Antimicrobial activity and fumonisins associated with cowpea (*Vigna unguiculata*)

BY

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SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

PHILOSOPHIAE DOCTOR

IN THE FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
DEPARTMENT OF MICROBIOLOGY AND PLANT PATHOLOGY

UNIVERSITY OF PRETORIA
PRETORIA

OCTOBER 2004

DECLARATION

I, the undersigned, declare that these studies, except where acknowledged in the text, is my own work and has not been previously submitted in any other form to this or any other tertiary institution.

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October 2004

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ABSTRACT

A survey involving 71 farmers from rural communities in Mpumalanga, South Africa was conducted to gather information regarding the importance and utilisation of cowpea. Cowpea was rated third most important in contributing to household security, preceded by maize and vegetable production. Cowpea was mainly produced for own consumption, as a source of income and as fodder for livestock to a lesser extent. The crop was used by 8.5% of the farmers for medicinal purposes. Results indicated that 20% of the farmers encountered problems with mouldiness during storage, with insect infestation to a lesser degree.

Cowpea seed samples from South Africa and Benin, West Africa were analysed for seed mycoflora and various fungal genera, particularly *Aspergillus*, *Phoma* and *Lasiodiplodia* were recorded. The results indicated an array of *Fusarium* spp. including *F. equiseti*, *F. chlamydosporum, F. graminearum, F. proliferatum, F. sambucinum*, *F. scirpi, F. semitectum* and *F. subglutinans*. The seed samples and the *F. proliferatum* isolates, cultured on maize patty media, were analysed for fumonisin production. 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 FB_1, FB_2 and FB_3. The cowpea cultivars from South Africa had levels of FB_1 ranging between 0.12 – 0.61 µg/g. All the *F. proliferatum* isolates produced FB_1, FB_2 and FB_3 with total fumonisin concentration levels between 0.80 - 25.30 µg/g. The highest level of FB_1 detected was 16.86 µg/g.

Surface-disinfected seeds were imbibed in sterile distilled water amended with FB_1 to yield final concentrations of 10, 25, 50 and 100 ppm. Percentage germination was determined in paper towels according to the International Seed Testing Association (ISTA) rules. Root and shoot length was measured after 9 days. All the toxin concentrations significantly decreased seed germination whilst root and shoot elongation was inhibited by the 50 and 100 ppm concentrations. Embryonic seed tissue treated with FB_1 indicated compaction of the protoplasm and separation of the plasma membrane from the cell wall. Lipid bodies accumulated and seemed to line the cell wall.

Acetone and ethanol extracts of the leaves of two cowpea cultivars exhibited significant inhibition of the growth of fungal plant pathogens at 5.0 mg/ml, with the exception of *Fusarium equiseti*. The growth of some fungi, in particular *Alternaria alternata*, was also reduced by lower concentrations of certain extracts. Acetone extracts of the Bechwana White cultivar inhibited growth of *Staphylococcus aureus* and *Enterococcus faecalis* at 2.5 mg/ml and *Bacillus cereus*, *B. subtilis* and *Enterobacter cloacae* at 5.0 mg/ml. Ethanol extracts of the same cultivar showed antibacterial activity against *E. faecalis* and *E. cloacae* at 5.0 mg/ml.

This study represents the first report on the natural occurrence of fumonisins on cowpea seed and the potential of *F. proliferatum* isolates from cowpea seed to produce fumonisins. The phytotoxic effects of FB_1 on cowpea seeds as well as the antimicrobial potential of cowpea leaf extracts were demonstrated for the first time.

Keywords: antimicrobial, cowpea, FB_1, fumonisins, *Fusarium proliferatum*, germination, medicinal, mycoflora, phytotoxic, storage, ultrastructure, *Vigna unguiculata*

ACKNOWLEDGEMENTS

I wish to thank:

- Prof. Terry Aveling, as primary supervisor, for guidance and motivation throughout the course of this study.
- Dr. Namrita Lall for valuable advice, especially with the antimicrobial investigations.
- The National Research Fund for providing financial assistance towards this study.
- The PROMEC Unit, Medical Research Council in Tygerberg, Cape Town for the provision of *Fusarium* cultures and fumonisin standards and for providing the facilities for HPLC analyses.
- Norma Leggott (formerly from the PROMEC Unit) for performing HPLC analyses.
- The Agricultural Research Council - Grain Crops Institute in Potchefstroom and Ecolink in Nelspruit for providing seeds for this study.
- Dr. Appolinaire Adandonon for supplying seeds from Benin.
- Claire Strange, Sarie Burger and Elizabeth Kola for assistance during the phytotoxicity studies.
- The personnel at the Laboratory of Microscopy and Microanalysis, University of Pretoria for assistance with the electron microscopy studies. Special thanks are due to Chris van der Merwe, Allan Hall and Lani Emslie.
- July Mhlabane, Felicia Mhlabane, David Lengwati and other field technicians from the Lowveld Research Station, Department of Agriculture, Mpumalanga for valuable help with the interviews.
- Dr. Cherian Matthews and Kgosi Dongo from the Department of Agriculture, Mpumalanga and for assistance during the survey conducted.
- The farmers visited in Mpumalanga for valuable co-operation during the survey.
- Friends and colleagues at the Department of Botany and Department of Microbiology and Plant Pathology, University of Pretoria for support and understanding during this study.
- My family for support and encouragement throughout the duration of the study.
- The Lord for giving me the strength and determination to accomplish this study.

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LIST OF ABBREVIATIONS

AIDS	-	Acquired immunodeficiency syndrome
ANOVA	-	analysis of variance
BW	-	Bechwana White
$CuCl_2$	-	copper chloride
DDT	-	dichloro diphenyl trichloroethane
DDAC		-dimethyl dodecyl ammonium chloride
FAO	-	Food and Agriculture Organisation of the United Nations
FB_1	-	fumonisin B_1
FB_2	-	fumonisin B_2
FB_3	-	fumonisin B_3
HPLC	-	high performance liquid chromatography
HIV-1	-	human immunodeficiency virus type 1
IARC	-	International Agency for Research on Cancer
ISTA	-	International Seed Testing Association
JECFA	-	Joint FAO/WHO Expert Committee on Food Additives
Kpod	-	Kpodjiguégué
LEM	-	leukoencephalomalacia
LTP	-	lipid transfer proteins
MEA	-	malt extract agar
MRC	-	Medical Research Council
MIC	-	minimum inhibitory concentration
NTD	-	neural tube defects
NA	-	nutrient agar
OPA	-	*o*-phthaldialdehyde
PDA	-	potato dextrose agar
PES	-	pulmonary edema syndrome
PMTDI	-	provisional maximum tolerable daily intake
NaH_2PO_4	-	sodium dihydrogen phosphate
SPE	-	solid phase extraction
SAX	-	strong anion exchange
TEM	-	transmission electron microscopy

TLC	-	thin layer chromatography
WHO	-	World Health Organisation

CHAPTER 1

GENERAL INTRODUCTION

1.1. BACKGROUND AND MOTIVATION OF THE STUDY

Cowpea (*Vigna unguiculata* (L.) Walp) is an indigenous African legume crop that is widely cultivated in the semi-arid tropical regions of Asia, Oceania, Africa, the Middle East, southern United States of America and Central and South America (Singh *et al.* 2002). In South Africa, 7000 t of cowpeas was produced from 13,500 ha during 2003 (FAOSTAT 2004). This production figure compares weakly with other legume crops including soybeans (*Glycine max* L.), groundnuts (*Arachis hypogaea* L.) green peas (*Pisum sativum* L.) and lupines (*Lupinus* spp.) (Table 1.1.).

Table 1.1. Production figures of different grain, pulse and oilseed crops in South Africa for 2003 (FAOSTAT 2004)

Commodities	Production (t) for 2003
Maize (*Zea mays* L.)	9,714,254
Wheat (*Triticum aestivum* L.)	1,600,000
Sunflower (*Helianthus annuus* L.) seed	708,000
Sorghum (*Sorghum bicolor* (L.) Moench.)	224,818
Barley (*Hordeum vulgare* L.)	150,000
Soybeans (*Glycine max* L.)	148,000
Groundnuts (*Arachis hypogaea* L.)	100,000
Oats (*Avena sativa* L.)	35,000
Peas (*Pisum sativum* L.), green	18,545
Millet (*Pennisetum glaucum* (L.) K Schum.)	12,000
Lupines (*Lupinus* spp.)	11,700
Cowpeas (*Vigna unguiculata* (L.) Walp)	7,000
Rice (*Oryza sativa* L.)	3,200
Peas, dry	1,033
Rye (*Secale cereale* L.)	550

Cowpea is of particular importance to many people living in less developed countries, where resource poor farmers and rural communities rely largely on the crop as a source of nutritious food. The seed contains on average 23-25% protein and 50-67% starch (Quin 1997). The young leaves, green pods and green seeds are used as vegetables whilst the dry seeds are used in the preparation of various food dishes (Singh *et al.* 2002). The haulms are also used as a quality feed for livestock (Singh *et al.* 2002). The plant is well adapted to hot and dry climates, has the ability to fix atmospheric nitrogen, curbs soil erosion through good ground cover and contributes to soil fertility through decayed residues (Singh *et al.* 2002). Furthermore, the trade of the fresh produce, seeds and foods processed from cowpea provides many farmers with a source of income (Singh *et al.* 2002). Medicinally, the leaves are used to treat burns, a root paste is used as an antidote for snake bites whilst the seeds are used to treat bilharzia, liver complaints and amenorrhoea (Hutchings *et al*. 1996; van Wyk & Gericke 2000). Previous studies have shown that cowpea leaves contain flavonoids (Lattanzio *et al*. 1997) of which some are known to exhibit antimicrobial properties (Aziz *et al*. 1998). However, the direct influence of cowpea extracts on microbes still remains to be established.

As is the case with other food crops, cowpea seeds are susceptible to fungal contamination especially when stored under inadequate and poor storage conditions. As a result, deterioration and spoilage of the seed occurs. It is under these conditions, together with high humidities and high temperatures that certain fungi tend to produce toxic secondary metabolites, namely mycotoxins. Serious health complications in both animals and humans can result from the ingestion of these metabolites through the consumption of infected seed and other foodstuffs (Moss 1996). Although there are reports relating to aflatoxin contamination (Zohri 1993; El-Kady *et al.* 1996), little information is known about other mycotoxins associated with cowpea seed.

Fumonisins, and in particular the analogue fumonisin B_1 (FB_1), is considered to be of major toxicological significance to animal and human health (Shephard *et al.* 1996). This toxin is produced mainly by *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg and is the causal agent of the diseases, leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome (PES) in pigs (Harrison *et al.* 1990; Kellerman *et al.* 1990). The International Agency for Research on Cancer (IARC) reported that the toxins produced by *F. verticillioides* are possibly carcinogenic to humans (IARC 2002). Fumonisin B_1 has been reported to be associated with other legume seeds (Tseng *et al.* 1995; Tseng & Tu 1997), but the *Fusarium* spp. responsible for the toxin production were not identified.

Numerous studies have indicated that FB_1 also exhibits phytotoxic activity towards several plants, including economically important crops (McClean 1996). The toxin is reported to cause deleterious effects in various crops including chlorosis and necrosis of leaves and reduction of root and shoot growth (Abbas & Boyette 1992; Doehlert *et al.* 1994; Lamprecht *et al.* 1994). Moreover, van Asch (1990) showed that FB_1 caused ultrastructural changes in maize (*Zea mays* L.) callus cells. These changes included thickening of the cell wall, the accumulation of phenolics in the vacuoles and accumulation of large starch grains in swollen plastids.

1.2. OBJECTIVES OF THE STUDY

The primary objectives of this study were firstly to investigate the presence and effects of fumonisins associated with cowpea seed and, secondly to evaluate the antimicrobial activity of the leaf extracts. The results that stem from these studies will provide valuable information with regard to further mycotoxin contamination of cowpea seed and the potential use of the plant extracts for antimicrobial purposes.

The specific objectives of this study were to:

- ❖ Conduct a survey amongst rural communities in the Mpumalanga Province of South Africa to establish the importance, role and utilisation of cowpea production in this region.
- ❖ Investigate the detection and quantification of the fumonisin mycotoxins in cowpea seeds.
- ❖ Identify the fumonisin-producing *Fusarium* spp. from cowpea seeds and to investigate their potential for fumonisin production.
- ❖ Investigate the phytotoxic effects of FB_1 on cowpea seed. These include the effect on germination, root and shoot elongation and on the ultrastructure of the cotyledon and embryonic tissue of the seed.
- ❖ Investigate the inhibitory effect of cowpea leaf extracts on the growth of selected bacterial and fungal pathogens.

1.3. STRUCTURE OF THESIS

Some of the chapters presented in this thesis have been prepared for publication in peer-reviewed journals. The reader will thus note some inconsistencies within the contents page and format of the chapters. Author citation and literature citation in Chapters 1, 2, 3 and 7 are according to the South African Journal of Botany.

Chapter 2 This chapter provides a concise review of the fungi and mycotoxins associated with cowpea seed. Information has been given on the fumonisin mycotoxins and their toxicological significance. The use of plant extracts for antimicrobial activity and secondary metabolites associated with cowpea have also been briefly discussed.

Chapter 3 The importance and role of cowpea in the livelihoods of rural communities was established by means of a survey conducted in the Mpumalanga Province of South Africa. The survey was aimed to gather information on the utilisation of the crop and to provide insight on the cultivation and storage practices followed.

Chapter 4 Cowpea seeds were analysed for storage fungi and fumonisin contamination. Possible fumonisin-producing *Fusarium* spp. isolated were investigated for potential to produce fumonisins.

Published as: Kritzinger Q, Aveling TAS, Marasas WFO, Rheeder JP, van der Westhuizen L, Shephard GS. (2003) Mycoflora and fumonisin mycotoxins associated with cowpea (*Vigna unguiculata* (L.) Walp) seeds. Journal of Agricultural and Food Chemistry **51**: 2188-2192.

Chapter 5 The phytotoxic effects of FB_1 on cowpea seed germination and on root and shoot elongation were investigated. The effect of the toxin on the ultrastructure of the cotyledon and embryonic tissue of the seed was also reported. (Submitted to Phytopathology for publication)

Chapter 6 Leaf extracts from two cowpea cultivars were tested for their antimicrobial potential against selected bacterial and fungal pathogens.

In press as: Kritzinger Q, Lall N, Aveling TAS (2004) Antimicrobial activity of cowpea (*Vigna unguiculata*) leaf extracts. South African Journal of Botany.

Chapter 7 In this chapter, the findings from the research conducted have been interpreted and discussed, and suggestions for future research made.

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CHAPTER 2

LITERATURE REVIEW

2.1. AN INTRODUCTION TO COWPEA: *VIGNA UNGUICULATA* (L.) WALP

Cowpea is an indigenous African legume crop belonging to the Fabaceae/Leguminosae family (Wiersema & León 1999). This widely cultivated and adapted crop is of great importance in the tropical and subtropical countries of Asia, Oceania, the Middle East, southern Europe, Africa, southern United States of America and Central and South America (Brader 2002). Cowpea is commonly known as black-eyed pea and southern pea. Other names for the crop include dinawa (Sotho, Tswana), munawa (Venda), akkerboon (Afrikaans), niébé (French), Frijol de costa (Spanish) and augenbohne (German) (Wiersema & León 1999).

2.1.1. Uses of cowpea

The crop is known to be economically important due to its soil improvement abilities by increasing soil nitrogen levels (Quin 1997; Wiersema & León 1999). Cowpea also suppresses weed growth and prevents soil erosion through excellent ground cover. Furthermore, a source of cash for rural communities is provided through the trade of seed (Quin 1997). Many subsistence farmers and rural communities living in less developed countries rely largely on the vegetable crop as a good source of nutritious food. Farmers also obtain fodder and forage for their animals (Wiersema & León 1999). It has been reported that all the parts of the cowpea plant (i.e. roots, leaves and seeds) are used medicinally (Nyazema 1987; van Wyk & Gericke 2000).

2.1.1.1. Importance as a food crop

The leaves, pods and seeds provide a good source of protein, vitamins and carbohydrates. The seed particularly contains on average 23-25% protein and 50-67% starch (Quin 1997). The seed provides an important source of nourishment, especially protein, for relatively poor people who cannot afford milk and meat products (Brader 2002). In Africa, the seeds are consumed either fresh or rehydrated, as an ingredient in soups or as a paste in steamed ('moin-moin') and fried dishes ('akara') (Ogunsanwo *et al.* 1989; Hung *et al.* 1990; van Wyk & Gericke 2000). In India, it is mainly eaten as cooked whole seeds or immature seeds (Kachare *et al.* 1988). In Nigeria, the seeds are eaten after boiling to softness and mixed with pepper, salt and palm oil to form a porridge (Ogunsanwo *et al.* 1989; Maduekwe &

Umechuruba 1992). Seeds can also be eaten with yams (*Dioscorea* sp.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.) (Maduekwe & Umechuruba 1992). The young pods and leaves are eaten as green vegetables (van Wyk & Gericke 2000).

2.1.1.2. Traditional medicinal uses

Cowpea has been identified by traditional healers in Zimbabwe to treat urinary schistosomiasis (bilharzia) (Nyazema 1987). The decoction made from the seeds of cowpea and the roots of *Euclea divinorum* Hiern or *Terminalia sericea* Burch ex DC. is taken orally to treat this illness (Nyazema 1987). Similarly, cowpea seeds and the roots of *Lannea edulis* (Sond.) Engl. can be used to treat blood in the urine and bilharzia (van Wyk & Gericke 2000). In East Africa, roots are used to expel the afterbirth (placenta, umbilical cord and ruptured membranes associated with the foetus) after childbirth (Kokwaro 1976, as cited by Hutchings *et al.* 1996). The leaves can be chewed and applied on burns and used as a snuff to treat headaches (Hutchings *et al*. 1996) whilst the Zulu's make emetics from the plant and are then taken to relieve fever (Gerstner 1939, as cited by Hutchings *et al.* 1996). The seeds also hold diuretic and anthelmintic (destruction of parasitic worms, e.g. tape worms) properties, and are used to treat liver complaints associated with jaundice (Noorwala *et al.* 1995). Further medicinal uses include: a decoction of the seed taken orally to treat the abnormal absence of menstruation (amenorrhoea); powdered roots eaten with porridge to treat painful menstruation, epilepsy and chest pain; a root paste applied to the bitten area caused by a snake bite as an antidote and a root infusion given to infants for constipation (van Wyk & Gericke 2000).

2.1.1.3. Useful compounds isolated from cowpea

In a study carried out by Ng *et al.* (2002) various antifungal proteins were isolated from legume seeds, including cowpea, and were assayed for ability to inhibit human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, protease and integrase enzymes. These enzymes are essential to the life cycle of HIV-1. The results concluded that cowpea P-antifungal protein had a high potency in inhibiting HIV-1 protease and HIV-1 integrase enzymes. Furthermore, cowpea a-antifungal protein was potent in inhibiting HIV-1 reverse transcriptase and HIV-1 integrase (Ng *et al.* 2002). Carvalho *et al.* (2001) reported that two cysteine-rich peptides isolated from cowpea seeds showed antimicrobial activity against the phytopathogenic fungi *Fusarium oxysporum* Schlecht.: Fr. and *F. solani* (Mart.) Appel and Wollenw. emend. Snyd. and Hans and the yeast *Saccharomyces cerevisiae* Hansen in an *in vitro* assay. The proteins, defensin and lipid transfer proteins (LTP) inhibited early growth and caused many hyphal morphological alterations of the fungi. The LTP's were immunolocalised in the cell walls and in intracellular compartments of the cotyledons and embryonic axes (Carvalho *et al.* 2001).

2.2. FUNGI AND MYCOTOXINS ASSOCIATED WITH COWPEA SEED

As in the case of many edible leguminous crops, the optimal utilisation of cowpea as a food crop is hampered by numerous constraints. Many losses of cowpea seed particularly are due to the inadequate post-harvest storage of the seeds (Uzogara & Ofuya 1992). Fungal infestation of stored seeds is highly dependent on seed moisture content and the storage temperature (Neergaard 1977). Seeds become susceptible to fungal infestation under conditions of relative high humidities and temperatures (Esuruoso 1975; Hitokoko *et al.* 1981; Seenappa *et al.* 1983). Some of these fungi are known to produce toxic secondary metabolites, namely mycotoxins, that can lead to severe health implications in both humans and animals when contaminated seed is ingested (Barrett 2000).

2.2.1. Storage fungi

There are numerous reports referring to storage fungi and seed-borne fungi associated with cowpea seed. A detailed list of the mycoflora associated with cowpea seed, which has been formulated from the available literature, is presented in Table 2.1. One of the earliest reports concerning seed-borne fungi associated with cowpea seed is from Singh & Chohan (1974). The authors analysed seed collected from local markets in Ludhiana, India for fungi using the agar plate and blotter method. Fungi including *Aspergillus niger* van Tieghem, *A. terreus* Thom, *Fusarium concolor* Reinking, *F. verticillioides* (Sacc.) Nirenberg (previously known as *F. moniliforme* Sheldon), *Penicillium crustosum* Thom and *Rhizopus arrhizus* Fischer were noted as new records of cowpea seed-borne fungi (Singh & Chohan 1974). In 1975, Esuruoso observed *Aspergillus flavus* Link ex. Fries, *A. niger*, *A. ochraceus* Wilhelm, *Penicillium digitatum* Sacc. and *R. arrhizus* to be associated with 81 samples of seed in western Nigeria. Other fungi isolated, including *Botryodiplodia theobromae* Pat, *Chaetomium globosum* Kunze ex. Fr., *Cladosporium cladosporioides* (Fresen.) de Vries., *C. herbarum* (Pers.) Link ex. S.F. Gray, *Colletotrichum lindemuthiamum* (Sacc. & Magh.) Bri. & Cav. *Curvularia lunata* (Wakker) Boedijn, *C. pallescens* Boedijn, *F. semitectum* Berk. & Rav., *F. solani* and *Phoma* sp. were new records for seed-borne fungi on cowpea (Esuruoso 1975). In 1981, Kumari & Karan detected *Trichothecium roseum* Link ex. Fries, *Verticillium* sp., *Circinella* sp., *Cladosporium* sp. and *Alternaria tenuis* Nees for the first time on cowpea seeds, collected from local markets of Hyderabad, India

Hedge & Hiremath (1987) noted that the frequency of cowpea seed mycoflora was less after storage than that of freshly harvested seed. Fungal genera including *Colletotrichum, Phoma, Curvularia, Trichothecium* and *Macrophomina* were not recorded in the seeds after storage, whereas *Aspergillus* dominated (Hedge & Hiremath 1987). Furthermore, the authors reported that more species of fungi were isolated from the seed coat and the cotyledons than the embryo. *Fusarium verticillioides* was isolated only from the embryo whereas *Aspergillus*, *Alternaria* and *Rhizopus* were mainly found in the seed coat (Hedge & Hiremath 1987).

Cowpea seed samples from India assayed for seed-borne fungi revealed that *F. verticillioides*, *F. oxysporum, Colletotrichum gleosporiodes* (Penzig) Penzig and Saccardo*, A. niger* and *Penicillium* sp. were the most dominant fungi (Shama *et al*. 1988). Similarly, Ushamalini *et al*. (1998) reported that *M. phaseolina, F. oxysporum, Alternaria alternata* (Fr.:Fr.) Keissler*, A. flavus, A. niger* and *Penicillium* sp. were isolated from seeds collected from different districts in Tamil Nadu, India. Previous studies concerning storage fungi associated with cowpea seeds carried out by Kritzinger (2000) showed that the genera *Aspergillus, Penicillium* and *Alternaria* were the most common fungi found from seed of nine cultivars. 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 (Kritzinger 2000).

A study done by Bulgarelli *et al.* (1988) showed that cowpea paste (prepared from dried seeds to make "akara") also supported an array of micro-organisms including bacteria, yeast and fungi. The paste, immediately collected after preparation from markets in Nigeria, showed the presence of *A. niger, F. sporotrichioides* Sherb.*, F. verticillioides*, *Acremonium* sp., *Moniella* sp. and *Geotrichum candidum* Link & Fries (Bulgarelli *et al*. 1988). From the above reports and results of previous studies concerning storage and seed-borne fungi associated with cowpea seed, it is evident that the seed supports a wide range of fungi. These fungi can play an important role in the quality and longevity of the seeds. Furthermore, important seed transmitted diseases are caused by some fungi, eg. *Colletotrichum* spp. (Shama *et al.* 1988). As stated earlier, several of these fungi are capable of producing mycotoxins, thus producing a possible potential health threat to the consumers.

Table 2.1. Mycoflora associated with cowpea seed

	Species	Reference(s)
en		
us		
Absidia	spp.	Gowda & Sullia 1987
Acremonium	*strictum* Gams	Jindal & Thind 1990
	spp.	Kritzinger 2000
Actinomucor	*repens* Schostak	Gowda & Sullia 1987
Alternaria	*alternata* (Fr:Fr.) Keissler	Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Jindal & Thind 1990; Zohri *et al.* 1992; Ushamalini *et al.* 1998
	cassiae Juriar & Khan	Van den Berg *et al.* 2002
	tenuis Nees	Kumari & Karan 1981
	tenuissima (Kunze: Fries) Wiltshire	Gowda & Sullia 1987
	spp.	Gowda & Sullia 1987; Kritzinger 2000
Ascochyta	spp.	Emechebe & McDonald 1979
Aspergillus	*awamori* Nakazawa	Zohri *et al.* 1992
	candidus Link ex. Fries	Kumari & Karan 1981
	carbonarius (Bainier) Thom	Zohri *et al.* 1992
	clavatus Desmazières	Gowda & Sullia 1987
	flavipes (Bainier & A. Sartory) Thom & Church	Zohri *et al.* 1992

flavus Link ex. Fries	Esuruoso 1975; Sinha & Khare 1977, 1978; Kumari & Karan 1981; Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Jindal & Thind 1990; Cabrales 1992; Maduekwe & Umechuruba 1992; Zohri *et al.* 1992; Ushamalini *et al.* 1998; Kritzinger 2000
fumigatus Fresenius	Kumari & Karan 1981; Zohri *et al.* 1992
glaucus Link ex. Gray	Gowda & Sullia 1987
janus Raper & Thom	Zohri *et al.* 1992
nidulans (Eidam) Wingate	Kumari & Karan 1981; Jindal & Thind 1990
niger van Tieghem	Singh & Chohan 1974; Esuruoso 1975; Sinha & Khare 1977, 1978; Kumari & Karan 1981; Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Jindal & Thind 1990; Maduekwe & Umechuruba 1992; Zohri *et al.* 1992; Ushamalini *et al.* 1998; Kritzinger 2000
ochraceus Wilhelm	Esuruoso 1975; Zohri *et al.* 1992; Kritzinger 2000
oryzae (Ahlburg) Cohn	Zohri *et al.* 1992
sulphureus Thom chruch	Kumari & Karan 1981
sydowii (Bainier & A. Sartory) Thom & Church	Zohri *et al.* 1992
tamarii Kita	Esuruoso 1975; Zohri *et al.* 1992
terreus Thom	Singh & Chohan 1974; Gowda & Sullia 1987; Shama *et al.* 1988; Maduekwe & Umechuruba 1992; Zohri *et al.* 1992
ustus (Bainier) Thom & Church	Zohri *et al.* 1992
spp.	Sinha & Khare 1977, 1978; Gowda & Sullia 1987; Cabrales 1992

Botryodiplodia	*theobromae* Pat.	Esuruoso 1975; De Barros *et al.* 1985
Botrytis	*cinerea* Persoon: Fries	Sinha & Khare 1977
	spp.	Sinha & Khare 1978; Gowda & Sullia 1987
Cacumisporium	spp.	Sinha & Khare 1977
Cephaliophora	*tropica* Thaxter	Zohri *et al.* 1992
Cephalosporium	spp.	Sinha & Khare 1977; Kumari & Karan 1981; Gowda & Sullia 1987; Shama *et al.* 1988
Cercospora	*canescens* Ellis & G. Martin	Emechebe & McDonald 1979
Chaetomium	*globosum* Kunze: Fries	Esuruoso 1975; Gowda & Sullia 1987; Shama *et al.* 1988; Zohri *et al.* 1992
	indicum Corda	Gowda & Sullia 1987
	spp.	Sinha & Khare 1977, 1978; Maduekwe & Umechuruba 1992; Kritzinger 2000
Circinella	spp.	Kumari & Karan 1981; Gowda & Sullia 1987
Cladosporium	*cladosporioides* (Fresenius) de Vries	Esuruoso 1975; Jindal & Thind 1990
	herbarum (Persoon: Fries) Link	Esuruoso 1975; Shama *et al.* 1988
	sphaerospermum Penzig	Zohri *et al.* 1992
	vignae Gardner	Hedge & Hiremath 1987
	spp.	Kumari & Karan 1981; Gowda & Sullia 1987; Kritzinger 2000
Cochliobolus	*lunatus* R.R. Nelson & Haasis	Singh & Chohan 1974
	spiciferus R.R. Nelson	Sinha & Khare 1977
Colletotrichum	*capsici* (Syd.) Butler & Bisby	Emechebe & McDonald 1979
	dematium (Persoon: Fries) Grove	Shama *et al.* 1988; Smith *et al.* 1999

	gleosporioides (Penzig) Penzig and Saccardo	Shama *et al.* 1988
	lindemuthianum (Saccardo & Magnus) Briosi & Cavara	Esuruoso 1975; Emechebe & McDonald 1979; Hedge & Hiremath 1987
	truncatum (Schw.) Andrus & Moore	Emechebe & McDonald 1979
	spp.	Gowda & Sullia 1987
Corticium	*rolfsii* Curzi	Emechebe & McDonald 1979
Corynespora	*cassiicola* (Berk. & Curt.) Wei	Esuruoso 1975
Curvularia	*lunata* (Wakker) Boedijn	Esuruoso 1975; Kumari & Karan 1981; Hedge & Hiremath 1987; Shama *et al.* 1988; Maduekwe & Umechuruba 1992; Ushamalini *et al*. 1998
	pallescens Boedijn	Esuruoso 1975; Shama *et al.* 1988
	tuberculata Jain	Jindal & Thind 1990
	verruculosa Tandon & Bilgrami	Singh & Chohan 1974; Sinha & Khare 1977, 1978
	spp.	Kritzinger 2000
Diaporthe	*phaseolorum* (Lehman) Wehmeyer	Sinha & Khare 1977, 1978
Diplodia	spp.	De Barros *et al.* 1985
Drechslera	*hawaiiensis* (Bugnicourt) Subramanian & Jain	Singh & Chohan 1974; Sinha & Khare 1977
	spp.	Shama *et al.* 1988
Emericella	*nidulans* (Eidam) Vuillemin	Zohri *et al.* 1992
	quadrilineata (Thom & Raper) C.R. Benjamin	Zohri *et al.* 1992

Epicoccum	*nigrum* Link	Jindal & Thind 1990
Eurotium	*chevalieri* Mang	Zohri *et al.* 1992
	heterocaryoticum Chris., Lop., and Benj.	Jindal & Thind 1990
Fusarium	*concolor* Reinking	Singh & Chohan 1974
	equiseti (Corda) Sacc.	Sinha & Khare 1977, 1978; De Barros *et al.* 1985; Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Jindal & Thind 1990
	fusarioides (Fragoso & Ciferri) C. Booth	Sinha & Khare 1977
	oxysporum Schlecht.: Fr.	Esuruoso 1975; Emechebe & McDonald 1979; De Barros *et al.* 1985; Gowda & Sullia 1987; Shama *et al.* 1988; Zohri *et al.* 1992; Varma *et al.* 1995
	oxysporum f.sp. *tracheiphilum* (E.F. Smith) Snyder & Hansen	Ushamalini *et al.* 1998
	semitectum Berk. & Rav.	Esuruoso 1975; De Barros *et al.* 1985; Gowda & Sullia 1987; Shama *et al.* 1988; Jindal & Thind 1990
	solani (Mart.) Appel and Wollenw. emend. Snyd. and Hans	Esuruoso 1975; Emechebe & McDonald 1979; Shama *et al.* 1988
	verticillioides (Sacc.) Nirenberg	Singh & Chohan 1974; Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Maduekwe & Umechuruba 1992
	spp.	Kumari & Karan 1981; Gowda & Sullia 1987; Cabrales 1992; Kritzinger 2000
Gibberella	*fujikuroi* (Sawada) Wollenweber	Zohri *et al.* 1992
Gilmaniella	spp.	Kritzinger 2000

Macrophomina	*phaseolina* (Tassi.) Goid.	Esuruoso 1975; Sinha & Khare 1977, 1978; Emechebe & McDonald 1979; De Barros *et al.* 1985; Gowda & Sullia 1987; Hedge & Hiremath 1987; Maduekwe & Umechuruba 1992; Ushamalini *et al.* 1998
Memnomiella	spp.	Sinha & Khare 1977; Kumari & Karan 1981
Mortierella	spp.	Gowda & Sullia 1987
Mucor	*hiemalis* Wehmer	Gowda & Sullia 1987
	spp.	Gowda & Sullia 1987; Hedge & Hiremath 1987
Nigrospora	spp.	Sinha & Khare 1977; Shama *et al.* 1988; Kritzinger 2000
Paecilomyces	spp.	Gowda & Sullia 1987
Penicillium	*aurantiogriseum* Dierckx	Zohri *et al.* 1992
	chrysogenum Thom	Jindal & Thind 1990; Zohri *et al*. 1992
	citrinum Thom	Jindal & Thind 1990; Zohri *et al.* 1992
	crustosum Thom	Singh & Chohan 1974
	digitatum Sacc.	Esuruoso 1975
	funiculosum Thom	Esuruoso 1975; Zohri *et al.* 1992
	oxalicum Currie & Thom	Jindal & Thind 1990; Zohri *et al.* 1992
	purpurogenum Stoll	Zohri *et al*. 1992
	spp.	Sinha & Khare 1977, 1978; Gowda & Sullia 1987; Hedge & Hiremath 1987; Shama *et al.* 1988; Cabrales 1992; Maduekwe & Umechuruba 1992; Ushamalini *et al*. 1998; Kritzinger 2000

Pestalotiopsis	*mangiferae* (Henn.) Steyaert	Sinha & Khare 1977
Phoma	*bakeriana* Henn.	Sinha & Khare 1977; Sinha & Khare 1978
	exigua Desmazières	Shama *et al*. 1988
	glomerata (Corda) Wollenw. and Hochapf	Jindal & Thind 1990
	vignae P. Henn.	Hedge & Hiremath 1987
	spp.	Esuruoso 1975; De Barros *et al.* 1985; Shama *et al.* 1988; Kritzinger 2000
Phomopsis	spp.	De Barros *et al.* 1985
Pithomyces	spp.	Sinha & Khare 1977; Kritzinger 2000
Pleospora	*infectoria* Fuckel	Singh & Chohan 1974; Sinha & Khare 1977, 1978
Pyrenochaeta	*decipiens* Marchal	Gowda & Sullia 1987
Rhizoctonia	*bataticola* (Taubenhaus) E. J. Butler	Singh & Chohan 1974
	solani Kühn	Emechebe & McDonald 1979; Gowda & Sullia 1987; Shama *et al.* 1988
	spp.	Kritzinger 2000
Rhizopus	*arrhizus* Fischer	Singh & Chohan 1974, Esuruoso 1975; Hedge & Hiremath 1987
	nigricans Ehrenberg	Gowda & Sullia 1987
	nodosus Namysl.	Gowda & Sullia 1987
	oryzae Went and Prinsen	Jindal & Thind 1990
	stolonifer (Ehrenberg: Fries) Vuillemin	Esuruoso 1975; Gowda & Sullia 1987; Zohri *et al.* 1992; Ushamalini *et al*. 1998
	spp.	Kumari & Karan 1981; Gowda & Sullia 1987; Kritzinger 2000
Scopulariopsis	*brumptii* Salvanet-Duval	Zohri *et al.* 1992
	halophilica Tubaki	Zohri *et al.* 1992

Septoria	*vignae* Henn.	Emechebe & McDonald 1979
Sphaceloma	spp.	Shama *et al.* 1988
Stachybotrys	spp.	Sinha & Khare 1977, 1978
Syncephalastrum	*racemosum* Cohn ex J. Schroeter	Sinha & Khare 1977; Gowda & Sullia 1987; Shama *et al.* 1988; Zohri *et al.* 1992
Syncephalis	spp.	Gowda & Sullia 1987
Thamnidium	*elegans* Link: Fries	Gowda & Sullia 1987
Torula	*viride* Persoon: Fries	Shama *et al.* 1988
	spp.	Gowda & Sullia 1987; Kritzinger 2000
Trichothecium	*roseum* (Persoon: Fries) Link	Kumari & Karan 1981; Gowda & Sullia 1987; Hedge & Hiremath 1987; Jindal & Thind 1990
Tripospermum	spp.	Kritzinger 2000
Ulocladium	*chartarum* (Preuss) E. Simmons	Sinha & Khare 1977
Verticillium	spp.	Kritzinger 2000
Zygorhynchus	spp.	Gowda & Sullia 1987

2.2.1.1. Effects of storage fungi on cowpea seed germination and seedling development

It is well known that storage fungi have a negative impact on the germination of various seeds and grains. Maheshwari *et al.* (1984) reported that *F. verticillioide*s and various *Aspergillus* spp. adversely affected the seed germination, root length and shoot length of cowpeas to various degrees. *Aspergillus nidulans* (Eidam) Winter was most effective in inhibiting seed germination (38.8%) and root length (82.8%) whilst *F. verticillioides* was the least effective. The inhibitory activity was due to the presence of amino acids, organic acids and phenols (Maheshwari *et al.* 1984). On the other hand, Rheeder *et al.* (2002) found that *F. verticillioides* infection alone of maize seeds did not affect seed germination. Furthermore, Jindal & Thind (1990) reported that *A. flavus, F. equiseti* (Corda) Sacc., *F. semitectum, Rhizopus oryzae* Went and Prinsen and *T. roseum* also significantly reduced germination of cowpea seeds.

2.2.2. Mycotoxins and their effect on cowpea seed

These secondary metabolites cause mycotoxicosis when ingested by higher vertebrates and other animals. Liver and kidney functioning can deteriorate when these metabolites are ingested through contaminated plant-based foods and animal-derived foods. Mycotoxins can also be neurotoxic, interfere with protein synthesis and can produce skin sensitivity or necrosis and extreme immunodeficiency (Sweeney & Dobson 1998).

Although legumes do not generally support the growth of toxigenic fungi and the production of mycotoxins (Webley *et al.* 1997), there are numerous reports regarding mycotoxins and legume seeds (Ahmad & Singh 1991; El-Kady *et al.* 1991; Saber 1992; Pitt *et al.* 1994; Tseng *et al.* 1995, Tseng and Tu 1997; Saber *et al.* 1998). However, the information pertaining to the production of various mycotoxins on cowpea seed is scant. Most of the literature regarding this aspect focuses on *Aspergillus* infection and aflatoxin production (El-Hag & Morse 1976; Seenappa *et al.* 1983; Zohri *et al.* 1992; El-Kady *et al.* 1996). Seenappa *et al.* (1983) reported that all cowpea seed samples collected in Tanzania were susceptible to *Aspergillus parasiticus* Speare infection and subsequent aflatoxin production. El-Hag & Morse (1976) investigated the production of aflatoxins by *Aspergillus oryzae* (Ahlburg) Cohn when grown on cowpeas or rice. It was found that this variant strain was capable to produce significant quantities of aflatoxin B_1, B_2, G_1 and G_2.

In 1992, Zohri *et al.* investigated the natural occurrence of citrinin, ochratoxin A, patulin, sterigmatocystin, T-2 toxin, diacetoxyscirpenol, zearalenone and aflatoxins B1, B_2, G_1 and G_2 in 20 cowpea cultivars. Thin layer chromatographic (TLC) analyses of the chloroform extracts showed that only four seed samples were naturally infected with aflatoxins B_1, B_2, G_1 and G_2. None of the other toxins tested for were detected in these cultivars (Zohri *et al.* 1992). Hitokoko *et al.* (1981) found aflatoxin B_1 (4 µg/g), sterigmatocystin (1 µg/g), ochratoxin A (50 µg/g) and T-2 toxin (3.8 µg/g) in cowpea seed samples. Zohri (1993) inoculated 16 mycotoxin-free cowpea seed samples with *A. flavus* to determine the varietal differences of aflatoxin production in the cultivars. Three cultivars showed high resistance whilst eight revealed partial resistance and the remaining cultivars were highly susceptible to toxin accumulation (Zohri 1993). It was reported that there was no relationship between morphological characters (seed colour, shape and size) or testa thickness and the amount of toxin produced by the different cultivars. The author concluded that the susceptibility or resistance of cowpea cultivars to *A. flavus* colonisation and aflatoxin production was influenced by an interaction of several factors. Zinc and sodium (essential trace elements for aflatoxin synthesis) levels were increased in susceptible cultivars when compared to the resistant cultivars (Zohri 1993).

Similarly, El-Kady *et al.* (1996) reported cowpea seed to be susceptible to *A. flavus* infection and aflatoxins were produced on two of the three cultivars analysed. Morphological and histological characters of the different cultivars tested did not show any relation to the amount of aflatoxin produced. The one cultivar Balady, however, was found to be very resistant to toxin production and this seed contained low levels of sodium and high levels of phosphate and potassium (El-Kady *et al.* 1996). Reddy *et al.* (1992) suggested that the higher the lipid content of seeds or seed components, the higher was the growth of *A. parasticus* and aflatoxin B_1 biosynthesis. This was demonstrated using seeds of crops with different lipid contents, including cowpea.

It has been reported by Adekunle & Bassir (1973) that aflatoxin B_1 and crude aflatoxins inhibited chlorophyll formation and seed germination of cowpea. Koehler & Woodworth (1938) induced chlorophyll deficiency in seedlings of citrus and maize. The authors suggested that the crude aflatoxins present in the walls of the fungal spores of *A. flavus* were responsible for this observation. The same trend was noted in mung seeds (*Vigna radiata* (L.) R. Wilcz), where seed germination, seedling growth, chlorophyll, protein and nucleic acid formation was inhibited by different concentrations of aflatoxin B_1 (Sinha & Kumari 1990). Maximum seed germination inhibition was caused by a 1000 µg/l concentration of aflatoxin B_1.

2.3. THE FUMONISIN MYCOTOXINS

2.3.1. Characterisation and toxicity

The fumonisins are recently characterised mycotoxins with significant toxicological consequences. It has been reported that 15 *Fusarium* species (Rheeder *et al.* 2002) are capable of producing fumonisins with the most important producers being *F. verticillioides* and *F. proliferatum* (Matsushima) Nirenberg (Rheeder *et al.* 2002). Other producers include *F. globosum* Rheeder, Marasas et Nelson (Sydenham *et al.* 1992) and *F. nygamai* Burgess and Trimboli (Thiel *et al.* 1991). *Alternaria alternata* f. sp. *lycopersici* is the only fungus that does not belong to the genus *Fusarium* that produces fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) in culture (Chen *et al.* 1992; Abbas & Riley 1996). The fumonisins are a structurally related group of diesters of propane-1, 2, 3-tricarboxylic acid and various 2-amino-12, 16-dimethylpolyhydroxyeicosanes in which the C14 and C15 hydroxyl groups are esterified with the terminal carboxyl group of tricarboxylic acid (Bezuidenhout *et al.* 1988). Twenty-eight fumonisin analogues have been characterised and have been placed into series A, B, F and P based on their chemical structure (Rheeder *et al.* 2002). FB_1, FB_2 and FB_3 (Figure 2.1.) are regarded to be the most abundant and most toxic of the naturally occurring analogues (Sydenham *et al.* 1992; Rheeder *et al.* 2002).

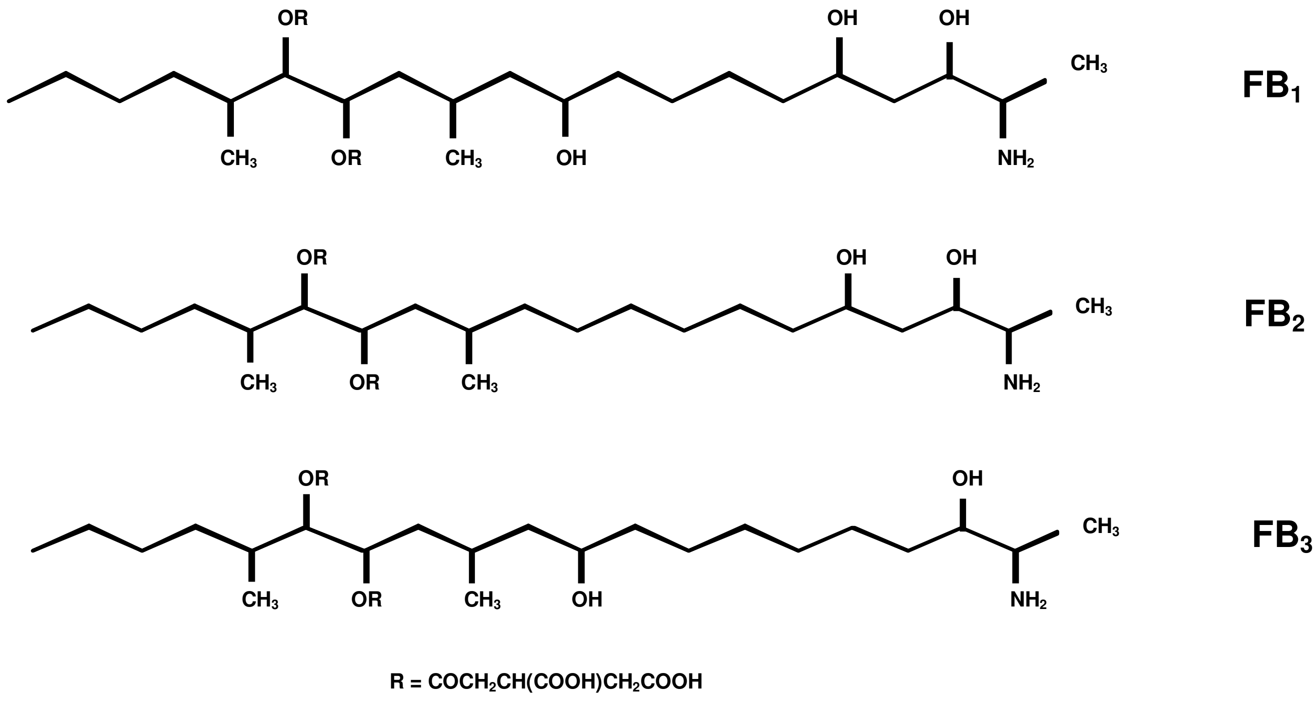


Figure 2.1. General chemical structure of fumonisins

Studies have shown that fumonisins have been known to cause various toxicological problems in animals. These include leukoencephalomacia (LEM), a fatal brain disease in horses, and pulmonary edema syndrome (PES) in pigs (Norred & Voss 1994; Marasas 1996). Recent studies have suggested that fumonisin consumption is a risk factor for neural tube defects (NTD) and other birth defects in humans. Fumonisins interfere with the utilization of folic acid, which is used to reduce the incidence of NTD (Marasas *et al.* 2004). Further toxicological effects include their hepatotoxicity and hepatocarcinogenicity to rats, and cytotoxicity to mammalian cell cultures (Marasas 1996). Fumonisin B_1 is statistically linked to the incidence of oesophageal cancer in humans in Transkei, South Africa and China (Marasas 1996) and evidence does suggest that it may play a role in the etiology of this disease in humans (Norred & Voss 1994; de Nijs *et al.* 1998). The International Agency for Research on Cancer (IARC) classed FB_1 in group 2B, which implies that it could possibly be carcinogenic to humans (IARC 2002). The joint FAO/WHO Expert Committee on Food Additives (JECFA) allocated a group provisional maximum tolerable daily intake (PMTDI) for fumonisins B_1, B_2, and B_3, alone or in combination, of 0.002 mg/kg body weight (JECFA 2001).

2.3.2. Occurrence on legume crops

Tseng *et al.* (1995) analysed three types of mouldy navy bean (*Phaseolus vulgaris* L.) samples for the *Fusarium* mycotoxins (diacetoxyscripenol, deoxynivalenol, T-2 toxin and FB_1), namely, healthy beans without discolouration, beans with pink discolouration and a mixture of beans with whitish grey and pink discolouration. FB_1 was found to be present in the two latter samples at 0.5 µg/g and 1.1 µg/g, respectively. *Fusarium* species isolated from the mouldy beans included *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe, *F. verticillioides*, *F. oxysporum* and *F. solani*. The *Fusarium* species responsible for the production of the toxin was not determined during this study. In a later study conducted by Tseng & Tu (1997), FB_1 was detected by TLC analysis in *Fusarium*-infected adzuki beans (*Phaseolus angularis* (Willd.) W.F. Wight) and mung beans (*Phaseolus aureus* Roxb.). *Fusarium avenaceum, F. culmorum, F. equiseti, F. graminearum, F. verticillioides, F. oxysporum, F. solani* and *F. sporotrichioides* were isolated from the mouldy and discoloured seeds. The quantification of FB_1 by high performance liquid chromatography (HPLC) revealed that the mouldy and discoloured adzuki and mung bean samples contained 261±43.8 and 230±21.6 µg/g of FB_1, respectively (Tseng & Tu 1997).

2.3.3. Phytotoxic effects of fumonisins

Not only do fumonisins play a negative role in the health of animals and possibly humans, they are known to show toxic effects towards plant species. Doehlert *et al.* (1994) conducted a study to assess the phytotoxic effects of FB_1 on maize seedlings. The germination of the seeds treated with zero to 1000 ppm FB_1 was unaffected. However, the toxin inhibited radical elongation by up to 75% after 48 h imbibition. Amylase production in the endosperm was also inhibited, and which could suggest that FB_1 interfered metabolically with germination (Doehlert *et al.* 1994). Danielsen and Jensen (1998) found a significant negative correlation (r = -0.52) between fumonisin content and maize seed germination. However, it was not established whether the fumonisins had a direct effect on germination. It was suggested that further research should study the effect of applying the purified toxin directly to the seeds (Danielsen & Jensen 1998). Van Asch *et al.* (1992) reported that maize callus growth was reduced as the concentration of FB_1 in the culture medium increased. The toxin significantly inhibited the growth of the calli at a concentration of 1.0 mg/l and at higher levels (van Asch *et al.* 1992).

Furthermore, FB_1 caused changes in the ultrastructure of treated maize callus cells, which included cell wall thickening, accumulation of phenolics in the vacuoles and accumulation of large starch grains in swollen plastids (van Asch 1990).

Abbas *et al.* (1991) reported that FB_1 could be exploited as a bioherbicide to control jimsonweed (*Datura stramonium* L.). Spores and mycelia of *F. verticillioides* isolated from jimsonweed incorporated into potted soil in which jimson weed plants were planted, caused local lesions and inhibited growth. The toxin caused the same symptoms on excised leaves (Abbas *et al.* 1991). These findings were supported by research carried out by Abbas & Boyette (1992). FB_1 sprayed onto jimsonweed plants at 10 to 200 µg/ml caused chlorosis and necrosis and reduced the height and biomass. Various other plants including sunflowers (*Helianthus anuus* L.), soybeans (*Glycine max* (L.) Merr.) and hemp (*Cannabis sativa* L.) showed varied degrees of symptoms (chlorosis, necrosis, black leaf lesions, tissue curl, stunting, defoliation, death) caused by the toxicity of FB_1. However, barley (*Hordeum vulgare* L.), maize, rice, sorghum (*Sorghum bicolor* (L.) Moench) and wheat (*Triticum aestivum* L.) were not visibly affected by the toxin (Abbas & Boyette 1992). Lamprecht *et al.* (1994) showed that FB_1, FB_2 and FB_3 caused leaf necrosis on detached tomato (*Lycopersicon esculentum* Mill.) leaves at the lowest concentration of 0.1 µM of each toxin and necrosis increased at higher concentrations. The fumonisins also caused reductions in shoot and root length and dry mass of maize and tomato seedlings at the varied concentrations (Lamprecht *et al.* 1994). The results from this study also showed that FB_1 was more phytotoxic to the seedlings than FB_2 and FB_3.

2.3.4. Mode of action of toxicity of fumonisins

The mechanisms responsible for the diseases caused by fumonisins in animals have been widely studied (Riley *et al.* 1994). *In vitro* studies have shown that fumonisins are potent inhibitors of the enzyme sphinganine (sphingosine) N-acyl transferase (ceramide synthase) (Riley *et al.* 1994). Animals or cultured cells exposed to fumonisins show a dramatic increase in the free sphingoid base, sphinganine, in tissues, serum and urine. Free sphingosine concentration increases, complex sphingolipid concentration decreases and sphingoid base degradation products and other lipid products also increase. Riley *et al.* (1994) hypothesised that this disruption in sphingolipid metabolism is an early molecular event in the onset and progression of cell injury and the diseases associated with the consumption of fumonisins e.g. LEM and PES. The exact mechanisms are not understood since the role of sphingolipids in cells is very complex.

Sphingolipids have various important functions in cell membranes including stabilisation of the membrane, sorting of lipids and proteins, binding to cytoskeletal elements and cell-cell recognition (Riley *et al.* 1994). A depletion of sphingolipids in membranes will lead to the disruption of the normal function of the membrane. In plants, however, there is little information regarding the function of sphingolipids. They do however play a role in cell signalling, membrane stability, stress response, pathogenesis and apoptosis (Sperling & Heinz 2003).

2.4. ANTIMICROBIAL EFFECTS OF PLANT EXTRACTS ON BACTERIAL AND FUNGAL PATHOGENS

With the dramatic increase in opportunistic systemic mycoses associated primarily with AIDS and treatment with immunosuppressive agents, new antifungal compounds are urgently required. There are numerous reports regarding the use of plant extracts to control human pathogens, and these include bacterial and fungal pathogens (Lall & Meyer 2000; Wiart *et al.* 2004). However, there is limited information on the use of plant extracts as an alternative means in controlling plant pathogens. With the decrease in the use of chemical formulations to control bacterial and fungal plant pathogens, plant extracts have been exploited as a novel means of control. Poswal *et al.* (1993) investigated the fungicidal properties of various plant parts of ten plant species against the fungal pathogens *M. phaseolina*, *Alternaria zinnae* Pape ex M.B. Ellis and *Sclerotium rolfsii* Sacc. Eksteen *et al.* (2001) reported that methanolic crude extracts of *Eucomis autumnalis* (Miller) Chitt. showed significant antifungal activity against fungal plant pathogens including *F. oxysporum, S. rolfsii, Rhizoctonia. solani* Kühn and *Pythium ultimum* Trow. This extract also compared favourably to the inhibition of the mycelial growth by a broad spectrum synthetic fungicide (carbendazim/difenoconazole). Barreto *et al.* (1997) found that ethanolic extracts of selected seaweed extracts including *Caulerpa filiformis* (Suhr) Hering, *Zonaria tournefortii* (Lamour.) and *Hypnea spicifera* (Suhr) Harv. inhibited the fungal growth by more than 50% of the phytopathogens *Verticillium* sp. and *R. solani*. Pretorius *et al.* (2003) tested crude extracts from 26 South African plant species *in vitro* for their potential to inhibit the growth of various plant pathogenic fungi and bacteria. None of the crude extracts showed growth inhibition of the fungi tested. The crude extracts of *Euclea crispa* (Thunb. Guerke) subsp. *crispa*, *Acacia erioloba* E. Mey, *Senna italica* Mill. subsp. *arachoides* and *Buddleja saligna* Willd. inhibited the growth of all five bacteria, namely, *Agrobacterium tumefaciens* Smith and Townsend, *Clavibacter michiganense* Spiekermann pv. *michiganense* Smith, *Erwinia carotovora* pv. *carotovora* Jones, *Pseudomonas solanacearum* Smith and *Xanthomonas campestris* Pammel pv. *phaseoli* Smith. The crude extract of *E. crispa* compared more favourably to that of dimethyl dodecyl ammonium chloride (DDAC), a commercial bactericide (Pretorius *et al.* 2003).

2.5. SECONDARY METABOLITES ASSOCIATED WITH COWPEA

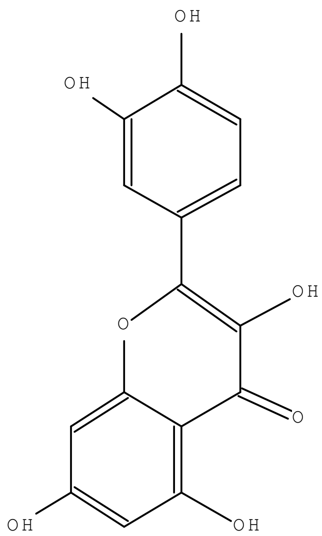
Flavonoids are low molecular weight 15-carbon secondary metabolites that are widely distributed in the vegetable kingdom (Salisbury & Ross 1992). They play vital roles in defence against pathogens and predators. However, some do have a negative impact on the use of seeds and grains in animal feed and human food e.g. proanthocyanidins (Shirley 1998). Flavonoids can be subdivided into groups, which include the flavonols and flavones (Salisbury & Ross 1992). Legumes are a particularly rich source of flavonoid compounds and could be explored for their increased use in medicine and disease control (Dakora 1995).

Isoflavonoids (which differ in chemical structure from flavonoids) (Salisbury & Ross 1992) formed in plants during attacks by plant pathogens, play an important role in host-plant resistance to diseases. Since these compounds are toxic to various microbes and the fact that their accumulation restricts microbial growth within plant tissue, they function as phytoalexins (Dakora 1995).

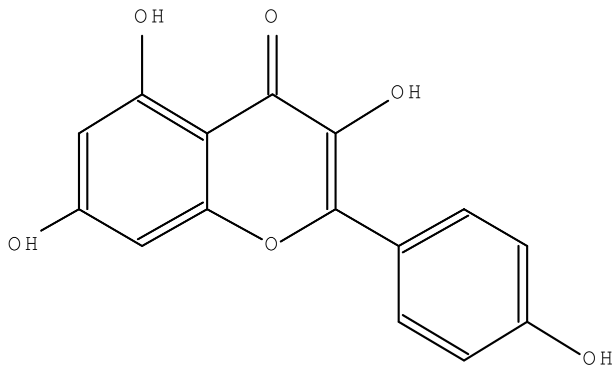
2.5.1. Secondary metabolites

Lattanzio *et al.* (1997) showed through flavonoid HPLC analyses of cultivated cowpea lines, that three flavonoid aglycones, namely, quercetin, kaempferol and isorhamnetin (Figure 2.2.), were always present in the leaves. Quercetin was noted as being the most abundant. The flavonoid glycoside pattern showed 10 different glycosides, including 2 *p*-coumaroylglycosides of kaempferol and five of quercetin (Lattanzio *et al.* 1997). Other phenolic aglycons that were identified from leaf extracts of cowpea lines and some wild species of *Vigna* include vanillic acid, *p*-coumaric acid, caffeic acid, cinnamic acid, ferulic acid, protocatechuic acid, sinapic acid and apigenin (Lattanzio *et al.* 2000; Cai *et al.* 2003). Isobe *et al.* (2001) found the flavonoids coumestral, daidzein and genistein in cowpea root extracts. A new pentacyclic triterpenoid saponin as well as other known compounds including cycloartenol, stigmasterol, 3-o-acetyl-oleanolic acid and 3-o-ß-D-glucoside were also isolated from cowpea seeds (Noorwala *et al.* 1995).

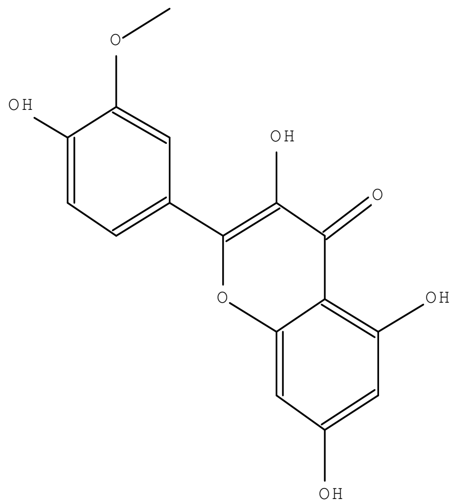
Various phytoalexins have been isolated from cowpea after fungal or virus infection. These include the isoflavonoids, kievitone (Bailey 1973; Keen 1975), phaseollin (Bailey 1973), phaseollidin (Bailey 1973), 2-0 methylphaseollidinisoflavan (Preston *et al.* 1975) and demethylhomopterocarpin (Lampard 1974). Vignafuran (Preston *et al.* 1975) is the first reported 2-aryl-benzofuran phytoalexin following inoculation with *C. lindemuthianum*. Vignafuran was active against two prevalent Nigerian races of *C. lindemuthianum* (Preston *et al.* 1975). Munn & Drysdale (1975) showed that kievitone can be induced in cowpea by abiotic treatments including topical application of copper chloride ($CuCl_2$), actinomycin D or cycloheximide solutions.



quercetin



kaempferol



isorhamnetin

Figure 2.2. Chemical structures of selected flavonoids associated with cowpea

2.5.2. Antimicrobial and medicinal activity of secondary metabolites

The isoflavonoids, pterocarpins, isoflavones and isoflavanones are very toxic to fungal pathogens. They can cause permanent damage to membrane systems, and therefore inhibit fungal spore germination, germ-tube elongation and hyphal growth (Dakora 1995). Quercetin is a naturally occurring bioflavonoid found in high concentrations in red wines, onions and green tea. Its properties include activity as an anti-oxidant and anti-inflammatory. Quercetin, extracted from the leaves of *Geranium dissectum* L. (cut-leaf geranium), showed a good inhibitory effect on the growth of fungi including *F. oxysporum*, *R. solani*, *M. phaseoli* and *Aspergillus carneus* Blochwitz (El-Gammal & Mansour 1986). This same trend could be seen regarding the bacteria *Staphlococcus aureus* Rosenbach, *Sarcina lutea* (Schroeter) Schroeter and various *Bacillus* species. Kaempferol, extracted from *Tribulus pentandrus* L. (devil's-thorn), showed similar results as with quercetin but did not inhibit the growth of *S. lutea* and *Bacillus mycoides* Flugge (El-Gammal & Mansour 1986). Aziz *et al*. (1998) reported that quercetin and *p*-coumaric acids inhibited the growth of *A. parasiticus* and *A. flavus* by 100% at 0.3 mg/ml while caffeic acid inhibited the fungal growth and aflatoxin production at 0.2 mg/ml. Furthermore, caffeic acid inhibited growth of the bacteria *Escherichia coli* (Migula) Castellani and Chalmers and *Klebsiella pneumoniae* (Schroeter) Trevisan at 0.3 mg/ml and *Bacillus cereus* Frankland and Frankland at 0.5 mg/ml. *P*-coumaric acid completely inhibited the growth of the three above-mentioned bacteria at 0.4 mg/ml (Aziz *et al*. 1998).

The increased inhibitory action of phenolic compounds is due to the presence of a phenolic OH group (Gourma *et al.* 1989). The OH group is much more reactive and can easily form hydrogen bonds with active sites of enzymes. Lueck (1980) reported that the antimicrobial action of these compounds was due to the inhibition of certain enzyme reactions or enzyme synthesis in the microbial cell by chemicals. This makes it possible to inhibit the enzyme involved in the basic metabolism of the cell or the synthesis of important cell constituents. El-Gammal & Mansour (1986) reported that quercetin was successful in inhibiting microbial growth of various pathogens used in medicinal and industrial fields. It has been reported that therapy with quercetin provides significant symptomatic improvement in most men with chronic pain syndrome (Shoskes *et al.* 1999).

2.6. THE INFLUENCE OF PHYSICAL FACTORS ON THE ULTRASTRUCTURE OF COWPEA SEEDS

The principle features of cowpea seed noted by transmission electron microscopy (TEM) examination include round, ellipsoidal or kidney shaped starch grains and thick cell walls with pit-pairs

(Saio & Monma 1993). Other cellular materials such as vacuoles, protein bodies and lipid bodies can also be observed. Lipid bodies are rarely found adjacent to the cell walls (Saio & Monma 1993).

Suspension culture cells of cowpea (unadapted and thermoadapted cells) underwent various structural changes when exposed to heat stress. These modifications included: almost complete loss of polyribosomes, rough endoplasmic reticulum and dictysomes; migration of intracellular waste material into the vacuole; retraction of the tonoplast from the cytoplasm into vacuoles and the swelling of the nucleolus with assumed accumulation of preribosomal RNP granules (Dylewski *et al.* 1991). Hung *et al.* (1990) found that severe heat treatment damaged the middle lamella of cotyledon cells and changed the birefringence property of starch granules. Enwere *et al.* (1998) investigated the effect of a drying treatment on the microstructure of cowpea seed and found that cavities occurred in the cotyledons of the 80°C and 120°C dried seeds. The high temperatures weakened the binding forces between the starch granules and protein matrix and the force applied during sectioning was enough to dislodge the granules (Enwere *et al.* 1998). In certain cases the entire cell content was lost after sectioning. It also appeared that the cell content was shrinking away from the cell wall (Enwere *et al.* 1998).

There are various reports of the effects of imbibition on cowpea seed structure. Thomson & Platt-Aloia (1982) reported that the plasmalemma in cowpea seeds is quite permeable during the early stages of imbibition. This was noted by the leakage of electrolytes and the localisation of chloride within the cells of NaCl-imbibed seeds. Freeze-fracture electron microscopy of the radical after 24 h of imbibition revealed a major change in the subcellular organisation. The endoplasmic reticulum and dictysome were easily visualised and the protein and lipid bodies were spherical. The plasmalemma was also more regular even when compared to the dry seeds (Thomson & Platt-Aloia 1982). The ultrastructure of the dry seeds, after a non-aqueous primary fixation, showed the cytoplasm containing numerous ribosomes. The organelles were ill defined and irregular in outline. Freeze-fracture of the dry embryos revealed that the lipid droplets were closely appressed to the plasmalemma (Thomson & Platt-Aloia 1982).

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CHAPTER 3

SURVEY ON THE IMPORTANCE AND UTILISATION OF COWPEA BY RURAL COMMUNITIES IN THE MPUMALANGA PROVINCE OF SOUTH AFRICA

3.1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp) is an indigenous African legume crop that is widely cultivated throughout tropical and subtropical parts of Africa, the Middle East, Oceania, southern United States of America, Asia, southern Europe and South America (Singh *et al.* 2002). According to the Food and Agriculture Organisation of the United Nations (FAO) (FAOSTAT 2004), cowpea production in South Africa compares weakly (7000 t) with major grain crops like maize (*Zea mays* L.) (9,714,254 t) and wheat (*Triticum aestivum* L.) (1,600,000 t). Production levels for 2003 for other legume crops including soybeans (*Glycine max* L.) (148,000 t), groundnuts (*Arachis hypogaea* L.) (100,000 t), green peas (*Pisum sativum* L.) (18,545 t) and lupines (*Lupinus* spp.) (11,700 t) were much higher than cowpea, with the exception of dry peas, which was substantially lower at 1,033 t (FAOSTAT 2004). Cowpea production in South Africa is fairly minor when compared to other African countries (FAOSTAT 2004). Nigeria is the largest producer and consumer of cowpea with about 5 million ha and over 2 million t production during 2003. Cowpea seed set aside for sowing or planting in South Africa was also lower (365 t) when compared to Nigeria with 175,000 t and Niger with 140, 000 t (FAOSTAT 2004).

Small-scale farmers and rural communities receive numerous benefits from the cultivation of this crop. These include the haulm used as fodder for animals, income through the trade of the seed and a source of nutritious food. The people obtain a good supply of proteins, carbohydrates and vitamins from all the plant parts used for food, especially from the seed (Quin 1997; Singh *et al.* 2002). Furthermore, it is a fast growing crop, prevents soil erosion by covering the ground and fixes atmospheric nitrogen making it a good intercrop (Singh *et al.* 2002). There are also reports on the use of the plant for medicinal purposes. Among these are seed decoctions taken to treat liver complaints associated with jaundice (Noorwala *et al.* 1995), blood in the urine and bilharzia (Nyazema 1987; van Wyk & Gericke 2000), and amenorrhoea (van Wyk & Gericke 2000).

Despite all the beneficial uses, there are numerous constraints that impede the optimal utilisation of the crop. Diseases induced by various pathogenic groups, including fungi, bacteria, viruses, nematodes and parasitic flowering plants, are considered as important constraints to cowpea production

(Emechebe & Lagoke 2002). Similarly, after harvesting when the seeds are stored, seed deterioration can occur as a result of physical (temperature, humidity), biological (fungi, bacteria, insects, rodents) and technical (method and duration of storage) factors (Appert 1987). When seeds are stored in conditions where high relative humidities and high temperatures prevail, certain fungi produce toxic metabolites, namely mycotoxins. These metabolites when ingested after consuming infected seed, can lead to dramatic adverse health conditions for both animals and humans (Moss 1996).

Previous surveys regarding cowpea were concerned with indigenous cowpea production practices (Kossou *et al.* 2001) and consumer preferences for cowpea (McWatters *et al.* 1990; Langyintuo *et al.* 2004). Kossou *et al.* (2001) conducted a survey in the Ouémé valley, Benin to investigate the importance of pests and diseases as constraints to cowpea production. Similarly, Alghali & Pratt (1995) gained insight into indigenous farming practices regarding pest management for cowpea in southern Sierra Leone.

This study was undertaken to gain insight into the current status of cowpea production and utilisation in rural communities in the Mpumalanga Province of South Africa. Given the focus areas of this thesis, particular emphasis was placed on gathering information on post-harvest storage practices and the problems encountered as well as possible medicinal uses of the crop.

3.2. METHODOLOGY

3.2.1. Survey area

The survey was carried out in various rural settlements in the Lowveld region in the Mpumalanga Province (Figure 3.1.). The areas included Nsikazi North, Malekutu, Clau-Clau, Tsonga, Buffelspruit, Numbi, Kahoyi, Mahushu and Goba. The survey was done during the period of September 2003 - August 2004.

3.2.2. Survey questionnaire

A questionnaire was designed to gather information firstly on the importance and role of cowpea in the livelihoods of the people, secondly on the cultivation and storage practices followed, and thirdly health implications encountered. The questionnaire is presented as Appendix A. Although most of the farmers were interviewed on their farms, some farmers were interviewed as a group in a community hall. Trained interviewers administered the questionnaire to the people (Figure 3.2.). In certain instances a translator was needed to facilitate communication between the interviewer and the person interviewed. Where possible, digital photographs were taken of seed storage containers and facilities.

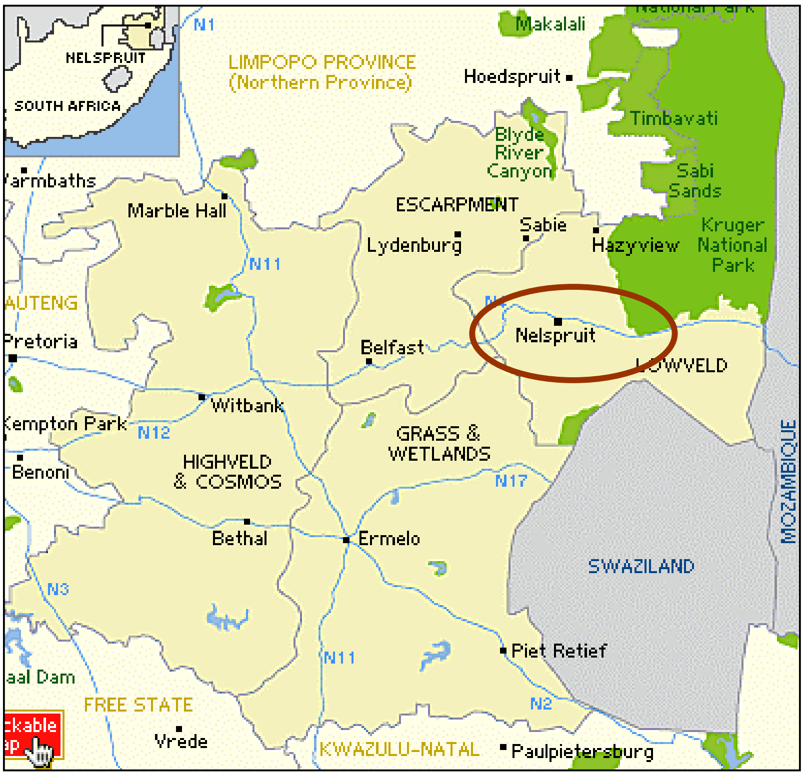

Figure 3.1. Survey area, indicated by the red ring, in the Mpumalanga Province, South Africa

Figure 3.2. Farmer and his family together with interviewers during an interview in Mpumalanga

3.2.3. Analysis of results

All the data gathered was entered in the Microsoft Excel Spreadsheet programme to calculate percentages and to construct graphical representations of the data.

3.3. RESULTS AND DISCUSSION

3.3.1. Biographical profile

A total of 71 people were interviewed in the survey, of which 43 were male and 28 were female. The age demographics of the people interviewed are indicated in Figure 3.3. The majority of the respondents (28.2%) were in the middle-age group (40-49 years) and the oldest people interviewed were between 80 and 89. A relatively large group (23.9%) stated that their age was unknown. All the farmers interviewed belonged to various farmer associations.

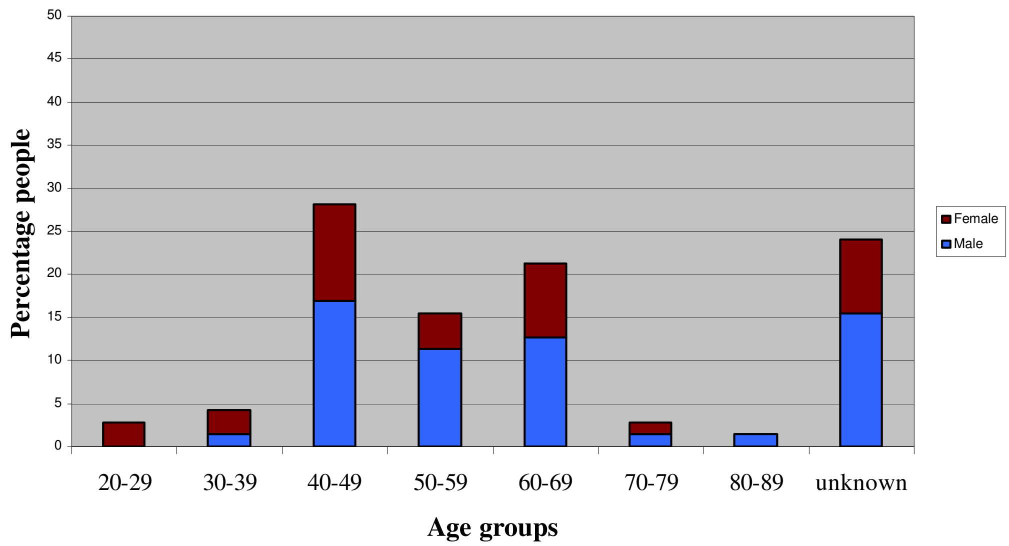

Figure 3.3. Age demographics of people interviewed in Mpumalanga

It was found that 29% of the respondents relied only on agricultural activities for a source of income (Figure 3.4.). Almost 70% of the people interviewed received income from agricultural activities together with other employment. Half the number of people who relied on agricultural activities as their major livelihood were pensioners. The additional jobs included teaching, taxi drivers, shop owners, social workers, mechanics, plumbers, ministers and selling clothes.

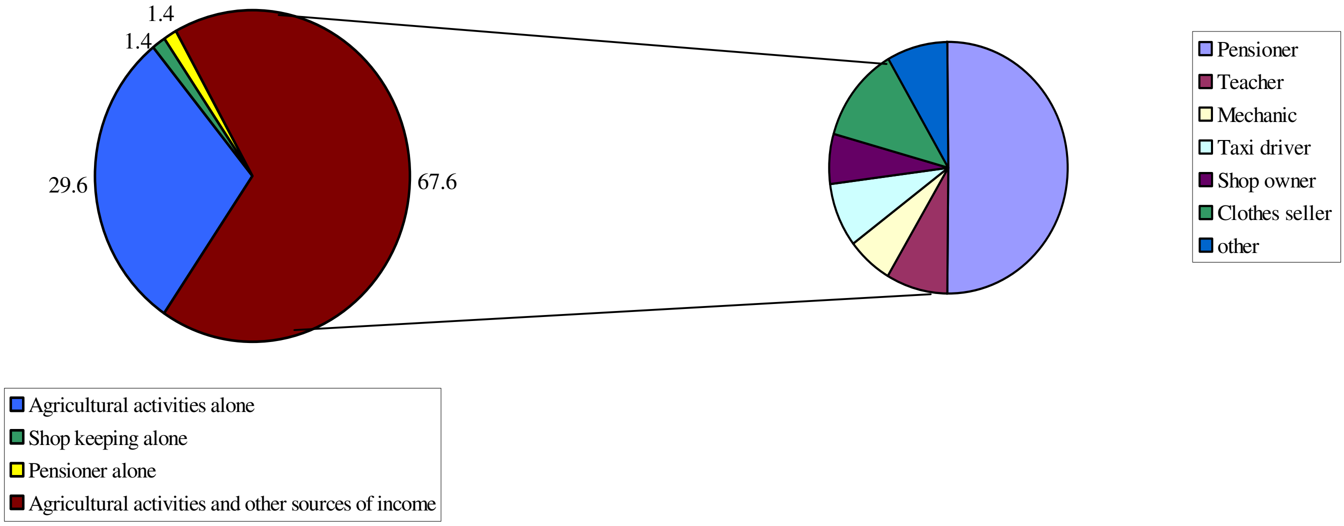

Figure 3.4. Source of income of the people interviewed in Mpumalanga

3.3.2. Agricultural profile

Crop production was ranked as the most important (98.5%) and only agricultural activity practiced whilst one farmer (1.4%) ranked livestock farming as most important. The size of land available for agricultural activities varied between less than 0.5 ha to more than 20 ha (Table 3.1.) with 81 ha being the largest piece of land for crop production. The majority of the farmers had small farms between 0.1 and 4 ha with many farms below 1 ha. The total area of land that was used in South Africa for cowpea production during 2003 was approximately 13,500 ha (FAOSTAT 2004).

Table 3.1. Size of land used for agricultural activities by farmers interviewed in Mpumalanga

	0.1-0.9*	**1-1.9**	**2-2.9**	**3-3.9**	**4-4.9**	**5-5.9**	**6-10**	**11-15**	**16-20**	**>20**
Male	17	4	4	6	4	2	1	2	1	2
Female	9	11	2	3	1	0	2	0	0	0
Total	26	15	6	9	5	2	3	2	1	2

* ha

3.3.3. Importance and role of cowpea

As expected, cowpea was generally not seen as a crop of major importance for household security. It ranked third highest together with a variety of other crops including groundnuts, fruits, cotton (*Gossypium* spp.) and other legumes. Maize and vegetable production was ranked as being most and second most important, respectively, by the respondents. This is in contrast with many African countries, especially in Central and West Africa, where cowpea plays a major role in the livelihoods of many subsistence farmers and rural communities (Singh *et al.* 2002). In Sierra Leone, cowpea is grown mostly as a secondary crop (Alghali & Pratt 1995).

A large number of the people chose to grow cowpea as it was a tradition (71.8%) passed on to them by their forefathers. The use of the crop for food consumption (23.9%) was also an important factor in their choice to produce cowpea. Other reasons why people chose to produce cowpea were; the right climate prevailed (4.2%), a source of good income (2.8%), drought resistant (1.4%) and a good crop for crop rotation (1.4%).

3.3.3.1. Use as a food crop

Of the 71 people interviewed, 98.6% use cowpea for own consumption. As indicated in Figure 3.5. cowpea was not readily consumed, as is the case in many other African countries. The majority of the respondents (52.1%) consumed cowpea less than once a week.

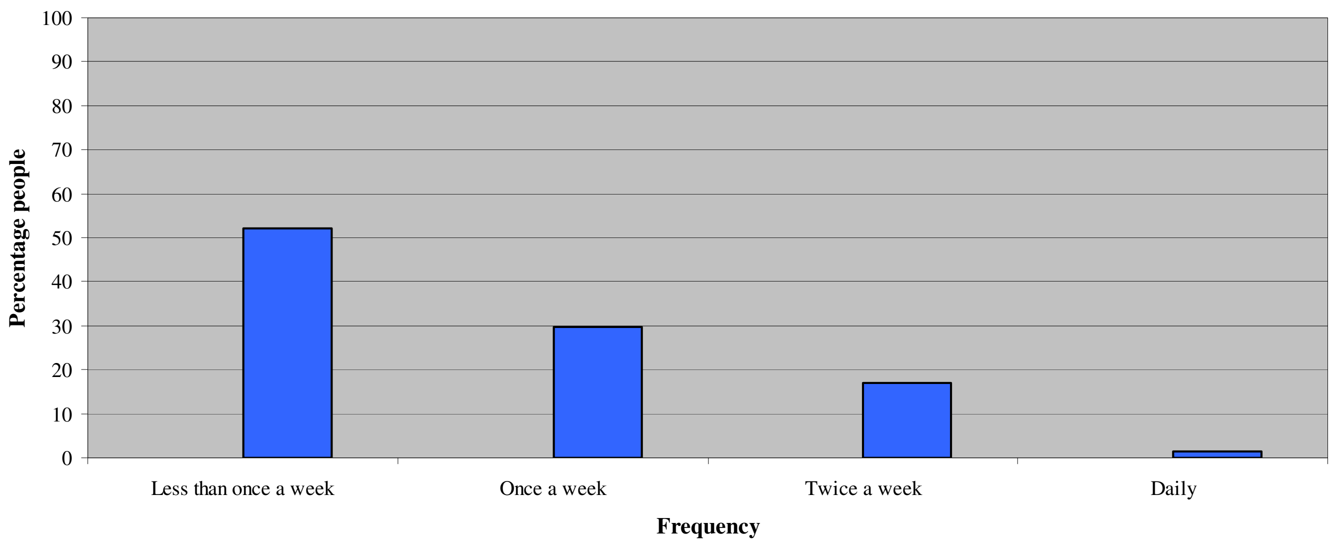

Figure 3.5. Frequency of cowpea consumption by people in Mpumalanga

The main source of cowpea for consumption was from the farmers' own produce (83.6%). Other places where cowpeas were obtained included hawkers and local markets (10.9%) and other shops (5.6%). The people that consumed cowpea preferred whole cooked seeds (88.7%). This same preference was also found amongst people in India (Kachare *et al.* 1988). Many people used the crop as an ingredient in dishes (69.0%). Responses indicated that it could be used as an ingredient in soups and could be added to samp (*Zea mays*). The leaves were used as a vegetable (26.8%), the seeds were ground to make a porridge (4.2%) and could be used as a baking product (1.4%). Seven percent of the people interviewed ate the fresh pods.

This study showed that a preference for seed colour existed amongst the people interviewed. Just over a half of the people preferred the light seed (50.7%), 33.8% preferred darker-coloured seeds whilst the remaining 15.5% did not seem to have any preference to seed colour. A respondent remarked that the plants that came from the dark seeds grew straight upwards, whilst another respondent did not know that seeds other than dark coloured ones even existed. Langyintuo *et al.* (2004) investigated the consumer preferences for cowpea in Cameroon and Ghana and found that the seed with a white seed coat was popular in only one market in Ghana. Black eyes were found to be premium in Ghana but not in Cameroon. On the other hand, grain size was found to be the most important characteristic in both countries as most consumers preferred large grain (Langyintuo *et al.* 2004). During another survey, 84.2% of Ghanaian mothers preferred cowpea varieties with light coloured seed coats for infant food preparation (Phillips *et al.* 2003).

3.3.3.2. Source of income

Just more than 40% of the respondents claimed that cowpea contributed in some way to their household income. The most stated that the contribution was small (less than 25%) whereas one person regarded cowpea as an important contribution (more than 50%) to the household income (Figure 3.6.). On average, a cup (± 250 ml) of seed was sold for three Rand. The price for one kilogram of seed varied between two and three Rand. Larger quantities reached prices of R 10 per 5 kg and R 20 per 10 kg. The excess produce was sold mainly to neighbours on a door-to-door basis or at pension pay points (84.9%) whereas a small percentage of the farmers sold the seed at the local markets (9.7%).

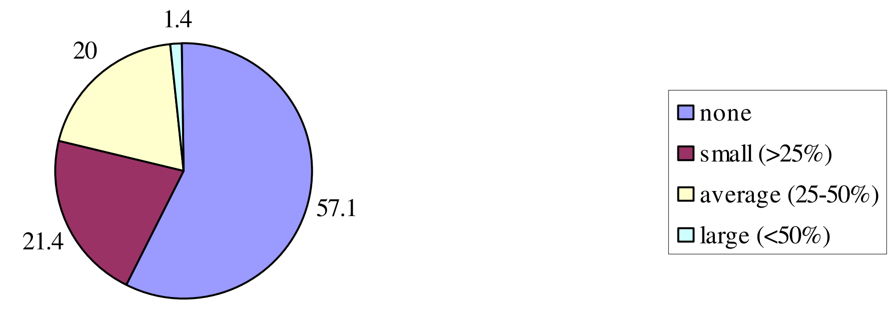

Figure 3.6. Percentage contribution of cowpea to household income in Mpumalanga

3.3.3.3. Feed for livestock

Cowpea was generally not used as feed for livestock. However, those that did use it preferred to use the fodder rather than the seed (Figure 3.7.). One respondent reported that she fed her pigs on a daily basis with cowpea fodder. The use of cowpea as fodder is most advanced in India, regarded primarily as a fodder crop in Australia since the late 1950's, and plays a major role in drier areas in West Africa (Tarawali *et al.* 1997). Cowpea haulms compare very well with other forage legumes, mostly with higher crude protein, digestibility and mineral content but has lower fibre (Tarawali *et al.* 1997).

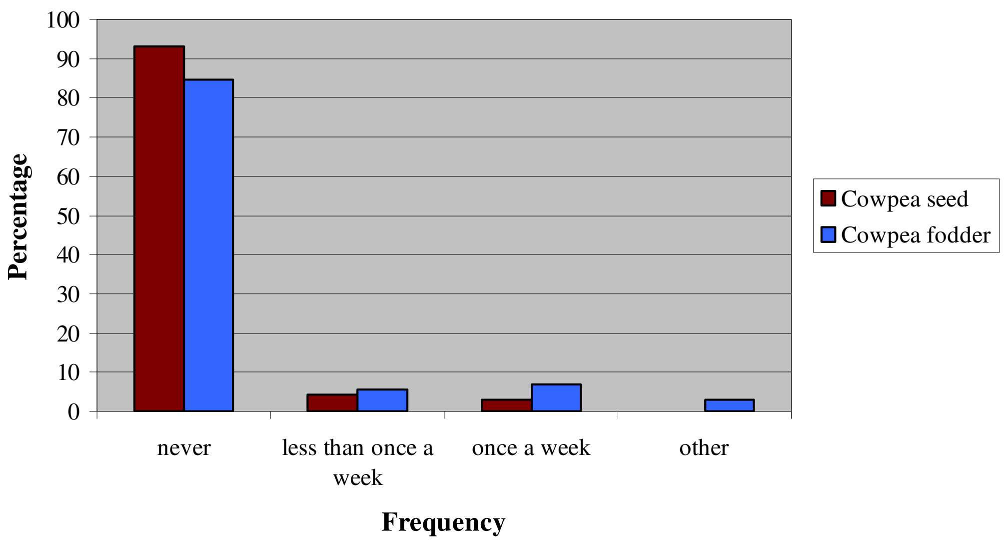

Figure 3.7. Frequency of cowpea consumption by animals in Mpumalanga

3.3.3.4. Medicinal uses

Due to the current interest in investigating plants for sources of new medicines, an aspect of particular interest of this survey was the use of cowpea for medicinal purposes. In this study, of the 10% of people who said that they use cowpea medicinally, the majority stated that they used the seeds. The seeds were used to make a paste and applied to open wounds, abscesses and tumours and the paste presumably healed the sores. Other researchers have identified the seeds to have diuretic and anthelmintic properties (Noorwala *et al.* 1995). One respondent indicated that she cooked the roots for medicinal purposes. A previous report indicated that decoctions of roots were used for treating painful menstruation, epilepsy and chest pain (van Wyk & Gericke 2000).

3.3.4. Cultivation practices

Less than half the people interviewed have been producing cowpea actively for less than five years (Figure 3.8.). However, 21.2% have been growing cowpea for more than 20 years where some farmers said they were continuing the tradition started by their forefathers.

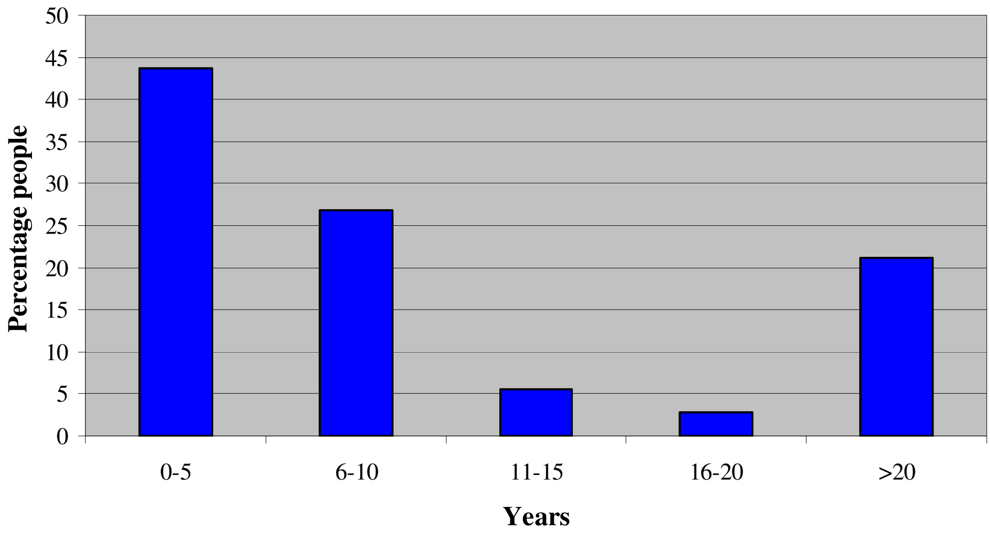

Figure 3.8. Number of years that the respondents have been producing cowpea in Mpumalanga

Most farmers received training with respect to the cultivation of cowpea (60.6%) and were mainly trained through the Department of Agriculture. This was done, for example, in the form of farmers' days where the farmers learnt more about the crop and cultivation procedures from scientists. A small percentage of the training was provided by a company known as Ecolink. Plant protection was not actively practiced by the people (65.7%). Those that did practice it (34.3%), used pesticides and ash to rid insects whereas some used the harmful chemical, dichloro diphenyl trichloroethane (DDT) to treat their crops in the field. A total of 53.5% of the farmers practiced intercropping, predominantly with maize and groundnuts, and to a lesser extent with Bambara groundnuts/jugo beans (*Vigna subterranea* (L) Verdc.). This is similar to intercropping practices followed in Benin (Kossou *et al.* 2001). More farmers should be encouraged to practice intercropping as cowpea actively fixates atmospheric nitrogen, thus increasing soil nitrogen levels for other crops. Almost 60% of the people interviewed did not practice crop rotation. In the Ouémé Valley in Benin, farmers practice various types of crop rotation. Cowpea is often sown second or third after maize as it improves soil fertility and is less labour intensive (Kossou *et al.* 2001).

3.3.5. Storage practices

Drying the seed prior to storage was followed by 98.5% of the people interviewed and this was done mainly by spreading the seeds onto a surface and letting them dry in the sun. Storage containers used by most farmers included calabashes and tins. Other storage mediums included cribs made from plant material, plastic buckets, glass bottles, cement rooms, sacks and wooden rooms (Figure 3.9.). This complements storage practices in Benin, where closed containers like casks and big jars are used to store dry grain (Kossou *et al.* 2001).

A low percentage of respondents encountered problems with mouldiness (19.7%), less had problems with insects (16.9%) and even less reported problems with both insects and mould (14.1%) during storage. Many of the farmers (47.9%) stated that they had no problems during seed storage. This was apparent since many of the farmers adequately dried their seeds prior to storage and many of them (67.1%) treated the seeds with different substances to deter pests.

Figure 3.9. Various storage containers observed during the survey in Mpumalanga; a. plastic bucket, b. cement room, c. wooden room and d. metal box

These substances included ash, pesticides and 8.5 % of the people treated the seeds with DDT during storage. In Benin, local plant-based preparations are used to protect the grains during storage (Kossou *et al.* 2001). In the results of a survey by Kossou *et al.* (2001), it was shown that the insects *Callosobruchus maculatus* Fab. and *Bruchidius atrolineatus* Fab. caused up to 100% loss to cowpea seeds within a few months of storage. The survey indicated that white coloured mould and black coloured mould were predominantly found on the seeds that were stored. Some people stated that green and pink coloured moulds were also growing on the seeds. From these observations, fungi like *Fusarium* spp. and *Aspergillus flavus* Link ex Fries could be present on the seeds. The majority of the farmers (85.9%) did not encounter any problems with the germination of the seeds after storage.

3.3.6. Health aspects

Cowpeas and beans are known to contain antinutritional factors that limit their consumption (Phillips *et al.* 2003). Indigestible oligosaccharides, such as raffinose and stachyose cannot be utilised by humans and monogastric animals since they lack the specific specific ∝-galactosidase enzyme needed to digest them (Phillips *et al.* 2003). In Ibadan, Nigeria mothers were interviewed concerning the problems encountered by children consuming cowpeas. The majority of the mothers (90%) reported no problems attributed to the consumption, but 9.9% of the mothers reported discomfort in their children including diarrhoea, vomiting, offensive stool, abdominal pains, bad breath, abdominal distension and flatulence. Apparently, these problems disappeared later in the lives of the children (Hussain *et al.* 1992). During the present survey, only one person complained of constipation after consuming cowpea. In the case of the livestock, one person noted that his livestock were bloated after eating the fodder.

A plausible explanation why not many problems were reported could be because cowpea was not consumed on a regular basis. It does not seem that there are any ill effects due to mycotoxin ingestion amongst the people and animals. This could also be due to the small intake of seeds and good storage practices. However, chronic effects due to mycotoxin ingestion can only be established by monitoring the people and animals on a long term basis.

3.3.7. Major constraints associated with cowpea production

About 36.6% of the people stated that they experienced no major constraints when it came to the production of cowpea (Figure 3.10.). The constraints identified were largely due to external factors. Thirty-five percent saw drought as a major constraint. Insects including black lice and aphids were also a cause for concern (22.5%) whereas lesser constraints included lack of irrigation water, loss of plants due to cattle grazing and wild animals, deep ploughing and poor flowering of the crop. In the Ouémé Valley, Benin amongst 129 farmers interviewed, the primary constraints to cowpea production were insects (bruchids during storage), birds and rodents (Kossou *et al.* 2001). Similarly, vertebrate and insect pests were identified by farmers in Sierra Leone as limiting cowpea grain production (Alghali & Pratt 1995).

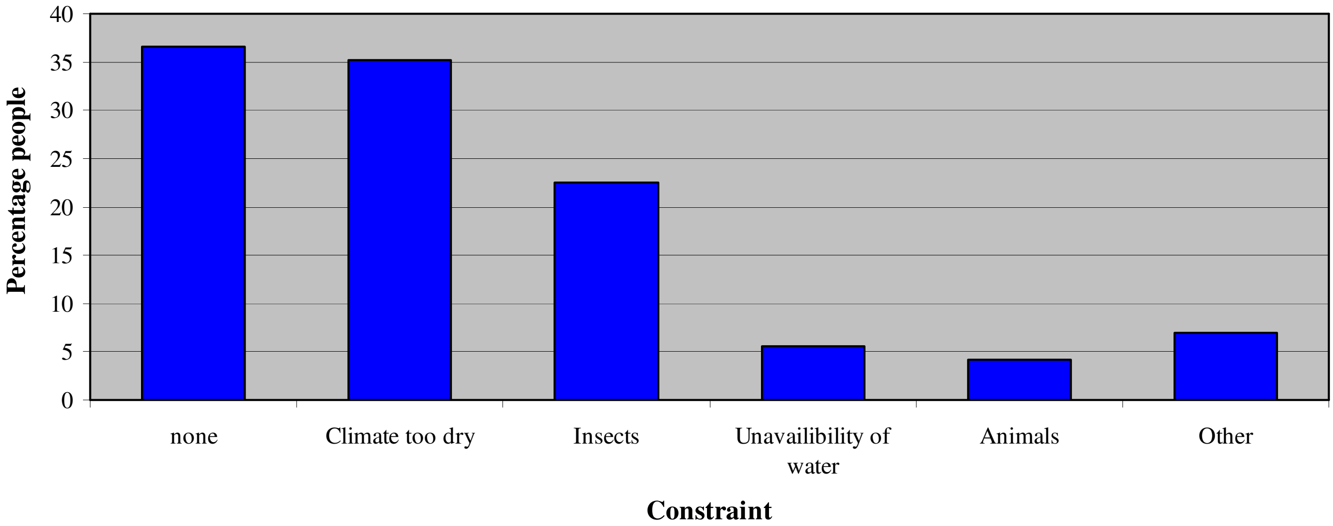

Figure 3.10. Major constraints associated with cowpea cultivation in Mpumalanga

3.4. CONCLUSION

Although cowpea is not considered as an important crop to cultivate, it is evident from this study that the rural communities do rely on it in many ways, mainly as a source of food and, to a lesser extent, as a commodity to trade with. The information gathered from this survey revealed that most of the farmers had a good understanding of the cultivation and benefits of cowpea. Although the majority of the farmers received formal training with respect to cowpea production a concern exists with the continuing use of DDT both in the field and during storage. The evidence of mould on the seeds led to the investigation of storage fungi and possible mycotoxin contamination associated with the seed (Chapter 4). A possible health risk could exist due to the consumption of contaminated feed. Interesting information regarding the medicinal uses of the crop confirms previous reports by other sources. This study prompted the investigation into the antimicrobial activity of cowpea extracts (Chapter 6), providing a good starting point into the scientific validation of the medicinal properties of cowpea.

The potential benefits from the cultivation of cowpea by small-scale farmers in South Africa are numerous. As a good source of protein, it can contribute positively towards food security. Although the farmers have established adequate cultivation and storage practices the input from researchers to rural communities to disseminate knowledge will play an integral part in the increased and sustainable use of cowpea in South Africa.

3.5. ACKNOWLEDGEMENTS

The authors are grateful to July Mhlabane, Felicia Mhlabane, David Lengwati and other field technicians from the Lowveld Research Station, Department of Agriculture, Mpumalanga for their valuable assistance with the interviews. Dr. Cherian Mathews and Kgosi Dongo from the Department of Agriculture, Mpumalanga are acknowledged for their help during the survey. Thanks are due to the farmers, who were the main contributors, for their cooperation.

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CHAPTER 4

Mycoflora and Fumonisin Mycotoxins Associated with Cowpea (*Vigna unguiculata* (L.) Walp) Seeds

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Title Running Header: Fumonisins associated with cowpea seed

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Abstract

Cowpea seed samples from South Africa and Benin were analyzed for seed mycoflora. *Fusarium* species detected were *F. equiseti*, *F. chlamydosporum, F. graminearum, F. proliferatum*, *F. sambucinum*, *F. semitectum* and *F. subglutinans*. Cowpea seed from South Africa and Benin and *F. proliferatum* isolates from Benin, inoculated onto maize patty medium, were analyzed for fumonisin production. Samples were extracted with methanol/water and cleaned-up on strong anion exchange solid phase extraction cartridges. HPLC with pre-column derivatization using *o*-phthaldialdehyde was used for the detection and quantification of fumonisins. Cowpea cultivars from South Africa showed the presence of fumonisin B_1 with concentrations ranging between 0.12 - 0.61 µg/g whilst those from Benin showed no fumonisins. This is believed to be the first report of the natural occurrence of FB_1 on cowpea seed. Fumonisin B_1, B_2 and B_3 were produced by all *F. proliferatum* isolates. Total fumonisin concentrations were between 0.80 - 25.30 µg/g and the highest level of FB_1 detected was 16.86 µg/g.

Keywords: cowpea, fumonisins, FB_1, *Fusarium proliferatum*, mycoflora, *Vigna unguiculata*

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a popular, nutritious and important legume crop of many subsistence farmers and rural communities living in less developed countries of tropical and sub-tropical Africa. This indigenous African legume is cultivated as a pulse, vegetable, fodder and cover crop (*1*), providing more than half of the plant protein in human diets in certain areas of the semi-arid tropics (*2*) and is also a valuable source of carbohydrates and minerals (*3*). Cowpea seeds have an average protein content of 23-25% and a carbohydrate content of 50-67% (*3*) and are consumed in various ways. In Nigeria, the seed is consumed after boiling to softness, and seasoned with salt and pepper with palm oil to form a porridge or after frying in oil to form "akara" (*4*). Dry seeds alone or as part of other dishes are popular in southern Africa (*5*).

Unfortunately, fungal contamination often prevails under conditions of relative high humidity and high ambient temperatures (*6-8*) and some of these fungi produce toxic secondary metabolites under these sub-optimum storing conditions. These mycotoxins, when ingested during the consumption of infected seed and other foodstuffs, can lead to serious health complications in animals as well as humans. It is well documented that various legume seeds are prone to fungal infestation and subsequent mycotoxin contamination (*9-14*). However, reports on mycotoxins associated with cowpea seed are scant and mainly refer to *Aspergillus* infection associated with aflatoxin production (*7, 8, 15-17*). Hitokoko et al. (*7*) reported that cowpea seeds inoculated with toxigenic fungi were contaminated with sterigmatocystin, ochratoxin A and T-2 toxin.

Fumonisins, produced primarily by *Fusarium verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg and *F. nygamai* Burgess and Trimboli (*18, 19*), are mycotoxins that have major toxicological significance in animal and human health (*20, 21*). Various analogues of fumonisins have been identified and characterized of which the most abundant are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (**Figure 1**) (*19, 21*). FB_1 causes equine leukoencephalomalacia in horses (*22*) and pulmonary edema in pigs (*23*). The incidence of *F. verticillioides* infection on homegrown maize is associated with the high incidence of human oesophageal cancer in Transkei, southern Africa and China (*24, 25*). The International Agency for Research on Cancer (IARC) classified the toxins produced by *F. verticillioides* as being possibly carcinogenic to humans (*26*). Furthermore, fumonisins have been detected in naturally infected mouldy navy beans (*Phaseolus vulgaris* L.) (*12*), and *Fusarium* infected adzuki beans (*Phaseolus angularis* (Willd.) W.F. Wight) and mung bean (*Phaseolus aureus* Roxb.) (*13*) and the phytotoxic activity of fumonisins to various plants has been reported (*27, 28*).

The objectives of this study were to investigate the detection and quantification of the fumonisin mycotoxins in cowpea seeds and to identify the fumonisin-producing *Fusarium* species from cowpea seeds and to investigate their potential for fumonisin production.

MATERIALS AND METHODS

Seed samples. Fumonisins were determined in cowpea seeds received from the Agricultural Research Council – Grain Crops Institute in Potchefstroom, South Africa consisting of four cultivars (Bechwana White, Glenda, Iron Grey and Rhino) and seed samples collected in street markets, Kpodjiguégué, Ghebami and Tawa, from Benin, western Africa. Potential fumonisin-producing *Fusarium* species were isolated from the Benin cowpea seed samples.

Isolation and identification of seed-borne fungi. One hundred seeds from each sample of the South African cultivars and two hundred seeds from each of the Benin samples were chosen randomly. The seeds were surface disinfected using 1% sodium hypochlorite for 1 min and rinsed three times in sterile distilled water. Fifty seeds from each South African cultivar were not surface disinfected. Thereafter, the seeds of the South African samples and Benin samples were directly plated out onto malt extract agar (MEA) and potato dextrose agar (PDA), respectively, (five seeds per plate, one seed in the center and one seed in each quadrant). The Petri dishes were incubated at ± 25 °C for 5-7 days with 12 h light/dark cycles after which the seeds were examined for fungal growth. The fungi were transferred to PDA plates for growth and fungal genera and species were identified with the aid of various references (*29-32*) and recorded.

Maize patty media. *Fusarium proliferatum* isolates from the cowpea seed samples were grown on maize patty medium in duplicate based on the method by Alberts et al. (*33*). These isolates (MRC 8275, 8276, 8277 and 8278) were deposited in the culture collection of the PROMEC Unit, Medical Research Council, Tygerberg, South Africa. Maize patty media was prepared in 90 mm Pyrex Petri dishes by adding 25 g finely ground maize kernels / 25 g water. The Petri dishes were autoclaved for 1 h at 121 °C and 120 kPa on two consecutive days. *F. proliferatum* suspensions were prepared in 100 mL sterile distilled water from cultures grown for 7–9 days. The maize patty media were inoculated with 1 mL of the suspension and the cultures were incubated at 25 °C for ± 21 days or until all the media were completely colonized by the fungus.

Sample extraction and clean-up. The samples included the inoculated maize patty cultures and ± 50 g of cowpea seeds of each of the four South African cultivars and the three Benin samples. After incubation, the maize patty cultures were allowed to dry overnight at ± 40 °C. The maize patty cultures and cowpea seeds were ground into a fine meal using a laboratory grinder. The sample extraction and clean-up was carried out according to the method described by Sydenham et al. (*34*). After the addition of 100 mL methanol/water (70/30) (v/v) to 20 g of the fine meal, the samples were homogenized for 3 min at 5000 rpm with an Ultra-Turrex homogenizer (Jankel-Kunkel, Ika-Werk, Germany). The homogenized samples were centrifuged for 10 min at 4000 rpm and the supernatant filtered through Whatman No. 4 filter paper. The pH of the filtrate was adjusted to 5.8–6.5 with 0.1 M sodium hydroxide.

Clean-up of the filtrates were carried out on Chromabond strong anion exchange (SAX) cartridges (Machery-Nagel, Dűren) attached to a solid phase extraction (SPE) vacuum manifold. Prior to loading 10 mL of the filtrate, the SAX cartridges were preconditioned by washing them successively with 5 mL methanol and 5 mL methanol/water (70/30) (v/v), whilst maintaining a flow rate of 1 mL/min. After loading, the cartridges were washed with 5 mL methanol/water (70/30) (v/v) and 3 mL methanol. This was followed by elution with 10 mL methanol/acetic acid (1/99) (v/v) at a flow rate of 1 mL/min and the eluate collected in vials. Eluates were evaporated to dryness in vials on a Reacti-Therm Heating module with a Reacti-Vap Evaporator (Pierce, Rockford, Illinois) at ± 50 ºC under nitrogen gas. The collection vials were washed with methanol and evaporated to dryness. The dry residues were stored at 4 ºC until analyzed.

Fumonisin analyses. The fumonisin analyses were performed at the PROMEC Unit, Medical Research Council, Tygerberg, South Africa, utilizing high performance liquid chromatography (HPLC) on a 150 x 4.6 mm Ultracarb 5 ODS (20) column (Phenomenex) with *o*-phthaldialdehyde (OPA) pre-column derivatization and fluorescence detection with a model 474 scanning fluorescence detector (Waters Corp., Milford) at 335 nm (excitation) and 440 nm (emission). Fumonisin standards were purified as described previously by Cawood et al. (*35*). OPA (225 µL) was added to 25 µL of the combined standard (FB_1, FB_2 and FB_3) and 10 µL injected, whilst 150 µL OPA was added to 100 µL sample (redissolved in 200 µL methanol) and 50 µL was injected. The mobile phase was methanol: 0.1 M sodium dihydrogen phosphate (NaH_2PO_4) (77/23) adjusted to pH 3.35 with *ortho*-phosphoric acid and run at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

The incidence of fungi isolated from each cultivar obtained in South Africa was higher in the untreated seeds than in the surface disinfected seeds (**Table 1**). In both the untreated and the surface disinfected seeds the highest infection was reported in the Iron Grey and Rhino cultivars. *Aspergillus* and *Phoma* species were present in all the cultivars and in both surface disinfected and untreated seeds. *Aspergillus glaucus* Link ex Gray was the most abundant *Aspergillus* species, occurring in three of the cultivars, followed by *A. flavus* Link ex. Fries and *A. niger* van Tieghem. Hitokoko et al. (*7*) reported *A. glaucus*, *Penicillium* and *Alternaria* species to be present in cowpea seed. Esuruoso (*6*) observed various fungi to be associated with 81 samples of cowpea seed in western Nigeria. These included *A. flavus*, *A. niger*, *A. ochraceus* Wilhelm, *Penicillium digitatum* Sacc., *Rhizopus arrhizus* Fischer, *Chaetomium*, *Cladosporium*, *Curvularia* and *Macrophomina* species. In the present study *Chaetomium* and *Cladosporium* species were isolated from two and three samples, respectively. Other fungal genera isolated from these samples included *Penicillium* and *Trichothecium* species. The most abundant fungi from cowpea seeds from India were *F. verticillioides*, *F. oxysporum* Schecht ex. Fries, *Colletotrichum gleosporiodes* Penz. and Sacc., *A. niger* and *Penicillium* sp. (*36*). Similarly, Ushamalini et al. (*1*) reported that *Macrophomina phaseolina* (Tassi.) Goid., *F. oxysporum, Alternaria alternata* (Fr.:Fr.) Keissler, *A. flavus, A. niger* and *Penicillium* sp. were isolated from seeds of different districts in Tamil Nadu, India. Cowpea samples analyzed by Seenappa et al. (*8*) were invariably infected by *Aspergillus* and subsequently contaminated by aflatoxin. In a study by El-Kady et al. (*17*) two of three cowpea seed samples artificially infected by *A. flavus* produced aflatoxins.

In the present study six *Fusarium* species were isolated, of which *F. equiseti* (Corda) Sacc. was the most abundant. Of these *Fusarium* species four were present in the Rhino, two in the Bechwana White and one in the Glenda cultivar. *Fusarium* species producing high concentrations of mycotoxins other than fumonisins, which include *F. equiseti, F. sambucinum* Fuckel and *F. subglutinans* (Wollenw. and Reink.) Nelson, Toussoun, and Marasas (*37*), were isolated.

FB_1 was detected in all four samples of the South African cultivars, while FB_2 and FB_3 were not detected. The Rhino cultivar had the highest average concentration of FB_1 (0.61 µg/g) followed by Glenda, Bechwana White and Iron Grey with low levels of 0.16, 0.13 and 0.12 µg/g, respectively. Even though the most important fumonisin-producing species are *F. verticillioides* and *F. proliferatum*, neither of these two species was isolated from the South African cowpea seed samples. No fumonisins, however, were detected in the Benin seed samples, which could be attributed to conditions being unfavorable for fumonisin production in these samples. Tseng et al. (*12*) detected FB_1 levels of 0.5 µg/g and 1.1 µg/g in naturally infected mouldy navy beans from Ontario, Canada. *Fusarium* species isolated from these beans included *F. avenaceum* (Fr.) Sacc., *F. culmorum* (W.G. Smith) Sacc., *F. graminearum* Schwabe, *F. verticillioides, F. oxysporum*, and *F. solani* (Mart.) Appel and Wollenw. emend. Snyd. and Hans. However, the *Fusarium* species responsible for FB_1 production was not identified. Furthermore, Tseng & Tu (*13*) investigated the presence of FB_1 in adzuki and mung beans from Ontario, Canada. It was found that the adzuki and mung bean samples contained average concentrations of 261 and 230 µg/g of FB_1, respectively. Identified *Fusarium* species isolated from mouldy beans included *F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. verticillioides, F. oxysporum, F. solani* and *F. sporotrichioides* Sherb. In an attempt to identify the *Fusarium* sp. responsible for FB_1 production, the beans were inoculated with *F. graminearum*, and analyzed for FB_1 and FB_2 and the results proved to be negative. The authors suggested that FB_1 found in diseased adzuki and mung beans was due to *F. verticillioides* infection (*13*).

The incidence of fungal infection of the cowpea seed of the three Benin market samples is presented in **Table 2**. The fungi isolated from these seeds differ quite substantially from those isolated from the South African cultivars. The highest percentage infection of the cowpea seeds was found in Kpodjiguégué, followed by Tawa and then Gbehami. *A. flavus* was detected in the Tawa and Gbehami samples and a large percentage of *Lasiodiplodia theobromae* (Patouillard) Griffon et Maublanc was detected in the Kpodjiguégué sample. Other fungal genera detected included *Curvularia, Penicillium* and *Mucor* species. The total percentage of *Fusarium* isolates was relatively low and included *F. equiseti* (2.5%), *F. semitectum* Berkeley & Ravenel (1.5%), *F. subglutinans* (0.5%) and *F. proliferatum* (2%). *F. equiseti*, *F. semitectum* and *F. subglutinans* were also isolated from the South African cowpea seed samples in this study as well as cowpeas from Nigeria and India in other studies (*6, 36*). However, in contrast to previous studies on cowpea seeds (*6, 36, 38*) *F. oxysporum, F. solani* and *F. verticillioides* were not detected in these samples. Esuruoso (*6*) recorded *F. verticillioides* infection on most of the 81 cowpea seed samples analyzed. In this study, four *F. proliferatum* isolates were detected (two each from Kpodjiguégué and Gbehami). *F. proliferatum* is a primary producer of fumonisins (*39*), and therefore these four were grown on maize patty medium in duplicate and analyzed for fumonisin production.

The data shown in **Table 3** represents the mean concentration of fumonisin production by the *F. proliferatum* isolates from the Benin cowpea samples. The highest concentration of FB_1 was produced by Gbehami Isolate 2 with a mean of 16.86 µg/g for the two replicates. In previous studies *F. proliferatum* isolated from various other cereals produced higher fumonisin levels than the current isolates (*18, 39*). Thiel et al. (*18*) found that *F. proliferatum* isolates from sorghum and maize produced 20 - 660 µg FB_1/g and 65 - 450 µg FB_2/g, respectively. *Fusarium proliferatum* maize cultures produced 1 670 - 2 790 µg FB_1/g and 150 - 320 µg FB_2/g, respectively (*39*). As far as the authors are aware this is the first report of the natural occurrence of FB_1 in cowpea seeds and this is also the first study that has shown that *F. proliferatum* isolates from cowpea seed has the potential to produce fumonisin mycotoxins. *F. verticillioides*, a major fumonisin producing fungus, has been isolated from cowpea seed (*6, 36*). Therefore, studies are needed to confirm whether *F. verticillioides* is associated with cowpea seed and whether it produces fumonisins in cowpea seed. Although the fumonisin levels shown in this study are relatively low, further screening for fumonisins in cowpea seeds intended for human consumption and animal feed is warranted.

ACKNOWLEDGEMENTS

The authors thank Norma Leggott for performing the HPLC analyses on the cowpea seed samples. The National Research Foundation (NRF) is acknowledged for financial support. The PROMEC Unit for the preparation and purification of the fumonisin standards.

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Table 1. Fungi isolated from four cultivars of cowpea seeds obtained in South Africa

Fungus	Incidence (%)							
	Cowpea cultivars							
	Glenda		Bechwana White		Rhino		Iron Grey	
	SD[a]	UT[b]	SD	UT	SD	UT	SD	UT
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 fungi	8	88	4	92	68	94	52	98

[a] surface disinfected seeds

[b] untreated seeds

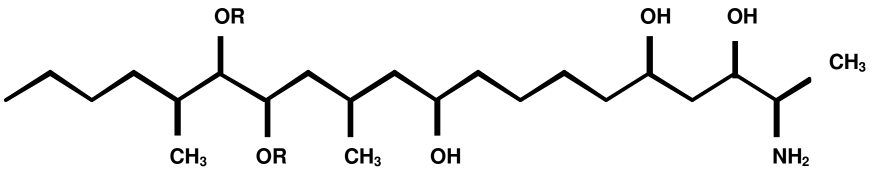
Table 2. Fungi isolated from cowpea seeds obtained from three localities in Benin

Fungus	Incidence (%)		
	Cowpea samples		
	Kpodjiguégué	Tawa	Gbehami
Aspergillus flavus	-	1.5	5.5
Chaetomium sp.	-	-	1.5
Curvularia sp.	0.5	4	0.5
Fusarium equiseti	1	-	1.5
F. proliferatum	1	-	1
F. semitectum	1	-	0.5
F. subglutinans	-	0.5	-
Lasiodiplodia theobromae	34	3	-
Mucor sp.	-	5	2.5
Penicillium chrysogenum	1	0.5	-
Other	32	17.5	2
Total fungi	70.5	32	15

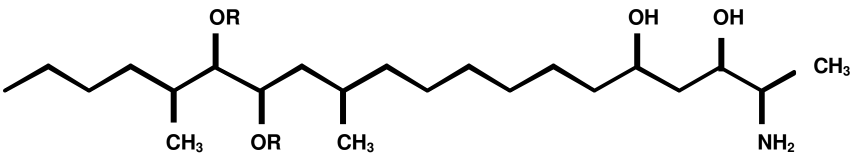
Table 3. Fumonisin production by *Fusarium proliferatum* isolates grown on maize patty medium

Isolates	Fumonisin concentration (µg/g)			
	FB_1	FB_2	FB_3	Total Fumonisins
Kpodjiguégué Isolate 1	9.33 ± 5.26[a]	1.54 ± 0.52	0.76 ± 0.40	11.62 ± 6.18
Gbehami Isolate 1	0.67 ± 0.55	0.11 ± 0.09	0.03 ± 0.04	0.80 ± 0.68
Kpodjiguégué Isolate 2	2.93 ± 0.45	0.65 ± 0.04	0.20 ± 0.02	3.77 ± 0.52
Gbehami Isolate 2	16.86 ± 3.97	6.61 ± 2.28	1.83 ± 0.16	25.30 ± 6.09

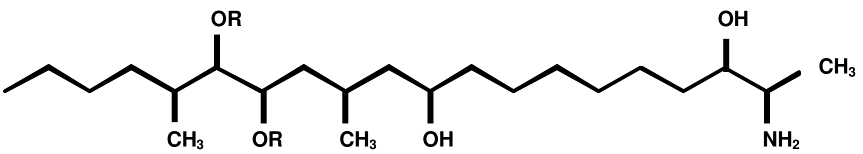
[a] mean ± standard deviation of two replicates



FB$_1$



FB$_2$



FB$_3$

R = $COCH_2CH(COOH)CH_2COOH$

Figure 1. Structures of fumonisin B$_1$, B$_2$ and B$_3$

CHAPTER 5

Phytotoxic Effects of Fumonisin B_1 on Cowpea Seed

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ABSTRACT

Kritzinger, Q., Aveling, T.A.S., and Van der Merwe, C.F. 2004. Phytotoxic effects of fumonisin B_1 on cowpea seed. Phytopathology

The cultivation of cowpea plays a vital role in the livelihood of many subsistence farmers and rural communities in tropical and sub-tropical countries. The seeds are prone to fungal infestation and mycotoxin contamination during sub-optimal storage conditions. Fumonisin B_1 (FB_1), produced by *Fusarium proliferatum,* has been detected in cowpea seeds. Surface-disinfected seeds were imbibed for 10 h in 50 ml sterile distilled water amended with FB_1 to yield final concentrations of 10, 25, 50, 100 ppm (concentrations based on previous studies on maize). Slow imbibed seeds (placed in moist paper towels) incubated at 25˚C for 10 h and seeds placed in sterile distilled water for the same period of time served as the positive and negative control, respectively. Percentage germination was determined according to the International Seed Testing Association (ISTA) rules. Root and shoot length was measured after 9 days. Parts of the embryonic axes and cotyledon tissues were removed and prepared for transmission electron microscopy. All the toxin concentrations significantly decreased seed germination. The 50 and 100 ppm FB_1 concentrations inhibited root and shoot elongation. FB_1 treated embryonic tissues indicated compaction of the protoplasm and separation of the plasmalemma from the cell wall. Lipid bodies accumulated, which seemed to be lining the cell wall. This is the first study to demonstrate the phytotoxic effects of FB_1 on cowpea seeds.

Additional keywords: germination; Fusarium spp.; Vigna unguiculata; ultrastructure

Cowpea (*Vigna unguiculata* (L.) Walp) is a widely cultivated indigenous African legume crop that is of great importance in tropical and sub-tropical countries of Asia, Africa, Oceania, the Middle East, southern Europe, southern United States of America and Central and South America (8). This crop has a variety of uses, which include providing excellent ground cover to prevent soil erosion, suppressing weed growth, its ability to improve soil nitrogen levels and is a source of cash for rural communities through trade of the seed (22). Furthermore, many subsistence farmers and rural communities residing in less developed countries rely greatly on the crop as a good source of nutritious food (22).

However, when the seeds are stored at high relative humidities and high ambient temperatures, fungal infestation usually occurs. It is under these conditions that some of these fungi may produce secondary toxic metabolites namely, mycotoxins (21). Mycotoxins are well known to have a negative impact on the health of animals and humans (7), but some are also known to have toxic effects on plants (11,17). Previous studies have shown that aflatoxin B_1 and crude aflatoxins inhibited chlorophyll formation and seed germination in cowpea (5).

The fumonisins, the most recently characterized mycotoxins, are produced by certain *Fusarium* spp. including *F. verticillioides* (Sacc.) Nirenberg, *F. proliferatum* (Matsushima) Nirenberg and *F. nygamai* Burgess and Trimboli (19,24). Fumonisin B_1 (FB_1) (Fig. 1) has been known to cause various toxicological problems in animals. These include leukoencephalomacia (LEM), a fatal brain disease in horses, and pulmonary edema syndrome (PES) in pigs (15,18). There is evidence that suggests fumonisins are associated with birth defects i.e. neural tube defects in humans (16). This toxin is statistically linked to the incidence of esophageal cancer in humans in Transkei, South Africa and China (18). Fumonisin B_1 is known to exhibit phytotoxic effects towards different plants, including economically important crops (1,2,3,4,9,14,17,26,27). Previous studies on other legume crops showed that soybeans (*Glycine max* L.) were severely damaged (necrosis and wilting) when sprayed with a 1000 µg/ml concentration of FB_1 (1).

Fumonisin B_1 has been found to be associated with cowpea seed from South Africa and Benin, West Africa (13). During this investigation, *F. proliferatum* was found to be responsible for the production of the toxin on cowpea seed. (13).

This paper reports on the effect of the FB_1 toxin on cowpea seed germination and on root and shoot elongation. The effect of the toxin on the ultrastructure of the cotyledon and embryonic tissue of the seed is also reported.

MATERIALS AND METHODS

Seed material. Cowpea seeds (cultivar IT 85F-867-5) were obtained from Ecolink, Nelspruit, South Africa. Three replicates of 100 seeds were used for each treatment. Prior to the treatments, the seeds were surface disinfected with 1% sodium hypochlorite for 1 min and thereafter rinsed three times with sterile distilled water.

Toxin. Dried FB_1 (batch A/01, 11.3 mg) was supplied by the PROMEC Unit, Medical Research Council (MRC), Tygerberg, South Africa. Methanol (20 ml) was added to FB_1 and 1 ml quantities were aliquoted into 20 vials. The aliquots were dried down under nitrogen gas and stored at ± 4°C until used.

Seed treatments. The required volume of fumonisin was added to 50 ml sterile distilled water to yield final concentrations of 10, 25, 50 and 100 ppm. The seeds were allowed to imbibe in the various solutions for a period of 10 h. Sterile distilled water (50 ml) was added to 7-day-old cultures of *F. verticillioides* (MRC 4315), *F. nygamai* (MRC 3997) and *F. proliferatum* (MRC 8278). The surface of each culture was scraped to free the spores and the spore suspensions were poured through muslin cloth into flasks. A spore concentration of 1×10^6 ml^{-1} was determined with the use of a haemacytometer. The seeds were added to the flasks and mixed thoroughly and thereafter allowed to dry for ± 5 min. Slow imbibed seeds (seeds placed in moist paper towels) were incubated at 25°C for 10 h (positive control). Seeds placed in sterile distilled water for the same period of time served as the negative control.

Seed germination. Percentage germination was determined by placing the seeds between moist paper towels which were rolled up and placed individually in polythene bags, held upright in plastic buckets and maintained at ± 25°C in an incubator. Percentage germination was determined after 5 and 9 days according to the International Seed Testing Association (ISTA) rules (12). Root and shoot length was determined after 9 days of growth.

Transmission electron microscopy. Representative seeds from each treatment (as described above) were removed after the 10 h period of imbibition. The seeds were dissected and the embryonic axes and cotyledon tissue were removed. The tissues were fixed overnight in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.4). The samples were rinsed three times in 0.075 M phosphate buffer and post-fixed in 1% aqueous osmium tetroxide. Thereafter, the samples were rinsed and dehydrated in an ethanol series and embedded in Quetol 651 resin at 60°C for 48 h. Ultra-thin sections were prepared using a Reichert Ultracut E ultramicrotome (Vienna, Austria) and stained for viewing with a Philips EM301 transmission electron microscope (Eindhoven, Netherlands). Sections were also stained for viewing with a Nikon Optiphot light microscope (Tokyo, Japan).

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 AND DISCUSSION

Effect on seed germination and root and shoot elongation. All four FB_1 concentrations significantly decreased seed germination when compared to both the positive and negative controls (Fig. 2). The lowest percentage (6.67%) of seed germination was at the 100 ppm concentration. It is apparent from these results that the toxin may block various biochemical reactions that are necessary for normal germination to take place. Danielsen and Jensen (9) found a significant negative correlation between fumonisin content and corn (*Zea mays* L.) seed germination. It was, however, not established whether the fumonisins had a direct effect on germination or not. On the other hand, Doehlert *et al.* (10) reported that FB_1 had no effect on corn seed germination but the toxin did, however, inhibit radical elongation in the seeds by up to 75% after 48 h of imbibition. The authors found that amylase production in the endosperm was also inhibited, which could suggest that FB_1 interfered metabolically with germination (10).

In this study, *F. verticillioides* and *F. proliferatum* inoculated seeds also showed significant reduction in germination (Fig. 2). It was not established whether the fungus alone affected germination or whether it was a combination between the production of a toxin and fungal infestation. Danielsen and Jensen (9) found no significant correlation between *F. verticillioides* infection and seed germination in corn. At nine days, a significant increase in ungerminated seeds was noted in the toxin treated seeds (Fig. 3). Correspondingly, these treatments had the lowest number of normal seeds. With the exception of *F. nygamai*, the *Fusarium* inoculated seeds and the 50 ppm and 100 ppm toxin treated seeds revealed the highest amounts of diseased seeds.

Only the 50 and 100 ppm toxin concentrations significantly inhibited root and shoot elongation (Fig. 4). In several cases, the toxin caused severe stunting of the roots. Lamprecht *et al.* (14) found that FB_1 and the FB_2 and FB_3 analogues caused dose dependant reductions in root and shoot length and dry mass in corn seedlings. The three *Fusarium* spp. artificially inoculated onto the cowpea seeds showed no inhibitory effect on the growth of the roots and shoots of the seedlings.

Effect on ultrastructure. The ultrastructure of both controls of the untreated embryonic axes (Fig. 5 a and b) and cotyledon tissues (Fig. 6 a and b) revealed neat, intact cells with clearly defined nuclei and other organelles. Numerous lipid bodies, ribosomes and vacuoles can also be seen. When looking at the micrographs of the embryonic axis tissues, there seemed to be no noteworthy differences in the ultrastructure of the lower toxin treated tissues (Fig. 5 c and d) when compared to the control. The 25 ppm treated tissue did, however, show an abundance of vacuoles containing protein bodies and lipid bodies throughout the protoplasm (Fig. 5 d). The only distinctive destructive effects caused by the toxin are shown in the 100 ppm treated embryonic tissues (Fig. 5 f to h). The plasma membrane has separated from the cell wall and irregular sized vacuoles (Fig. 5 f to h) have formed due to the contraction of the protoplasm. Some of the contents of the cytoplasm have passed through the plasma membrane as it separated away from the cell wall (Fig. 5 f). The compacted protoplasm appears very dense and darker in color when compared to the control micrographs. An abundance of lipid bodies was noted next to the cell wall in the 50 and 100 ppm treated seed tissues (Fig. 5 e and h). The treated cotyledon tissue (25, 50 and 100 ppm) showed similar patterns with regard to the accumulation of lipid bodies (Fig. 6 d to f). Baird et al. (6) found lipid bodies to be conspicuous at the margins of the cytoplasm, outlining the cell walls in dry radical cells of soybean. Similarly, lipid droplets were closely appressed to the plasma membrane in dry cells of cowpea embryo tissue (25). During imbibition, lipid droplets become less as they dissolve to become part of the membranous system of the cell. It is possible that FB_1 could prevent or reduce the normal metabolism of the cell so that the cell does not take up the lipids. The 100 ppm treated cotyledon tissue did not reveal any noticeable effects caused by the toxin (Fig. 6 f) as noted in the 100 ppm treated embryonic axis tissue (Fig 5 f to h).

These destructive effects seen in the ultrastructure of the 100 ppm treated embryonic axis tissue could possibly play a role in the significant reduction in germination and root and shoot length at the same concentration. Van Asch (26) treated corn callus with different doses of FB_1, which produced deteriorative alterations in the cell ultrastructure. These included cell wall thickening and the accumulation of large starch grains within a swollen plastid.

Fumonisin B_1 inhibits the enzyme ceramide synthase in plants, which leads to the reduced formation of sphingolipids and accumulation of free sphingoid bases (3). Sphingolipids are highly bioactive compounds of cellular membranes that have profound effects on cell regulation (28). In plants, sphingolipids play a role in cell signaling, membrane stability, stress response, pathogenesis and apoptosis, but little is known about their precise functions (23). Studies on animal cells have also shown that fumonisins interfere with the synthesis of sphingolipids, which results in disturbances in cell growth, differentiation and morphology (20).

Although the mode of action of FB_1 in plant cells is uncertain, the interference of the metabolism of the sphingolipids by FB_1 could play a role in the alterations noted in the ultrastructure of the toxin treated seeds. It is evident from this study that the toxin has interfered with the cell morphology in some of the treated tissues and this interference has caused a negative impact on the germination of the seeds as well as the growth of the seedlings. However, further research is necessary to determine the precise toxic effects of the toxin on the seed tissue.

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Fig. 1. Chemical structure of fumonisin B_1.

Fig. 2. Effect of FB_1 and *Fusarium* spp. on cowpea seed germination. Each bar is a mean of 3 replicates. Values of the bars not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 3. Effect of FB_1 and *Fusarium* spp. on cowpea seeds and seedlings. Values of the lines with the same symbol not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 4. Effect of FB_1 and *Fusarium* spp. on root and shoot elongation. Values of the lines with the same symbol not followed by the same letter are significantly different (P=0.05) according to the student's *t* test.

Fig. 5. TEM micrographs of the embryonic axes of cowpea seed, a) imbibed for 10 h in sterile distilled water, b) imbibed for 10 h in moist paper towels, c) imbibed for 10 h in sterile distilled water with the addition of FB_1 at 10 ppm, d) 25 ppm, e) 50 ppm, f, g and h) and 100 ppm.
(CW = cell wall, L = lipid, N = nucleus, PM = plasma membrane, V = vacuole, arrows – plasma membrane separated from cell wall). Bar = 1 μm.

Fig. 6 TEM micrographs of cotyledon tissue of cowpea seed, a) slow imbibed for 10 h in moist paper towels, b) imbibed for 10 h in sterile distilled water, c) imbibed for 10 h in sterile distilled water with the addition of FB_1 at 10 ppm, d) 25 ppm, e) 50 ppm, f) and 100 ppm.
(CW = cell wall, L = lipid, N = nucleus, V = vacuole, arrows – plasma membrane separated from cell wall). Bar = 1 μm.

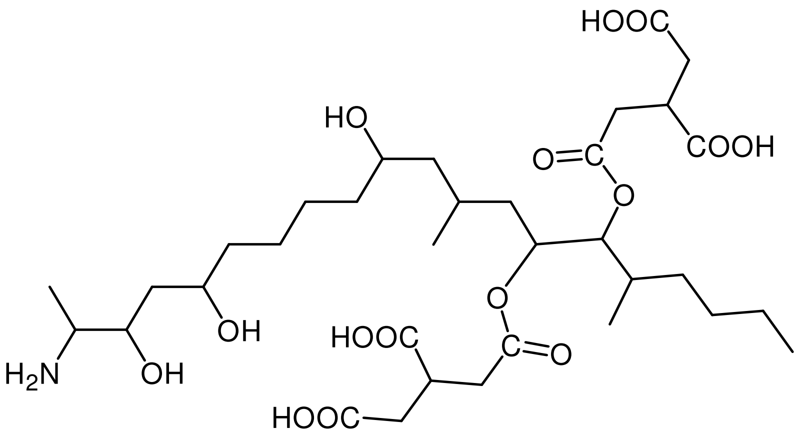
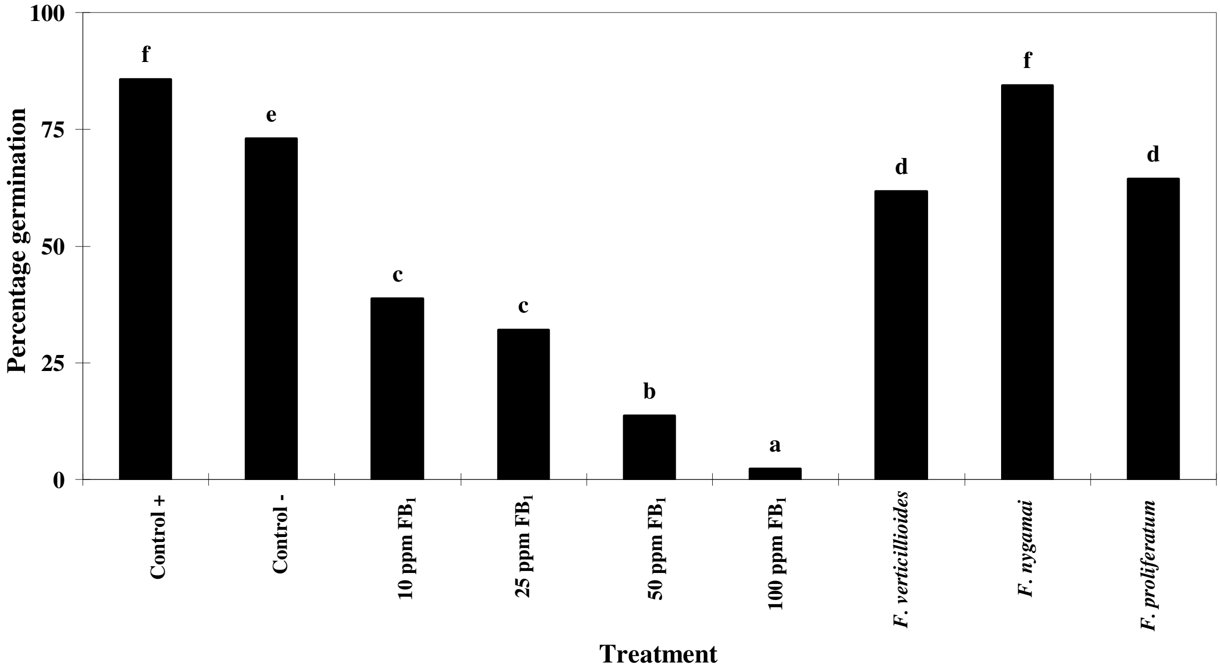


Fig. 1., Kritzinger, *Phytopathology*



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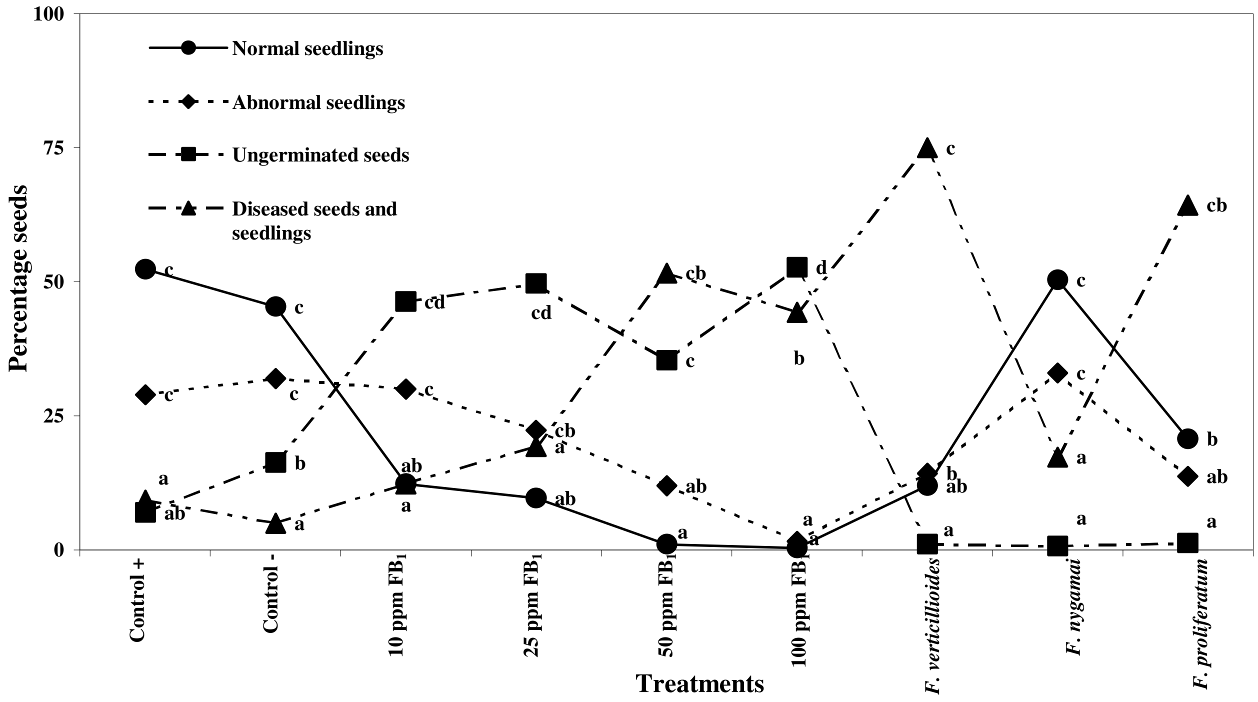

Fig. 3., Kritzinger, *Phytopathology*

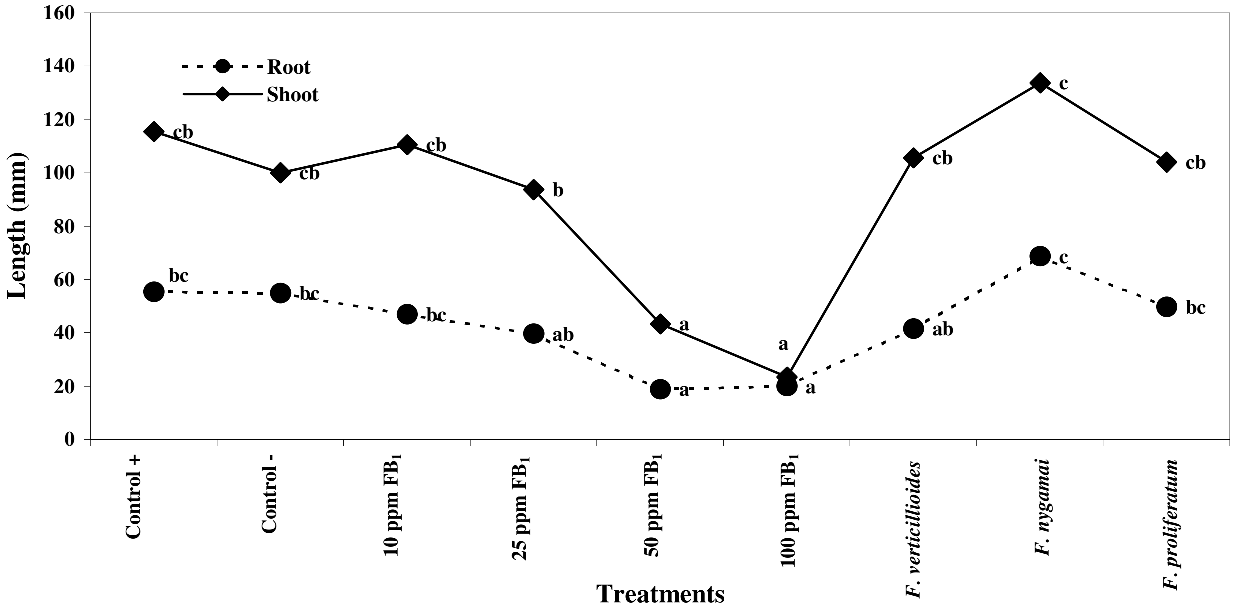

Fig. 4., Kritzinger, *Phytopathology*

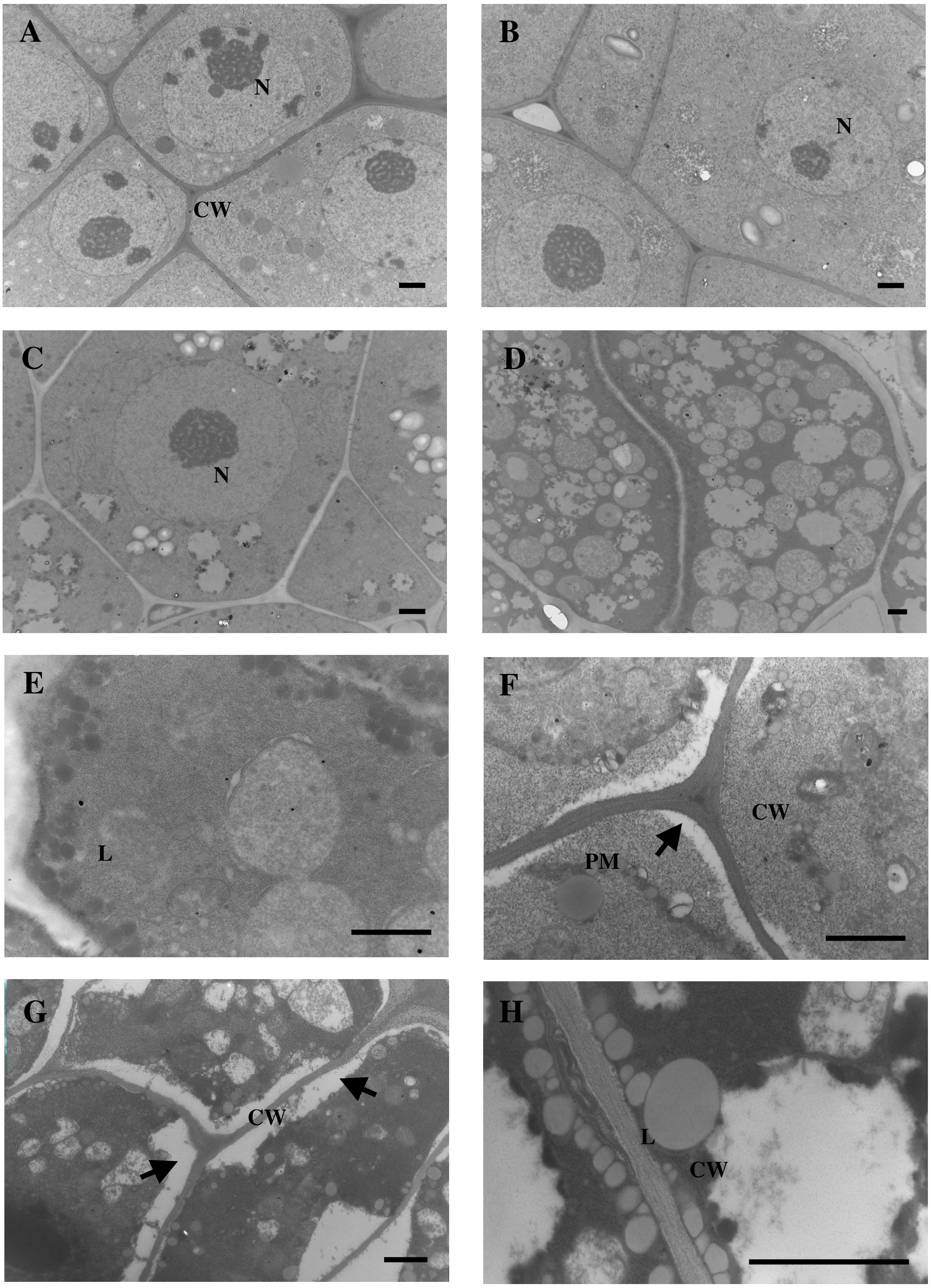

Fig. 5., Kritzinger, *Phytopathology*

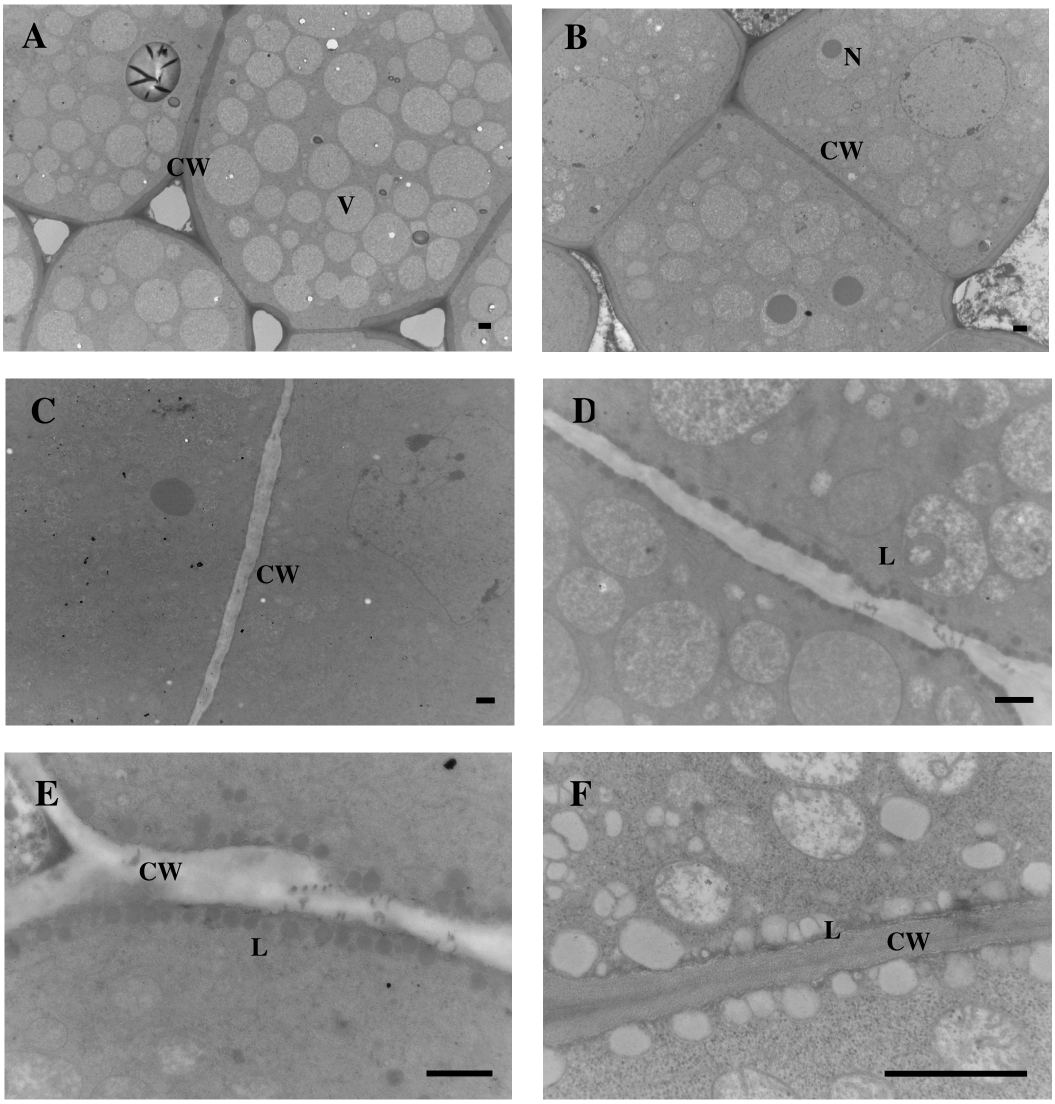

Fig. 6., Kritzinger, *Phytopathology*

CHAPTER 6

Antimicrobial activity of cowpea (*Vigna unguiculata*) leaf extracts

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Abstract

Cowpea (*Vigna unguiculata* (L.) Walp), an indigenous African legume crop, is used to treat epilepsy, bilharzia, chest pains and constipation. Acetone and ethanol extracts of the leaves of Bechwana White (BW) and Kpodjiguégué (Kpod) cultivars were investigated for their antimicrobial properties against bacterial and fungal pathogens. With the exception of *Fusarium equiseti*, all the extracts significantly inhibited growth of the fungal pathogens at 5.0 mg/ml. *Alternaria alternata* was significantly reduced by both BW extracts at 2.5 mg/ml whereas only the ethanol extract showed antifungal activity against *Fusarium proliferatum* at the same concentration. The acetone extract from Kpod inhibited the growth of *A. alternata* at 2.5 mg/ml. BW acetone extracts inhibited growth of the Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis* at 2.5 mg/ml and *Bacillus cereus*, *B. subtilis* and *Enterobacter cloacae* at 5.0 mg/ml. Ethanol extracts of the same cultivar only showed antibacterial activity against *Enterococcus faecalis* and *Enterobacter cloacae* at 5.0 mg/ml. The Kpod extracts exhibited no inhibitory effect on the bacteria. This is the first report on the inhibitory effect of cowpea leaf extracts on the growth of bacterial and fungal pathogens.

Introduction

Bacterial and fungal pathogens play a negative role with regard to the nutritional and economical value of various important crop plants. These pathogens cause severe damage to the roots and aerial parts of the plants. Many pesticides as well as various other chemical formulations used to control plant pathogens are inaccessible to small-scale farmers due to financial constraints. The use of plant extracts provides a less expensive means of controlling these pathogens (Poswal *et al.* 1993). Furthermore, since chemicals pose a danger to the environment and non-targeted organisms, the use of plant extracts as an alternative means of controlling plant fungal and bacterial pathogens has been widely exploited (Poswal *et al.* 1993). There have been numerous investigations into the use of plants extracts in controlling plant pathogens (NRC 1992, Eksteen *et al.* 2001).

Moreover, bacterial and fungal pathogens are also capable of causing serious diseases in humans and animals (Van Burik and Magee 2001, Worthington and Bigalke 2001). Some fungal pathogens infest the seed during storage and produce toxic secondary metabolites, mycotoxins, which when ingested can cause acute and chronic toxicities in humans and animals (Barrett 2000). Due to problems with the toxicity of existing antimicrobial agents as well as the emergence of drug-resistant strains, the use of plant extracts can be exploited as an alternative way to control these pathogens. Many of these plant extracts contain secondary compounds that have an inhibitory effect on harmful bacterial and fungal human pathogens (Afolayan and Meyer 1997, Lall and Meyer 2000, Mathekga *et al.* 2000).

Cowpea, (*Vigna unguiculata* (L.) Walp), is an important legume crop that is widely grown in many countries of sub-Saharan Africa and Latin America (Lattanzio *et al.* 2000). This versatile crop has various uses, which include a good source of nutritious food, animal fodder and a source of cash through trade of the seed (Singh *et al.* 1997). It also increases soil nitrogen levels and prevents soil erosion (Singh *et al.* 1997). Furthermore, an infusion of the seed can be taken orally to treat amenorrhoea whilst powdered roots eaten with porridge are believed to treat painful menstruation, epilepsy and chest pain by the indigenous people of South Africa (Van Wyk and Gericke 2000). Leaves are applied on burns and can be used as a snuff to treat headaches (Hutchings *et al.* 1996). The Zulu's (a South African tribe) make emetics from the plant, which are taken to relieve fever (Gerstner 1939, as cited by Hutchings *et al.* 1996). Cowpea has also been identified as a plant that traditional healers use to treat urinary shistomiasis (bilharzia) in Zimbabwe (Ndamba *et al.* 1994). Cowpea seeds cooked with the roots of *Lannea edulis* (Sond.) Engl. (Van Wyk and Gericke 2000), *Euclea divinorum* Hiern or *Terminalia sericea* Burch ex DC. (Nyazema 1987) are used by South Africans to treat blood in the urine and bilharzia.

However, as far as the literature is concerned, no report on the antimicrobial activity of cowpea has been found thus far. This is the first report on the inhibitory effect of extracts made from cowpea leaves on the growth of various bacterial and fungal pathogens.

Material and methods

Plant material

Seeds of two cowpea cultivars, "Bechwana White" (BW) obtained from the Grain Crops Institute – Agricultural Research Council, Potchefstroom (South Africa) and "Kpodjiguégué" (Kpod) collected from a market in Cotonou, Benin (West Africa) were planted under greenhouse conditions. The plants were harvested after ± two months of growth.

Preparation of extracts

Two solvents, namely acetone and ethanol were used for the extractions of both the cultivars. Air dried plant material (100 g) was homogenised with 250 ml of the solvent for 1 min and then filtered. This process was repeated three times. The filtrate was concentrated to dryness at reduced pressure with a rotary evaporator (Büchi Laboratoriums, Technik AG, Germany). The resultant residues were later dissolved with the respective solvent to 100 mg/ml. In the case of the antibacterial tests, the ethanol extract was re-dissolved using dimethyl sulphoxide since prior investigations showed ethanol to be toxic to the bacteria.

Micro-organisms

The fungal pathogens used in this investigation were *Alternaria alternata* (Fr: Fr.), *Aspergillus flavus* Link ex. Fries, *Fusarium equiseti* (Corda) Sacc., *F. proliferatum* (Matsushima) Nirenberg and *Penicillium chrysogenum* Thom. The fungal cultures were maintained on potato dextrose agar (PDA) at ± 25°C. The bacteria used in this study to determine antibacterial activity of the extracts included five Gram-positive bacteria: *Bacillus cereus* Frankland and Frankland, *B. pumilus* Meyer and Gottheil, *B. subtilis* (Ehrenberg) Cohn, *Staphylococcus aureus* Rosenbach, *Enterococcus faecalis* (Andrews and Horder) Schleifer and Kilpper-Balz and five Gram-negative bacteria: *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, *Escherichia coli* (Migula) Castellani and Chalmers, *Klebsiella pneumoniae*

(Schroeter) Trevisan, *Pseudomonas aeruginosa* (Schroeter) Migula and *Serratia marcescence* Bizio. All the bacteria were obtained from the bacterial collection at the Department of Microbiology and Plant Pathology, University of Pretoria. Bacterial cultures were recovered for testing by culturing in nutrient broth for 24 hr at 37 ºC.

Antimicrobial tests

For the antifungal assay, the required amount of extract was added to sterile PDA in 65 mm Petri-dishes before congealing to yield final concentrations of 0.5, 1.0, 2.5, and 5.0 mg/ml. Unamended PDA plates served as controls. Once the agar had solidified, a 5 mm plug of a 7-d-old fungal culture was placed in the centre of the Petri-dish containing the extract-amended and unamended PDA plates. The plates were sealed with Parafilm and placed in an incubator at 25 °C. Fungal growth was measured on two preset diametral lines after 3, 6, and 9 d of growth. Each treatment was analysed in triplicate. The results of the 6-d growth was statistically analysed using two-way analysis of variance (ANOVA) and least significant differences (P=0.05) were determined according to the students t test.

Prior to streaking, each bacterial culture was diluted 1:100 with fresh sterile nutrient broth. The minimum inhibitory concentration (MIC) of the extracts was determined by incorporating various amounts (0.5 - 5.0 mg/ml) of the extract into 65 mm Petri-dishes containing sterile nutrient agar (NA). Petri-dishes containing only the culture medium served as controls. The bacteria were streaked out in radial patterns onto the extract-amended NA and unamended NA plates. The Petri-dishes were sealed with Parafilm and incubated for 24 hrs at ± 37 °C. The MIC was regarded as the lowest concentration of an extract where no growth of a bacterium was visible. Each treatment was replicated three times.

Results and Discussion

The results pertaining to the antifungal investigations revealed that both the acetone and ethanolic extracts of the leaves of BW and Kpod cultivars, with the exception of *F. equiseti*, significantly inhibited growth of the fungal pathogens at 5.0 mg/ml [Figures 1(a-d)]. Only the BW ethanolic extract inhibited the growth of *F. equiseti* at the same concentration when compared to the control [Figure 1(b)]. *Alternaria alternata* was significantly reduced by both BW extracts at 2.5 mg/ml whereas only the ethanolic extract exhibited antifungal activity against *F. proliferatum* at the same concentration. The acetone extract from Kpod also inhibited the growth of *A. alternata* at 2.5 mg/ml when compared to the control. The acetone and ethanolic extracts of both cultivars showed no inhibitory activity at 1.0 mg/ml.

The Gram-positive bacteria were found to be more susceptible than the Gram-negative bacteria (Table 1) as previously reported by earlier researchers (Kuhnt *et al.* 1994, Meyer and Afolayan 1995). The weak activity shown by the extracts against the Gram-negative bacteria could be due to lipophilic characteristics displayed by certain compounds in the extracts (Werner *et al.* 1979). However, a minimum inhibition concentration of 5.0 mg/ml was observed when the acetone and ethanol extracts of BW were tested against *E. cloacae* (Table 1).

The results from this study have shown that cowpea extracts do have the potential to inhibit the growth of certain bacterial and fungal pathogens. This is likely to occur since it is known that cowpea leaves do contain flavonoids and these same flavonoids, isolated from other plant species, have shown antimicrobial activity. Lattanzio *et al.* (1997) found three flavonoid aglycones, namely, quercetin, kaempferol and isorhamnetin, always to be present in the leaves of cultivated cowpea lines. Quercetin, a naturally occurring bioflavonoid, is known to inhibit the growth of various fungi and bacteria (El-Gammal and Mansour 1986; Aziz *et al.* 1998). Further phenolic aglycons including p-coumaric acid and caffeic acid have also been isolated from cowpea leaves (Lattanzio *et al.* 2000) and it has been shown that p-coumaric acid, caffeic acid and kaempferol do exhibit antimicrobial activity against various bacterial and fungal pathogens (El-Gammal and Mansour 1986, Aziz *et al.* 1998).

It was noted in this study that the growth of *F. equiseti* was actually stimulated by the cowpea leaf extracts. Morris and Ward (1992) noted that an isoflavone, daidzein, acted as a germination stimulant for the zoospores of *Phytophthora sojae* (Kaufman & Gerdemann) and *Pythium irregulare* Buisman on soybean (*Glycine max* (L.) Merrill). However, Dakora (1995) reported that daidzein did not have the same effect on *Phytophthora vignae* Purss. on cowpea.

This study shows the potential of using cowpea extracts to control fungal pathogens that cause problems to various agricultural crops by causing disease and those that are capable of producing mycotoxins. The isolation of these active compounds from cowpea and other legumes, due to their rich source of flavonoid compounds (Dakora 1995), should be explored further for their use in disease control which can lead to the increase in the yield of agricultural commodities. Further exploitation of this activity could also increase the use of these extracts in the medicinal field.

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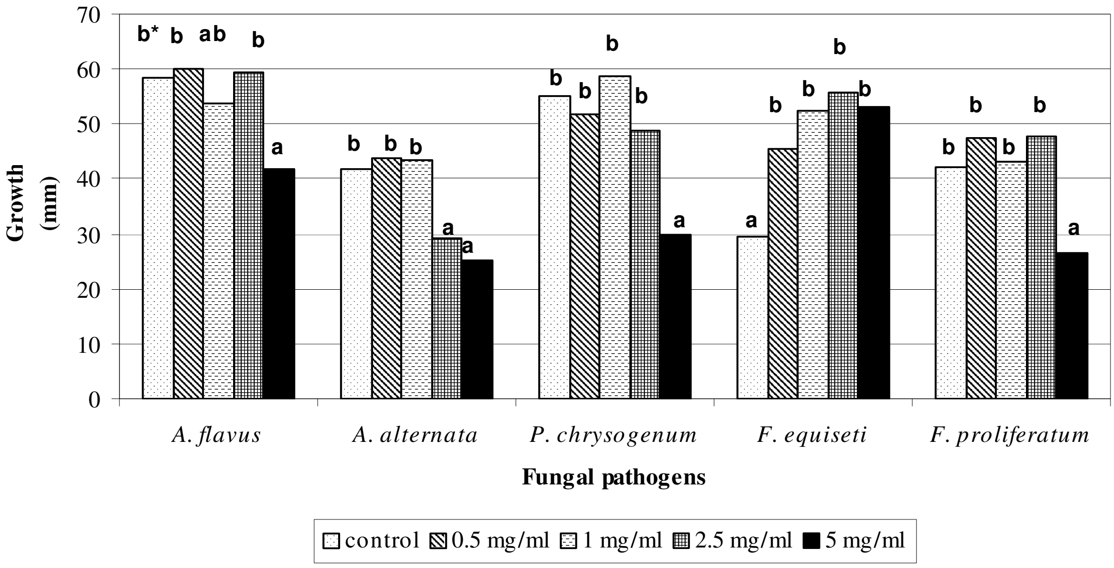
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50
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Growth (mm)
b* b ab b a
b b b a a
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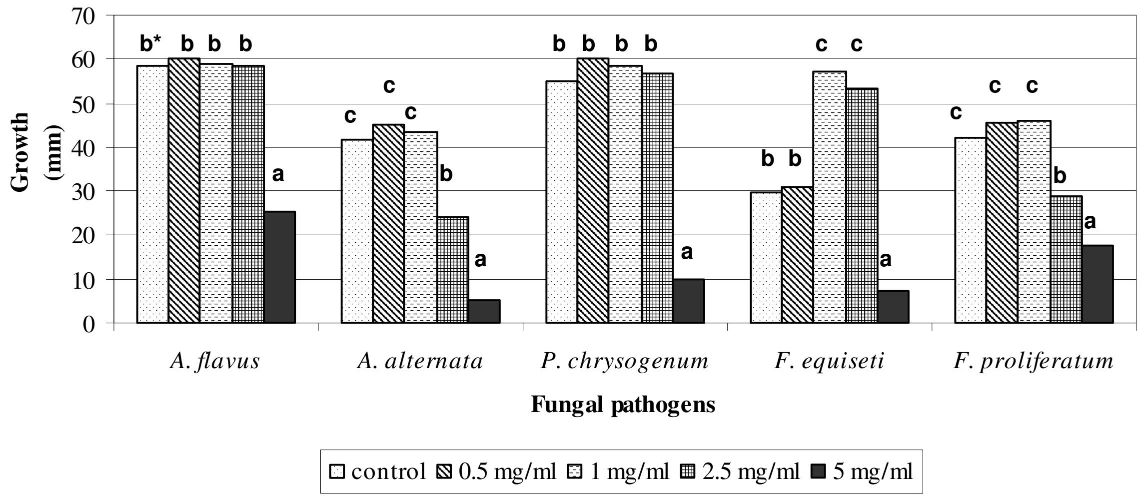
b b b b a
A. flavus
A. alternata
P. chrysogenum
F. equiseti
F. proliferatum
Fungal pathogens
control
0.5 mg/ml
1 mg/ml
2.5 mg/ml
5 mg/ml

(a)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Growth (mm)
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c
c
a
c
c
c
b
a
A. flavus
A. alternata
P. chrysogenum
F. equiseti
F. proliferatum
Fungal pathogens
control
0.5 mg/ml
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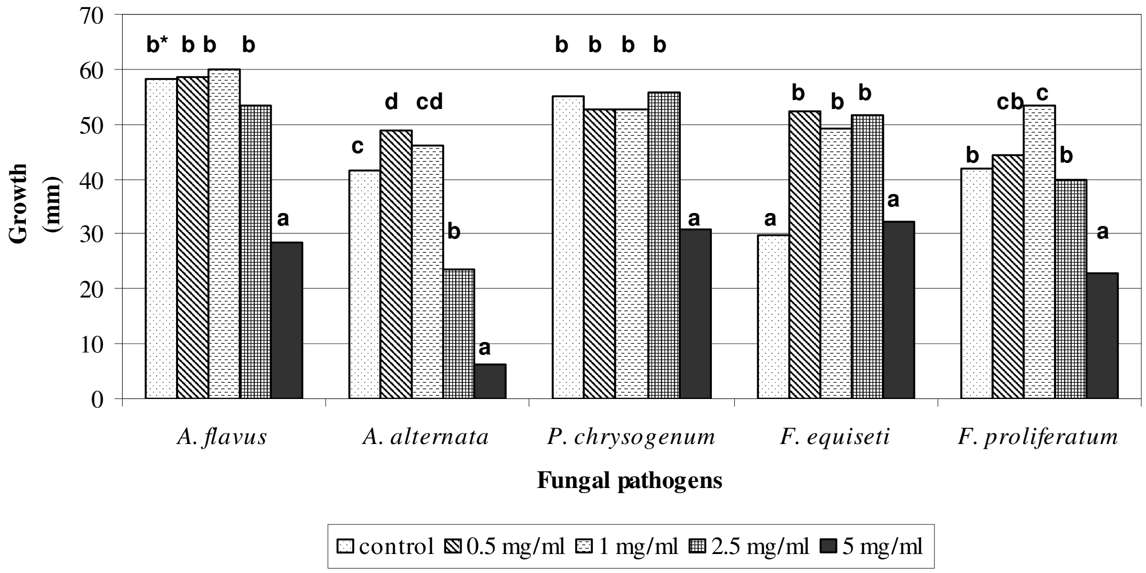
5 mg/ml

(b)



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Growth (mm)
b* b b b a
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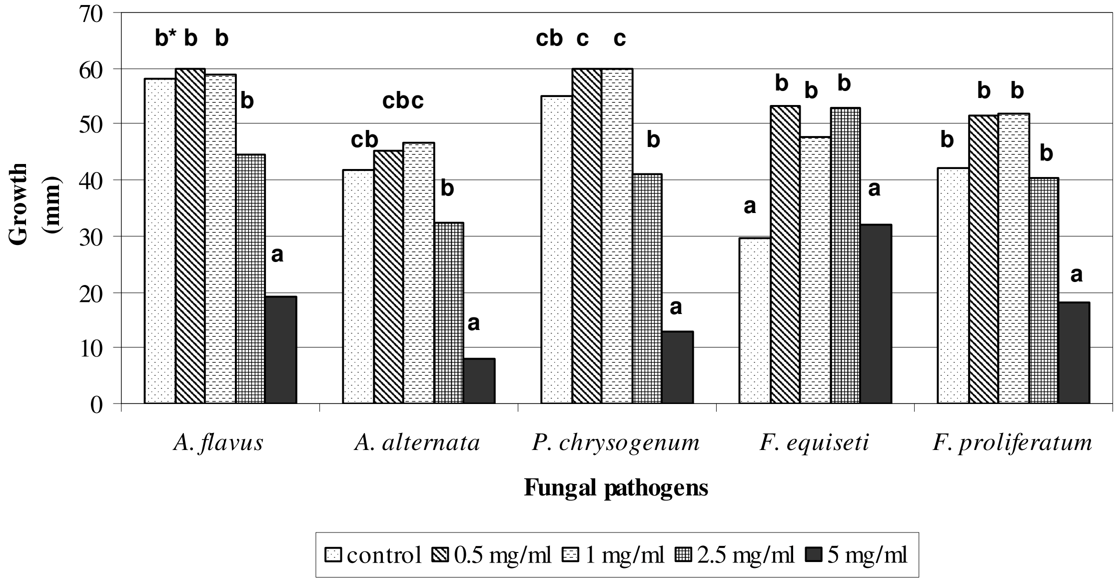
A. flavus
A. alternata
P. chrysogenum
F. equiseti
F. proliferatum
Fungal pathogens
control
0.5 mg/ml
1 mg/ml
2.5 mg/ml
5 mg/ml

(c)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Growth (mm)
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a
a
b b b
a
b
b b
b
a
A. flavus
A. alternata
P. chrysogenum
F. equiseti
F. proliferatum
Fungal pathogens
control
0.5 mg/ml
1 mg/ml
2.5 mg/ml
5 mg/ml

(d)

Figure 1: Antifungal activity of Bechwana White acetone leaf extracts (a), Bechwana White ethanol leaf extracts (b), Kpodjiguégué acetone leaf extracts (c) and Kpodjiguégué ethanol leaf extracts (d) on selected fungal pathogens. * Each value of a bar is a mean of 3 replicates. Values of the bars within each fungal species not followed by the same letter are significantly different (*P*=0.05) according to the student's *t* test.

Table 1: Antibacterial activity of acetone and ethanol leaf extracts of two cowpea cultivars

Bacterial species	Gram + / -	MIC[a] (mg/ml)			
		Acetone		Ethanol	
		BW[b]	Kpod[c]	BW	Kpod
Bacillus cereus	+	5.0	na	na	na
Bacillus pumilus	+	na[d]	na	na	na
Bacillus subtilis	+	5.0	na	na	na
Staphylococcus aureus	+	2.5	na	na	na
Enterococcus faecalis	+	2.5	na	5.0	na
Enterobacter cloacae	-	5.0	na	5.0	na
Escherichia coli	-	na	na	na	na
Klebsiella pneumoniae	-	na	na	na	na
Pseudomonas aeruginosa	-	na	na	na	na
Serratia marcescence	-	na	na	na	na

[a] Minimum inhibitory concentration

[b] Bechwana White cultivar

[c] Kpodjiguégué cultivar

[d] Not active

CHAPTER 7

GENERAL DISCUSSION

It is well known that cowpea (*Vigna unguiculata* (L.) Walp) is considered to be a beneficial crop to cultivate and as a nutritious food crop it contributes positively towards food security to many rural communities living in less developed countries. This thesis highlights the potential problems and future prospects associated with this legume crop.

Although cowpea forms an essential part of cropping systems and nutrition in many countries of the tropics and sub-tropics (Singh *et al.* 2002), it is not regarded as a major crop contributing to household security in South Africa, in particular by rural communities of the Mpumalanga Province (Chapter 3). As predicted, the production of maize (*Zea mays* L.) ranked highest with regard to household security. Even though cowpea was grown predominantly for own consumption, the majority of the respondents ate cowpea less than once a week. The crop was used to a lesser extent as feed for livestock and as a source of income. In other countries, and especially in Central and West Africa, cowpea is utilised largely for all of these purposes (Singh *et al.* 2002). Interestingly, the survey indicated that 8.5% of the farmers used the crop for medicinal purposes. Generally, post-harvest storage problems were well perceived by the farmers and storage practices seemed adequate, with the exception of the use of harmful pesticides like DDT (dichloro diphenyl trichloroethane). However, 20% of the farmers encountered problems with fungal contamination during storage and in view of this, it is probable that contamination of the seed by mycotoxins could exist. Although the survey indicated that 60.6% of the farmers received training with regard to cowpea cultivation, relatively low percentages of the farmers practiced intercropping (53.5%) and crop rotation (40%).

An increasing awareness should be made amongst the farmers and rural communities of the advantages associated with cowpea production. These include the nutritional benefits as a food crop and the use of the plant in intercropping and crop rotation farming systems to increase soil fertility. The knowledge of the potential problems arising from fungal contamination of the seeds during storage and the harmful effects of mycotoxin ingestion should be disseminated to the farmers. This can be achieved through the continual interaction between the rural farmers and researchers, which has already been established. Other regions in South Africa where farmers cultivate cowpea should be surveyed in order to contribute additional information to the existing knowledge on the importance and role of cowpea in the livelihoods of rural communities.

As indicated in the literature review (Chapter 2) cowpea seed supports an extensive array of storage fungi and the presence of aflatoxin associated with cowpea seed has been established (El-Kady *et al.* 1996) thus presenting a potential health risk to consumers. In Chapter 4, cowpea seed samples from South Africa and Benin, West Africa were investigated for mycoflora infestation and fumonisin contamination. Amongst various fungal genera recorded, including *Aspergillus*, *Phoma* and *Lasiodiplodia*, the results indicated an array of *Fusarium* spp. including *F. equiseti* (Corda) Sacc., *F. chlamydosporum* Wollenweber and Reinking*, F. graminearum* Schwabe*, F. sambucinum* Fuckel, *F. scirpi* Lamb. et Fautr., *F. semitectum* Berkeley & Ravenel and *F. subglutinans* (Wollenw. and Reink.) Nelson, Toussoun, and Marasas. Four isolates of *F. proliferatum* (Matsushima) Nirenberg were isolated from the seeds. The cowpea cultivars from South Africa showed the presence of FB_1 with concentrations ranging between 0.12 - 0.61 µg/g. Fumonisin B_1, B_2 and B_3 were produced by all *F. proliferatum* isolates. Total fumonisin concentrations were between 0.80 - 25.30 µg/g and the highest level of FB_1 detected was 16.86 µg/g. This was lower than FB_1 levels produced by *F. proliferatum* isolates isolated from the seed of other crops (Ross *et al.* 1990; Thiel *et al.* 1991). These studies represent the first report of the natural occurrence of FB_1 on cowpea seed and of the potential of *F. proliferatum* isolates from cowpea seed to produce fumonisin mycotoxins. In light of the substantial use of cowpea seed for food by people in many tropical and sub-tropical countries, the susceptibility of cowpea to *Fusarium* infection and fumonisin contamination remains a potential health hazard. Further screening for fumonisins is necessary to establish the degree of fumonisin contamination in cowpea seeds. Further studies on the other mycoflora associated with the seed are needed since some of the fungi found during these investigations, including *Aspergillus flavus* Link ex. Fries, *F. equiseti*, *F. graminearum*, *F. sambucinum* and various *Penicillium* spp. are known to produce other mycotoxins of toxicological significance (Desjardins & Hohn 1997).

Given the occurrence of FB_1 on cowpea seeds and the phytotoxic accounts relating to FB_1 (Abbas & Boyette 1992; Doehlert *et al.* 1994; McClean 1996), it was found necessary to investigate the effects of the toxin on cowpea seed (Chapter 5). Cowpea seeds were treated with purified FB_1 at concentrations of 10, 25, 50 and 100 ppm. The results indicated that all the FB_1 concentrations significantly decreased seed germination. This complimented the findings of Danielsen & Jensen (1998) where a significant negative correlation was found between fumonisin content and maize seed germination. On the other hand Doehlert *et al.* (1994) reported that FB_1 had no effect on maize seed germination. Known FB_1 producing strains of *F. verticillioides* (Sacc.) Nirenberg and *F. proliferatum*, which were inoculated onto the seeds also showed a significant reduction in seed germination. Further studies are required to establish if this was a result of fungal infestation alone or of both fungal infestation and the production of a toxin. This study also supported findings from other studies (Doehlert *et al.* 1994; Lamprecht *et al.* 1994) that FB_1 plays an inhibitory role in root and shoot growth of plants. Cowpea root and shoot elongation were significantly inhibited by the 50 and 100 ppm FB_1 concentrations and apparent physical abnormalities were seen amongst these seedlings. In an attempt to understand the actions of FB_1 at a cellular level, the ultrastructural changes caused by the toxin were studied by means of transmission electron microscopy (TEM). Damaging effects by FB_1 were only noted in the seeds treated with the highest concentration of toxin (100 ppm). These included the contraction of the protoplasm, which resulted in the formation of numerous vacuoles within the much more dense protoplasm. The plasma membrane also separated from the cell wall. These findings constitute the first report of the phytotoxic effects of FB_1 on cowpea seed.

Fumonisin B_1 interferes with the synthesis of sphingolipids through the inhibition of the enzyme, ceramide synthase (Riley *et al.* 1994). Sphingolipids are important components of cell membranes and play an important role in cell regulation, membrane stability and stress response in plants (Riley *et al.* 1994; Sperling & Heinz 2003). The interference in their metabolism results in disturbances in growth, differentiation and morphology of cells (Riley *et al.* 1994). This disruption of sphingolipids is a probable explanation for the destructive effects noticed in the seed tissue. However, further research is necessary to determine the precise toxic effects of the toxin in order to understand why seed germination and root and shoot growth of the seedlings were adversely affected.

As indicated from findings from this study and from other reports (van Wyk & Gericke 2000), cowpea is used for medicinal purposes. To provide the foundation from which to pursue the scientific substantiation of these reports, the antimicrobial activity of leaf extracts of two cowpea cultivars were evaluated (Chapter 6). The results indicated that the acetone and ethanol extracts of the leaves of both cowpea cultivars (Bechwana White (BW) and Kpodjiguégué (Kpod), significantly inhibited the growth of all the fungal plant pathogens at 5.0 mg/ml, with the exception of *F. equiseti*. *Alternaria alternata* (Fr: Fr.) Keissler was further inhibited by the acetone extract of Kpod and both BW extracts at 2.5 mg/ml. *Fusarium proliferatum* was inhibited by the BW ethanol extract at 2.5 mg/ml. Antibacterial activity was predominantly displayed by the BW acetone extract. A minimum inhibitory concentration (MIC) of 2.5 mg/ml was recorded for *Staphylococcus aureus* Rosenbach and *Enterococcus faecalis* (Andrews and Horder) Schleifer and Kilpper-Balz whilst *Bacillus cereus* Frankland and Frankland, *B. subtilis* (Ehrenberg) Cohn and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards were inhibited at a concentration of 5.0 mg/ml. The BW ethanol extracts inhibited the growth of *E. faecalis* and *E. cloacae* at 5.0 mg/ml. This is the first report on the inhibitory effect of cowpea leaf extracts on the growth of human bacterial and fungal plant pathogens. It is concluded from this investigation that the crude extracts from cowpea be further fractionated in order to purify and identify the active compounds responsible for antimicrobial inhibition.

The results presented in this thesis indicate the challenges facing future research on cowpea. The use of cowpea seed as a source of nutritious food can be hampered through fungal infestation and subsequent fumonisin contamination of the seed. Further research is imperative to establish the extent of this potential problem, especially amongst rural communities where storage practices are often inadequate. Not only does FB_1 contamination pose a threat to human and animal health, but it also holds a negative impact for the plant itself. The toxin could impede cowpea seed germination and the ability to emerge from the ground during germination in the field. This can be investigated through experiments in the glasshouse and in the field. Further investigation into the antimicrobial compounds associated with cowpea extracts will enhance the existing benefits of the crop.

The value of cowpea in sub-Saharan Africa can be amplified through the effective prevention and control of storage fungi and subsequent mycotoxin contamination, and through the exploitation of the beneficial compounds in the plant as potential alternative sources of medicine.

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SUMMARY

Cowpea is an indigenous African legume crop that is of great importance to the livelihood of many subsistence farmers and rural communities in tropical and subtropical countries. This resourceful legume crop is used as a nutritious source of food by people, as fodder for livestock, as a means to generate income through the trade of the seed and for medicinal purposes.

Cowpea was ranked third against maize and vegetable production with regard to the importance to the livelihoods of farmers surveyed in the Mpumalanga Province of South Africa. The crop was mainly grown for own consumption, with the purposes as a source of income and feed for livestock to a lesser extent. Some of the farmers used the roots and seeds for medicinal purposes. Although the farmers practiced adequate post-harvest practices, mouldiness of the seed was reported by 20% of the farmers with insect infestation to a lesser extent.

Cowpea seeds of seven cultivars (four from South Africa and three from Benin, West Africa) were analysed for seed mycoflora and fumonisin contamination. *Aspergillus*, *Phoma* and *Lasiodiplodia* spp. where predominantly isolated from these seed samples. The results indicated low percentages of *Fusarium* spp., including *F. equiseti*, *F. chlamydosporum, F. graminearum, F. sambucinum*, *F. semitectum*, *F. subglutinans* and *F. proliferatum*. The seed samples and *F. proliferatum* isolates, cultured on maize patty media, were extracted with methanol/water and cleaned-up on strong anion exchange solid phase extraction cartridges. Fumonisins were quantified as *o*-phthaldialdehyde pre-column derivatives by high performance liquid chromatography (HPLC) and fluorescence detection. Fumonisin B_1 (FB_1) was present in four seed samples with a range of 0.12 - 0.61 µg/g. The results revealed that all the *F. proliferatum* isolates produced FB_1, FB_2 and FB_3. Total fumonisins ranged between 0.80 - 25.30 µg/g and the highest FB_1 level was 16.86 µg/g.

In order to investigate the phytotoxic effects of FB_1 on cowpea seed, surface disinfected cowpea seeds were imbibed in sterile water amended with pure FB_1 to yield final concentrations of 10, 25, 50, and 100 ppm. Percentage germination, as determined according to the International Seed Testing Association (ISTA) rules, was significantly decreased at all toxin concentrations. Root and shoot length was measured after 9 days and the 50 and 100 ppm toxin concentrations showed an inhibitory effect on their growth. Embryonic axes and cotyledon tissues were removed and prepared for transmission electron microscopy (TEM) studies. Ultrastructural deteriorations included compaction of the protoplasm and separation of the plasmalemma from the cell wall. Furthermore, lipid bodies accumulated and lined the cell wall.

Acetone and ethanol extracts of the leaves of the Bechwana White and Kpodjiguégué cultivars of cowpea were investigated for their antimicrobial properties against human bacterial and fungal plant pathogens. All the extracts significantly inhibited the growth of all the fungal pathogens at 5.0 mg/ml, with the exception of *Fusarium equiseti*. Growth of *Alternaria alternata* and *F. proliferatum* was significantly reduced by certain extracts at 2.5 mg/ml. Acetone extracts of Bechwana white inhibited the growth of *Staphylococcus aureus* and *Enterococcus faecalis* at 2.5 mg/ml and *Bacillus cereus*, *B. subtilis* and *Enterobacter cloacae* at 5.0 mg/ml whilst the ethanol extracts of the same cultivar only showed antibacterial activity against *E. faecalis* and *E. cloacae* at 5.0 mg/ml.

This study is the first report of the natural occurrence of fumonisins on cowpea seed and of the production of fumonisins by *F. proliferatum* isolates isolated from cowpea seed. Furthermore, reports of the phytotoxic effects of FB_1 on cowpea seeds and the antimicrobial activity of cowpea leaf extracts were established.

APPENDIX A SURVEY ON COWPEAS

INTRODUCTION

The survey aims at collecting information on the current status of cowpea production in target communities in Mpumalanga. Emphasis is placed on the agricultural practices used for storage of the seed after harvest, uses of the crop and health aspects due to the consumption of the seed and food products thereof.

Name of interviewer	
Date of interview	
Respondent name (group)	
Name of production locality	

[Where applicable, mark by "x" in the ☐ . If more than one option is applicable, mark more than one]

A. Biographic profile of respondent

1. Gender: Male ☐ Female ☐

2. Age of respondent …………………

3. What is your major livelihood/source of income?

Agricultural activities ☐

Other Employment ☐ (Please specify)

…………………………………………………………………………………………….................

B. Agricultural profile

4. If various agricultural activities are practiced, please rank these activities according to their economic importance.

   a. ………………………………. d. ……………………………………..
   b. ………………………………. e. ……………………………………..
   c. ………………………………. f. ……………………………………..

5. What is the size of land available for agricultural activities?

   ……………………. unit …………

C. Importance and role of cowpea

6. Which crops do you rank as the 5 most important for household food security and for cash sale as income generation? Ranking (1 - 5)

Crop	Food	Cash
Maize	……..	……..
Cowpea	……..	……..
Other legumes	……..	……..
Fruits	……..	……..
Vegetables	……..	……..
Other ……………………	……..	……..

7. Why do you choose to produce cowpeas? (i.e. tradition, good at it, right climate, soil, good income, crop rotation? etc.)

   ……………………………………………………………………………………………………
   ……………………………………………………………………………………………………
   ……………………………………………………………………………………………………

8. For what purpose do you use your cowpeas?

   Sell to generate income ☐ Own consumption ☐
   Animal feed ☐ Medicinal purposes ☐

9. If you are selling your cowpea seed, to what extent does it contribute to your household income?

Small ☐ Average ☐ Large ☐

Percentage contribution ....%

10. What is the annual income of your household from cowpea?

< R100 ☐ R100 - R400 ☐

R500 - R900 ☐ More (specify) ....

11. How often do you eat cowpea?

Daily ☐ Twice a week ☐

Once a week ☐ Less than once a week ☐

12. Where do you get cowpea to eat?

Local market ☐ Hawkers ☐

Own produce ☐ Shop ☐

Other ....

13. How do you consume cowpea?

Whole cooked seeds ☐ Ingredient in dishes ☐

Leaves as a vegetable ☐ Fresh pods ☐

Ground seeds as a porridge ☐ Baking product ☐

Other ....

14. Do you have a preference of cowpea seed type?

Light coloured seed ☐ Dark coloured seed ☐

15a. How often do you use cowpea seed as feed for your livestock?

Never	☐	Once a week	☐
Less than once a week	☐	Twice a week	☐

More (specify) ……………………....................

b. How often do you use cowpea fodder as feed for your livestock?

Never	☐	Once a week	☐
Less than once a week	☐	Twice a week	☐

More (specify) ……………………....................

16. Which part of cowpea do you use for medicinal purposes?

Roots	☐	Leaves	☐
Seeds	☐		

17. For what purposes do you use cowpea medicinally?

……………………………………………………………………………………………..............................

……………………………………………………………………………………………..............................

……………………………………………………………………………………………..............................

……………………………………………………………………………………………..............................

<u>D.</u> Cultivation practices

18. How many years have you been actively producing cowpea? …………………..

19a. Have you ever received formal training in the cultivation and storage of cowpea?

Yes ☐ No ☐

b. If yes, from whom and when did you receive training?

……………………………………………………………………………………………..............................

……………………………………………………………………………………………..............................

20a. Are you practicing plant protection? Yes ☐ No ☐

b. If yes, what method or type of protection is used?

………

………

21a. Are you practicing intercropping with regard to cowpea?

Yes ☐ No ☐

b. If yes, please name other crops

………

22a. Do you rotate your crop with others?

Yes ☐ No ☐

b. If yes, what crops? ……………………………………………………………………………

23. What yield do you get from cowpea?

.... ton or kg per .... area

.... crates per .... area (Size of crates: ....)

.... buckets per .... area (Size of buckets: ....)

24. If you are selling your excess produce, please indicate to whom you are selling your produce.

Neighbours ☐ Door to door ☐

Local market ☐

Other, please specify

………

25. What is the average price obtained for the produce sold?

………… R per …………………. unit …………..

E. Storage practices

26. Where do you obtain your cowpea seed from?

Own ☐ Relatives ☐

Neighbours/Friends ☐ Shop ☐

Department of Agriculture ☐

Other………………………..

27a. Do you dry your seed prior to storage?

Yes ☐ No ☐

b. If yes, how do you do it?

…………………………………………………………………………………………………

…………………………………………………………………………...............................

28a. How do you store your seed?

Cribs (rooms) made from plant material ☐

Clay / earthenware pots ☐

Calabashes ☐

Tins ☐

Miniature silos ☐

Other: (please specify) ☐

…………………………………………………………………………...........................

…………………………………………………………………………...........................

b. Photograph of storage structures

Digital camera number: ………..

29. During which month did you last store your seed? …………………

30. For how long has the seed been in storage? ....

31. What problems do you encounter with the seed during and after storage?

Insects ☐ Mouldiness ☐

Other ....

32a. Do you treat the seed with some type of medium during storage?

Yes ☐ No ☐

b. If yes, what do you use?

....

....

....

33. What colour-type of mouldiness occurs on the seed?

White ☐ Black ☐

Pink ☐ Green ☐

Other ....

34. Do you encounter any problems with germination of stored seeds?

Yes ☐ No ☐

....

....

<u>F. Health aspects</u>

35a. Do you or your family members experience any type of ill effects after consuming cowpeas or products thereof?

Yes ☐ No ☐

b. If so, what types of symptoms are experienced?

....

……………………………………………………………………………………………...................

……………………………………………………………………………………………...............

36a. Do your farm animals experience any ill effects after consuming feed made from cowpea?

Yes ☐ No ☐

b. If so, what types of symptoms are experienced?

……………………………………………………………………………………………...................

……………………………………………………………………………………………...................

……………………………………………………………………………………………...................

G. Major constraints

37. What are the main problems you encounter in producing cowpeas?

………

..………………………………………………………………………………...................................

……………………………………………………………………………………………...................

THANK YOU FOR YOUR INPUT AND CO-OPERATION!

APPENDIX B LIST OF PUBLICATIONS AND PRESENTATIONS

The following publications and presentations at national and international conferences resulted from the research presented in this thesis.

Publications:

Peer-reviewed journals

Kritzinger, Q., Lall, N. and Aveling, T.A.S. 2004. Antimicrobial activity of cowpea (*Vigna unguiculata*) leaf extracts. *South African Journal of Botany* (in press)

Kritzinger, Q., Aveling, T.A.S., Marasas, W.F.O., Rheeder, J.P., van der Westhuizen, L. and Shephard, G.S. 2003. Mycoflora and fumonisin mycotoxins associated with cowpea (*Vigna unguiculata* (L.) Walp) seeds. *Journal of Agricultural and Food Chemistry* 51: 2188-2192.

Published conference proceedings

Kritzinger, Q., Aveling T.A.S., Marasas W.F.O., Rheeder J.P. and van der Westhuizen L. 2004. Occurrence and phytotoxicity of fumonisin B_1 associated with cowpea seed. *Phytopathology* 94: S55

Kritzinger, Q., Aveling, T.A.S. and Van der Merwe, C.F. 2003. Effect of fumonisins on the ultrastructure of cowpea seed. *Microscopy Society of Southern Africa – Proceedings* 33: 64.

Kritzinger, Q., Lall, N. and Aveling, T.A.S. 2003. Antimicrobial properties of cowpea (Vigna unguiculata (L.) Walp) leaf extracts. *South African Journal of Botany – Conference abstracts* 69: 224.

Kritzinger, Q., Aveling, T.A.S., Marasas, W.F.O., Shephard, G.S. and Leggott, N. 2002. Detection of fumonisin B_1 in cowpea seed. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamò M (eds) Challenges and opportunities for enhancing sustainable cowpea production. Proceedings of the World Cowpea Conference III held at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, 4-8 September 2000. IITA, Ibadan, Nigeria. pp. 147-153.

Presentations:

Internationally

Kritzinger, Q., Aveling T.A.S., Marasas W.F.O., Rheeder J.P. and van der Westhuizen L. 2004. Occurrence and phytotoxicity of fumonisin B_1 associated with cowpea seed. Annual Meeting of The American Phytopathological Society, 31 July – 4 August, Anaheim, California, United States of America. (poster)

Kritzinger, Q. and Aveling, T.A.S. 2004. Ultrastructural damage and germination reduction in cowpea seeds due to fumonisin B_1. 27th International Seed Testing Association Triennial Congress, 13 – 24 May, Budapest, Hungary. (poster)

Kritzinger, Q., Aveling, T.A.S., Rheeder, J.P. and van der Westhuizen, L. 2003. Fumonisin production in cowpea by *Fusarium proliferatum*. 8th International Congress of Plant Pathology, 2-7 February, Christchurch, New Zealand. (poster)

Aveling, T.A.S., Kritzinger, Q., Pakela, Y.P. and Regnier, T. 2003. Seed pathological aspects of cowpea. 8th International Congress of Plant Pathology, 2-7 February, Christchurch, New Zealand. (poster)

Aveling, T.A.S., Adandonon, A., Kritzinger, Q., Pakela, Y.P., Smith, J.E. and Van Den Berg, N. 2001. Research on cowpea diseases in South Africa. 2nd International Workshop of African Network of Research on Bruchids: Recent Developments in Crop Pre-and Post-harvest Pest Management Practices in Africa, 12-17 November, Cotonou, Republic of Benin. (oral)

Aveling, T.A.S., Adandonon, A., Kritzinger, Q., Pakela, Y.P., Smith, J.E. and Van den Bergh, N. 2001. Research developments on cowpea diseases in South Africa. Australasian Plant Pathology Society 13th Biennial Conference, September, Cairns, Australia. (oral)

Kritzinger, Q., Aveling, T.A.S. and Marasas, W.F.O. 2001. Effect of essential plant oils on storage fungi associated with cowpea seeds. 26th International Seed Testing Association Triennial Congress, 14-22 June, Angers, France. (poster)

Kritzinger, Q., Aveling T.A.S. and Marasas, W.F.O. 2001. Contamination of cowpea (*Vigna unguiculata* (L.) Walp) seed by storage fungi and mycotoxins. British Mycological Society International Symposium on Bioactive Fungal Metabolites – Impact and Exploitation, 22- 27 April, Swansea, Wales. (oral)

Nationally

Kritzinger, Q. and Aveling, T.A.S. 2004. Phytotoxicity of fumonisin B_1 to cowpea seeds. 42nd Congress – Southern African Society for Plant Pathology, 18 – 21 January, Cathedral Peak Hotel, Winterton. (oral)

Kritzinger, Q., Aveling, T.A.S. and Van der Merwe, C.F. 2003. Effect of fumonisins on the ultrastructure of cowpea seed. MSSA Conference, 3-5 December, University of Cape Town, Cape Town. (poster)

Kritzinger, Q., Aveling, T.A.S., Marasas, W.F.O., Rheeder, J.P., van der Westhuizen, L. and Shephard, G.S. 2003. Mycoflora and fumonisin mycotoxins associated with cowpea (*Vigna unguiculata* (L.) Walp) seeds. 41st Congress – Southern African Society for Plant Pathology, 19 – 22 January, Baines Game Lodge, Bloemfontein. (oral)

Kritzinger, Q., Lall, N. and Aveling, T.A.S. 2003. Antimicrobial activity of cowpea (*Vigna unguiculata* (L.) Walp) leaf extracts. Joint SAAB/ISE Congress, 7-11 January, University of Pretoria, Pretoria. (poster)