 1990) and extracts from garlic (*Allium sativum* L.) bulbs, green garlic and green onions (*Allium fistulosum* L. var. caespitosum, scallion) (Yin and Cheng, 1998). Neem (*Azadirachta indica* A. Juss) leaf extract has been reported to protect chickpea (*Cicer arietinum* L.) seeds against serious fungal diseases (*Rhizoctonia solani* (Ehrenb.:Fr) Vuill., *Sclerotium rolfsii*), to slow down growth of

Fusarium oxysporum Schecht ex. Fries and completely stopped *Aspergillus flavus* Link ex. Fries from producing aflatoxin (National Research Council, 1992). Some investigations have been carried out to determine the effectiveness of various essential plant oils on the inhibition of fungi causing cowpea seed spoilage and possible mycotoxin production. Adegoke and Odesola (1996) reported that samples of cowpea seeds treated with lemon grass (*Cymbopogon citratus* (DC. ex Nees) Stapf.) powder and essential oil showed no physical deterioration or mouldiness. Furthermore, it inhibited the fungal growth of fungi like *A. flavus* and *Penicillium chrysogenum* Thom. Montes-Belmont and Carvajal (1998) recorded that thyme, clove, peppermint, basil and cinnamon oils did not inhibit germination of maize seeds or subsequent seedling growth. Similarly, Cruz and Cardona (1981) found that soybean (*Glycine max* (L.) Merrill) oil caused no reduction of germination in cowpea seeds.

This paper deals with the inhibitory effect of five essential plant oils on the growth of potentially mycotoxigenic and other storage fungi causing cowpea seed spoilage. The control of these fungi by treating cowpea seed with the essential oils of clove, thyme and peppermint is evaluated and the effect on the germination and emergence of the treated seed is investigated.

Materials and methods

1. Fungal species

The fungi used in these investigations were chosen from the range of fungi isolated from cowpea seeds during previous experiments carried out at the Department of Botany, University of Pretoria, Pretoria, South Africa. These fungi were found to be the predominant fungi associated with cowpea seed and could be potential mycotoxic species. The fungi chosen were *A. flavus*, *A.*

niger van Tieghem, *P. chrysogenum*, *F. oxysporum* and *F. equiseti* (Corda) Sacc. These fungal cultures were maintained on potato dextrose agar (PDA) at ± 25 °C under constant light.

2. *Seed samples*

Two cowpea cultivars, PAN 311 (brown seed) and PAN 325 (white seed), were obtained from the Vegetable and Ornamental Plant Institute (VOPI), Roodeplaat, Pretoria, South Africa. The cultivars, CH 14 (brown seed) and IT 93K452-1 (white seed), were obtained from the Agricultural Research Council (ARC) - Grain Crops Institute, Potchefstroom, South Africa.

3. Essential plant oils

The essential oils used during these investigations were thyme, clove, peppermint (*Mentha piperita*), peanut (*Arachis hypogaea* L.) and soybean. The oils were purchased at The Health Shop, Atterbury Value Mart, Pretoria, South Africa. The thyme, clove and peppermint oils were Escentia products and the peanut and soybean oils, Natures Choice products.

4. Effect of oils on seed-borne and storage fungi

a. In vitro *tests*

The *in vitro* tests were based on the method described by Zambonelli *et al.* (1996). The oils were dissolved in 100% ethanol and then immediately added to autoclaved PDA, before being poured into sterile Petri dishes (9 mm diameter.). The oils were initially tested at concentrations of 100 and 1000 ppm. Based on these results, the thyme and clove oils were further tested at 500 ppm and peppermint, peanut and soybean, at 2000 ppm. There were two controls used during this investigation. The one received the same quantity of ethanol used to dissolve the oils, mixed with the PDA. In the second control, no ethanol was mixed with the PDA. There were four replicates

for each oil concentration. Inoculation of the prepared Petri dishes was carried out by placing a disk (5 mm diameter) from the rim of a 7-day-old culture in the centre of the Petri dish. The Petri dishes were then incubated at ± 25 °C. Fungal growth was measured on two preset diametral lines after three, six and nine days. The 6-day growth measurements (mm) were statistically analysed.

b. In vivo *tests*

Naturally infected cowpea seed

Based on the results from the *in vitro* tests, it was decided to test the thyme, clove and peppermint oil *in vivo*. The 4 replicates of 25 seeds were surface-sterilised for 1 min using 1% sodium hypochlorite, rinsed three times with sterile distilled water and allowed to dry. Seeds of PAN 311 and PAN 325 were treated with the thyme and clove oils at a concentration 1000 ppm and peppermint at a concentration of 2000 ppm. The required volume of oil (dissolved in 100% ethanol) was added to sterile distilled water and the seeds were allowed to soak for approximately 5 min. The control received the same amount of 100% ethanol. After a brief drying period (± 5 min), the seeds were plated directly onto PDA plates (five seeds per plate and six plates per replicate). After five days of incubation at ± 25 °C, the seed-borne fungi present were recorded.

Artificially infected seed

Four replicates of 25 seeds (cultivar CH 14) were surface-sterilised as described above. Seeds were inoculated with each of the five test fungi as follows: For each fungus, a spore suspension of 1 x 10^6 spores ml^{-1} was prepared by adding 20 ml sterile distilled water to a 7-day-old culture. The surface of the culture was scraped to free the spores. The spore suspensions were poured through muslim cloth into flasks. The seeds were added to the suspensions and mixed thoroughly. Thereafter, the seeds were allowed to dry for ± 5 min. The seeds were treated with thyme and clove oils at a concentration of 1000 ppm and peppermint at 2000 ppm according to the following procedure: The required volume of oil (dissolved in 25 µl 100% ethanol) was added to sterile

distilled water and the seeds were allowed to soak for ± 5 min. The control was also inoculated and was treated with the same amount of ethanol used to dissolve the oils. After a brief drying period, the seeds were plated directly onto PDA plates (five seeds per plate with twenty plates per replicate). The fungal colonies were counted after five days of incubation at ± 25 °C.

5. Effect of seed-borne and storage fungi and oils on seed germination

Seeds of CH 14 and IT 93K452-1 were surface-sterilised as previously described. After a short drying period, seeds were inoculated with a 1 x 10^6 spore suspension of a mixture of the five test fungi in equal proportions. Thereafter, the seeds were dried and treated with thyme and clove oils, at a concentration of 1000 ppm and peppermint, at 2000 ppm. The method used for seed inoculation and oil treatment of the seeds was as for the *in vivo* tests on artificially inoculated seeds. The controls included inoculated seed without oil treatment (simulating seed naturally infected with storage fungi) and the uninoculated control. Uninoculated seeds were also treated with each of the three oils. Percentage germination was determined by placing four replicates of 100 seeds each between moist paper towels which were rolled up and placed individually in polythene bags, held upright in plastic beakers and maintained at ± 25 °C in an incubator. Percentage germination was determined after 4 and 8 days according to the International Seed Testing Association (ISTA) rules (International Seed Testing Association 1999).

6. Effect of seed-borne and storage fungi oils on seed emergence

Seeds of CH 14 and IT 93K452-1 were surface-sterilised as previously described. Seed inoculation and oil treatments were carried out according to the method used in the germination tests. The two controls included inoculated seed without oil treatment and uninoculated seeds. Uninoculated seeds were treated with each of the three oils as described above. Potting soil was

used to fill 128-cell plastic seedling trays (10 cm x 3 cm x 3 cm). A single seed was planted at a depth of 2 cm in each cell. There were four replicates of 25 seeds per treatment. The seedling trays were placed in a controlled environment room at 25 °C with a 12h light/dark regime. Seedling trays were watered daily. After 10 d the emerged seedlings were counted. Thereafter, the seedlings were cut at soil level and the wet and dry mass of the upper parts (stem and leaves) of seedlings within each replicate were determined and the percentage dry mass calculated.

7. *Statistical analysis*

Two-way analysis of variance (ANOVA) was performed on all the data and least significant differences ($P = 0.05$) were determined according to the student's *t* test.

Results

The results of the effect of essential oils on the five fungi *in vitro* are shown in Table 1. The essential oil of thyme significantly inhibited the growth of *A. flavus*, *P. chrysogenum*, *F. oxysporum* and *F.equiseti* at 100, 500 and 1000 ppm when compared with the controls. Thyme reduced growth of *A. niger* significantly at 500 and 1000 ppm but not at 100 ppm. Clove oil significantly inhibited growth of *P. chrysogenum*, *F. oxysporum* and *F. equiseti* at all three concentrations tested. Only concentrations of 500 and 1000 ppm significantly reduced growth of *A. flavus* and *A. niger*. The essential oil of peppermint only showed a successful inhibitory effect on all the fungi at a concentration of 2000 ppm when compared with the controls. Peppermint oil, at a concentration of 100 ppm, did not appear to have any inhibitory effect on the fungal growth. On the contrary, growth of *F. equiseti* was stimulated by this oil. The variable results obtained at 100 ppm could possibly be due to the fact that since the volume of oil used was small, the oil

dispersed unevenly through the plate. The oils of both peanut and soybean had no inhibitory effect on the growth of the five fungi tested.

The results of the effect of thyme, clove and peppermint oils on the five tested fungi and other storage fungi present on naturally infected cowpea seed are represented in Table 2. There was no significant inhibition of any of the five fungi tested by the three oils in both cultivars when compared with the control.Thyme oil did, however, reduce the total incidence of fungi significantly on both cultivars and was especially effective on PAN 311. Peppermint and clove oil also significantly reduced the total incidence of fungi in PAN 311. The other seed-borne fungi included *Rhizopus*, *Phoma*, *Alternaria* and other *Fusarium* and *Aspergillus* species.

The results of the percentage of fungi isolated from artificially inoculated cowpea seed are presented in Table 3. There was no inhibitory effect by any of the essential oils on the growth of *A. flavus* and *A. niger* when compared with the control. Clove, peppermint and thyme did, however, significantly inhibit the growth of *P. chrysogenum* and growth of *F. oxysporum* and *F.equiseti* was significantly inhibited on seeds treated with thyme oil. Peppermint and thyme oil significantly inhibited the growth of *F. oxysporum* when compared with the control.

There were no significant differences noted in percentage germination of the brown cowpea seeds (CH 14) when compared to the controls (Table 4.) In the white cultivar (IT 93K452-1), the percentage germination of the uninoculated seeds treated with the oils (thyme, peppermint and clove) did not differ significantly from the uninoculated control but was significantly higher than that of all the inoculated treatments. The percentage germination of the inoculated brown seeds was significantly higher than that of the inoculated white seeds. In the uninoculated treatments, brown seeds treated with thyme and clove had a significant higher percentage germination than those of the white seeds. The latter had a higher percentage germination when treated with peppermint and clove.

The percentage of diseased seeds was generally lower in the brown seeds than in the white seeds. No significant difference in diseased seeds is shown in the brown seeds whereas a significant increase is shown in all the treatments of the white seeds when compared with both the controls. The percentage diseased seeds was significantly lower in the brown seed when compared with the white seed, except in the case of the uninoculated control and the uninoculated seeds treated with clove and peppermint oils.

Only inoculated brown seed treated with clove showed a significant decrease in emergence when compared with the other treatments (Table 4.). All inoculated treatments in the white seed showed a significant increase of percentage emergence when compared with the inoculated control. There was no significant differences in percentage emergence of both the brown and white seed in the uninoculated treatments when compared to the uninoculated controls. In the white seed, percentage emergence of inoculated treatments was, however, severalfold more than that of the inoculated control. The essential oils had no effect on the uninoculated treatments. No significant differences are shown in the percentage dry mass between the treatments of both cultivars when compared with the uninoculated control and between the two cultivars (Table 4.).

Discussion

The trend shown in the results of the effect of plant essential oils on the growth of fungi *in vitro*, was that at all the concentrations tested in the present study, thyme oil significantly suppressed fungal growth. It was reported by Zambonelli *et al.* (1996) that thyme was the most effective oil against the inhibition of fungal growth of pathogenic fungi. Its fungicidal activity is attributable to the compound thymol, which was found at 50.06% in the oil tested (Zambonelli *et al.*, 1996). In the present study, clove oil also significantly inhibited the fungal growth of all the fungi tested

except for *A. flavus* and *A. niger* at a concentration of 100 ppm. These results of inhibition by thyme and clove oils confirms those of Thompson and Cannon (1986) who found that these two oils inhibited mycelial growth of *A. flavus* at both 500 and 1000 ppm.

In the present study, peppermint oil appeared to have a significant inhibitory effect at the higher concentrations (1000 and 2000 ppm) tested, with the exception of *A. niger* at 1000 ppm. Zambonelli *et al.* (1996) documented that mint oil was more effective at higher concentrations (1600 ppm). During the preent investigation, it was evident that the oils of both peanut and soybean were generally not effective at inhibiting fungal growth, which contradicts McGee and Misra's findings (1988) that soybean oil decreased the incidence of storage fungi in maize (*Zea mays* L.) and soybean.

In the *in vivo* tests on naturally infected cowpea seeds, the essential oils of thyme, clove and peppermint showed significant antifungal activity on PAN 311 seeds, while only thyme oil showed significant antifungal activity on the PAN 325 seeds. Although these oils did not significantly inhibit growth of the five fungi tested for in this test, they did reduce the total amount of fungi in both cultivars. In artificially inoculated seeds *P. chrysogenum* incidence was reduced by clove, peppermint and thyme whereas no inhibition of the incidence of *A. flavus* and *A. niger* occurred. Thyme and peppermint showed antifungal activity against *F. oxysporum* and with the exception of peppermint oil, *F. equiseti*. Chatterjee (1990) found that clove oil (30 µl g^{-1} grain and above) inhibited the *in vivo* growth of *A. flavus*, *Curvularia pallescens* Boedijn and *Chaetomium indicum* Corda on maize grains during storage. Furthermore, Montes-Belmont and Carvajal (1998) reported that cinnamon, peppermint, basil, origanum (*Origanum vulgare* L.), clove and thyme oils caused total inhibition of *A. flavus* in maize.

It is evident from the results of the percentage germination and emergence of the two cultivars that the oils did not reduce or adversely affect the germination or emergence of seeds, supporting

the findings of by Montes-Belmont and Carvajal (1998). Those authors found that clove, thyme and peppermint oils did not have an inhibitory effect on maize seed germination at a dosage of 10%. In the white cultivar, the percentage germination in uninoculated seeds was higher than the treatments that were inoculated. Since the invasion of storage fungi in seeds causes a decrease in seed germination (Neergaard 1977), storage fungi could have been responsible for the severe decrease in germination and impaired emergence of the white seed. Treating the inoculated white seed with the oils appeared to greatly reduce the effect of the storage fungi on the seeds, resulting in an increase in the percentage emergence. The lower percentage germination than seeds that actually emerged in the greenhouse experiments was probably due to the warm moist conditions in the paper towels, which favoured seed deterioration by the storage fungi.

It was also noted that PAN 311 (brown seeds) had a lower incidence of fungi than the white seeds, PAN 325. The percentage diseased seeds of CH 14 (brown seeds) was also generally lower than the white seeds (IT 93K452-1). Legesse and Powell (1992) found that cream/beige seeds of cowpea were more susceptible to infection by *Pythium* sp. than coloured (brown) seed. This could be attributed to the higher tannin content in dark seeds (Morrison *et al.*, 1995) since Mixon and Sanders (1979) reported that seed coat tannins of *Arachis hypogaea* inhibited growth of *Aspergillus parasiticus* Speare. Furthermore, Legesse and Powell (1992) reported that differences in the rate of water uptake and incidence of inbibition damage in cowpea cultivars was associated with testa colour, cream/beige seeds being more prone to imbibition damage than dark seeds. Imbibition damage also enhances predisposition of cowpea to pre-emergence mortality in unsterilised soil (Legesse and Powell 1992).

Although the essential oils of clove and peppermint controlled the storage fungi in one of the cultivars tested in naturally infected seed and inhibited growth of *P. chrysogenum*, thyme oil seemed more effective in controlling storage fungi and more specifically, *P. chrysogenum, F.*

oxysporum and *F. equiseti*. Since thyme also does not show any harmful effects on the germination and emergence of the seed, it has potential as a natural treatment for the control of fungi on cowpea seed.

Table 1. Effect of plant essential oils on the growth of selected fungi *in vitro*.

Treatments	Fungi (colony diameter in mm)																			
	A. flavus				*A. niger*				*P. chrysogenum*				*F. oxysporum*				*F. equiseti*			
	100[1]	500[1]	1000[1]	2000[1]	100	500	1000	2000	100	500	1000	2000	100	500	1000	2000	100	500	1000	2000
Control 1	8.0*^{B}	7.9^{C}	8.0^{C}	7.9^{B}	7.9^{A}	8.0^{C}	7.9^{CB}	8.0^{C}	3.5^{CD}	2.9^{C}	3.5^{D}	2.9^{CB}	8.0^{D}	6.5^{C}	8.0^{D}	6.5^{CB}	4.4^{C}	4.5^{B}	4.4^{C}	4.5^{B}
Control 2	8.0^{B}	7.9^{C}	8.0^{C}	7.9^{B}	8.0^{A}	6.9^{B}	8.0^{C}	6.9^{BC}	3.3^{C}	3.2^{D}	3.3^{CD}	3.2^{D}	6.2^{C}	8.0^{D}	6.2^{C}	8.0^{D}	4.7^{C}	4.6^{B}	4.7^{CD}	4.6^{B}
Clove	8.0^{B}	2.7^{B}	0.0^{A}	nt	7.5^{A}	1.3^{A}	0.0^{A}	nt	2.4^{B}	0.7^{B}	0.0^{A}	nt	4.0^{B}	0.9^{B}	0.0^{A}	nt	3.6^{B}	0.0^{A}	0.0^{A}	nt
Peanut	8.0^{B}	nt	8.0^{C}	7.9^{B}	8.0^{A}	nt	7.4^{B}	8.0^{C}	4.0^{D}	nt	2.9^{C}	2.7^{B}	6.5^{C}	nt	7.7^{D}	6.8^{C}	5.4^{D}	nt	4.5^{CD}	7.4^{D}
Pepper-mint	8.0^{B}	nt	7.6^{B}	0.0^{A}	7.9^{A}	nt	7.9^{CB}	4.7^{A}	3.1^{C}	nt	2.3^{B}	0.0^{A}	6.6^{C}	nt	4.0^{B}	0.00^{A}	5.7^{D}	nt	2.2^{B}	0.0^{A}
Soybean	8.0^{B}	nt	8.0^{C}	7.9^{B}	8.0^{A}	nt	8.0^{C}	5.6^{AB}	3.7^{CD}	nt	3.4^{D}	3.0^{CD}	8.0^{D}	nt	5.7^{C}	6.3^{B}	5.8^{D}	nt	5.2^{D}	6.5^{C}
Thyme	6.9^{A}	1.4^{A}	0.0^{A}	nt	7.6^{A}	0.7^{A}	0.00^{A}	nt	1.5^{A}	0.0^{A}	0.0^{A}	nt	3.5^{A}	0.0^{A}	0.0^{A}	nt	2.7^{A}	0.0^{A}	0.0^{A}	nt

* Each value is a mean of 4 replicates after 6 d of growth. Values within a column not followed by the same letter are significantly different (P = 0.05) according to the student's t test.

[1] Concentration in ppm
Control 1 - PDA unamended
Control 2 - PDA amended with ethanol
nt = not tested

Table 2. Number of storage fungi isolated from naturally infected cowpea seed (cultivars PAN 311 and PAN 325).

		Treatment			
Fungi	Cultivar	Control	Clove 1000 ppm	Peppermint 2000 ppm	Thyme 1000 ppm
A. flavus	PAN 311	0.0*^{A}	0.0^{A}	0.0^{A}	0.0^{A}
	PAN 325	17.3^{A}	13.3^{A}	14.7^{A}	12.0^{A}
A. niger	PAN 311	0.7^{A}	0.0^{A}	0.0^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.7^{A}	0.0^{A}
P. chrysogenum	PAN 311	0.7^{A}	0.0^{A}	0.7^{A}	0.0^{A}
	PAN 325	0.7^{AB}	0.7^{AB}	2.0^{B}	0.0^{A}
F. oxysporum	PAN 311	2.0^{A}	0.0^{A}	2.0^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}
F. equiseti	PAN 311	0.0^{A}	0.0^{A}	0.7^{A}	0.0^{A}
	PAN 325	0.0^{A}	0.0^{A}	0.0^{A}	0.0^{A}
Total[1]	PAN 311	24.0^{C}	14.0^{B}	14.0^{B}	5.3^{A}
	PAN 325	30.0^{B}	26.7^{AB}	26.0^{AB}	18.7^{A}

* Each value is a mean of 3 replicates of 30 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to student's t test.

[1] Represents all the storage fungi isolated from the cultivars, including the five fungi tested.

Table 3. Percentage of storage fungi isolated from artificially inoculated cowpea (cultivar CH 14) seeds

Fungi	Treatments			
	Control	Clove 1000 ppm	Peppermint 2000 ppm	Thyme 1000 ppm
A. flavus	100^{*A}	100^{A}	100^{A}	100^{A}
A. niger	100^{A}	100^{A}	100^{A}	100^{A}
P. chrysogenum	100^{D}	0^{A}	93^{C}	85^{B}
F. oxysporum	81^{B}	74^{B}	39^{A}	55^{A}
F. equiseti	14^{B}	10^{AB}	5^{AB}	3^{A}

* Each value is a mean percentage of 4 replicates of 25 seeds. Values within a row not followed by the same letter are significantly different ($P = 0.05$) according to student's t test.

Table 4. Effect of storage fungi and essential oils on germination and emergence of cowpea seeds (cultivars CH 14 and IT 93K452-1).

Treatment	% Germination		% Diseased seeds		% Emergence		% Dry mass	
	CH-14	IT 93K452-1	CH-14	IT 93K452-1	CH-14	IT 93K452-1	CH-14	IT 93K452-1
Inoculated Control	72.0*^{AB}**^{Y}	1.0^{AX}	19.0*^{AB}**^{X}	95.0^{DY}	86$^{\dagger BC\dagger\dagger Y}$	16^{AX}	12.13$^{A\dagger X\dagger\dagger}$	11.67^{ABX}
Clove	71.8^{ABY}	32.0^{BX}	21.3^{BX}	68.0^{CY}	75^{AY}	36^{BX}	19.47^{ABX}	8.88^{AX}
Peppermint	72.0^{ABY}	28.0^{BX}	18.3^{ABX}	72.0^{CY}	78^{ABY}	40^{CBX}	25.33^{ABX}	12.73^{ABX}
Thyme	69.0^{ABY}	23.5^{BX}	21.8^{BX}	76.8^{CY}	79^{ABY}	35^{BX}	20.92^{ABX}	10.07^{ABX}
Uninoculated Control	73.0^{ABX}	77.0^{CDX}	10.8^{AX}	18.3^{ABX}	93.0^{CX}	90.0^{DX}	26.1^{ABX}	17.1^{ABX}
Clove	76.0^{BX}	80.8^{DX}	11.3^{AX}	19.0^{ABX}	91^{CX}	83^{DX}	18.77^{ABX}	15.65^{ABX}
Peppermint	64.8^{AX}	75.3^{CDY}	14.0^{ABX}	15.8^{AX}	92^{CY}	52^{CX}	28.10^{BX}	16.25^{ABX}
Thyme	70.3^{ABX}	68.3^{CX}	11.8^{AX}	27.0^{BY}	88^{BCX}	90^{DX}	16.45^{ABX}	20.43^{BX}

* Each value is a mean percentage of 4 replicates of 100 seeds. Values within a column not followed by the same letter are significantly different (P = 0.05) according to the student's t test.

** Each value is a mean percentage of 4 replicates of 100 seeds. Values within a row not followed by the same letter are significantly different (P = 0.05) according to the student's t test.

† Each value is a mean percentage of 4 replicates of 25 seeds. Values within a column not followed by the same letter are significantly different (P = 0.05) according to the student's t test.

†† Each value is a mean percentage of 4 replicates of 25 seeds. Values within a row not followed by the same letter are significantly different (P = 0.05) according to the student's t test.

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CHAPTER 6

GENERAL DISCUSSION

The importance of cowpeas as a source of nutritious food, animal fodder and income for many relatively poor people living in countries of the tropics cannot be overemphasised. Cowpea seeds are susceptible to fungal contamination during storage under sub-optimum conditions including high temperatures and relative humidities. Due to the subsequent production of mycotoxins by certain fungi, a potential health risk exists when infected food is ingested. This problem can, however, be reduced or perhaps alleviated through the control of these fungi with the use of essential oils from various plants.

It was found that the predominant fungi associated with newly harvested cowpea seeds were *Aspergillus*, *Penicillium* and *Alternaria* spp. Almost all of the nine cultivars analysed in Chapter 3 were infected with these three fungal genera. Various other species including *Fusarium*, *Rhizopus* and *Phoma* were also isolated. These results are in agreement with those recorded by Esuruoso (1975) and Shama *et al.* (1988.) Esuruoso (1975) recorded that *Aspergillus flavus*, *A. niger*, *Fusarium verticillioides*, *F. solani*, *Penicillium digitatum* and *Rhizopus arrhizus* occurred on the 12 cowpea varieties tested. Shama *et al.* (1988) found *F. verticillioides*, *F. oxysporum* Schelcht ex. Fries, *A. niger* and *Penicillium* spp. to be the predominant fungi isolated from cowpea seed.

After the seeds were subjected to a cold (± 5 °C) storage period of three years it was noted that *Aspergillus* and *Alternaria* spp. showed a significant increase in two of the nine cultivars whilst *Penicillium* spp. showed an significant increase in one cultivar. In certain cultivars, there was a decrease in the number of fungi isolated. According to Neergaard (1977), certain fungi are

capable of remaining viable in storage for long periods of time. In the present study, some species did not occur after the cold storage period. This could be attributed to the fact that the low temperature slowed down and/or inhibited growth or caused the death of certain fungal species (Christensen, 1973). Furthermore, Lutey and Christensen (1963) found that *Fusarium* and *Alternaria* stored for a few months at 20 °C, died out and was reduced, respectively. After the three year cold storage period, the main fungal genera isolated were *Alternaria*, *Penicillium* and *Aspergillus* with *Gilmaniella*, *Cladosporium*, *Curvularia*, *Nigrospora*, *Pithomyces*, *Torula*, *Tripospermum* and *Verticillium* not being detected.

Since some of the fungi isolated from the cowpea seeds in the present study are known to be mycotoxigenic (e.g. *Aspergillus*, *Penicillium* and *Fusarium* spp.) (Desjardins and Hohn, 1997) and since there are few reports concerning mycotoxins associated with cowpea seeds, it was important to analyse the seeds for the presence of mycotoxins. Seenappa *et al.*, (1983) did however, document the susceptibility of cowpea seeds to *Aspergillus* infection, with subsequent aflatoxin production. Four cultivars of cowpea seeds were analysed during the present study for the presence of FB_1, FB_2, and FB_3. Only FB_1 was detected in all eight samples analysed. The levels ranged from 81-1002 ng g^{-1}. Further studies are necessary to determine the This is to be believed to be the first report of a new natural occurrence of FB_1 in cowpea. Further investigations are required to determine the *Fusarium* sp. responsible for the FB_1 production, since the known toxin-producing *Fusarium* spp. (*F. verticillioides* and *F. proliferatum*) were not isolated from any of the cowpea seeds used during this investigation. The *Fusarium* spp. isolated in this study included *F. equiseti*, *F. sambucinum* Fuckel, *F. chlamydosporum* Wollenweber and Reinking, *F. graminearum*, *F. scirpi* Lambert and Fautrey and *F. subglutinans* (Wollenw. and Reink) Nelson, Toussoun, and Marasas.

The investigation into the antifungal activity of plant essential oils showed promising results. Of the five essential oils tested during the *in vitro* study, thyme and clove significantly inhibited the growth of *Aspergillus flavus*, *A. niger*, *P. chrysogenum*, *F. oxysporum* and *F. equiseti*. at concentrations of 500 and 1000 ppm. Thompson and Cannon (1986) previously reported that thyme and clove oils inhibited mycelial growth of *A. flavus* at 500 and 1000 ppm. Furthermore, Zambonelli *et al.* (1996) found thyme to be the most effective oil against the inhibition of fungal growth of pathogenic fungi, while peppermint oil successfully reduced growth of all five fungi at a concentration of 2000 ppm. Soybean and peanut oil did not show any significant antifungal activity, contradicting McGee and Misra's (1988) findings that soybean oil decreased storage fungi incidence in maize and soybean. Thyme (1000 ppm), clove (1000 ppm) and peppermint (2000 ppm) oil significantly reduced the number of fungi isolated from the cowpea cultivar PAN 311 while only the essential oil of thyme (1000 ppm) showed an inhibitory effect on fungal growth on the PAN 325 cultivar. Thyme, clove and peppermint significantly inhibited the growth of *P. chrysogenum* on artificially inoculated cowpea seeds. *F. oxysporum* and *F. equiseti* were significantly reduced by thyme oil and *F. oxysporum* was also inhibited by peppermint oil. The percentage germination and emergence of cowpea seeds were not significantly reduced by thyme, peppermint or clove oils. According to Montes-Belmont and Carvajal (1998), thyme, clove and peppermint oils did not seem to affect the germination of maize seeds and the growth of the seedlings. It was found that in white cowpea seeds, fungi reduced percentage germination and emergence. Treatment of these infected seeds with the essential oils of thyme, clove and peppermint, however, greatly enhanced germination. Brown cowpea seeds, however, did not seem to be affected by the presence of the storage fungi and there was no increase in germination or emergence when seeds infested with these fungi were treated with oils. These results confirm those of Legesse and Powell (1992), who found that brown/dark cowpea seeds were less

susceptible to imbibition damage than white/light seeds. Furthermore, dark seeds were less susceptible to damping-off than white seeds (Legesse and Powell, 1992).

These records of storage fungi and FB_1 associated with cowpea seeds provides further valuable information which complements the existing knowledge of the problems associated with the post-harvest storage of cowpea seeds. Cowpeas are consumed on a daily basis in many tropical and sub-tropical countries and the presence of FB_1 in the seed can pose a serious health threat (Gelderblom *et al.*, 1988; Marasas, 1994) for those consuming the seed. These results provide a basis for further research into fumonisin contamination of cowpea seeds or seed products and the health implications that may result from cowpea seed ingestion that are contaminated with mycotoxins. Another focus point is improving storage practices and the treatment of seeds with plant essential oils to inhibit fungal contamination of the seed. This provides an alternative approach to chemical control, which can have harmful effects on the environment and human health.

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