THE PHYSIOLOGICAL AND MOLECULAR EFFECTS OF FUMONISIN B_1 ON COWPEA (*VIGNA UNGUICULATA* (L.) WALP)

By

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DECLARATION

I, Richard Gavin Kotze declare that the dissertation, which I hereby submit for the degree of Magister Scientiae at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution

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October 2015

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SUMMARY

Cowpea (*Vigna unguiculata*) is an edible annual leguminous crop cultivated by many subsistence and rural communities in developing countries in subtropical areas of the world. Cowpea is a very versatile, well adapted and nutritious grain legume. However, cowpea seed is prone to fungal infestation under suboptimal storage conditions. Some of these fungi, including *Aspergillus* and *Fusarium* spp., produce their associated mycotoxins under these conditions. Fumonisins are produced primarily by *Fusarium verticillioides* and *F. proliferatum* and are known to be toxic to vertebrates and plants. Fumonisin B_1 is phytotoxic to cowpea seeds and is speculated to inhibit ceramide synthase in the sphingolipid pathway in plants. This study was done to determine the phytotoxic effects of FB_1 on cowpea seedlings and to provide insight on the mode of action of the toxin at a molecular level.

Surface-disinfected seeds were imbibed for 10 h in sterile distilled water amended with FB_1 to yield final concentrations of 2, 20 and 40 mg/L. Slow imbibed seeds (placed in moist paper towels) and seeds placed in sterile distilled water for 10 h served as the positive and negative controls, respectively. Additionally cowpea seeds were inoculated with the conidia of three different FB_1-producing *Fusarium verticillioides* strains. Percentage emergence was determined after seven dpi whereas, seedling mass, length and seedling vigour index where

determined after seven and 21 dpi. Total chlorophyll content was measured after 14 and 21 dpi. Stunted growth was observed in FB_1 treated seedlings. Emergence was reduced by all three FB_1 concentrations as well as in seeds treated with *F. verticillioides* strain MRC 8265. Seedlings imbibed in 40 mg/L FB_1 had reduced seedling length. Seedling mass was reduced by all three FB_1 concentrations as well as all three *Fusarium* strains. Total chlorophyll content was higher for seeds imbibed in all three FB_1 concentrations when compared to both controls. This is in contrast to other studies which reported that FB_1 causes chlorosis in plants.

In order to evaluate the effect of FB_1 on ceramide synthase gene expression seeds were imbibed in a 20 mg/L FB_1 solution and the control seeds were imbibed in sterile distilled water. RNA was extracted from untreated and treated samples after 0, 3 and 12 dpi with the latter being divided into shoot and roots samples. cDNA was synthesised from the extracted RNA samples. Amplification of the ceramide synthase gene was done using primers that were designed to the conserved regions of *Glycine max* and *Phaseolus vulgaris* ceramide synthase gene homologues. Sequence analysis revealed that the designed primers did amplify the correct gene from cowpea seedlings. The expression levels of the ceramide synthase gene from cowpea were thus tested using semi-quantitative PCR amplification. No significant differences in ceramide synthase gene expression were observed between the control and toxin treated samples. Two FB_1 unrelated differences were observed. A possible developmental difference was observed as ceramide synthase gene expression decreased over time in both the control and treated samples. It thus seems that FB_1 did not influence ceramide synthase gene expression in cowpea.

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ABBREVIATIONS

ANOVA	Analysis of variance
Asc	*Alternaria* stem canker
bp	base pairs
cDNA	complimentary DNA
Cers	ceramides
CGI	Cowpea genomics initiative
CGKB	Cowpea Genespace/Genomics Knowledge Base
CIA	chloroform: isomyl alcohol
CTAB	hexadecyltrimethylammonium bromide
Da	daltons
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dpi	days post imbibtion
EDTA	ethylenediaminetetraacetic acid
EDTA-Na_2	ethylenediaminetetraacetic acid disodium salt solution
ER	endoplasmic reticulum
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
GPI	glycosylphosphatidylinositol anchored proteins
GSS	gene-space sequence
IP	inositol phosphate group
IPCers	inositolphosphorylceramides
ISTA	International Seed Testing Association
kbp	kilo base pairs
kDa	kilo daltons
LAG	longevity assurance gene
LAG1	longevity assurance gene 1

LB	Luria Bertani
LCB	long chain base
LiCl	Lithium chloride
LOH	LAG one homologue
LOH1	LAG one homologue 1
LOH2	LAG one homologue 2
LOH3	LAG one homologue 3
Mbp	mega base pairs
$MgCl_2$	magnesium chloride
$MgCO_3$	magnesium carbonate
MRC	Medicinal Research Council
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information
NMR	nuclear magnetic resonance
PCD	programmed cell death
PCR	polymerase chain reaction
PDA	potato dextrose agar
pI	isoelectric point
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SAM	sphinganine analogue mycotoxins
sdH_20	sterile distilled water
SVI	seedling vigour index
TAE	tris-acetate ethylenediaminetetraacetic acid
UV	ultraviolet

CHAPTER 1

General introduction

1.1. Background and motivation for the study

Cowpea (*Vigna unguiculata* (L.) Walp) is a very important annual leguminous crop that originated in southern Africa (Padulosi & Ng, 1997) and is utilized in the drier areas of the tropical and subtropical regions of Africa, the Americas, Oceania, the Middle East, Europe and Asia (Brader, 2002; Singh *et al*., 2002). Nigeria is the biggest producer of cowpea in the world followed by Niger, with these countries having produced 2 950 000 and 1 300 000 tons of grain in 2013, respectively (FAOSTAT, 2015). The main cowpea producing areas in South Africa are Limpopo, Kwazulu-Natal, North-West, Mpumalanga and the Free State provinces (Directorate Plant Production, 2011). The crop is cultivated by both subsistence and commercial farmers since it is a multifunctional, widely adapted and nutritious crop (Ehlers & Hall, 1997; Timko & Singh, 2008). It can grow in a variety of soils and soil conditions, has the ability to fix nitrogen, can prevent soil erosion due to it being a fast grower and is a shade tolerant crop (Ehlers & Hall, 1997; Singh *et al*., 2003). It is a highly valued crop as it can grow with little water, and can thus be cultivated productively during long seasons of drought (Hall, 2004).

The production of cowpea by farmers in rural communities is of great importance as it can be used as a source of food and animal feed, as a source of income through the selling of its seed and for its medicinal uses (Kritzinger *et al*., 2004; Quin, 1997). The crop also provides food security early in the season as certain varieties can produce grain within 55 days (Timko *et al*., 2007). The most important product of cowpea utilized by humans for consumption is the dry grain, however, the other parts of the plant are also used as a vegetable (Ehlers & Hall, 1997; Quin, 1997). The seed of cowpea contains on average between 23–25% protein and 50–67% carbohydrates (Quin, 1997). Cowpea is a cheaper alternative source of protein than expensive conventional animal protein for many rural communities throughout Africa (Brader, 2002). Cowpea is also medicinally important and is often used in treating various ailments such as epilepsy, chest pain and amenorrhea (Van Wyk & Gericke, 2000).

Cowpea is, however, susceptible to a variety of pests and diseases, which is a major problem for both subsistence and commercial farmers. Pests and diseases include fungal diseases, bacterial diseases, viral diseases, insect pests, nematodes and parasitic plants, which affect all parts of the plant (Davis *et al.*, 1991; Singh & Allen, 1979). Cowpea is also negatively influenced by abiotic factors such as low temperature, high rainfall, herbicides and pesticides (Singh, 2005; Singh & Allen, 1979; Timko *et al.*, 2007; Timko & Singh, 2008). A variety of cowpea diseases arise due to seed storage problems (Kritzinger *et al.*, 2003). In particular, cowpea seeds are prone to fungal infestation due to suboptimal storage conditions, which include high temperature and high humidity (Kritzinger *et al.*, 2003). Numerous reports exist on the storage fungi associated with the seeds (Emechebe & McDonald, 1979, Kritzinger *et al.*, 2003; Zohri *et al.*, 1992). These include *Fusarium verticillioides* (Sacc.) Nirenberg, *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, *Fusarium oxysporum* Schltdl., *Fusarium solani* (Mart.) Sacc., *Aspergillus flavus* Link, *Aspergillus niger* Tiegh., and *Alternaria alternata* (Fr.) Keissl.. Many of these fungi have the ability to produce secondary metabolites called mycotoxins, which are toxic to humans, animals and plants (Richard, 2007).

Fumonisins are one of the major groups of mycotoxins that are primarily produced by *Fusarium* spp. (Rheeder *et al.*, 2002). *Fusarium verticillioides*, *Fuasrium nygamai* L.W. Burgess & Trimboli, and *F. proliferatum* are the main producers of fumonisins with *A. alternata* also having the ability to produce fumonisins (Abbas & Riley, 1996, Rheeder *et al.*, 2002). The most toxicologically and abundant fumonisin analogue, fumonisin B_1 (FB_1), is generally known to causes diseases in animals such as leukoencephalomalacia in horses and swine lung edema as well as oesophageal cancer in humans (Marasas *et al.*, 1979; Norrad & Voss, 1995; Richard, 2007; Steyn, 1995). Furthermore, it has also been found that FB_1 is phytotoxic to variety of important agricultural plants as well as weeds (Abbas & Boyette, 1992; Kritzinger *et al.*, 2006, Kroschel & Elzein, 2004). Phytotoxic effects include necrosis, chlorosis and growth inhibition leading to death of the plant (Abbas & Boyette, 1992; Abbas *et al.*, 1995; Williams *et al.*, 2007). Affected plants include agricultural crops such as cowpea (Kritzinger *et al.*, 2006), sunflower (*Helianthus annuus* L.), tomato (*Solanum lycopersicum* L.) (Abbas & Boyette, 1992) and weeds such as witchweed (*Striga* spp.) (Kroschel & Elzein, 2004), spurred anoda (*Anoda cristata* (L.) Schltdl.) (Abbas & Boyette, 1992) and duckweed (*Lemna aequinoctialis* Welw.) (Abbas *et al.*, 1998).

Kritzinger *et al*., (2003) reported that cowpea seed samples from South Africa were infested with fumonisin-producing *Fusarium* spp. and that the seed samples contained FB_1 at concentrations ranging between 0.12 and 0.61 µg/g. Houssou *et al*., (2009) also detected FB_1 in cowpea seed samples with relatively low concentrations of 0.01, 0.012 and 0.068 µg/g for three different seed samples, respectively. Kritzinger *et al*., (2006) indicated that FB_1 had deleterious effects on the germination of cowpea seed, and caused stunting, malformation leading to the death of cowpea seedlings. Ultrastructural studies of the cells of FB_1 treated cowpea seed tissue, with the use of transmission electron microscopy (TEM), revealed irregular sized vacuoles and an accumulation of lipid bodies against cell walls (Kritzinger *et al*., 2006). In the study by Kritzinger *et al*., (2006) the effect of FB_1 on cowpea seed germination was assessed using the between paper method. It is important that the phytotoxic effects are studied further in a glasshouse environment and in the field. The use of a glasshouse environment is necessary as the majority of the conditions such as lighting, temperature and water can be controlled and thus only the effects of FB_1 on the cowpea seedlings are studied. A glasshouse environment also limits the contamination and infestation of plants under study by pest and diseases.

It is also necessary to study the mechanism of action of FB_1 on cowpea as the crop plays an important role in the livelihood of many poor and rural farmers throughout Africa in the light of its variety of uses and benefits. The majority of the work done on the mechanism of action of FB_1 are human and animal based studies (Desai *et al*., 2002; Kim *et al*., 2012; Schmelz *et al*; 1998; Soriano *et al*., 2005; Voss *et al*., 2002; Voss *et al*., 2007; Wang *et al*., 2013) whereas only a few articles have been published with regards to the mechanism of action of FB_1 in plant cells (Abbas *et al*., 1994; Abbas *et al*., 1998; Lynch, 1999). A possible explanation as to how FB_1 acts could be that it inhibits the enzyme ceramide synthase, which is a key enzyme in the sphingolipid pathway in both plants and animals (Abbas *et al*.,1998; Merrill *et al*., 2001). Merrill *et al*., (2001) reported that FB_1 and spinganine are similar in structure and that they compete for the same binding site in ceramide synthase. It is speculated that when FB_1, which is a spinghanine analog mycotoxin (SAM), inhibits ceramide synthase it results in the accumulation of free sphinghoid bases such as sphinganine, which could result in growth arrest and cell apoptosis (Abbas *et al*., 1998; Merrill *et al*., 2001; Schmelz *et al*; 1998; Wang *et al*., 1991; Zhang *et al*., 1991). According to Lynch & Dunn, (2004) ceramide synthase catalyses the reaction of sphinganine into ceramide, which in turn, is catalysed into complex sphingolipids further down in the

bionsynthetic pathway. Ceramide is a very important molecule in the sphingolipid pathway as it is a central intermediate in this pathway (Ternes *et al*., 2011). Spingolipids and complex spingolipids play a major role in membrane stability, membrane permeability, cell signalling, phytopathogenesis and programmed cell death (Lynch & Dunn, 2004; Sperling & Heinz, 2003).

Although previous studies have proposed that FB_1 inhibits ceramide synthase, the effect of FB_1 on the expression of the ceramide synthase gene in plants has not been investigated. We hypothesised that FB_1 directly inhibits the ceramide synthase transcript in cowpea. This study could be potentially useful in determining the effect of FB_1 on other plant species.

1.2. Aims and objectives

The aim of the current research study was to evaluate the physiological and molecular effects of FB_1 on cowpea seedlings.

The specific objectives of the study are described below.

1. Investigate the phytotoxic effect of pure FB_1 and FB_1-producing *Fusarium* spp. on cowpea seedlings under phytotron conditions. Parameters that were evaluated included seedling emergence, root and shoot length, root and shoot dry weight, seedling vigour index and chlorophyll production.

2. Investigate the mode of action of the toxicity of FB_1 in cowpea seedlings by determining whether FB_1 inhibits the gene expression of ceramide synthase, which forms part of the sphingolipid pathway in cowpea.

1.3. Structure of dissertation

This dissertation is divided in six chapters as set out below.

Chapter 1: This chapter provides a brief justification for the study and states the aim and objectives.

Chapter 2: This chapter provides a concise review of cowpea, its importance, constraints to cowpea production, fungi associated with cowpea seed and their associated mycotoxins. A general introduction to mycotoxins with emphasis on fumonisins is given. Information is also provided on the phytotoxic effects of FB_1 and its possible mode of action, which includes the sphingolipid pathway and a key enzyme in the pathway, ceramide synthase.

Chapter 3: In this chapter, the phytotoxic effect of FB_1 and FB_1 producing *F. verticillioides* strains on cowpea emergence, seedling length, root and shoot weight, and seedling vigour is investigated. Furthermore the effect of FB_1 on the total chlorophyll content of the seedlings is also assessed.

Chapter 4: This chapter examines whether or not FB_1 inhibits the gene expression of ceramide synthase in FB_1 treated seedlings. Sequence analysis of the cowpea ceramide synthase gene fragment is also reported in this chapter.

Chapter 5: This chapter includes a general discussion, interpretation of the experimental results achieved, shortcomings of the study and suggestions for future research.

Chapter 6: Literature cited.

1.4. Conferences contributions

Aspects of this study were presented at the following conferences/symposia:

39th Annual Conference of the South African Association of Botanists (SAAB), University of Kwazulu-Natal, 20–24 January 2013, Drakensberg. **Phytotoxic effects of fumonisin B_1 on cowpea (*Vigna unguiculata* (L.) Walp**. Kotze, R.G., Crampton, B.G., Kritzinger, Q. (Poster presentation).

41st Annual Conference of the South African Association of Botanists (SAAB), University of Venda, 11–15 January 2015, Tshipise Resort. **The molecular and physiological effects of fumonisin B_1 on cowpea (*Vigna unguiculata* L. Walp)**. Kotze, R.G., Crampton, B.G., Kritzinger, Q. (Oral presentation).

CHAPTER 2

Literature review

2.1. Introduction to cowpea (*Vigna unguiculata* (L.) Walp)

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important annual summer food legume belonging to the family Fabaceae and is indigenous to Africa (Padulosi & Ng, 1997). The crop forms part of the Phaseoleae tribe, which also contains other important crops such as soya bean (*Glycine max* (L.) Merr.), common bean (*Phaseolus vulgaris* L.), mung bean (*Vigna radiata* (L.) R.Wilczek) and bambara groundnut (*Vigna subterranea* (L.) Verdc.) (Fery, 2002; Timko *et al.*, 2007; The Plant List, 2015). The legume was an important source of fodder for cows in the south-eastern United States and is thus probably where the name originated from and is also referred to as black-eye pea or bean, crowder pea or southern pea (Davis *et al.*, 1991; (Timko *et al*., 2007)). Other names for cowpea include akkerboon (Afrikaans), dinawa (Sepedi, Tswana), imbumba (Zulu), niébé (French), caupi (Brazil) and augenbohne (German) (Directorate Plant Production, 2011; Timko *et al*., 2007). Cowpea is often referred to as *Vigna sinensis* (L.) Savi in older literature (Quinn, 1999).

2.1.1. Taxonomy

Cowpea is a dicotyledonous plant belonging to the order Fabales, family Fabaceae, sub-family Faboideae, tribe Phaseoleae, subtribe Phaseolinae and genus *Vigna* (Padulosi & Ng, 1997). The database, The Plant List, (2014) suggests there are 323 species in the genus *Vigna* but only 108 are accepted species. Other economically important *Vigna* species include urd beans (*Vigna mungo* (L.) Hepper), adzuki beans (*Vigna angularis* (Willd.) Ohwi & H.Ohashi) and maize bean (*Vigna umbellata* (Thunb.) Ohwi & H.Ohashi)) (Fery, 2002; The Plant List, 2015).

Padulosi & Ng, (1997) states that the genus is divided into several subgenera, which is based on morphological characteristics, extent of genetic hybridization/reproductive isolation and geographical distribution of the species. The various subgenera include the African subgenera *Vigna* and *Haydonia*, the Asian subgenus *Ceratotropsis* and the American counterparts *Sigmoidotropis* and *Lasiopron* (Timko & Singh, 2008). The classification and

nomenclature of cowpea and its associated subspecies and varieties is somewhat confusing and complicated due to different authors classifying cowpea differently from each other. All cultivated cowpeas are grouped under *V. unguiculata* spp. *unguiculata* and are sub-dived into four cultigroups. The four groups are Unguiculata, Biflora, Sesquipedalis and Textilis (Ng & Marechal, 1985). Padulosi, (1993) divided the wild *V. unguiculata* species into five subspecies with each subspecies containing its varieties (Table 2.1).

Table 2.1. Classification and nomenclature of *Vigna unguiculata* according to Padulosi, (1993).

V. unguiculata
spp. *dekindtiana*
var. *dekindtiana*
var. *huliensis*
var. *congolensis*
var. *grandiflora*
var. *ciliolata*
spp. *protracta*
var. *protracta*
var. *kgalagadiensis*
var. *rhombiodea*
spp. *pubescens*
spp. *stenophylla*
spp. *tenuis*
var. *tenuis*
var. *oblonga*
var. *parviflora*

2.1.2. Genomics

Cowpea is a diploid plant which contains 2n=2x= 22 chromosomes and its genome size has been estimated at 620 mega base pairs (Mbp) (Arumuganathan & Earl, 1991; Timko & Singh, 2008). Chen *et al*., (2007) reported that cowpea is a rather underexploited crop with little genomic information available for use in plant biotechnology. The Cowpea Genomic

Initiative (CGI) undertook the laborious task to sequence the genome of cowpea. Rather than to sequence the complete genome of cowpea, the CGI opted to sequence the gene rich region of the cowpea genome (termed gene-space) by the means of methylation filtering (Chen *et al*., 2007; Timko *et al*., 2008). The project is now complete and over 250 000 gene-space sequence reads (GSRs) with an average length of 610 bp were generated, which in turned yielded 160 Mbp of sequence information (Timko *et al*., 2007). These results can be found in the CGKB, Cowpea Genespace/Genomics Knowledge Base, which was created under the CGI (Chen *et al*., 2007; Timko *et al*., 2008). The CGI created the database as there was no database of genomic resources for the cowpea community at that time (Chen *et al*., 2007). The database is open to researchers all over the world and is accessible via a web-based interface (Chan *et al*., 2007; Cowpea Genomics Knowledge Base (CGKB), 2014).

2.1.3. Origin, diversity and domestication

The determination of the precise origin of cowpea has, for many years, been of high speculation and difficult to determine. De Candolle, (1886) stated that the origin of a cultivated plant is where wild relatives are found. It has been suggested that Africa and Asia could be centres of origin due to cowpea varieties being very diverse and morphologically different from each other, respectively (Timko & Singh, 2008). Due to the absence of wild cowpea in Asia, the validity of the Asian centre of origin has been questioned (Timko & Singh, 2008). Wild cowpeas only exist in Africa and Madagascar (Steel, 1976). It is thought that the former Transvaal region of the Republic of South Africa is the centre of origin due to the presence of the most primitive of the wild varieties of cowpea (Padulosi & Ng, 1997). Padulosi & Ng, (1997) also reported that some of the primitive forms radiated to other parts of southern and eastern Africa.

Vavilov, (1926) postulated that a crop which contains intense variation in a certain area can be due to the crop being cultivated for long time in that area, for example maize (*Zea mays* L.) originating in Mexico. This ensures that there would have been enough time for large numbers of mutations and gene recombination to take place due to inbreeding of different varieties of the same plant species. Large number of varieties or high variation within species is usually found towards the centre of distribution, with less variability towards the fringes of an area (Padulosi & Ng, 1997). West Africa has been reported to be the centre of diversity for cultivated cowpea (Padulosi & Ng, 1997). The process of

domestication and cultivation resulted in the loss of seed dormancy and pod dehiscence, which correlates with an increase in seed and pod size (Padulosi & Ng, 1997). Baudoin & Maréchal, (1985) reported south and east Africa as the primary centres of diversity and west and central Africa to be the secondary centre of diversity. They also proposed Asia to be the third centre of diversity. This could be as a result of cowpea being introduced during the Neolithic period to India (Pant *et al*., 1982).

2.1.4. Distribution, production and cultivation

Cowpea is often cultivated in hot low elevation equatorial and subtropical areas of the world, usually below 1300 m above sea level (Ehlers & Hall, 1997). Cowpea is extensively cultivated in Africa and also in areas such as Asia and the Americas with several smaller areas spread over Europe, Oceania and the Middle East (Ehlers & Hall, 1997; Quin, 1997). Roughly 70% of cowpea production occurs in sub-Saharan Africa (Brader, 1997). Cowpea is produced in countries such as Nigeria, Niger, Burkina Faso, Mozambique, Kenya, Tanzania, Cameroon, Botswana and South Africa in Africa; India, Sri Lanka, Myanmar and China in Asia and Brazil, Haiti, Peru and USA in the Americas (Ehlers & Hall, 1997; Singh *et al*., 2003).

In South Africa the four main producing provinces are Limpopo, Mpumalanga, North-West and KwaZulu-Natal. The main producing districts in Limpopo are: Bohlabela, Vhembe, Mopani, Capricorn, Sekhukhune and Waterberg; in Mpumalanga: Gert Sibande, Nkangana and Ehlanzeni; in North-West: Central, Bophirima and Southern and in KwaZulu-Natal: Umgungundlovu (Directorate of Plant Production, 2014). Cowpea is also produced in the Free State but not on a large scale.

Cowpea is an excellent crop for the more arid regions of the world due to being a drought tolerant and warm weather crop (Singh *et al*., 2003). It is able to maintain some growth or at least survive under drought conditions partly due to its deep rooting habitat (Quin, 1997). Cowpea is cultivated under both non-irrigated and irrigated regimes (Davis *et al*., 1991). It grows well under 400 to 700 mm of rainfall but is often grown with less than 400 mm of rainfall (Directorate of Plant Production, 2014; Valenzuela & Smith, 2002). Hall, (2004) indicated that the “Ein El Gazal” cultivar produced 1091 kg/ha of grain with only 181 mm of rainfall with an evaporative demand of 6 mm/day at Louga, Senagal. Cowpea cannot

withstand waterlogged conditions as is the case with most leguminous crops (Davis *et al*., 1991; Valenzuela & Smith, 2002).

A base temperature of 8–11°C is appropriate for all cowpea developmental stages with 28°C being the optimal temperature for developmental stages (Craufurd *et al*., 1997). They are often replaced by the common bean at 1300 meter above sea level, although cowpeas have been grown at higher altitudes (Ehlers & Hall, 1997). Cowpea is often intercropped with several cereal and root crops as well as with cotton (*Gossypium hirsutum* L.), sugarcane (*Saccharum* spp.) and several plantation crops due to it being shade tolerant (Singh *et al*., 1997).

Worldwide production for 2013 was estimated at 6.2 million tons from 11.9 million hectares, which results in an average grain yield of 0.54 tons/hectare (FAOSTAT, 2015). Nigeria is the biggest producer by area followed by Niger and Burkina Faso (Table 2.2) according to FAOSTAT, (2015). The top cowpea producing countries are all located in Africa with the exception of Myanmar, which is located in Southeast Asia (Table 2.2). Almost none of the cowpea produced in Brazil is exported and all of it is consumed within the country itself, however not a lot of data exists in terms of the production values.

Table 2.2. Top cowpea producing countries in the world in 2013 (FAOSTAT, 2015).

#	Country	Grain Production (t)	Production Area (ha)	Yield (t/ha)
1	Nigeria	2950000	3800000	0.776
2	Niger	1300000	4700000	0.277
3	Burkina Faso	580000	1200500	0.483
4	Tanzania	188717	234233	0.806
5	Myanmar	177000	148000	1.196
6	Mali	168274	254384	0.661
7	Cameroon	165350	236214	0.700
8	Kenya	122682	192345	0.638
9	Uganda	94200	75000	1.257
10	Democratic Republic of the Congo	80000	150000	0.533
11	Mozambique	80000	320000	0.250

Due to the demand of cowpea in urban areas, the cultivation of cowpea is moving from intercropping to sole cropping in order to meet the demands (Ehlers & Hall, 1997). The commercialization of cowpea correlates with the increase in area and grain production of cowpea over the last 50 years (Figure 2.1). The increase of cowpea over the last 50 years is due to long term drought conditions in West Africa since cowpea is a drought resistant crop (Figure 2.1) (Van Duivenbooden *et al*., 2002). The average cowpea grain yield is given as 0.39 t/ha for the last 50 years, with both area and grain productions increasing six fold (Figure 2.1) (FAOSTAT, 2015). Yearly cowpea yields have almost doubled from 0.28 t/ha in the 1960's to over 0.50 t/ha in the 2000's (FAOSTAT, 2015).

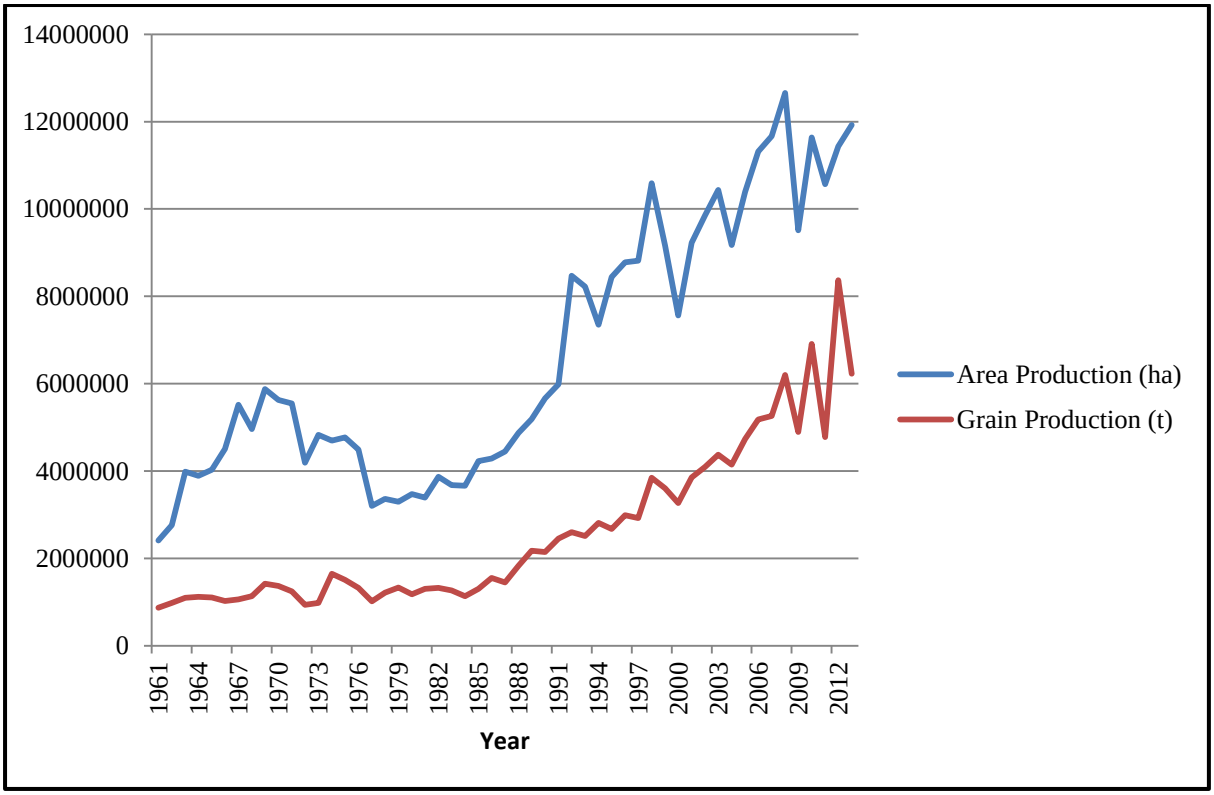


Figure 2.1. Cowpea production over the last 50 years (Adapted from FAOSTAT, 2015).

2.1.5. Morphological characteristics

Cowpea is described as an annual, warm season herbaceous plant with a variety of growth habits that can be either erect, semi-erect, prostrate (trailing) or climbing (Davis *et al*., 1991; Timko & Singh, 2008). Cowpea has a very strong taproot, which can grow up to a depth of 240 cm, with numerous lateral roots emerging from the tap root (Davis *et al*., 1991; Directorate of Plant Production, 2014). The stem of cowpea is smooth or slightly hairy with

purple shades (Directorate of Plant Production, 2014). The first pair of leaves after emergence are opposite, sessile and entire, while remaining leaves are trifoliate, alternate and petiolated (Timko & Singh, 2008). The flowers of cowpea are borne in multiple racemes on 20–50 cm long peduncles arising from the leaf axil (Davis *et al*., 1991). Cowpea is primarily autogamous in that it mainly fertilizes itself. Usually two to three pods per peduncle are common but if growing conditions are favourable, four or more pods can be present (Timko *et al*., 2007). Cowpea pods are cylindrical and can be either curved or straight with between eight and 20 seeds per pod (Timko & Singh, 2008). The seeds can be either smooth or wrinkled and can be various colours including cream, white, brown, black, buff and red (Figure 2.2) (Timko *et al*., 2007). Emergence of cowpea seedlings after germination is epigeal, where the cotyledons emerges from the ground first during germination (Davis *et al*., 1991).

Figure 2.2. Cowpea seed with a diversity of testa colours (Timko *et al*., 2007).

2.1.6. Utilization and importance of cowpea

Cowpea is known to be one of the most versatile, widely adapted and nutritious grain legumes (Ehlers & Hall, 1997). It grows well in most types of soil textures from heavy clays, when well drained, to sandy soils but prefers sandy loam or sandy soils, which tends to be less restrictive on root growth (Davis *et al*., 1991; Directorate of Plant Production, 2014; Valenzuela & Smith, 2002). The crop can grow in about any type of soil condition, but

responds best in slightly acidic to slightly alkaline soils (pH 5.5–8.3) (Valenzuela & Smith, 2002).

The crop also has the ability to grow in infertile soils due to its ability to fix atmospheric nitrogen through symbiosis with the root nodule bacteria *Bradyrhizobium* spp (Ehlers & Hall, 1997; Singh *et al*., 2003; Zhang *et al*., 2007). It has the ability to contribute about 40–80 kg nitrogen/ha back into the soil with the total amount of nitrogen fixation by cowpea being 70–350 kg/ha (Quin, 1997). The roots of cowpea also form a symbiotic association with mycorrhiza, which improves the soil's available phosphorous content when cowpea is grown in soils which are low in phosphorous (Valenzuela & Smith, 2002). Subsistence farmers benefit from this since they do not have to buy fertilizers to grow cowpea.

Additional benefits of cowpea include the incorporation of organic matter that improves soil structure and fertility, water infiltration and soil water holding capacity (Valenzuela & Smith, 2002). Cowpea has the ability to control and prevent soil erosion due to being shade tolerant, a quick grower and being a rapid ground covering species (Singh *et al*., 2003). These attributes make cowpea an important crop component of subsistence farmers around the world.

2.1.6.1. Uses as a food crop

Cowpea is an important crop for the survival of millions of poor people living in the equatorial and subtropical areas of the world (Quin, 1997). It is a valuable crop in that it provides food and fodder for humans and animals, respectively, and is also a source of income to subsistence farmers and grain traders (Langyintuo *et al*., 2003; Timko & Singh, 2008). Earlier maturing varieties of cowpea, which can mature in 55 days after planting, have the ability to provide the first food of the season and thus shortening the hunger period that occurs between the previous season harvest and the current season crop (Timko *et al*., 2007).

The most important part of the cowpea plant for human consumption is the seeds of the plant or the grain as it is often referred to, but fresh leaves, immature pods, and peas are used as vegetables with the leaves being prepared as a pot herb like spinach (Quin, 1997; Timko *et al*., 2007; Timko & Singh, 2008). The majority of subsistence farmers harvest the seeds and dry them for storage, which is then later used in a variety of dishes (Timko & Singh, 2008).

Cowpea seeds on average contains between 23–25% protein and 50–60% starch (Quin, 1997). The protein of cowpea seeds are rich in the amino acids lysine and tryptophan when compared to cereal grains but lack the amino acids methionine and cysteine, both of which are high in animal proteins (Davis *et al*., 1991). The nutritional profile of cowpea and the common bean are similar except that cowpea has higher level folic acid and lower levels of flatulence producing factors (Ehlers & Hall, 1997). The higher level of folic acid (which is a B vitamin) is needed during pregnancy to prevent brain and spine defects in the unborn baby (Folic Acid, 2015; Timko & Singh, 2008).

For millions of people throughout the developing world cowpea plays a major part in their livelihoods as it provides them with cheap major source of protein which complements other low protein crops (Timko & Singh, 2008). The diet of people living in rural and urban poor Africa consists mainly of starchy foods such as cassava (*Manihot esculenta* Crantz), yams (*Dioscorea* spp.), millet (*Panicum miliaceum* L.), sorghum (*Sorghum bicolor* (L.) Moench) and maize and the addition of cowpea improves the nutritional value of the diet (Singh *et al*., 2003). Protein quality is greatly enhanced due to the synergistic effect between the protein from cowpea and energy from the foods rich in carbohydrates (Singh *et al*., 2003). When compared to other cereal and tuber crops, cowpea has a lower fat content and its protein content is about two- to four fold higher (Timko & Singh, 2008). Due to the high protein content and the use of cowpea in the diets of relatively poor people, it makes an excellent crop for food security (Coulibaly & Lowenberg-DeBoer, 2002).

Cowpea is used in wholegrain form or milled form and is used in over 50 dishes (Langyintuou *et al*., 2003). Milled cowpea is commonly used in West Africa in dishes such as “akara” or “kosai”, which is fried cowpea balls or in steam cowpea cakes called “moin-moin” or “ole-le” (Langyintuou *et al*., 2003). Ehlers and Hall, (1997) state that cowpea can be cooked very quickly and is also thus a favourite for people in developing countries where fuel for cooking is in short supply.

Rural and urban woman earn a valuable income by the trading of freshly produced and processed cowpea food and snacks in Africa (Singh *et al*., 2003). Mature pods of cowpea are harvested and the haulms are harvested still while green. These haulms are then rolled into bundles and are sold as “Harawa”, which is a feed supplement for livestock in the dry season (Singh *et al*., 2003). Cowpea forms a major part of animal forage during the dry season when

the demand for animal forage reaches its peak (Coulibaly & Lowenberg-DeBoer, 2002). It has been reported that the seed contains protease inhibitors but the use of cowpea seed as fodder does not present any problems in animals (Timko & Singh, 2008).

2.1.6.2. Medicinal importance

It is known that cowpea plants possess various medicinal properties and have been used traditionally in the treatment of various ailments. Powered roots of cowpea are eaten with porridge to treat painful menstruation, epilepsy and chest pain (Van Wyk & Gericke, 2000). Sreerama *et al*., (2012) stated that cowpea flour made from the seeds has enzyme inhibitory and antioxidant properties linked with hyperglycemia and hypertension diseases. Siddhuraju & Becker, (2006) also stated that processed cowpea seed extract has antioxidant and free radical scavenging activities. The seed of cowpea is taken orally as a decoction to treat amenorrhea (Van Wyk & Gericke, 2000). Chharbra *et al*., (1990) reported that people chew the leaves of cowpea when they have toothache. Both the aqueous and ethanol extracts of cowpea have antidrepanocytary (anti-sickle cell anaemia) activity, which supports the claims of traditional healers in the Congo (Mpiana *et al*., 2007).

2.1.7. Constraints to cowpea production

Cowpea is negatively influenced by a number diseases, pests and abiotic factors such as fungal diseases, bacterial diseases, viral diseases, insects, nematodes, parasitic plants, water and temperature (Singh & Allen, 1979). All parts of the plant including the roots, shoots, leaves and seed bearing pods are affected by pests, diseases and abiotic factors. The production of cowpea in the developing world is severely hampered by too much and too little rainfall, the absence of soil fertilizers, herbicides and pesticides and proper production methods (Singh, 2005; Timko *et al*., 2007; Timko & Singh, 2008). Water and temperature are the biggest abiotic factors affecting cowpea production. Timko & Singh, (2008) indicated that erratic rainfall at the beginning and the end of the rain season severely affects cowpea growth and flowering resulting in a decrease in grain yield. Too much rain also affects cowpea production as it cannot withstand waterlogged conditions (Davis *et al*., 1991; Valenzuela & Smith, 2002). High night temperature results in abscission of flowers and thus lowering the production of cowpea (Warrag & Hall, 1984).

Hampton *et al*., (1997) reported that there are over 20 viral diseases present in cowpeas. A few of the most destructive viral pathogens are transmitted through seed from one generation to the next (Hampton *et al*., 1997). Important viruses in cowpea include: blackeye cowpea mosaic potyvirus (BICMV), cowpea aphid-borne mosaic potyvirus (CABMV), cowpea chlorotic mottle bromovirus (CCMV), cowpea golden mosaic geminivirus (CGMV), cowpea yellow mosaic (CPMV), cucumber mosaic cucumovirus (CMV) and southern bean mosaic sobemovirus (SBMV) (Ehlers & Hall, 1997; Singh & Allen, 1979). A majority of the seed-borne viruses are destructive since infection of plants with the virus can result in spreading of the virus to neighbouring plants by means of insect vectors such as aphids or beetle species (Ehlers & Hall, 1997; Singh & Allen, 1979)

Root-knot nematodes are a major constraint of cowpea in certain parts of the world. Four species of nematode infect cowpea. The four *Meloidogyne* species that parasitise cowpea are *M. arenaria, M. hapla, M. incognita* and *M. javanica*. *Meloidogyne ingognita* and *M. javanica* are the most widely studied and important of the nematodes that infect cowpea (Roberts *et al.,* 1997). Singh & Allen, (1979) reported that *M. incognita* may cause major crop losses in cowpea, whereas *M. javanica* may make cowpea more susceptible to *Fusarium* wilt.

Insect pests target all parts of the cowpea plant, including its leaves, flowers and pods. Insect damage is one of largest constraints to cowpea production in most cowpea producing regions (Ehlers & Hall, 1997). These insects include aphids (*Aphis craccivora*), thrips (*Megalurothrips sjostedti*), maruca pod borer (*Maruca vitrata*), pod sucking insects (*Clavigralla tomentosicollis*, *Acanthomia* spp., *Riptortus* spp.) and storage weevils (*Callosobruchus* spp.) (Jackai & Adalla, 1997; Timko & Singh, 2008).

Two types of weeds, *Striga gesnerioides* (Willd.) Vatke and *Alectra vogelii* Benth. parasitize cowpea plants, leading to an increase in yield loss. Both these weeds occur throughout Africa (Singh & Emechebe, 1997). They parasitize by forming a haustorium with root tissue of the host after which the shoot emerges (Singh & Emechebe, 1997). Both these weeds are difficult to control due to the fact that they produce many seeds and that damage to the plant is already done by the time the weeds emerge from the ground (Timko & Singh, 2008).

Two major bacterial diseases of cowpea are bacterial blight (canker) and bacterial pustule (bacterial spot) (Emechebe & Florini, 1997; Singh & Allen, 1979). Bacterial blight is induced by *Xanthomonas campestris* pv. *vignicola* and bacterial pustule by *Xanthomonas* sp. (Emechebe & Florini, 1997; Singh & Allen, 1979).

Fungal diseases include diseases such as stem, root and foot rots, wilts, leaf spots and rusts, various pod and seed diseases (Singh & Allen, 1979). Major fungal diseases associated with cowpea include anthracnose (*Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara), leaf smut (*Protomycopsis phaseoli* (Patel, Y.S. Kulk. & G.W. Dhande) K. Ramakr. & Subram.) and powdery mildew (*Erysiphe polygoni* DC.) (Madamba *et al*., 2006). Cowpea seeds are prone to fungal infestation due to the suboptimal conditions they are stored in, which include high temperature and high humidity (Kritzinger, 2004). There are numerous articles that report the occurrence of mycoflora on cowpea seed (Emechebe & McDonald, 1979; Hedge & Hiremath, 1987; Kritzinger *et al*., 2003; Zohri *et al*., 1992). Some of the most common cowpea seed mycoflora are presented in Table 2.3.

Emechebe & McDonald, (1979) reported that they isolated fungi from cowpea seeds obtained from markets in Northern Nigeria. The fungi included *Ascochyta* sp., *Fusarium oxysporum* Schltdl., *Fusarium solani* (Mart.) Sacc., *Macrophomina phaseolina* (Tassi) Goid. and *Athelia rolfsii* (Curzi) C.C. Tu & Kimbr. (Emechebe & McDonald, 1979). It was thought that cowpea was not susceptible to fungal attack due to the dry conditions they were grown in, but *M. phaseolina* attacks cowpea when the plants are under severe stress due to drought conditions and high temperature (Adam, 1990). Houssou *et al*., (2009) reported that they isolated 23 fungal species from 92 cowpea representatives. They analysed the cowpea samples when they were collected from the field and after three months of storage. It was reported that the incidence of infection of *Aspergillus flavus* Link increased in these three months (Houssou *et al*., 2009). Cowpea seed samples from India were assayed for seed-borne fungi, with *Fusarium verticillioides* (Sacc.) Nirenberg, *Penicillium* sp. *Fusarium oxysporum* Schltdl., *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., and *Aspergillus niger* Tiegh. being the dominant fungi (Shama *et al*., 1988). Shama *et al*., (1988) also indicated that *F. verticillioides* inhibited cowpea seed germination.

Zaidi, (2012) isolated 11 fungal species from cowpea seed, which he used as inocula to test the germination rate of cowpea seed infected with these fungi. All the fungal species

reduced the germination rates of cowpea seed and disease symptoms included reduction in roots and shoot length, yellowing of the leaves and deformation of the leaves (Zaidi, 2012). *Alternaria alternata* (Fr.) Keissl., *Alternaria dianthi* J.V. Almeida & Sousa da Câmara, *A. flavus, F. verticillioides* had the greatest effect on the reduction of root and shoot length of cowpea out of the 11 fungal species tested (Zaidi, 2012). Studies by Kritzinger *et al*, (2004), indicated that cowpea seed germination was reduced by *F. verticillioides* and *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg, but had no effect on the root and shoot lengths.

Table 2.3. The common seed mycoflora of cowpea.

Genus	Species	References
Alternaria	*alternata*	(Shama *et al*., 1988; Shah *et al*., 1992; Zaidi, 2012)
***Ascochyta* sp.**		(Emechebe & McDonald, 1979)
Aspergillus	*flavipes*	(Zohri *et al*., 1993)
	flavus	(Hedge & Hiremath, 1987; Houssou *et al*., 2009; Kritzinger *et al*., 2003; Shama *et al*., 1988; Zaidi, 2012; Zohri *et al*., 1992)
	niger	(Hedge & Hiremath, 1987; Houssou *et al*., 2009; Kritzinger *et al*., 2003; Shama *et al*., 1988; Singh & Chohan, 1974; Zohri *et al*., 1992)
	ochraceus	(Houssou *et al*., 2009; Zohri *et al*., 1992)
	oryzae	(Zohri *et al*., 1992)
	sydowii	(Zohri *et al*., 1992)
	tamarii	(Zohri *et al*., 1992)
	terreus	(Shama *et al*., 1988; Singh & Chohan, 1974; Zohri *et al*., 1992)
Collectotrichum	*dematium*	(Smith *et al*., 1999)
	lindemuthianum	(Emechebe & McDonald, 1979)
	truncatum	(Emechebe & McDonald, 1979)
Fusarium	*anthophilum*	(Rodrigues & Menezes, 2005)
	chlamydosporum	(Kritzinger *et al*., 2003)
	equiseti	(Houssou *et al*., 2009; Kritzinger *et al*., 2003; Shama *et al*., 1988; Zaidi, 2012)
	oxysporium	(Biemond *et al*., 2013; Emechebe & McDonald, 1979; Houssou *et al*., 2009; Shama *et al*., 1988)
	proliferatum	(Kritzinger *et al*., 2003)

	semitectum	(Rodrigues & Menezes, 2005)
	solani	(Biemond *et al*., 2013; Emechebe & McDonald, 1979; Shama *et al*., 1988)
	sporotrichioides	(Rodrigues & Menezes, 2005)
	verticillioides	(Biemond *et al*., 2013; Hedge & Hiremath, 1987; Kritzinger *et al*., 2003; Shama *et al*., 1988; Singh & Chohan, 1974; Zaidi, 2012)
Emericella	*nidulans*	(Zohri *et al*., 1992)
Macrophomina phaseolina		(Adam, 1990; Hedge & Hiremath, 1987; Emechebe & McDonald, 1979; Shama *et al*., 1988)
Penicillium	*chrysogenum*	(Houssou *et al*., 2009; Zohri *et al*., 1992)
	spp.	(Kritzinger *et al*., 2003; Shama *et al*., 1988)
Rhizoctonia	*solani*	(Emechebe & McDonald, 1979; Shama *et al*., 1988)
Rhizopus	*stolonifer*	(Zohri *et al*., 1992)
Septoria	*vignae*	(Emechebe & McDonald, 1979)

2.2. Mycotoxins

Mycotoxins have probably existed for as long as man has grown crops throughout the ages. It has only become clear in the last 30 years that fungi that live in foods and feeds have the ability to produce mycotoxins (Pitt, 2000; Richard, 2007). Food can be contaminated with mycotoxins before or after harvesting, during drying of grain and in storage (Dombrink-Kurtzman, 2008). Mycotoxins are secondary metabolites produced by filamentous fungi and appear to have no role in the normal growth of these fungi (Bennett & Klich, 2003; Pitt, 2000). These secondary metabolites are varied molecules in that their structures range from single heterocyclic rings to irregularly arranged six to eight membered rings (Pitt, 2000; Steyn, 1995). Mycotoxins are low molecular weight compounds (50 Da to 500 Da or greater) and are toxic to wide range of organisms such as mammals, plants, birds and fish (Bennett & Klich, 2003; Pitt, 2000; Richard, 2007; Steyn, 1995) The term “Mycotoxin” was coined after the death of 100 000 turkey polts near London in 1962 due to aflatoxin contamination of the peanut meal the turkeys consumed (Blount, 1961; Bennett & Klich, 2003). The period between 1960 and 1970 was termed the mycotoxin gold rush due to many scientists who joined a well-funded search for mycotoxins (Bennett & Klich, 2003).

The toxicity of mycotoxins can be classified into four groups: acute, chronic, mutagenic (agent that changes the genetic material of an organism) and teratogenic (agent that acts on the foetus to cause congenital abnormality) (Pitt, 2000). Mycotoxin poisoning causing acute toxicity usually results in deterioration of liver or kidney function, whereas chronic mycotoxin poisoning causes no immediate effect but in the long run can cause cancer (Pitt, 2000). It is widely known that grain contaminated with mycotoxins causes a toxic effect in humans consuming these grains (Desjardins & Hohn, 1997). Mycotoxin poisoning can also interfere with DNA replication and protein synthesis (Pitt, 2000). The majority of mycotoxins are highly stable during food processing and are thus a great threat to humans (Barkai-Golan & Paster, 2008). Diseases caused by the indigestion of mycotoxins by humans and animals are termed mycotoxicoses (Steyn, 1995). Mycotoxins have caused major epidemics in man and animals over the ages. Such epidemics include St Anthony's fire which caused gangrenous and convulsive effects in humans; alimentary toxic aleukia (ATA), which was responsible for death of at least 100 000 Russians between 1942 and 1948; and stachybotryotoxicosis which killed thousands of horses in the Union of Soviet Socialist Republics (USSR) in the 1930's (Pitt, 2000; Richard, 2007).

There are about seven principle types of mycotoxins that appear in human food and animal feeds and they are primarily produced by five genera of fungi (Edite Bezerra da Rocha *et al*., 2014; Steyn, 1995). The mycotoxin producing fungi include genera such as *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria* and *Claviceps* (Table 2.4) (Richard, 2007; Steyn, 1995) *Aspergillus*, *Fusarium* and *Penicillium* are the major mycotoxin producers that are linked to food production (Pitt, 2000). *Fusarium* spp. which produce mycotoxins before or immediately after harvest are one of the most destructive pathogens on cereal crops (Pitt, 2000). The main mycotoxins produced by these fungi include aflatoxins, fumonisins, trichothecenes, ergot alkaloids, AAL-toxins and citrinin (Table 2.4) (Bennett & Klich, 2003; Richard, 2007; Steyn, 1995).

Cereal grains, oil seeds, tree nuts and dehydrated fruit are very susceptible to fungal contamination and thus mycotoxin formation (Steyn, 1995). It was previously perceived that mycotoxin contamination of grains was a postharvest problem, but that perception has changed and it is now considered that mycotoxin contamination occurs under field conditions (Desjardins & Hohn, 1997). Many secondary metabolites produced by either fungi or bacteria play a role in causing or exacerbating plant diseases (Bennett & Klich, 2003). Toxins

produced by fungi that have a deleterious effect on plants are called phytotoxins (Bennett & Klich, 2003). Mycotoxins can be both mycotoxic (toxic to animals) and phytotoxic (toxic to plants) (Desjardins & Hohn, 1997).

Table 2.4. The major mycotoxins and their producers (Bennett & Klich, 2003; Desjardins & Hohn, 1997; Hoagland et al., 2007; Richard, 2007; Steyn, 1995; Ueno & Hsieh, 1985, Varga *et al*., 2010).

Mycotoxin type	Producers
AAL-toxins	*Alternaria alternata* f. sp. *lycopersici*
Aflatoxins	*Aspergillus flavus, A. parasiticus, A. bombycis, A. ochraceus, A. nomius, A. pseudotamari*
Citrinin	*Aspergillus* spp., *Monascus* spp., *Penicillium* spp.
Ergot alkaloids	*Claviceps purpurea, C. fusiformis, C. paspali*
Fumonisins	*Fusarium verticilioides, F.proliferatum, F. nygamai, Alternaria alternata f. sp. lycopersici, Aspergillus niger*
Trichothecenes	*Fusarium, Myrothecium, Cephalosporium, Cylindrocarpon, Dendrochium, Stachybotrys, Trichoderma*

2.2.1. Phytotoxins

Phytotoxins are natural chemical compounds that have deleterious effects on plants and are produced either by plants, fungi or bacteria (Duke & Lydon, 1987; Kenfield *et al*., 1988). Phytotoxins that are produced by microbes are of great interest especially those living on weedy plants because it is here that necrosis, chlorosis and wilt occur first (Kenfield *et al*., 1988). The mycotoxins produced by fungi are a major source of phytotoxins. Mycotoxins such as AAL-toxins, alfatoxins, fumonisins and trichothecenes are considered to be major phytotoxins (Hoagland *et al*., 2007). Effects of phytotoxins include inhibition of seed germination, growth inhibition, chlorosis and necrosis leading to death in plants (Abbas & Boyette, 1992; Abbas *et al*., 1995; Kritzinger *et al*., 2004; Zonno & Vurro, 2002).

2.2.2. Mycotoxins associated with cowpea

Literature has indicated that only fumonisins and aflatoxins have been isolated from naturally infected cowpea seed (Adekunkle & Bassir, 1973; Houssou *et al*., 2009; Kritzinger

et al., 2003; Zohri *et al*., 1992). Houssou *et al*., (2008) reported that 23 fungal species were identified on cowpea seed samples with *A. flavus* encountered the most frequently. Furthermore they stated that three of the 92 samples tested positive for fumonisin B_1 (FB_1) while six of the 92 samples tested positive for aflatoxin B_1. They also reported that seed mycoflora increased with storage time of cowpea seed. Kritzinger *et al*., (2003) analysed cowpea seed samples from South Africa and Benin for seed mycoflora and fumonisin mycotoxins. They stated that seed samples from South Africa contained FB_1 at concentrations of 0.12–0.61 µg/g whereas samples from Benin contained no fumonisins.

In 1993, Zohri inoculated the seed of 16 mycotoxin free cowpea cultivars with *A. flavus* to determine varietal differences in aflatoxin production in the different samples. The study indicated that three samples were highly resistant and did not accumulate any aflatoxins, eight samples showed partial resistance and the remaining five samples were highly susceptible to aflatoxin accumulation. The study reported that there was no relationship between morphological character (seed colour, shape and size), testa thickness and the amount of aflatoxin produced on the different cowpea samples. Seenappa, (1983) inoculated the seeds of 22 cowpea varieties with *Aspergillus parasiticus* Speare and the aflatoxin content was measured. They estimated that between 466.6 µg/kg to 1805 µg/kg aflatoxin B and 20.8 µg/kg to 82.7 µg/kg aflatoxin G was produced per kilogram of cowpea seed. The results suggested that certain lines have resistance to aflatoxin contamination. Zaidi, (2012) reported that cowpea seed inoculated with fungi had a reduced germination rate. He argued that this was due to secondary metabolites produced by the fungi that have a deleterious effect on the seeds. Furthermore, Adekunle & Bassir, (1973) reported that aflatoxins and palmotoxins (produce by *A. flavus* growing on palm sap), produced by *A. flavus* inhibited chlorophyll formation and seed germination to a certain extent.

2.3. Fumonisin group mycotoxins

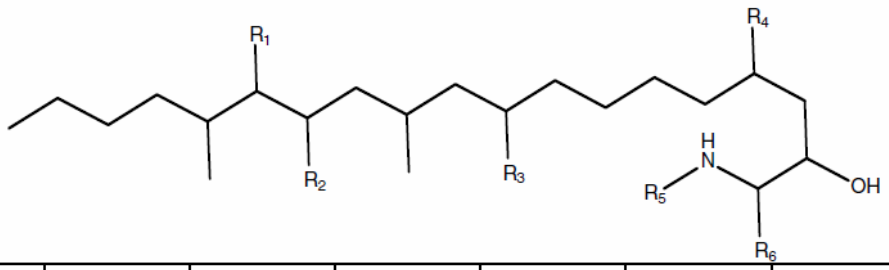
2.3.1. General information of fumonisins

Fumonisins are a class of non-fluorescent mycotoxins that are toxic and carcinogenic, and were first isolated in 1988 (Gelderblom *et al*., 1988; Richards, 2007). Rheeder *et al*., (2002) reported that 15 species of *Fusarium* produce fumonisins but especially *Fusarium verticillioides* (which was formerly known as *F. moniliforme*), *F. proliferatum*, *F. nygamai*

and certain strains of *Alternaria alternata* f. sp. *lycopersici* (Table 2.4) (Kroschel & Elzein, 2004). *Alternaria alternata* f. sp. *lycopersici* was until recently the only other fungal species apart from *Fusarium*, shown to produce fumonisins *(*Rheeder *et al.*, 2002). However, Varga *et al*., reported in 2010 that *A. niger* isolates were able to produce fumonisins in high quantities. *Alternaria alternata* f. sp. *lycopersici* also produces AAL toxin, which is similar in structure to FB_1 (Abbas *et al.*, 1998). Not all strains of *F. verticillioides* produce fumonisins and thus if the fungus is present it does not necessary mean fumonisins are present (Bennet & Klich, 2003; Plumlee & Galey, 1995). *Fusarium verticillioides* and *F. proliferatum* are the *Fusarium* species that are the most important in terms of the highest production of fumonisins (Rheeder *et al*., 2002). Fumonisins and AAL-toxins are structural analogs of each other, are sphinganine analogue mycotoxins (SAM) and they are associated with a variety of plant, human and mammalian diseases (Abbas *et al.*, 1998; Brandwagt *et al*., 2000; Caldas *et al*., 1998; Desjardins & Hohn, 1997). Fumonisins and AAL-toxins have been extensively studied due to their phytotoxicity, their potential to be used as an herbicide and their ability to induce disease in various organisms (Abbas *et al.*, 1991; Abbas *et al.*, 1995; Bennett & Klich, 2003; Caldas *et al.*, 1998).

2.3.2. Chemistry of fumonisins

There have been 28 fumonisin analogues that have been characterized since 1988 and can be classified into four main groups as fumonisin A, B, C and P series (Rheeder *et al.*, 2002). The fumonisin B (FB) group consists of FB_1, FB_2 and FB_3 with FB_1 being toxicologically important and the most abundant of the fumonisins. Fumonisin B_1 accounts for 70–80% of fumonisins produced with FB_2 and FB_3 accounting for 15–25% and 3–8%, respectively (Rheeder *et al*., 2002). Other analogues apart from the FB series may be present in levels lower than 5% (Rheeder *et al*., 2002). The structure of fumonisins was elucidated by Bezuidenhout *et al.*, in 1988 by means of mass spectrometry and NMR spectroscopy as the diester of propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,10,14,15-penta-hydroxyicosane (Figure 2.3). In each case the C14 and C15 hydroxy groups are esterfied with terminal carboxy group of propane-1,2,3-tricarboxylic acid. (Figure 2.3). Fumonisins are hydrophilic and thus makes them difficult to study (Bennett & Klich, 2003).



	R_1	R_2	R_3	R_4	R_5	R_6
FB1	TCA	TCA	OH	OH	H	CH_3
FB2	TCA	TCA	H	OH	H	CH_3
FB3	TCA	TCA	OH	H	H	CH_3
FB4	TCA	TCA	H	H	H	CH_3
AP1	OH	OH	OH	OH	H	CH_3

Tricarballylic Acid (TCA)

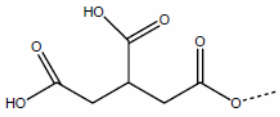


Figure 2.3. Chemical structure of the most common types of fumonisins (Soriano *et al*., 2005).

2.3.3. Toxicity to humans and mammals

Fumonisin B_1 is toxic to both humans and mammals and can induce a number of diseases. They have been known to cause equine leukoencephalomalacia, swine lung edema, tumours in the liver of rats and are associated with oesophageal tumours in humans (Norrad & Voss, 1995; Richard, 2007; Steyn, 1995). These diseases are usually caused by the consumption of maize, sorghum and rice (*Oryza sativa L.*) that are contaminated with fumonisin producing species (Richard, 2007). Fumonisin B_1 can also be teratogenic since they interfere with the utilization of folic acid, which is a dietary supplement that reduces the incidences of neural tube defects (NTD) (Marasas *et al*., 2004). It is also known that FB_1 is hepatocarcinogenic and hepatotoxic to rats (Voss *et al*., 1993). The International Agency for Research on Cancer

found that FB_1 could be a possible carcinogen but was not mutagenic or genotoxic (IARC, 1993).

2.3.4. Phytotoxicity of fumonisin B_1

Fumonisin B_1 not only has a toxic effect on humans and animals but also exerts a toxic effect on plants. Fumonisin B_1 causes phytotoxicity in a variety of agricultural crops and weeds such as cowpea (Kritzinger *et al*., 2006), tomato (*Solanum lycopersicum* L.), sunflower (*Helianthus annuus* L.), maize, soya bean, jimson weed (*Datura stramonium* L.), creeping cucumber (*Melothria pendula* L.), prickly sida (*Sida spinose* L.) and spurred anoda (*Anoda cristata* (L.) Schltdl.) (Abbas & Boyette, 1992) and duckweed (*Lemna aequinoctialis* Welw.) (Abbas *et al*., 1998). Phytotoxic effects on these plants include chlorosis, necrosis, growth inhibition, leaf curl, abnormal leaf development, reduced root development, wilting leading to death (Abbas *et al*., 1995; Abbas & Boyette, 1992; Williams *et al*., 2007). Members from the Poaceae family were initially damaged by FB_1 but it seems that they out-grow the toxin, which suggests that fumonisin can be used as a herbicide such as glyphosate (Abbas & Boyette, 1992).

Abbas & Boyette, (1992) reported that chlorosis, necrosis, stunting and death occurred when plants such as cotton, tomato and prickly sida were sprayed with 50 and 1000 µg/ml FB_1 solutions. Abbas & Boyette, (1992) also reported that barley (*Hordeum vulgare* L.), maize, rice and alfalfa (*Medicago sativa* L) were resistant to FB_1. In 1992, Van Asch *et al*., reported that FB_1 was phytotoxic to maize callus cultures at FB_1 levels of 1.0 mg/L and higher. Fumonisin B_1 did not influence the germination of maize seeds but did inhibit radicle elongation by up to 75% after 48 h imbibition at various concentrations of FB_1 ranging from 0 to 100 mg/L (Doehlert *et al*., 1994). It was also reported that amylase production in the maize endosperm was inhibited by FB_1 and that it could be that FB_1 interferes with the germination process in the seeds (Doehlert *et al*., 1994). Fumonisin B_1 caused bleaching symptoms in duckweed and was found to be eight times more potent than australifungin (anti-fungal agent) (Abbas *et al*., 1998).

Williams *et al*., (2007) reported that maize seeds that were inoculated with the conidia of a pathogenic *F. verticillioides* strain had seedling disease, which was apparent as early as seven days. Typical visual symptoms of the maize seedlings included abnormal leaf development

and reduced root development was observed after seven days post inoculation. A FB_1 watering assay was also done by Williams *et al*., (2007) where they watered maize seed with different concentrations of FB_1 solutions (1.4, 6.9, 13.9 and 27.7 nmol/mL FB_1). They indicated that the seedlings only showed signs of stunted growth after day 13. Reduction in root mass and increase in necrotic leaf lesions were observed with a increase in FB_1 concentration.

In 2006, Kritzinger *et al*., investigated the effect of fumonisins on the germination of cowpea seeds. They imbibed cowpea seeds in various solutions containing four different concentrations of FB_1 (10, 25, 50 and 100 mg/L) and also in solutions containing the conidia of *F. verticillioides, F. proliferatum* and *F. nygamai*. They suggested that cowpea seed germination decreased with an increase in FB_1 concentration, and the seeds inoculated with the conidia of *F. verticillioides* and *F. proliferatum* showed significant reduction in germination. Fumonisin B_1 also caused stunted growth in seeds that did germinate (Figure 2.4). There was almost complete inhibition of the germination of cowpea seeds at 100 mg/L of FB_1. Significant differences in root and shoot inhibition were observed by Kritzinger *et al*., (2006) when seeds were imbibed in 50 and 100 mg/L FB_1 toxin concentrations. These findings were reconfirmed by Kafua, (2011) and Kotzé, (2012). Through the use of transmission electron microscopy (TEM), distinctive differences between the controls and the toxin treated tissue were noted. Irregular sized vacuoles due to the contraction of the plasma membrane from the cell wall and lipid bodies lining the cell wall were observed. Kotzé, (2012) studied the root anatomy using light microscopy since morphological abnormalities (Figure 2.4) of the root were observed. However, no distinct differences were observed between the treated and control root tissue.

Figure 2.4. A- Normal growth of a cowpea seedling. B- Stunted growth of a cowpea seedling treated with FB_1 toxin (Photo: Richard Kotze).

2.3.5. Mode of action of fumonisin B_1

Information of the mode of action of FB_1 in plants is scant. Majority of the work done on the mode of action of FB_1 is either on human, animal or on cultured cells (Kim *et al.*, 2012; Soriano *et al.*, 2005; Voss *et al.*,2002; Voss *et al.*, 2007; Wang *et al.*, 2013). It has been proposed by numerous researchers that FB_1 inhibits the enzyme ceramide synthase, in both plants and animals, which is a key component in the sphingolipid pathway (Kim *et al.*, 2012; Lynch, 1999, Mandala *et al.*, 1994; Marasas *et al.*, 2004; Soriano *et al.*, 2005; Ternes *et al.*, 2011; Voss *et al.*, 2002; Voss *et al.*, 2007; Wang *et al.*, 2013; Williams *et al.*, 2007). Further detail on the inhibition of ceramide synthase by FB_1 is discussed under 2.3.5.2.

2.3.5.1. Sphingolipids and the sphingolipid pathway

The sphingolipid pathway is important in the sense that it produces complex sphingolipids that are necessary for maintenance and growth of both plant and animal cells (Merrill *et al.*, 2001; Sperling & Heinz, 2003). The sphingolipid pathway is very intricate pathway and contains numerous enzymes that take part in the formation of sphingolipids (Figure 2.6)

(Lynch, 1999; Sperling & Heinz, 2003). Sphingolipids are ubiquitous membrane lipids in eukaryotic cells and also in a few bacteria (Sperling & Heinz, 2003). Lynch & Dunn, (2003) have reported that sphingolipids and complex sphingolipids have various functions in plants and are involved in membrane stability, play a role in cell signalling and regulation and in cell-cell interactions. Additional functions include abiotic stress response, phytopathogenesis and programmed cell death (Sperling & Heinz, 2003).

A German scientist Johann Ludwig Wilhelm Thudichum was the first person to name sphingolipids after the mythological Sphinx due to their complex nature (Soriano *et al.*, 2005). Sphingolipids consists of a sphingoid base, which is an amino alcohol with a long hydrocarbon chain and a long chain fatty acid which is attached to the amino group of the sphingoid base through an amide bond (Figure. 2.5) (Merrill; *et al*., 2001; Soriano *et al*., 2005; Sperling & Heinz, 2003). This is also known as the ceramide backbone, which forms part of complex sphingolipids (Figure 2.5). In mammals the predominately sphingoid base is sphingosine whereas in plants, the sphingoid composition is more variable and can be composed of up to eight C18-sphingoid bases, which are derived from sphinganine (Sperling & Heinz, 2003). The only difference in the sphingoid bases are the position and number of the double bonds and other functional groups such as hydroxyl groups (Merrill *et al*., 2001; Sperling & Heinz, 2003). The fatty acid also varies in chain length (C14–C24), and the degree of unsaturation (Merrill *et al*., 2001; Sperling & Heinz, 2003). The ceramide backbone can be further modified on its primary hydroxyl group of the sphingoid base resulting in a polar head group (Figure 2.5) (Kim *et al*., 2012; Sperling & Heinz, 2003). The polar head group can be either, a phosphoryl group (ceramide phosphate), mono- or pluri-hexose (glycosylceramides) or an inositol phosphate group (IP) (Figure 2.5) (Lynch & Dunn, 2004; Sperling & Heinz, 2003). The last two molecules mentioned are complex sphingolipids and can be further modified to form more complex sphingolipids. Glucosylceramides, complex glycophosphosphingolipids and inositolphosphoceramides are the predominant complex sphingolipids in plants (Lynch, 1999).

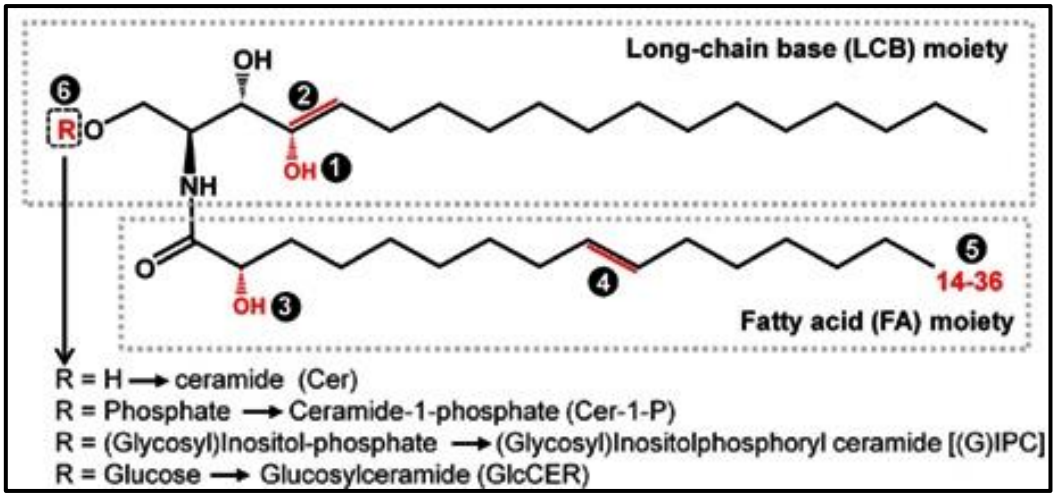


Figure 2.5. An illustration of a complex sphingolipid and all of its components (Adapted from Berkey *et al*., 2012).

The biosynthesis of the sphingolipid pathway starts with the condensation reaction of palmitoyl-CoA and the amino acid serine, which is catalysed by the enzyme serine palmitoyl-transferase to yield 3-ketosphinganine (Figure 2.6) (Delgado *et al.*, 2002; Soriano *et al*., 2005; Sperling & Heinz, 2003). In the second step of the pathway, 3-ketosphinganine is reduced with NADPH by 3-ketosphinganine reductase to yield sphinganine (Figure 2.6) (Delgado *et al.*, 2002; Soriano *et al*., 2005; Sperling & Heinz, 2003). The next step in the pathway is the formation of ceramide, which can be formed by two mechanisms, the fatty acid-CoA dependent pathway and the free fatty acid dependent pathway (Figure 2.6) (Lynch, 1999; Pata *et al*., 2009; Sperling & Heinz, 2003). Ceramide is of great importance in the sphingolipid pathway since it is a central intermediate (Ternes *et al*., 2011). It is here that *de novo* biosynthesis, degradation and recycling of the sphingolipid pathway are interconnected (Ternes *et al* ., 2011).

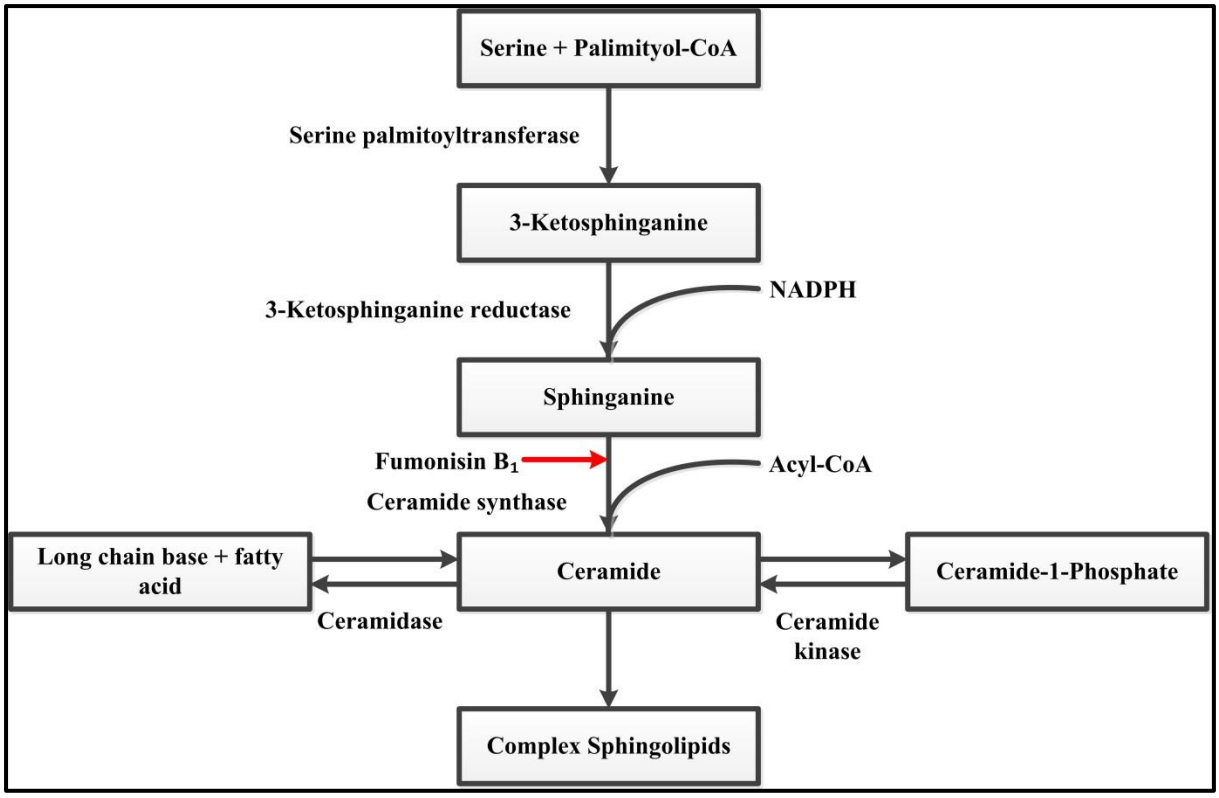


Figure 2.6. A simplified representation of the sphingolipid pathway in plants containing major enzymatic steps. The red arrow indicates where FB_1 inhibits ceramide synthase (adapted from Sperling & Heinz, 2003).

The reaction of sphinganine to ceramide can be catalysed by two enzymes: ceramide synthase (LAG1) or ceramidase (Figure 2.6) (Merrill *et al*., 2001; Sperling & Heinz, 2003). Ceramide synthases are encoded by the longevity assurance gene or *LAG*1 gene family, which occurs in the majority of all eukaryotic organisms studied this far (Winter & Ponting, 2002). In the first mechanism (fatty acid-CoA dependent pathway), ceramide synthase catalyses the reaction of sphinganine and a fatty acyl-CoA into ceramide (Figure 2.6) (Lynch, 1999; Merrill *et al*., 2006; Sperling & Heinz, 2003). In the second mechanism (free fatty acid dependent pathway), ceramidase catalyses the same reaction but instead of using a fatty acyl-CoA, it utilizes free fatty acids (Figure 2.6) (Sperling & Heinz, 2003). The fatty acid-CoA dependent pathway is the primary pathway by which the formation of ceramide occurs (Merrill *et al*., 2001; Sperling & Heinz, 2003; Ternes *et al*., 2011; Voss *et al*, 2002; Voss *et al*., 2007) .

It can be noted in many reports, that the words ceramide synthase and sphinganine N-acyltransferase are used interchangeably (Wang & Merrill, 2000). Lynch, (1999) and Lynch

& Dunn (2003) indicated that sphinganine N-acyltransferase is the enzyme that catalyses the reaction of sphinganine and fatty acyl-CoA to form ceramides and that ceramide synthase catalyses the reaction where a fatty acid instead of a fatty acyl-CoA is used. They also indicated that ceramidase catalyses the reverse reaction of ceramide synthase. They further mentioned that it can be possible that ceramide synthase and ceramidase activities could reflect the activity of a single enzyme operating in forward and reverse directions.

Arabidopsis thaliana contains three ceramide synthase isoforms which are localized in the ER (endoplasmic reticulum) (Marion *et al.*, 2008). The three ceramide synthase gene homologues are often referred to as *LOH1*, *LOH2* and *LOH3* (LAG One Homologue) (Markham, 2011). LOH1 and LOH3 are 77% identical and thus are highly similar, whereas as LOH2 is 45% identical and is thus less similar than LOH1 and LOH3 isoforms (Ternes *et al.*, 2011). The difference between the homologues is due to the length of the fatty acyl-CoA they prefer as a precursor molecule (Ternes *et al.*, 2011). There are two major differences between LOH2 and LOH1 and LOH3. First, LOH1 and LOH3 can accept fatty acyl-CoA with chains lengths ranging from 20 to 28 carbons, whereas LOH2 predominately accepts acyl-CoA with chains consisting of 16 carbons (Ternes *et al.*, 2011). Markham *et al.*, (2011) also reported that LOH2 is specific for 16 carbons whereas LOH1 and LOH3 are specific for long carbon chains. Secondly LOH1 and LOH3 accept primarly trihydroxy sphingoid bases, whereas LOH2 accepts dihyroxy- and trihydroxy sphingoid bases (Ternes *et al.*, 2011). Thirdly LOH1 and LOH3 produce ceramides with a very broad chain length distribution whereas LOH2 produces only C_{16}-ceramide (Ternes *et al.*, 2011).

2.3.5.2. Effect of FB_1 on the sphingolipid pathway

The proposed mode of action of FB_1 is that it inhibits ceramide synthase, which would lead to an increase in sphinganine levels (Figure 2.6) (Abbas *et al.*, 1994; Abbas *et al.*, 1998; Delgado *et al.*, 2002; Lynch, 1999; Merrill *et al.*, 2001; Wang & Merrill, 1999). Fumonisins and sphingolipids are structurally very similar, suggesting that fumonisin could disrupt the sphingolipid mechanism (Figure 2.7) (Riley *et al.*, 1996; Soriano *et al.*, 2005, Wang *et al.*, 1991). Merrill *et al.*, (2001) suggested that FB_1 interacts with the binding sites for both sphinganine and fatty acyl-CoA on the enzyme (Figure 2.7). Abbas *et al.*, (1994) indicated that when FB_1 inhibits ceramide synthase that an increase in the levels of sphinganine and phytoaphinanine were observed. These sphingoid bases are usually present in low

concentrations due to it being an intermediate precursor molecule in the sphingolipid pathway and blockage of the complex sphingolipid biosynthesis (Figure 2.6). Fumonisin B_1 is a competitive inhibitor with respect to both substrates (sphinganine and fatty acyl-CoA) (Soriano *et al.*, 2005).

Abbas¸ *et al.*, (1994) stated that they observed electrolyte leakage and observed necrotic spots on leaves of the plants following FB_1 application. Asai *et al.*, reported in 2000 that the death of *Arabidopsis* by FB_1 is light dependent and requires the salicylic acid, jasmonate acid and ethylene mediated signalling pathways as well as one or more unidentified factors. Shi *et al.*, (2007) showed that FB_1 induced cell death was accompanied with the deposition of callose in *Arabidopsis* leaves. They also indicated that FB_1 caused a variety of phytotoxic effects such as fragmentation of nuclear DNA and the accumulation of reactive oxygen intermediates (ROI). Ternes *et al.*, (2011) stated that *loh1* mutant *Arabisopsis* plants (lacked *LOH1* gene) that were cultivated under short-day conditions showed spontaneous cell death accompanied with increased expression levels of the pathogeneisis-related gene *PR-1*. It was mentioned that the levels of free trihydroxy sphingoid bases and C_{16}-Ceramides were elevated, while the levels of $C_{20\text{-}28}$-Ceramides were reduced. These authors suggested that spontaneous cell death could be due to the increase levels of free trihydroxy sphingoid bases or ceramide synthase species with 16 carbon fatty acid.

In 2011, Markham *et al.*, studied the dose dependent effect of FB_1 on *Arabidopsis* seedlings and seed germination. Seedlings that were germinated on FB_1 concentrations of 1µM or higher, primary root length was reduced by 50% and more, whereas seedlings that were grown on 0.5 µM FB_1, had a reduced root length of only 30%. They also reported that lateral roots were more sensitive to FB_1 than the primary roots as lateral root emergence of seedlings treated with 1 µM FB_1 was reduced by 80% when compared to the control. Seedlings grown on FB_1 contained higher levels of sphingolipids than the control plants, but there was an increase in 16 carbon species and a reduction in 18 carbon longer species (Markham *et al.*, 2011). Markham *et al.*, (2011) reported that FB_1 targets LOH1 and LOH3 ceramide synthases rather than LOH2 ceramide synthases. This was determined by treating *Arabidopsis* mutant plants lacking *LOH1* and *LOH3* genes and only containing the *LOH2* gene with FB_1. The amount of long chain bases (LCB) were then measured with high performance liquid chromatography (HPLC) after FB_1 treatment. Inhibition of both *LOH1* and *LOH3* ceramide synthase isologues by FB_1 resulted in the accumulation of high levels

LCB and drove the synthesis of an excessive amount of 16 carbon sphingolipids (Markham *et al*., 2011).

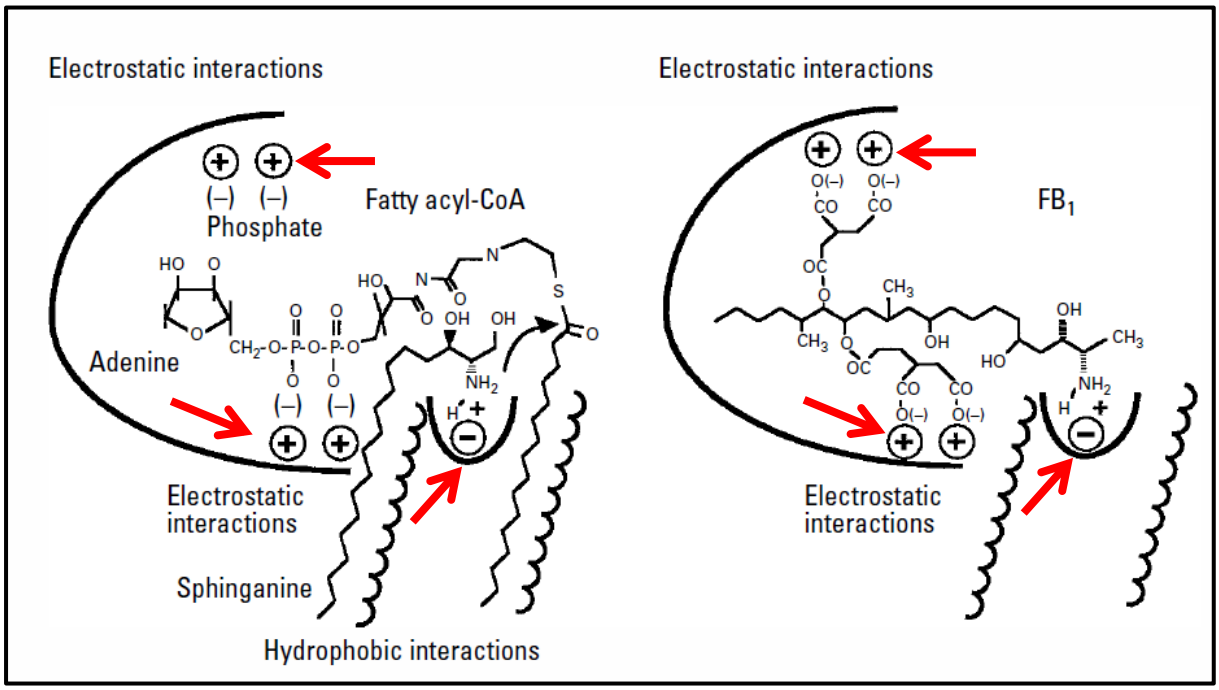


Figure 2.7. A proposed model on how FB_1 binds with the active site in ceramide synthase. The red arrows indicates the likely contact points between the enzyme and substrate or FB_1 (Merrill *et al*., 2001).

The *Alternaria* stem canker (*Asc*) locus, which mediates resistance to sphinganine analogue mycotoxins (SAM) (FB_1) induced apoptosis was studied by Brandwagt *et al*., in 2000. Resistance or susceptibility to both SAM's and AAL-toxin in tomato is determined by the *Asc* locus (Brandwagt *et al*., 1998). Plants that are heterozygous for the *Asc* locus (*Asc, asc*), have an intermediate sensitivity to SAM's (Brandwagt *et al*., 2000). They reported that *Asc-1* is responsible for SAM-resistance in tomatoes and that *Asc-1* gene is homologous to the yeast *LAG1*. Rice, soya bean and poplar tree were also found to transcribe the *Asc-1* homologs, which suggests that *Asc-1* homologs are functional in most plants, particular monocot species (Brandwagt *et al*., 2000). In yeast ceramides (Cers) and inositolphosphorylceramides (IPCers) are synthesised on the ER membrane, whereas glycosylphosphatidylinositol (GDI)-anchored proteins are synthesised in the ER itself (Figure 2.8) (Brandwagt *et al*., 2000). The Cers and IPCers are covalently linked to GPI in the ER (Figure 2.8) (Schultz *et al*., 1998). In yeast, the ER-to-Golgi transport of GPI-anchored proteins (EGGAP transport) is facilitated by LAG1 and delayed ER-to-Golgi transport

(*DGT1*) functional homolog (Figure 2.8) (Brandwagt *et al*., 2000). Brandwagt *et al*., (2000) suggested that after plants were treated with SAM and *de novo* ceramide synthesis was inhibited, that *Asc-1* prevented apotosis in resistant plants by means of restoring EGGAP transport due to alternative production of ceramides (Cers* in Figure 2.8) (Figure 2.8). Brandwagt *et al*., (2000), thus proposed that *Asc-1* plays a role in a salvage mechanism of sphingolipid-depleted cells.

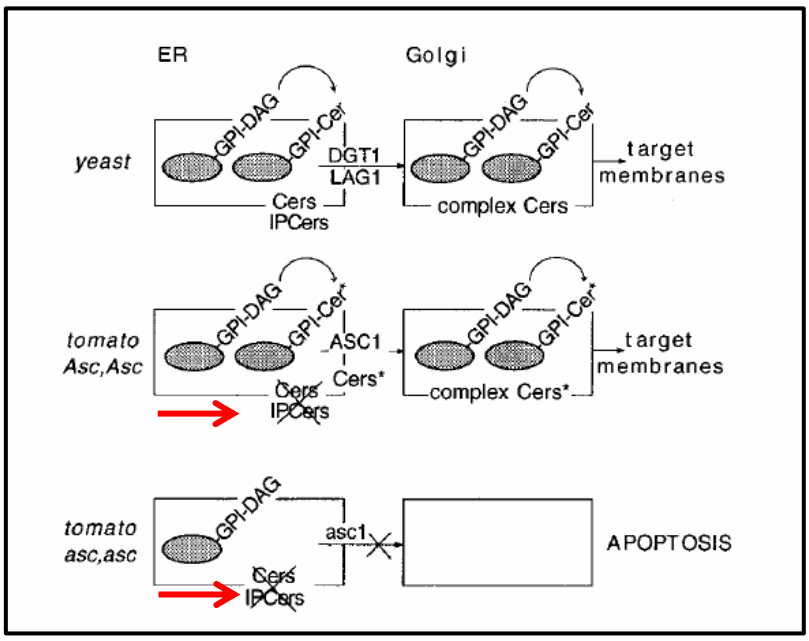


Figure 2.8. A working hypothesis for resistance of SAM in tomato plants proposed by Brandwagt *et al*., (2000). In the yeast cells the ceramides (Cers) and inositolphosphorylceramides (IPCers) are synthesises on the ER membrane (rectangle), whereas the GDI anchors are synthesised in the ER and is then covalently link to Cers and IPCers (grey ovals). In the yeast cells the LAG1 and DGT1 facilitates ER-to-Golgi transport of glycosylphosphatidylinositol (GPI)-anchored proteins (EGGAP). The red arrow as indicate where SAM's will inhibit ceramide synthesis. In resistant tomato plants the ASC1 could restore the ER-to-Golgi transport of GPI-anchored proteins (EGGAP) by producing alternative ceramides (Cers*) in the absence of of SAM sensitive ceramide synthesis after SAM exposure. In sensitive tomato plants, the EGGAP transport could be halted due to the inhibition of ceramide synthesis (asc1) leading to apoptosis (Adapted from Brandwagt *et al*., 2000).

2.4. Conclusion

This review highlights the importance of cowpea to many subsistence farmers and rural communities throughout the subtropical and tropical regions of the world. It is evident that the crop is hampered by numerous pests and diseases, with fungal contamination of the seed being a major problem. Fumonisin B_1 is known to be phytotoxic to various crops, including cowpea. The phytotoxic studies have been performed on cowpea and other important crops

but largely focus on seed germination and above ground application of the toxin and not seedling emergence and growth. Additionally it has been reported that FB_1 inhibits the enzyme ceramide synthase but information is limited and there is no evidence that any studies have been conducted on the expression levels of the gene encoding ceramide synthase.

CHAPTER 3

The effect of fumonisin B_1 on the morphological and physiological aspects of cowpea seedlings

Abstract

Cowpea (*Vigna unguiculata*) is an important crop grown in the tropics and subtropical parts of the world by both subsistence and commercial farmers. However, cowpea seed is often susceptible to fungal infestation and mycotoxin contamination under the suboptimal conditions they are often stored in. Fumonisin B_1, primarily produced by *Fusarium verticillioides*, *F. proliferatum* and *F. nygamai*, is known to display phytotoxic effects on cowpea seed. The aim of this chapter was to investigate the phytotoxic effects that pure FB_1 and FB_1-producing *F. verticillioides* strains exert on the emergence and growth of cowpea seedlings under phytotron conditions. Surface-disinfected seeds were imbibed for 10h in sterile distilled water amended with FB_1 to yield final concentrations of 5, 20 and 40 mg/L. Seeds were additionally imbibed in conidial suspension of FB_1-producing *F. verticillioides* strains. Slow imbibed seeds (sown in seedling trays) and seeds placed in sterile distilled water for 10h served as the non-imbibed and imbibed controls, respectively. Percentage emergence was determined after seven and 21 days dpi, whereas the seedling mass, length and seedling vigour index where determined after 21 days dpi. Total chlorophyll content was determined after 14 and 21 dpi. Morphologically, the toxin caused stunted growth in seedlings. Percentage emergence was reduced by all three FB_1 concentrations as well as one *F. verticillioides* strain, MRC 8265. Seedling length was only reduced by seedlings whose seed was imbibed in 40 mg/l FB_1. Seedling mass was reduced by all FB_1 concentrations and *F. verticillioides* strains. The seedling vigour index (SVI) was reduced by all three FB_1 concentrations and *F. verticillioides* strains. In terms of both SVI indices (SVI 1: % emergence X seedling length; SVI 2: % emergence X seedling mass), the pure toxin and the fungal strain did negatively influence the growth of cowpea seedlings. Total chlorophyll content was the lowest in the non-imbibed control whereas it was the highest in the seedlings that were treated with 40 mg/l FB_1. This is in contrast to other studies which reported that FB_1 causes chlorosis in plants. This study thus confirms that FB_1 is phytotoxic to cowpea and that the three *F.verticillioides* strains did have an effect on the growth of cowpea seedlings.

Keywords: cowpea seed, *Fusarium* spp., phytotoxic effects, fumonisin B_1, seedling vigour index (SVI), total chlorophyll, *Vigna unguiculata*.

3.1. Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an annual legume crop that is indigenous to Africa and is of great importance not only to subsistence farmers, but also to commercial farmers, since it is a multipurpose and beneficial crop (Brader, 2002; Ehlers & Hall, 1997). The crop has a variety of uses such as being a source of food, animal fodder and income through the sale of the seed (Ehlers & Hall, 1997; Kritzinger *et al*., 2006; Singh *et al*., 2003). The seeds are high in protein and thus offer a cheap alternative source of protein, when compared to meat, for people living in rural areas (Singh *et al*., 2003, Timko & Singh, 2008). However, numerous constraints to the optimal utilisation of cowpea exist (Singh & Allen, 1979). Many of these constraints emanate from inadequate storage of cowpea seed after harvesting of the seed (Uzogara & Ofuya, 1992). Cowpea seeds are especially prone to fungal infestation when they are stored at high temperatures and humidities (Kritzinger *et al*., 2003). The seed are susceptible to a variety of fungal pathogens such as *Fusarium verticillioides* (Sacc.) Nirenberg (Hedge & Hiremath, 1987; Kritzinger *et al*., 2003; Zaidi, 2012), *Fusarium proliferatum* (Matsush.) Nirenberg (Kritzinger *et al*., 2003), *Fusarium oxysporum* Schltdl. (Kritzinger *et al*., 2003) and *Aspergillus flavus* Link (Adekunle & Bassir, 1973). It is well-known that these fungi produce mycotoxins under suboptimal storage conditions (Richard, 2007).

Fumonisins are a major class of mycotoxins produced by *Fusarium* spp. but especially by *F. verticillioides* and *F. proliferatum* (Rheeder *et al*., 2002; Richard, 2007). Fumonisins are known to be phytotoxic to a wide range of agriculturally important crops as well as weeds, which include cowpea (Kritzinger *et al*., 2003), sunflower (*Helianthus annuus* L.), tomato (*Solanum lycopersicum* L.), soya bean (*Glycine max* (L.) Merr.), jimson weed (*Datura stramonium* L.), creeping cucumber (*Melothria pendula* L.), prickly sida (*Sida spinose* L.), spurred anoda (*Anoda cristata* (L.) Schltdl.) (Abbas & Boyette, 1992) and duckweed (*Lemna aequinoctialis* Welw.) (Abbas *et al*., 1998). The phytotoxic effects of FB_1 in plants include chlorosis, necrosis, wilting, reduced root growth leading to the death of the plant (Abbas & Boyette, 1992; Abbas *et al*., 1995; Williams *et al*., 2007). Maize (*Zea mays* L.) seeds that were imbibed in solutions containing FB_1 toxin, showed signs of stunted growth, which included reduction of the seedlings root mass (Williams *et al*., 2007). Moreover, Doehlert *et al*., (1994) reported that FB_1 inhibited maize seed radicle elongation by up to 75% after 48 hours post imbibition at various concentrations of the toxin.

In 2006, Kritzinger *et al*., investigated the phytotoxic effect of FB_1 on cowpea seed. The authors reported that cowpea seed germination decreased with an increase in FB_1 concentration and the toxin caused stunted growth of cowpea seedlings. The authors also studied the ultrastructure of the embryonic axis and cotyledon tissue of untreated and FB_1 treated tissue using transmission electron microscopy (TEM). They reported that the plasma membrane separated from the cell wall, irregular size vacuoles formed and an abundance of lipid bodies was observed next to the cell wall. Thus, FB_1 has shown to have serious deleterious effects on the germination and growth of cowpea seeds and seedlings. This is problematic to subsistence farmers as it could influence not only the growth of their crops, but also their livelihood since they use the seed as well as the rest of the plant for human and animal consumption.

In the study by Kritzinger¸*et al*., (2006), the phytotoxic effect of FB_1 was investigated by using the between paper method but further work was found to be necessary to assess the effects under more natural controlled conditions, such as in a phytotron or greenhouse. The between paper method focuses largely on seed germination and not seedling emergence and growth. In a phytotron environment, abiotic factors such as light, humidity and temperature can be kept constant and thus the effects of the toxin and/or fungus can be assessed more thoroughly. Kafua, (2011), carried out a preliminary study to investigate the effect of FB_1 on cowpea emergence under greenhouse conditions. A reduction of emergence was noted in seeds treated with FB_1 at concentrations of 25, 50 and 100 mg/L. Greenhouse studies of the effect of FB_1 on plant growth have been done on other plants such as maize, tomato, sunflower (*Helianthus annuus* L.) and jimson weed (Abbas & Boyette, 1992; Abbas *et al*., 1994). However, these studies focussed rather on the effects due to the application of the toxin on above ground parts and not on seed germination and seedling establishment.

The main aim of this chapter was to investigate the phytotoxic effects of both pure FB_1 and FB_1-producing *F. verticillioides* strains on cowpea plants under phytotron conditions. The parameters that were analysed in this study included seed emergence, root and shoot lengths and dry mass of seedlings. The effect on total chlorophyll content was also examined, since it has been previously reported that FB_1 causes chlorosis, which leads to a reduction in photosynthetic rates as a result of reduced chlorophyll in the leaves (Abbas & Boyette, 1992; Abbas *et al.*, 1995). The seedling vigour of cowpea seedlings was also determined as it constitutes the sum total of the properties which determine the level of activity and seed

performance during germination and seedling emergence (ISTA, 2005). The results of this study will provide further valuable insight into the phytotoxic effects caused by FB_1 on cowpea, and it will assist in providing a better understanding of how the seedlings respond to the toxin.

3.2. Materials and Methods

3.2.1. Materials

All materials and reagents were purchased from Sigma-Aldrich (Johannesburg, South Africa) unless otherwise stated.

3.2.2. Seed material

Cowpea seeds (cultivar PAN 311) were obtained from Mr C. Mathews of the Lowveld College of Agriculture (Marapyane Campus, Department of Agriculture, Mpumalanga, South Africa). The seeds showed no visible signs of disease or insect infestation/damage. The seeds were stored in a brown paper bag at 4°C in a cold room in the laboratories provided by the Department of Plant Science, University of Pretoria until further use.

3.2.3. Fumonisin B_1 toxin

Dried FB_1 (Batch A/13, 10.53 mg) was obtained from the PROMEC unit, Medical Research Council (MRC), Tygerberg, South Africa. Methanol (20 ml) was added to the FB_1 and 1 ml was aliquoted into each of 20 vials. The methanol was dried down under nitrogen gas and the vials were stored at ± 4°C until used.

3.2.4. Fungal cultures

The cultures of three known FB_1-producing strains of *F. verticillioides* (MRC 8265, 8271, 8272) were obtained from the PROMEC unit of the MRC. The cultures were re-suspended in sterile distilled water, plated on potato dextrose agar (PDA) plates and incubated at 25°C in an incubator. They were sub-cultured fortnightly on fresh PDA plates. For long term preservation, the fungi were either cultured in McCartney bottles containing PDA or small

pieces of PDA containing the fungi were stored in McCartney bottles containing distilled water. The bottles were then stored at ±4°C.

3.2.5. Seed treatment

Prior to seed treatment with FB_1, the seeds were surface disinfected with 1% sodium hypochlorite solution after which they were rinsed thrice with sterile distilled water (sdH_2O). Four replicates of 25 seeds per replicate were used per treatment. In total 100 seeds were thus used per treatment. The required amount of FB_1 was added to 50 ml sterile distilled water to yield final concentrations of 5, 20 and 40 mg/L. In previous studies, Kafua, (2011) used FB_1 concentrations of 10, 25, 50 and 100 mg/L to evaluate the effect of FB_1 on cowpea seed germination, whereas Kritzinger *et al*., (2006) used concentrations of 20, 40, 60, 80 and 100 mg/L. Williams *et al*., (2007) did a fumonisin watering assay on maize seedlings using FB_1 concentrations of 1, 5, 10 and 20 mg/L. Lower concentrations were used as we wanted seeds to germinate so as to evaluate the effect of the toxin on emergence and seedling growth. It is known that concentrations of above 50 mg/L FB_1 resulted in almost complete inhibition of germination. Seeds were imbibed for a period of 10 hours in the respective FB_1 solutions. Sterile distilled water (50 ml) was added to 14 day old cultures of the *F. verticillioides* strains. The surface of each culture was scraped with a pipette tip to free the conidia, after which the conidial suspension was poured through a muslin cloth into beakers. The conidial suspensions were adjusted to a concentration of $1 \text{ X } 10^6$ conidia/ml using a haemocytometer. Similarly, seeds were imbibed in the conidial suspension (50 ml) for four hours. The seeds were removed after the allocated time, placed on sterile paper towels in a laminar flow cabinet and allowed to dry. For the imbibed control, seeds were imbibed in 50 ml sterile distilled water for the same period of time as the FB_1 treatments. The non-imbibed control seeds were sown in seedling trays containing autoclaved soil obtained from Hygrotech (Pretoria) at the same time that the other seeds were imbibed. The same number of seeds per replicate was used for both non-imbibed and imbibed controls.

3.2.6. Phytotron trial

After drying, the imbibed seeds were sown in seedlings trays containing autoclaved soil (Hygrotech, Pretoria). A randomised block design utilizing five seedling trays were used in total to accommodate four replicates of eight treatments as illustrated in Appendix, Figure

7.1. The seedling trays were placed in a phytotron room (Department of Plant Sciences, University of Pretoria) and the seedlings were grown for 21 days at 25° C ±1° C with a 16/8 hour light/dark cycle. The seedlings were watered daily in order to keep the soil moist. The percentage emergence was determined after seven and 21 days post imbibition (dpi). The seedlings were harvested after 21 dpi and the excess soil was washed off. The roots and shoots of the seedlings were separated whereafter the root and shoot lengths and dry mass were determined. Plant samples were dried overnight at 65° C using an air drier oven (Vivid Air, Pretoria). The seedling vigour indices (SVI) of all eight treatments were calculated according to the equations determined by Abdul-Baki & Anderson, (1973):

SVI 1 = % emergence X seedling length

SVI 2 = % emergence X seedling dry mass

The trial was repeated twice.

3.2.7. Determination of total chlorophyll content of cowpea seedlings

Seedlings from the different treatments and controls that were grown under the same conditions as described in 3.2.5 were used for this experiment. After 14 and 21 dpi, leaves were removed from the plants and weighed to an amount of between 0.5 – 0.6 g. The leaf samples were quickly cut into very small pieces using scissors and added to a mortar. The leaf tissue was sprinkled with a small amount of $MgCO_3$, to prevent degradation of chlorophyll into the degradation product pheophytin, where after 10 ml of ice cold 80% acetone was added. The tissue was then ground to a fine pulp with a mortar and pestle. The homogenate was filtered through a Whatman No. 1 filter paper using a Buchner filter and vacuum flask system. The filter paper was rinsed thrice with small volumes of 80% acetone to remove all the extract from the filter paper. The chlorophyll extract was transferred to a measuring cylinder and 80% acetone was added to yield a final volume of 40 ml. The extracts were kept cold and dark. Spectrophotometric readings were made using a Beckman DU 720 General Purpose UV/Vis Spectrophotometer (Brea, USA). Cuvettes with diameter of 10 mm were used. Readings were taken at 645 and 663 nm with 80% acetone used as the blank. Total chlorophyll was calculated using the following equation (Arnon, 1949):

$$TC = \frac{Q\ X\ V}{1000\ ml\ X\ W}$$

Where:

TC = mg total chlorophyll/g tissue

Q = 20.2 A_{645} + 8.02 A_{663}

V = final volume of extract in 80% acetone

W = fresh mass of tissue extracted

The chlorophyll extractions were repeated twice.

3.2.8. Statistical analysis

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

3.3. Results and Discussion

3.3.1. The effect of FB_1 on seedling emergence and growth

Cowpea seed emergence was reduced significantly after seven and 21 dpi for all three FB_1 concentrations as well as for one *F. verticillioides* strain (MRC 8265) when compared to the non-imbibed and imbibed controls (Figure 3.1). No significant differences were observed between the non-imbibed and imbibed control (Figure 3.1). For each treatment there were no major differences in the emergence rates after seven and 21 dpi except for seeds imbibed in 40 mg/L FB_1. The emergence rate in the MRC 8265 seeds remained the same after seven and 21 dpi.

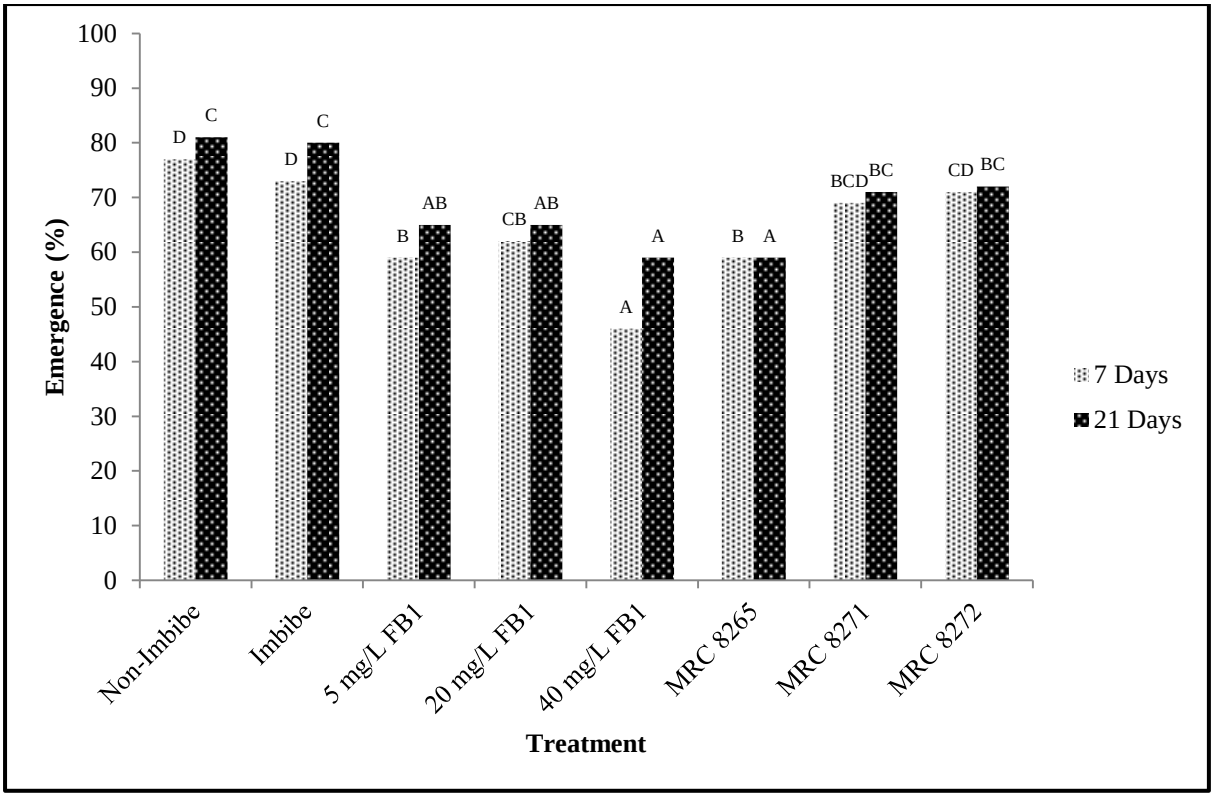


Figure 3.1. The effect of FB_1 and *Fusarium* spp. on cowpea seed emergence after seven and 21 dpi. Values of the bars of the same shade, not designated with the same letter are significantly different (P=0.05) according to the student's *t* test.

Seeds imbibed in different FB_1 concentrations had similar emergence rates after 7 dpi except for the seeds imbibed in 40 mg/L FB_1. Seeds imbibed in 40 mg/L FB1 showed the lowest emergence rates of 46% and 59% after seven and 21 dpi, respectively. However, at 21 dpi the emergence of seeds imbibed in 40 mg/L FB_1 was not significantly different from seeds imbibed in 5 and 20 mg/L FB_1. Seeds that were inoculated with the conidia of MRC 8271 and MRC 8272 *Fusarium* strains, had similar emergence rates (Figure 3.1). Seeds imbibed in 40 mg/L FB_1 and inoculated with the conidia of the *Fusarium* strain MRC 8265, showed similar emergence rates and they were significantly the same after 21 dpi. MRC 8265 had the greatest phytotoxic effect on seed emergence of the three fungi at 21 dpi.

Kritzinger *et al*., reported in 2006 that cowpea seeds imbibed in various concentrations of FB_1 (10, 25, 50 and 100 mg/L) showed a decrease in germination rate with an increase in FB_1 concentration. There was almost complete inhibition of seed germination, when the seeds were imbibed in 100 mg/L FB_1 solution. There is a difference in emergence/germination rate when comparing the results of this study with that of Kritzinger *et al*., (2006) especially when

comparing seeds imbibed in the FB_1 solutions. When seeds imbibed in 20 and 40 mg/L FB_1 of this study were compared, respectively, with seeds imbibed in 25 and 50 mg/L FB_1 of Kritzinger *et al*., (2006), which are almost similar concentrations of FB_1, a major difference can be seen in emergence/germination rates. Seeds imbibed in 25 and 50 mg/L FB_1 had germination rates of almost 35 and 20%, respectively. Emergence rate for seeds treated with 20 and 40 mg/L FB_1 was 65 and 59%, respectively (Figure 3.1). There is also a large difference in cowpea seed emergence percentage when the seeds imbibed in 20 mg/L FB_1 of this study were compared to the preliminary greenhouse study of Kafua, (2011) where the seeds were imbibed in 25 mg/L FB_1. Kafua, (2011) had almost zero percentage emergence whereas this study had 65% emergence. It is interesting to note that the seeds imbibed in 25 mg/L FB_1 had lower germination rates than the seeds imbibed in the 40 mg/L FB_1 used in this study. Different emergence rates could possibly be attributed to the difference in the seed coat colour. Aveling & Powell, (2005) stated that resistance against fungal pathogens in pigmented cowpea seed could be attributed to the presence of compounds such as tannins, phenols and lignins.

Generally, in this study, the emergence rates were greater than the germination rates of Kritzinger *et al*., (2006). This could possibly be due to the fact that Kritzinger *et al*., (2006), used paper towels to germinate the seed whereas in this study seedling trays with soil were used. The paper towels could have had deleterious effects on the cowpea seed due to the confined humid space they were germinated in, compared to the seedling tray method which contains soil with high aeration levels. It seems that using a seedling tray is favoured when seed emergence and seedling establishment is studied and when only seed germination is studied the between paper method or even a sand tray is preferred. This study confirmed the findings of Kritzinger *et al*., (2006) that FB_1 has a negative effect on cowpea seed germination as well as the *in vivo* and *in vitro* experiments performed by Kafua, (2011). Zonno & Vurro, (1999) tested the phytotoxic effect of 14 fungal toxins on the emergence of the weed *Striga hermonthica* (Delile) Benth.. The authors reported that FB_1 inhibited 50% of *Striga* seed emergence, when the seeds were treated with 10^{-4}M FB_1 solution. Danielsen & Jensen, (1998) found a significantly negative correlation between fumonisin content and seed emergence, but they could not determine whether fumonisins affected seed emergence. Doehlert *et al*., (1994) and Williams *et al*., (2007) reported that FB_1 did not have any effect on maize seed emergence, but that it did inhibit radicle elongation.

Doehlert *et al*., (1994) also studied the effect of FB_1 on amylase production in maize seedlings. They reported that that radicle and low pI amylase secretions were inhibited by the presence of FB_1 up to 100 ppm. Other amylases were not affected to the same extent by FB_1 as the low pI class amylases. The low pI amylases are effective in hydrolysing granular starch which plays a key part during maize seed germination (Knutson, 1993). Gutierrez-Najera *et al*., (2005) studied the action of FB_1 on the plasma membrane H^+-ATPase in maize embryos. H^+-ATPase was not only inhibited by the toxin but also by its structural analogs the sphingoid intermediates such sphinganine under *in vivo* conditions. The inhibition of the H^+-ATPase is detrimental to the growth of the plant since ATP is an important energy molecule. The reduced emergence rates observed for seeds treated with the toxin could be possible due to a lack of the energy molecule ATP, since ATP is needed for everyday processes in the cells of the plants.

Kritzinger *et al*., (2006) also studied the effect of seed inoculated with the conidia from *F. verticillioides*, *Fusarium nygamai* L.W. Burgess & Trimboli and *F. proliferatum* on cowpea seed germination. The emergence of seeds that were inoculated with the conidia of *F. verticillioides* and *F. proliferatum* was significantly reduced when compared with the controls. These results correspond well with the seeds inoculated with MRC 8265 but not with MRC 8271 and 8272, which were used in this study. Zaidi, (2012) reported that cowpea seeds inoculated with the conidia of both *Fusarium equiseti* (Corda) Sacc. and *Fusarium. moniliforme* J. Sheld. (*F. verticillioides*) had emergence rates of 30% and 42%, respectively. Desjardins *et al*., (1994) performed a fumonisin and virulence study of *F. verticillioides* on maize seedlings. They reported that the high virulence of the fungi was associated with the production of fumonisin. Strains of *F. verticillioides* have the ability to produce between between 60–6400 ppm FB_1. Percentage emergence range of maize seed inculated with the conidia of *F. verticillioides* strains range between 17 to 70 %. Zaidi, (2012) reported that the reduced emergence of seeds inoculated with the fungal conidia could be due to the fungi having the ability to produce secondary metabolites that could have a deleterious effect on the emergence of the seed. This study supports findings by Desjardins *et al*., (1994) and Zaidi, (2012) that some *Fusarium* species do have deleterious effects on seed emergence.

In this study the majority of seedlings from all the treatments were well developed except for seedlings that were imbibed in 20 and 40 mg/L FB_1 (Figure 3.2). A well-developed seedling consisted of a well-developed root system having a tap root with numerous root

hairs and a shoot with leaves (A, Figure 3.2). Numerous other studies indicated that the phytotoxic effects of FB_1 include effects such as necrosis, leaf curl, abnormal leaf development, growth inhibition, reduced root development and stunted growth (Abbas & Boyette, 1992; Abbas *et al*., 1995; Williams *et al*., 2007). The only developmental effects observed in this study were reduced root development and stunted growth. Stunted or delayed growth (D and E, Figure 3.2) was observed in some of the seedlings that were treated with 20 mg/L and 40 mg/L FB_1 when compared to the two controls and the rest of the treatments (A–C and F–H, Figure 3.2). A possible explanation for stunted/delayed growth could be that there was not sufficient uptake of nutrients due to the lack of root hairs. Trials with longer growth periods would be needed to establish if stunted or delayed growth is indeed consistent. No deformed seedlings were observed, but seedlings with underdeveloped root systems were observed (Figure 3.3). Kritzinger *et al*., (2006) indicated that in several cases FB_1 caused severe stunting of the roots. Williams *et al*., (2007) stated that maize seeds grown in soil and watered with solutions of FB_1, had stunted growth, reduced root development and leaf lesions. Zaidi, (2012) reported that cowpea seeds inoculated with the conidia of *F. equiseti* and *F. moniliforme* seedlings had negative symptoms, which included stunted growth. No malformed seedlings were observed in this study, which could be due to the seeds being germinated in seedling trays. No necrosis, chlorosis, leaf curl, abnormal leaf development or spontaneous death of seedlings occurred in this study.

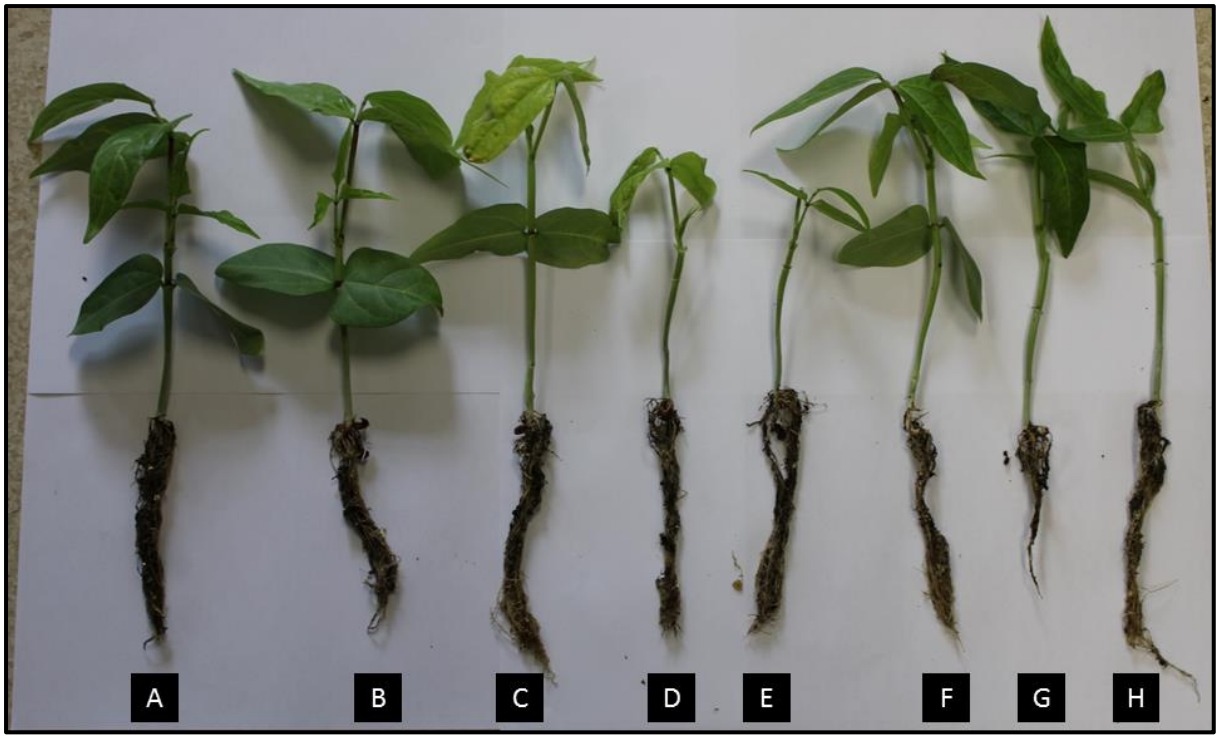


Figure 3.2. The effect of FB_1 and *Fusarium* spp. on the growth of cowpea seedlings. A- Non-imbibed control; B- Imbibed control; C- 5 mg/L FB_1; D- 20 mg/L FB_1; E- 40 mg/L FB_1; F- MRC 8265; G- MRC 8271; H- MRC 8272. (Photo: R.G Kotze).

Figure 3.3. A- An example of an underdeveloped root system of a cowpea seedling that was treated in 40 mg/L FB_1; B- A well-developed cowpea root system. (Photo: R.G Kotze).

Generally, no significant reduction in root/shoot length was seen across all treatments and the controls (Figure 3.4). However, in terms of shoot length, seedlings whose seeds were imbibed in the 40 mg/L FB_1 solution was significantly different from the rest except for seeds imbibed in 20 mg/L FB_1 and and seeds treated with MRC 8271. The majority of the treatments had root and shoot lengths of approximately 12 and 16 cm, respectively. The seedlings that were treated with 40 mg/L FB_1 had the shortest root and shoot lengths of 10.75 cm and 12.1 cm, respectively. This was expected of the seeds that were imbibed in the highest concentration of FB_1. It was anticipated that the controls would have had the longest root and shoot lengths but this was not the case. The seedlings whose seeds were imbibed in the MRC 8265 spore solution had the longest root and shoot lengths but was not significantly longer than the controls. Both this study and the study done by Kritzinger *et al*., (2006), showed similar trends in terms of the root and shoot lengths of the seedlings. In both studies the root and shoot lengths of the seedlings were almost the same for all treatments including the controls. The only exception was for seedlings treated with high concentrations of FB_1. In this study seedlings treated with 40 mg/L FB_1 were significantly shorter compared to 5 mg/L FB_1 and this was also the same for the study by Kritzinger *et al*., (2006) where seedlings treated with 50 and 100 mg/L FB_1 were significantly shorter than those of the of the other lower concentration treatments. Thus high concentrations of FB_1 do have an effect on the root and shoot lengths of cowpea seedlings.

Williams *et al*., (2007) also observed that with an increase in FB_1 concentration, the plant height of maize seedlings decreased. Doehlert *et al.*, (1994) reported that although FB_1 did not have any effect on maize seed germination, it inhibited radicle elongation by up to 75% after 48 hours. In the study maize seedlings were watered with different concentrations of FB_1 (1.4, 6.9, 13.9, 27.7 nmol/ml FB_1). They also indicated that maize seeds inoculated with *F. verticillioides* conidia had reduced seedling length. The authors further reported that they germinated maize seed on Murashige and Skoog medium supplemented with 13.9 nmol/ml FB_1 to better assess the roots visually since transparent containers were used. Reduced root development as well as reduced shoot developmental was observed in maize seedlings grown in culture. Lamprecht *et al*., (1994), reported that FB_1, FB_2 and FB_3 caused dose-dependent reductions in root and shoot lengths in maize and tomato seedlings with FB_1 being the most potent of the three toxins. Zaidi, (2012) reported that cowpea seedlings inoculated with the spore suspension of *F. equiseti* and *F. moniliforme* (*F. verticillioides*) had reduced root and shoot lengths when compared to the controls. This indicates that fungal pathogens can

negatively influence the length of cowpea seedlings, which is in contradiction with what was observed in this study. It could be that the fungal strains used by Zaidi, (2012) were more pathogenic than the strains used in this study and that the fungi of this study had lost their pathogenicity.

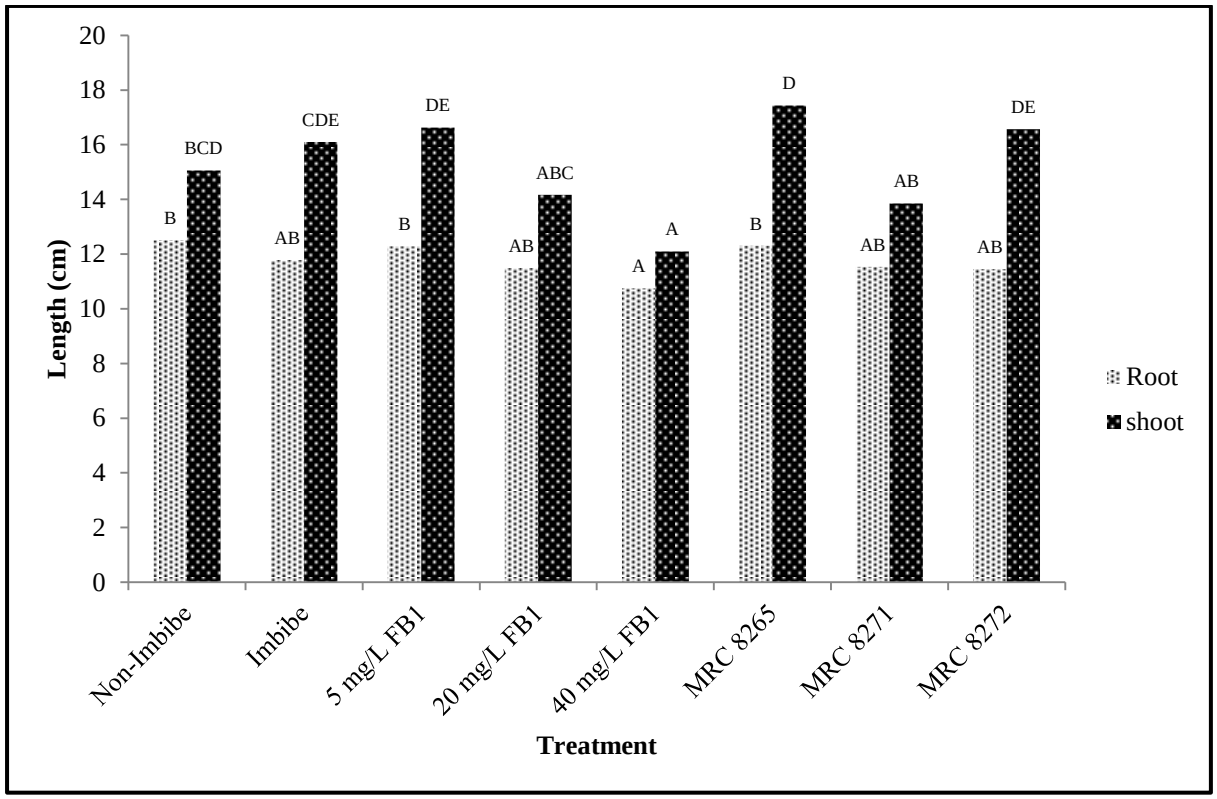


Figure 3.4. Root and shoot length of FB_1 and *Fusarium* spp. treated cowpea seeds after growing 21 dpi in a phytotron room. Values of the bars of the same shade, not designated with the same letter are significantly different (P=0.05) according to the student's t test.

After 21 dpi the root and shoot dry masses of the seedlings from each treatment, including both controls, were determined. The root and shoot masses ranged from 2.8–9.5 g and 2.7 to 11.8 g, respectively (Figure 3.5). It was observed, in all three FB_1 treatments as well as the both controls, that the shoot mass was higher than the root mass. The non-imbibed control had the highest root and shoot masses whereas MRC 8271 treatment had the lowest root and shoot masses. The two controls were significantly different from each other (Figure 3.5). This could possibly be due to the imbibed seeds having a greater electrolyte leakage than non-imbibed seeds indicating that the imbibed seeds had an initial setback in growing properly compared to the seeds that were not imbibed (Hampton, 1995). Root and shoot masses

decreased with an increase in FB_1 concentration indicating that FB_1 has an influence on the growth of the seedling.

Weighing plants is a useful measurement to determine if FB_1 has an effect on plant growth as some of the mentioned effects of FB_1 include reduced root development and stunted growth. Measuring the mass of a plant is important for plant growth and biology (Golzarian *et al*., 2011). It is interesting that FB_1 did not have such a profound influence on the root and shoot lengths of the seedlings, as it had on the root and shoot masses in this study. As earlier stated, Williams *et al*., (2007) did a watering assay on maize seedlings using different concentrations of FB_1. They reported that root mass of maize seedlings decreased with an increase in FB_1 concentration. Lambrecht *et al*., (1993) reported that FB_1, FB_2 and FB_3 caused a reduction in the mass of maize and tomato seedlings. Williams *et al*., (2007) tested the effect of the fumonisin producing *F. verticillioides* strain (MRC 826) on maize seedlings grown in different types of soil. The study indicated that there were significant reductions in both root and shoot masses. The authors suggested that FB_1 and its effects on sphingolipids could contribute to *F. verticillioides* disease in maize seedlings. This corresponds well to the *F. verticillioides* strains used in this study.

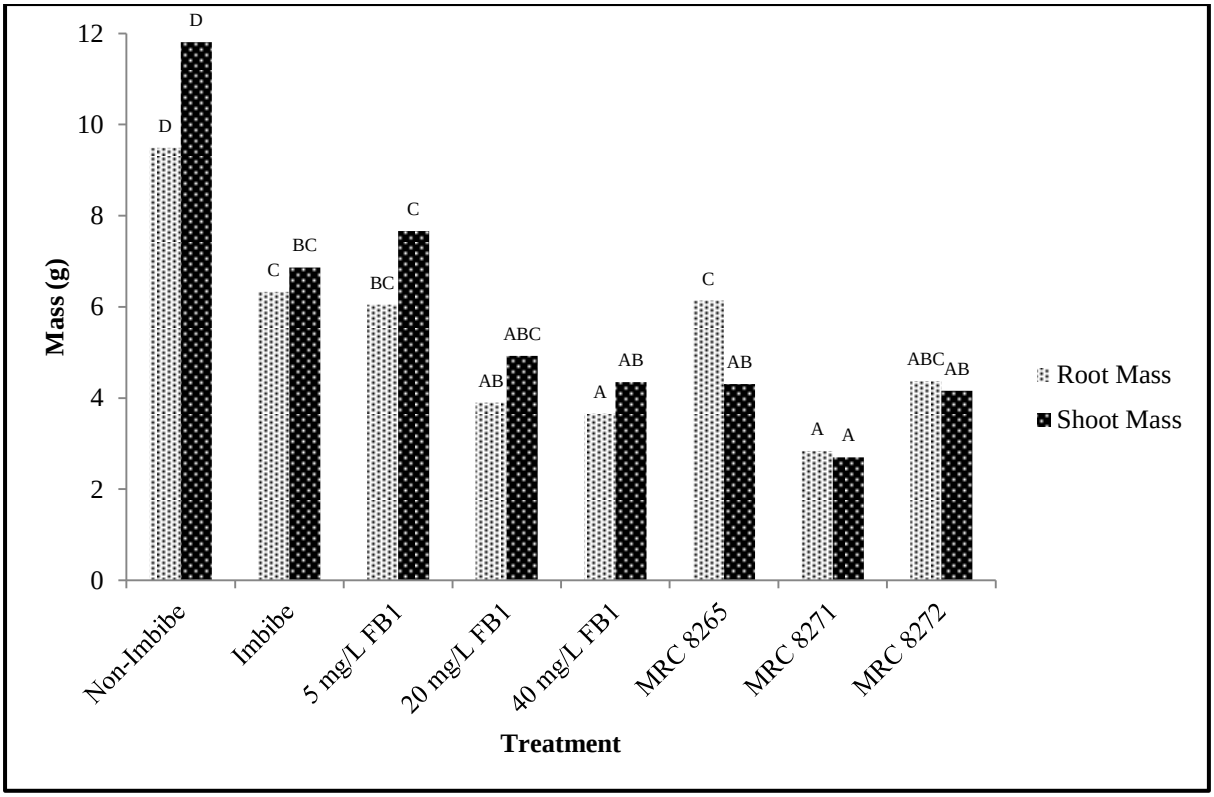


Figure 3.5. Root and shoot dry mass of FB_1 and *Fusarium* spp. treated cowpea seeds after growing for 21 dpi in a phytotron room. Values of the bars of the same shade, not designated with the same letter are significantly different (P=0.05) according to the student's t test.

The seedling vigour index (SVI) as described by Abdul-Baki & Anderson, (1973) is a useful tool to compare more than one factor such as both emergence and seedling lengths (SVI 1) or both emergence and seedling dry mass (SVI 2). While these factors on their own give valuable insight on how the seedling reacts to the toxin or the fungal pathogens they do not give a global perspective on how the seedling as a whole reacts to the treatments.

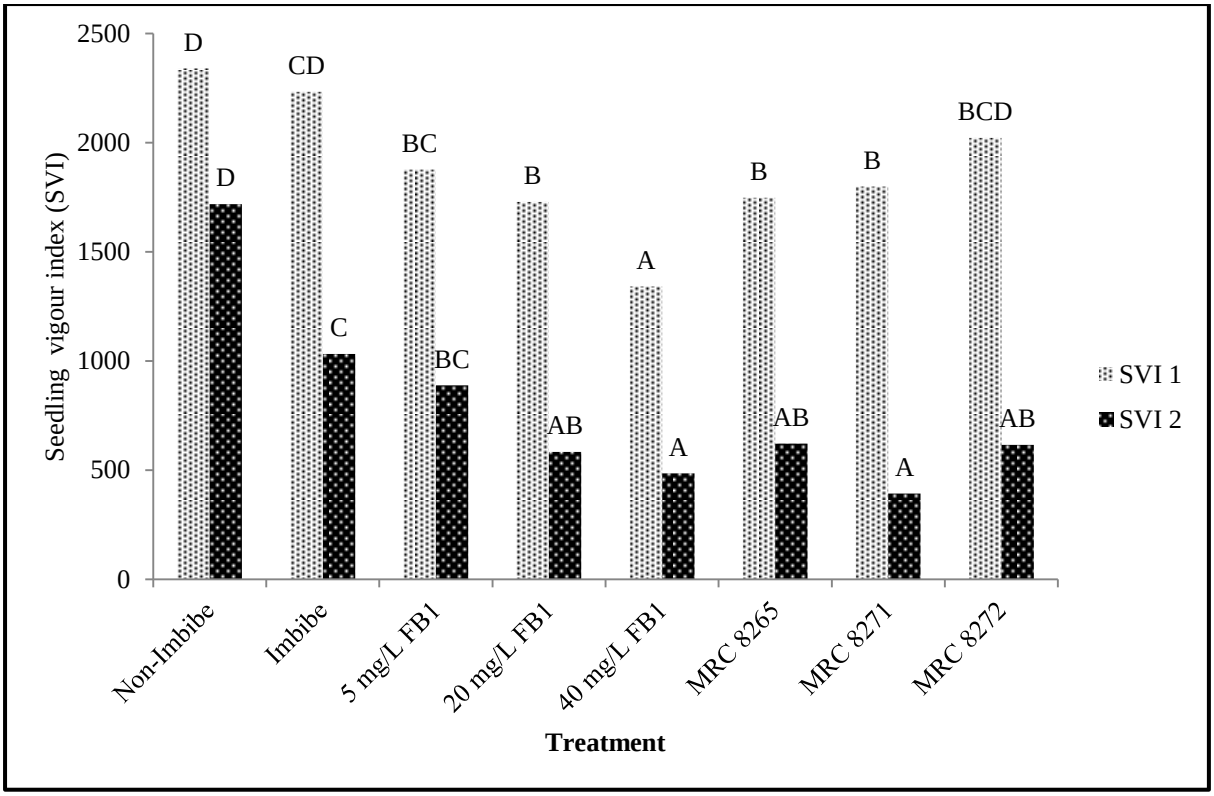


Figure 3.6. The seedling vigour indexes (SVI) of cowpea seedlings. Seedling vigour index 1 (SVI 1) = % emergence X seedling length; SVI 2 = % emergence X seedling mass. Values of the bars of the same shade, not designated with the same letter are significantly different (*P*=0.05) according to the student's *t* test.

There is a clear difference across all treatments and controls between SVI 1 and SVI 2, indicating there is a difference in using either length or mass of the seedlings in determining the SVI (Figure 3.6). Thus it justifies using both indices to determine the vigour of the plant. Both the non-imbibed control and imbibed control were significantly the same for SVI 1 but were not for SVI 2. The non-imbibed control had a SVI of 2340 and 1718 for SVI 1 and SVI 2, respectively, and the imbibed control 2233 and 1032, respectively. As earlier mentioned, the imbibition of the imbibed control seeds could possibly negatively influence the growth due to electrolyte leakage. Seedlings whose seeds were imbibed in 40 mg/L FB_1 solution were significantly different to both controls with a SVI of 1341 and 485 for SVI 1 and SVI 2, respectively (Figure 3.6). The SVI for both SVI 1 and SVI 2 of seeds imbibed in FB_1 solutions decreased with an increase in the concentration of FB_1 (Figure 3.6). In terms of both indices FB_1 does negatively influence the growth of cowpea seedlings. With seedlings inoculated with the conidia of MRC 8265 and 8271, SVI for both indices were lower and significantly different from both controls. When parameters such as emergence and seedling

lengths are compared on their own, it seems that the fungal strains did not have an effect on the seedlings but in terms of the SVI they did in fact have an effect on the wellbeing of the seedlings. Anjorin *et al*., (2008) tested the effect *F. verticillioides* mycelium and a metabolite extract made from the fungus had on SVI 1 for maize seedlings. They reported that maize seeds treated with the mycelium of *F. verticillioides* had the third lowest SVI 1 value whereas seeds treated with both the mycelium and metabolite extract had the lowest SVI 1 value when compared to the control. This study follows a similar trend in terms of cowpea seeds inoculated with the conidia of FB_1 producing *F. verticillioides* strains in that the SVI 1 value was lower and significantly different from both the controls except for MRC 8272. Thus the fungal pathogens, the secondary metabolites they produced, or both, do influence the growth and vigour of cowpea seedlings.

3.3.2. The effect of FB_1 on total chlorophyll content of cowpea seedlings

Chlorophyll extractions from leaves were done after 14 and 21 dpi for all treatments including both controls. A major difference could be observed in the total chlorophyll content between the two time points across all eight treatments (Figure 3.7). The total chlorophyll content was lower for all eight treatments after 21 dpi when compared to 14 dpi. A possible explanation for the difference between the two time points for all eight treatments could be that the cotyledons were used for the extractions done 14 dpi while the first true leaves were used for the extractions done 21 dpi. Wilson and Cooper, (1969), reported that chlorophyll content was greater in thin leaves with smaller mesophyll cells than in thicker leaves in *Lolium* spp.. They also stated that photosynthetic rates were higher in unfolding leaves due to the still small expanding mesophyll cells. Chlorophyll content followed the same trend. In this study the leaves were still expanding at 14 dpi and were fully developed at 21 dpi. It is thus possible that the mesophyll cells were still expanding in the leaves when the first round of extractions was made.

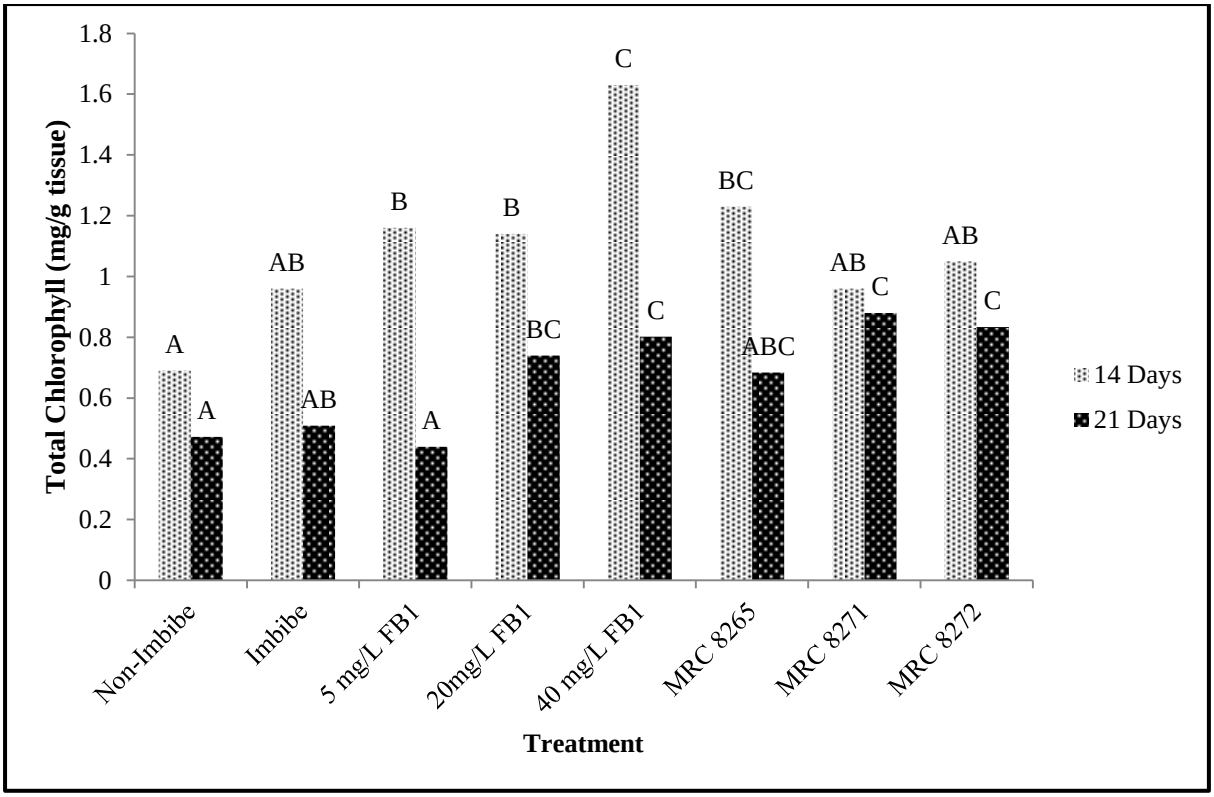


Figure 3.7. The total chlorophyll content of FB_1 and *Fusarium* spp. treated cowpea seedlings after 14 and 21 dpi . Values of the bars of the same shade not designated with the same letter are significantly different (P=0.05) according to the student's t test.

When comparing the different treatments with one another the non-imbibed control had the lowest total chlorophyll content and 40 mg/L FB_1 treatment had the highest chlorophyll content (Figure 3.7). The leaves from plants of the three FB_1 seed treatments had higher total chlorophyll contents compared to the two controls. The 40 mg/L treatment was significantly different from the rest of the treatments except MRC 8265 at 14 dpi. Total chlorophyll content from the three *F. verticillioides* strains treatments was also greater than both the controls but not significantly different. This is rather unexpected as it would have been expected that the chlorophyll levels would have been less than in the controls since numerous studies reported that FB_1 causes chlorosis in plants. This could possibly be that the plants are photosynthesising more due to the stressed conditions they are experiencing and that the cotyledons are more active since they are responsible for all primary growth.

Nadubinska *et al*., (2003) tested the effect of various mycotoxins produced by *Fusarium* spp. such as FB_1 and moniliformin on the chlorophyll content of maize seedlings. The roots of 14 day old maize seedlings were submerged for 72 hours in different mycotoxin solutions

containing 30 mg/L of the particular mycotoxin. The effect of each mycotoxin on the chlorophyll a and b content of the leaves of the maize seedlings was determined after the 72 hour treatment. The authors reported that chlorophyll a and b content of FB_1 treated maize seedlings were 15.9% and 17.8% higher, respectively, than that of the control which consisted of distilled water. The same trend was seen with seedlings treated with the toxin moniliformin where chlorophyll a and b levels were 21% and 18.9% higher respectively than that of the control. The maize study correlates well with the results obtained in this study since the authors also found an increase in chlorophyll levels in FB_1 treated maize seedlings. The results of this study and that of Nadubinska *et al*., (2003) suggest that the FB_1 toxin and the fungi could possibly stimulate the plant to produce more chlorophyll. In response to the FB_1 toxin and the fungal pathogens, the plant could possibly produce more chlorophyll in an attempt to overcome deleterious effects of the toxin and fungal pathogens. Nadubinska *et al*., (2003) justified their results by reporting that the FB_1 concentration they used was possibly too low or that the exposure time was too short to cause an deleterious effect and that the plant was able to suppress the effects of the toxin by its defence mechanisms.

Numerous studies report that FB_1 caused chlorosis in plants treated with the toxin (Abbas & Boyette, 1992; Abbas *et al*., 1995). Adekunle & Bassir, (1973) reported that two other mycotoxins, the well-known aflatoxin and palmotoxins, inhibited chlorophyll formation. Zearalenone, deoxynivalenol and fusaproleferin had a negative effect on the chlorophyll levels of maize seedlings (Nadubinska *et al*., 2003). The results found in this study on cowpea are rather contradictory as the previous studies reported that mycotoxins either inhibited chlorophyll formation or caused chlorosis (Abbas & Boyette, 1992; Abbas *et al*., 1995; Adekunkle & Bassir, 1973). In this study on cowpea the toxin increased chlorophyll production, and no chlorotic spots were observed. It could be that the concentration of the toxin used in this study was not sufficiently high to have an effect on chlorophyll production or to cause chlorosis. What is evident from this study is that FB_1 does have a positive effect on chlorophyll formation at low concentrations.

3.4. Conclusion

In this study it was confirmed that fumonisin B_1 is phytotoxic to cowpea seed and seedlings under phytotron room conditions. The toxin as well as the FB_1-producing *Fusarium* spp. significantly influenced the emergence of the seed and seedling establishment as it was

evident from both seedling vigour indices. The toxin treated seedlings also had higher total chlorophyll levels than the untreated samples which is odd since numerous studies reported that FB_1 caused chlorosis. The sole limitation of the study was that the concentration level of toxin that is produced by the fungi was unknown. Aspects that should be considered in future studies include determining the concentration levels of the toxin of the FB_1 producers prior to a study, and assessing if mature plants are still infected with the fungus after their seeds were inoculated with the *F.verticillioides* conidia. Field studies are also needed to determine how the plants react to the toxin and FB_1-producing *Fusarium* spp. under natural conditions. Further research is required to provide more insight into the effects of FB_1 on chlorophyll production in toxin treated seedlings

CHAPTER 4

The effect of fumonisin B1 on the ceramide synthase gene expression in cowpea seedlings

Abstract

The sphingolipid pathway is responsible for the formation of sphingolipids and complex sphingolipids which are necessary for everyday processes in plants such as membrane stability, cell signalling and regulation and programmed cell death (PCD. It has been proposed that FB_1 inhibits the enzyme ceramide synthase, which is an important and key enzyme in the sphingolipid pathway. The mode of action of FB_1 was investigated by determining if the toxin had an effect on the expression of the ceramide synthase gene in cowpea. Surface-disinfected seeds were imbibed for 10 h in sterile distilled water amended with FB_1 to yield a concentration of 20 mg/L FB_1. Seed imbibed in sterile distilled water served as the control. Samples were harvested after 0, 3 and 12 dpi with the latter seedlings being split into shoot and root samples. RNA was extracted from untreated and treated samples where after cDNA was synthesised. The ceramide synthase gene was then amplified using primers that were designed to the conserved regions of *Glycine max* and *Phaseolus vulgaris* ceramide synthase gene homologues. Sequence analysis of the cowpea ceramide synthase gene fragment confirmed that the designed primers did amplify the correct gene from cowpea. The expression levels of the gene could thus be tested after FB_1 treatment using semi-quantitative PCR amplification. No significant differences were seen in ceramide synthase gene expression between the control and treated samples at each time point. The only significant difference that was observed was that ceramide synthase gene expression decreased over time. Fumonisin B_1 did not influence the expression levels of the ceramide synthase gene in cowpea and thus exerts an effect at some other level.

Keywords: FB_1, ceramide synthase, sphingolipid pathway, cowpea, gene expression, mechanism of action

4.1. Introduction

Cowpea seed is prone to fungal infestation due to the suboptimal conditions they are often stored in. Numerous fungi have been associated with cowpea seed especially *Fusarium* species. *Fusarium* species that have been detected on cowpea seed include *Fusarium oxysporum* Schltdl., *Fusarium proliferatum* (Matsush.) Nirenberg ex Gerlach & Nirenberg *Fusarium solani* (Mart.) Sacc. and *Fusarium verticillioides* (Sacc.) Nirenberg (Emechebe & McDonald, 1979; Kritzinger *et al*, 2004; Shama *et al*., 1988). Many of these fungal species have the ability to produce mycotoxins. Kritzinger *et al*., (2003) reported that fumonisin B_1 (FB_1) has been isolated from cowpea seed samples from South African at concentrations of 0.12–0.61 µg/g. Numerous studies as well as results presented in chapter 3, have reported on the phytotoxic effects of FB_1 on plants, but there are few reports on the molecular mechanism of action of the toxin (Abbas *et al*., 1995; Abbas & Boyette, 1992; Krtitzinger *et al*., 2006; Williams *et al*., 2007)

It has been proposed that a possible mode of action of FB_1 is that the toxin inhibits the enzyme ceramide synthase, which is a key enzyme in the sphingolipid pathway in both plants and animals (Abbas *et al*., 1994; Brandwagt *et al*., 2000; Soriano *et al*., 2005; Ternes *et al*., 2011; Williams *et al*., 2007). The sphingolipid pathway produces complex sphingolipids such as glucosylceramides, glycophosphosphingolipids and inositolphosphoceramides, which all contain a ceramide backbone (Lynch, 1999; Sperling & Heinz, 2003). These complex sphingolipids play a role in membrane stability, cell signalling and regulation, phytopathogenesis and programmed cell death (PCD) in plants (Lynch & Dunn, 2003; Sperling & Heinz, 2003). Ceramide synthase is a key enzyme in the formation of complex sphingolipids as it is involved in the synthesis of ceramide, which forms the backbone of all sphingolipids and complex sphingolipids (Pata *et al.*, 2009; Sperling & Heinz, 2009; Ternes *et al*., 2011). Ceramide synthase catalyses the reaction of sphinganine and fatty acyl-CoA into ceramide (Lynch, 1999; Merrill *et al*., 2006; Ternes *et al*., 2011). Fumonisin B_1 is a sphinganine analogue mycotoxin due to its similarity in structure to sphinanine, and it has been proposed that it could inhibit the enzyme ceramide synthase (Abbas *et al*., 1994; Abbas *et al*., 1998; Brandwagt *et al*., 2000; Williams *et al*., 2007). In addition, an accumulation of sphingoid bases such as sphinganine and phytosphingosine has been observed when FB_1 inhibited ceramide synthase (Abbas *et al.*, 1994; Desjardins & Hohn, 1997; Soriano *et al*., 2005; Williams *et al*., 2007)

Ceramide synthase is encoded by the longevity assurance gene or *LAG1* gene family in the majority of eukaryotic organisms (Winter & Ponting, 2002). *Arabidopsis thaliana* (L.) Heynh. contains three ceramide synthase isoforms, which are localised in the endoplasmic reticulum (ER) (Marion *et al*., 2008). The three isoforms are often referred to as LOH1, LOH2 and LOH3 (LAG one homologue) (Markham, 2011). The difference in the isoforms is due to the length of the fatty acyl-CoA they preferentially bind (Ternes *et al*., 2011). It has been suggested by Markham *et al*., (2011) that FB_1 targets LOH1 and LOH3 ceramide synthase isoforms rather than the LOH2 ceramide synthase isoform. The disruption of LOH1 and LOH3 by FB_1 resulted in the accumulation of free sphingoid bases and the excessive production of the specific ceramides that are formed by the LOH2 isoform of ceramide synthase since FB_1 does not influence the LOH2 isoform (Markham *et al*., 2011).

The aim of this chapter was to establish if FB_1 has an effect on the expression of the ceramide synthase gene in cowpea seedlings. This was done by determining if there was a difference in the ceramide synthase transcript levels between untreated and FB_1 treated cowpea embryos/seedlings across three different time points. In addition, sequencing of the cowpea ceramide synthase polymerase chain reaction (PCR) products was performed to confirm the identity of the gene.

4.2. Materials and Methods

4.2.1. Materials

All materials and reagents were purchased from Sigma-Aldrich (Johannesburg, South Africa) unless otherwise stated.

4.2.2. Seed material

Cowpea seeds (Cultivar: PAN311) were obtained from Mr C. Matthews from the Lowveld College of Agriculture (Marapyane Campus, Department of Agriculture, Mpumalanga, South Africa). The seeds had no visible signs of infestation or damage. The seeds were stored in a brown bag at 4°C in a cold room in the laboratories provided by the Department of Plant Science, University of Pretoria until used.

4.2.3. Fumonisin B_1 toxin

Dried FB_1 (Batch A/13, 10.53 mg) was obtained from the PROMEC unit, Medical Research Coucil (MRC), Tygerberg, South Africa. Methanol (20 ml) was added to the FB_1 and 1 ml was aliquoted into 20 vials. The methanol was dried down under nitrogen gas and the vials stored at ± 4°C until use.

4.2.4. Seed treatment

Cowpea seeds were surface disinfected prior to treatment with FB_1, by treating them with a 1% sodium hypochlorite solution for one minute after which they were rinsed thrice with sterile distilled water (sdH_2O). The required amount of FB_1 was added to 50 ml sdH_2O to yield a concentration of 20 mg/L FB_1. Seeds were imbibed in a concentration of 20 mg/L FB_1 for 10 hours, whereafter they were removed, placed on sterile paper towels in a laminar flow cabinet and allowed to dry. The same procedure was followed for the control seeds except that the control seeds were imbibed in 50 ml sdH_2O for the same length of time. Each treatment and control consisted of three biological replicates and each biological replicate comprised three plants each, which were harvested and pooled for downstream analysis. Seeds were sowed in seedling trays containing vermiculite and were incubated at 25° C ±1° C with a 16/8h light/dark cycle, respectively, in an incubator.

Cowpea embryos/seedlings were harvested 0, 3 and 12 days post imbibition (dpi) and immediately placed in liquid nitrogen (flash frozen). Seedlings harvested 12 dpi were split into root and shoot samples prior to flash freezing in liquid nitrogen. All the samples were labelled and stored in conical tubes at -72°C until used. In total 24 samples were obtained, 12 each for the treatment and control respectively (Table 4.1).

Table 4.1. Summary of cowpea samples used in the study. Twelve samples each for both the control and treatment were obtained. Both the control and treatment were done in triplicate. For both the control and treated plants were harvested at three time points: 0 dpi, 3 dpi, 12 dpi. Seedlings harvested on 12 dpi were split into shoots and roots respectively. These samples were labelled 1–24 for ease of identification.

	Sample number	Time harvested	Sample type	Replicate
Control	1	0 dpi	embryo	1
	2	3 dpi	seedling	1
	3	12 dpi	shoots	1
	4	12 dpi	roots	1
	5	0 dpi	embryo	2
	6	3 dpi	seedling	2
	7	12 dpi	shoots	2
	8	12 dpi	roots	2
	9	0 dpi	embryo	3
	10	3 dpi	seedling	3
	11	12 dpi	shoots	3
	12	12 dpi	roots	3
Treatment	13	0 dpi	embryo	1
	14	3 dpi	seedling	1
	15	12 dpi	shoots	1
	16	12 dpi	roots	1
	17	0 dpi	embryo	2
	18	3 dpi	seedling	2
	19	12 dpi	shoots	2
	20	12 dpi	roots	2
	21	0 dpi	embryo	3
	22	3 dpi	seedling	3
	23	12 dpi	shoots	3
	24	12 dpi	roots	3

4.2.5. DNA extraction

Genomic DNA was extracted from one day old cowpea embryonic tissue using the CTAB isolation protocol adapted from Doyle & Doyle, (1987) and Cullings, (1992). Approximately 150 mg of ground frozen plant material was placed in a 1.5 ml centrifuge tube where after 500 µl of CTAB buffer (2 % CTAB (w/v), 0.1 M Tris (pH 8.0), 0.02 M, EDTA (pH 8.0), 1.4 M NaCl, 2 % PVP (polyvinyl pyrolidone, w/v), 0.5 % β-mercaptoethanol (v/v)) was added to the tube. The sample was incubated for 1.5 h and after 30 min, 2 µl of RNase A (Thermo Scientific) was mixed with the solution to degrade the RNA in the sample. Five hundred

microlitres of ice cold chloroform was added to the sample, mixed by inverting the tube, incubated at room temperature for 3 min and then centrifuged for 15 min at 15 000 rpm. The upper layer of lysate was collected and transferred to a new centrifuge tube together with 250 µl of ice cold isopropanol. The tube was mixed by inversion and incubated at -20°C for 1.5 h. The sample was centrifuged for 20 min at 15 000 rpm and the supernatant was discarded. The sample was washed with 150 µl 96 % ethanol and was then centrifuged for 3 min at 15 000 rpm. The supernatant was discarded and the resultant pellet was air dried to remove all the ethanol from the sample. Thereafter the pellet was re-suspended in 50 µl nuclease-free water (Thermo Scientific). The DNA concentration and purity was measured using a Nano Drop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA was analysed by agarose gel electrophoresis (1%, 100 V) using TAE buffer (40 mM Tris base, 20 mM glacial acetate, 2 mM EDTA-Na_2) and was visualised under UV light through the addition of ethidium bromide (10 mg/ml). The samples were stored at -20°C until used.

4.2.6. RNA isolation

RNA isolations were performed using the RNA isolation protocol as described by Chang *et al*., (1993) with modifications. All plant samples (samples 1–24) were individually ground to a fine powder with a mortar pestle using liquid nitrogen to ensure that the plant material remained frozen. Fifteen millilitres of CTAB buffer (2 % CTAB (w/v), 0.1 M Tris (pH 8.0), 0.02 M, EDTA (pH 8.0), 1.4 M NaCl, 2 % PVP (polyvinyl pyrolidone), 0.5 g/L spermadine 0.5 % β-mercaptoethanol) was warmed to 65°C in a 50 ml conical tube and activated by the addition of 300 µl of β-mercaptoethanol. Two to three grams of ground plant material was added to the warmed active CTAB buffer and mixed completely by vortexing the tube. The sample was then incubated for 10 min at 65° C, with shaking every two minutes. Fifteen millilitres of chloroform:isomyl alcohol (24:1) (CIA) was added to the conical tube, mixed well and centrifuged for 10 min at 10 000 rpm at 4°C The supernatant was transferred to a new cooled conical tube. This step was repeated four times. The supernatant was then transferred to a new conical tube, and a quarter volume 10 M lithium chloride (LiCl) added. The tube was mixed by inverting and precipitated overnight at 4° C. The LiCl/RNA solution was centrifuged for 1 hour at 10 000 rpm at 4° C and the supernatant decanted carefully to retain the pellet. The pellet was washed with 1.5 ml ice cold 70 % ethanol, transferred to a 2 ml centrifuge tube and centrifuged for 10 min at 10 000 rpm at 4° C. The supernatant was decanted, with the residual pellet being re-suspended in a small volume of ice cold 70 %

ethanol and centrifuged for 10 min at 10 000 rpm at 4° C. The supernatant was removed, the pellet then air dried and re-suspended in 50 µl nuclease free water. The RNA concentrations of the samples were determined with a NanoDrop 2000 spectrophotometer. The RNA was analysed with agarose gel electrophoresis (1 %, 80 V) and visualised under UV light through the addition of 1 µl ethidium bromide (10 mg/ml).

The RNA samples were deoxyribonuclease (DNase) treated to remove unwanted genomic DNA. DNase digestion was performed using DNase I, RNase-free (Thermo Scientific) according to manufacturer's instructions. A single reaction consisted of 1 µg RNA, 1 µl reaction buffer with $MgCl_2$, 1 U DNase I, RNase-free and DEPC-treated water to obtain a final volume of 10 µl. The samples were then incubated at 37°C for 10 min and the reaction stopped with the addition of 1 µl 50 mM EDTA, whereafter the samples were incubated for a further 10 min at 65°C .The samples were stored at -72° C until used.

4.2.7. cDNA synthesis

Complementary DNA (cDNA) was synthesised from extracted total mRNA samples, free of DNA, and subsequently used in polymerase chain reactions (PCR) to test the expression of ceramide synthase gene levels in cowpea. Complimentary DNA synthesis was performed using the Maxima First Strand cDNA Synthesis kit (Thermo Scientific) and following the manufacturer's instructions. A single reaction consisted of 4 µl 5X reaction mix, 2 µl maxima enzyme mix, 1000 ng RNA and nuclease free water made up to obtain a final volume of 20 µl. This was done for all 24 samples. The samples were then incubated for 10 min at 25° C, 15 min at 50° C with the reaction being terminated by heating for 10 min at 85° C. The samples were stored at -20°C until used.

4.2.8. Primer designing

Primers were designed to the conserved region of *Glycine max* (L.) Merr. (Glyma.16G091200.1) and *Phaseolus vulgaris* L. (Phvul.003G281100.1) ceramide synthase genes using the software CLCBio (Aarhus, Denmark) (Table 4.2). Primers for bean-specific glyceraldehyde-3-phospahte dehydrogenase C2 (*gapdh*) were obtained from Gazendam, (2012) (Table 4.2). The *gapdh* was used as a reference gene for normalisation of ceramide synthase expression. Primers were synthesized by Inqaba Biotechnology (Pretoria, South

Africa). Primers were re-suspended in the required amount of nuclease-free water (Thermo Scientific) to yield a final concentration of 100 µM.

Table 4.2. Primers designed and used for gene amplification of cowpea gDNA and cDNA. Y represents either cytosine or thymine.

Primer name	Gene Target	Sequence (5'–3')	Tm (°C)	PCR product (bp)	
				cDNA	gDNA
GAPDH_F	Bean-specific Glyceraldehyde-3-Phosphate dehydrogenase C2	ATCAGCCAAGGACTGGAGAG	62.5	130	233
GAPDH_R	Bean-specific Glyceraldehyde-3-Phosphate dehydrogenase C2	ACGGAATGCCATACCAGTCA	60.4		
LOH1_F	Ceramide synthase	GGAATCAGCYTGGAAATGT	57	458	
LOH1_R	Ceramide synthase	GGTAGTAAATGAGGCGCAA	58		

4.2.9. Cloning of LOH gene fragment into pJET cloning vector

Polymerase chain reaction amplification of the cowpea ceramide synthase gene was performed using both gDNA and cDNA as templates. A single PCR reaction of 25 µl consisted of 1X KAPA2G Robust Hotstart ReadyMix^2 (Wilmington, USA), 0.5 µM forward primer, 0.5 µM reverse primer, 1.5 µg template and made up to a final volume of 25 µl with nuclease-free water. Water was used as a template in the negative control. The reactions were conducted using a Gene Amp PCR system 2700 thermo cycler (Applied Biosystems, Foster City, USA) at an initial denaturing step of 5 min at 94°C, followed by 35 cycles with each cycle comprising a denaturing step at 94°C for 30 s, an annealing step at 60°C for 30 s and an elongation step at 72°C for 45 seconds. A final elongation step at 72°C for 5 min was included. A 5 µl sample of the amplified products was electrophoresed through a 1% agarose gel (100 V) using TAE buffer and was visualised under UV light to confirm the presence of the correct size of ceramide synthase cDNA and gDNA fragments. Once amplification was

confirmed, the remaining PCR samples (20 µL) were electrophoresed through a 1 % agarose gel using a 100 bp ladder (Fermentas).

Amplified products containing the ceramide synthase gDNA (sample 1) and cDNA (sample 2) fragments, respectively, were excised from the agarose gel on a UV light box using a clean scalpel blade. The gel slices were placed in pre-weighed 1.5 ml tubes and subsequently weighed. The PCR products were then extracted from the gel slices using the GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. The only deviation from the manufacturer's instructions was that 25 µl of elution buffer was used instead of 50 µl. The sample concentration and purity was measured using a NanoDrop 2000 spectrophotometer. The samples were stored at -20°C until used.

The purified cDNA and gDNA ceramide synthase fragments were subsequently cloned into the pJET1.2/blunt Cloning Vector (Appendix, Figure 7.1), from the CloneJET PCR Cloning Kit (Thermo Scientific). This was performed according to the manufacturer's instructions with variations. The sticky-end protocol was followed instead of blunt-end protocol due to the 3'-dA overhangs generated by using *Taq* polymerase in the PCR reactions. The blunting reaction for both samples was set up to a final reaction volume of 18 µl as follows: 10 µl 2X reaction buffer, 1 µl DNA blunting enzyme, 1 µl PCR product and made up to a final volume 18 µl with nuclease free water. For both gene fragments, 7 µl PCR product were used instead of the prescribed 2 µl. This was done as both samples had very low DNA concentrations (gDNA – 7.5 ng/µl; cDNA – 1.4 ng/µl). The kit ligation control experiment was also setup in the same manner as the two putative ceramide synthase gene fragment samples. The samples were then briefly vortexed and centrifuged for 5 s, incubated at 70°C for 5 min and then chilled on ice. For the ligation reaction, 1 µl pJET1.2/blunt cloning vector and 1 µl T4 DNA ligase were added to each sample to make each samples up to a final volume of 20 µl. The samples were then briefly vortexed, centrifuged and incubated at room temperature for 5 min. The samples were stored at -20° C.

The calcium chloride transformation method by Lamitina Lab Protocols 2007 (http://www.med.upenn.edu) was used for the transformation of the plasmids into *Escherichia coli*. Five µl of the ligation mixture was added to 50 µl of competent *E. coli* DH5α cells, which were prepared by Miss K Botha. The *E. coli*/ligation mix was incubated on ice for 30 min, was exposed to a heat shock treatment in a water bath at 42°C for exactly

30 s and placed on ice for 2 min. This was done for both cDNA and gDNA PCR fragments, the ligation control and the transformation control. For the transformation control, the plasmid pUC19 was used instead of the pJET vector. One thousand µl of warmed (37° C) Luria Bertani broth (LB) (1% tryptone (w/v), 0.5 % yeast extract (w/v) and 0.5% NaCl (w/v) pH 7.0) without any antiobiotics was added to the samples containing the transformed *E. coli* cells and placed in a shaking incubator at 37°C for 1 h. The samples were centrifuged for 30 s and the supernatant was removed, whereafter the pellet was re-suspended in 200 µl fresh warmed LB broth for each sample. A 100 µl volume of each sample was plated on LB ampicillin agar plates (50 ng/µl ampicillin, 1.5 % agar (w/v)). The remaining 100 µl of each sample was plated on a second plate. The plates were then incubated at 37°C for 24 h. Cells were removed from single *E. coli* colonies from the transformed plates and streaked on new LB ampicillin agar plates. This was only done for the two samples and the ligation control. The plates were incubated at 37°C for 24 h.

A colony PCR was performed using the primers that came with the ligation kit to screen for the successful ligation of the ceramide synthase fragments into the pJET cloning vector. A single colony PCR reaction consisted of 1X KAPA2G Robust Hotstart ReadyMix[2] (Wilmington, USA), 0.2 µM pJET1.2 forward primer, 0.2 µM µM pJET1.2 reverse primer and made up to a final volume of 20 µl with nuclease-free water. Six PCR reactions per ligation transformant were setup including six for the ligation control. For each reaction (with the exception of the negative control), a single colony was picked using a pipette tip and streaked onto a fresh LB ampicillin agar plate, with the tip then placed in a PCR tube containing the reaction mixture for a couple of minutes. The agar plates were incubated at 37°C for 24 h. The reactions were conducted using a Gene Amp PCR system 2700 thermo cycler (Applied Biosystems) at an initial denaturing step of 3 min at 95°C, followed by 25 cycles, each comprising a denaturing step at 94°C for 30 s, an annealing step at 60°C for 30 s and an elongation step at 72°C for 1 min. Fifteen microlitres of each PCR reaction were electrophoresed as outlined in 4.2.4 to analyse that the correct DNA inserts were present. A single colony with the correct DNA insert was chosen, streaked on a fresh LB ampicillin agar plate and incubated at 37°C for 24 hours. This was only done for samples, which contained the gDNA and cDNA inserts. The LB ampicillin agar plates were collected by Inqaba Biotechnology (Pretoria, South Africa) for sequencing of the ceramide synthase gene inserts.

4.2.10. DNA sequencing

Both the pJET1.2 cloning vectors containing the gDNA and cDNA inserts were sequenced by Inqaba Biotech, (Pretoria, South Africa) using pJET1.2 forward and reverse sequence primers. Both forward and reverse sequences of both gDNA and cDNA were aligned to each other respectively, using CLCBio to obtain the full length nucleotide sequences of the PCR products. A sequence alignment was performed in CLCBio to compare the cowpea ceramide synthase cloned cDNA sequence with the cDNA sequence from *G. max*, *P. vulgaris* and *A. thaliana*.

4.2.11. Ceramide synthase gene expression

Cowpea ceramide synthase gene expression was tested using cDNA as template in the PCR reactions. The reference gene *gapdh* was used to normalise the expression of ceramide synthase following the treatment of FB_1. A single PCR reaction of 10 µl consisted of 1X KAPA2G Robust Hotstart ReadyMix[2] (Wilmington, USA), 0.5 µM forward primer, 0.5 µM reverse primer, 0.4 µg template and made up to a final volume of 10 µl with nuclease-free water. Water was used as a negative control. The reactions were conducted using a Applied Biosystems Gene Amp PCR system 2700 thermo cycler (Foster City, USA) at an initial denaturing step of 5 min at 94°C, followed by 30 cycles, each comprising a denaturing step at 94°C for 30 s, an annealing step at 60°C for 30 s and an elongation step at 72°C for 45 seconds and a final elongation step at 72°C for 5 min. The amplified PCR products were analysed by 1% agarose gel electrophoresis (100 V). The expression of the control samples was compared to the expression of the treated samples. This was done for both the *gapdh* and ceramide synthase genes. Both *gapdh* and ceramide synthase gels were analysed using the software ImageJ (National Institutes of Health, Bethesda, USA). The values obtained from the software were used to calculate the relative expression rations for all samples between ceramide synthase and *gapdh*. The data was then statistically analysed using Graphpad Prism (San Diego, USA).

4.3. Results and Discussion

4.3.1. Primer design for amplification of the cowpea *LOH1* gene

To test whether or not FB_1 inhibits or reduces cowpea ceramide synthase gene expression, primers were designed to amplify a part of the ceramide synthase gene from cowpea DNA. Ternes *et al*., (2011) reported that the enzyme ceramide synthase exists in three isoforms in *Arabidopsis thaliana*: LOH1 (At3g25540), LOH2 (At3g19260) and LOH3 (At1g13580). Longevity assurance gene homologue one (LOH1) and LOH3 are highly similar (77% identical), while LOH2 is less similar (45% identical) to LOH1 and LOH3 (Ternes *et al*., 2011). Ternes *et al*., (2011) suggested that spontaneous cell death occurred in *Arabidopsis loh1* knockout plants, which is triggered by accumulation of free sphingoid bases or the accumulation of C16 ceramides. Fumonisin B_1 main chain consists of 20 carbons and thus LOH1 was chosen for this study, due to its similarity to LOH3, and the fact that both LOH1 and LOH3 accept a wide range of different lengths of acyl-CoAs whereas the LOH2 isoform only accepts acyl-CoA's consisting of 16 carbons. The *Arabidopsis* ceramide synthase LOH1 (AF198179) isoform mRNA sequence was obtained from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). This sequence was used in BLAST analyses (NCBI) to search for the cowpea equivalent on the Cowpea Genomics Knowledge Base (CGKB) (http://cowpeagenomics.med.virginia.edu). The CGKB forms part of the Cowpea Genomics Initiative (GCI) at the University of Virginia. A total of 250 000 genespace sequence (GSS) reads and 160 mbp of total sequences have been sequenced. However BLAST searches suggested that this database did not contain the cowpea ceramide synthase counterpart.

The closest relatives of cowpea with available genome sequences are *Glycine max* and *Phaseolus vulgaris*. The *Arabidopsis* ceramide synthase (*LOH1*) mRNA sequence was therefore used in the BLAST analysis function on the plant genome database Phytozome v10.2 (http://phytozome.jgi.doe.gov) to search for the *G. max* and *P. vulgaris* ceramide synthase (*LOH1*) transcript homologue equivalents. Phytozome is a database that contains a number of plant genomes and gene family data. The *G. max* and *P. vulgaris* ceramide synthase mRNA sequences were downloaded to the bioinformatics software CLCBio (Aarhus, Denmark) and were aligned to each other (Figure 4.1). Conserved regions in both the *G. max* (Glyma.16G091200.1) and *P. vulgaris* (Phvul.003G281100.1) *LOH1* transcripts

sequences were identified, and forward and reverse primers were designed to these regions (Figure 4.1). The *P. vulgaris* ceramide synthase sequence is shorter than that of *G. max* (Figure 4.1). This is so since there is only a partial gene fragment for *P. vulgaris* ceramide synthase is available. The forward primer of ceramide synthase was degenerate with the degeneracy being on nucleotide position 10 (Table 4.2). Using these primers it was determined in CLCBio that a fragment of approximately 458 bp is expected to be amplified from cowpea cDNA. Alignment of both species gDNA, cDNA and primer sequences to each other, using CLCBio indicated that a 916 bp and 927 bp genomic fragments would be amplified from *G. max* and *P. vulgaris* gDNA respectively, and a 458 bp fragment cDNA would be amplified from both species.

Bean-specific glyceraldehyde 1 phosphate dehydrogenase (*gapdh*) was used as a housekeeping gene, as it is often used as a housekeeping gene in gene expression studies to confirm that equal amounts of cDNA have been added to the PCR reactions since it is usually expressed at similar levels at different developmental stages (Table 4.2) (Kim *et al*., 2003). Reference genes should be expressed constantly regardless of the plant treatment and the gene product is required for basic cellular function. Housekeeping genes should be constantly expressed in plants cells even under different treatments otherwise it can lead to inaccurate gene expression results (Jain *et al*., 2006). The forward and reverse primer sequences for *gapdh* gene were obtained from Gazendam, (2012). A 130 bp fragment was expected to be amplified from *P. vulgaris* cDNA and 233 bp from gDNA using the *gapdh* primers. Similar size fragments were expected following amplification from cowpea gDNA and cDNA. The gDNA fragments are larger than those of cDNA due to the gDNA containing introns and exons, whereas the cDNA contains only exons.

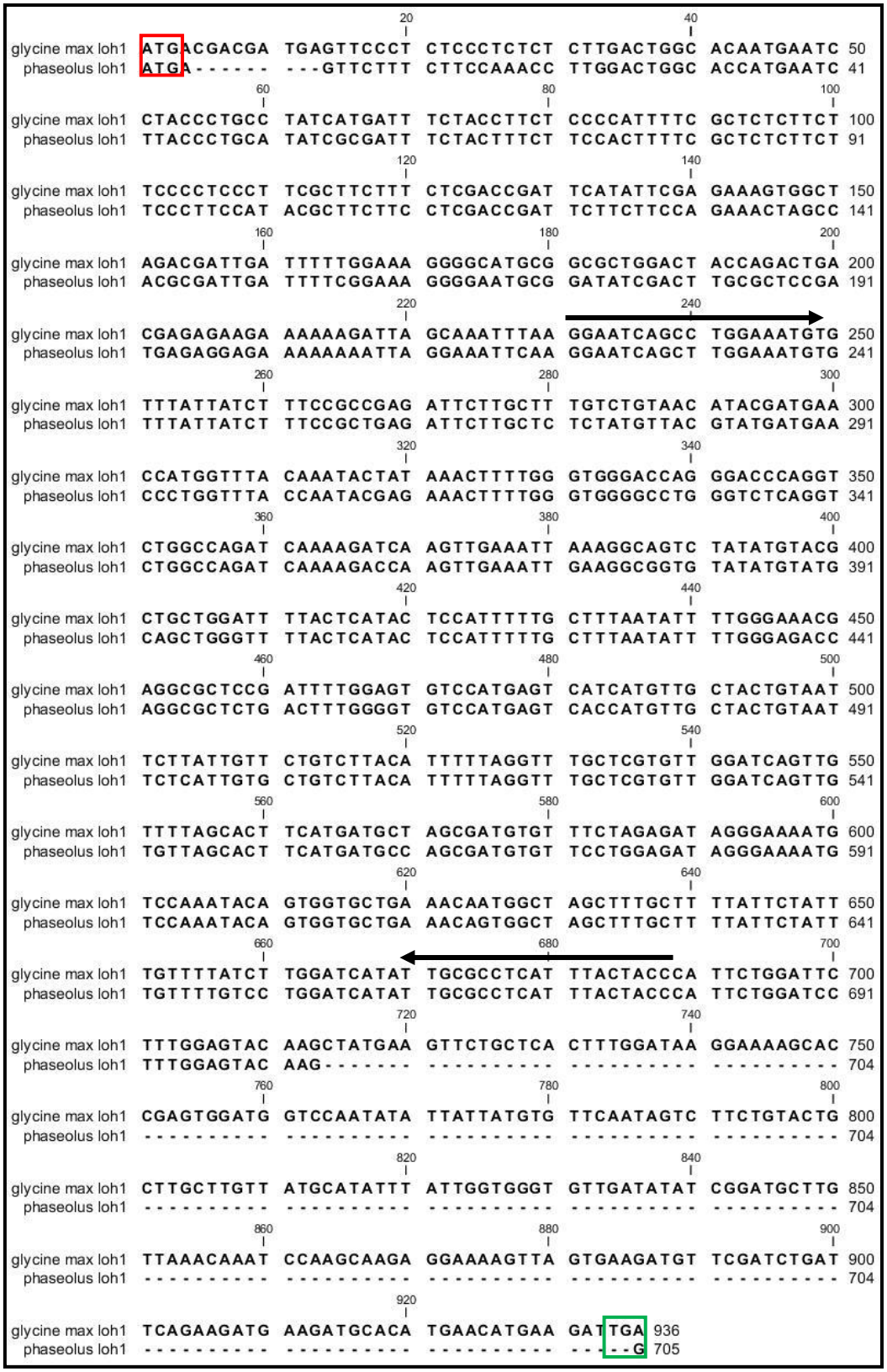


Figure 4.1. Sequence alignment of *Glycine max* and *Phaseolus vulgaris LOH1* gene. Forward and reverse primers were designed to conserved regions (see arrows). Forward and reverse primers are indicated by black arrows and the start and stop codons of *G. max* and *P. vulgaris* are indicated by red and green boxes, respectively.

4.3.2. DNA extraction from cowpea

Genomic DNA was extracted from untreated cowpea embryonic tissue samples, in order to test whether or not the designed primers did amplify from cowpea DNA. Genomic DNA was first extracted using the Nucleon Phytopure Genomic DNA Extraction Kit (GE Healthcare). The concentration and the A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios of the RNA were determined using a spectrophotometer. The A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios were used as purity indices to determine the quality of the RNA samples. The A_{260}/A_{280} absorbance ratio specifies whether the samples were contaminated with proteins and should be between 1.8–2.2 to be free of protein contamination (Manning, 1991; http://www.nanodrop.com). The A_{260}/A_{230} absorbance ratio specifies whether the samples are contaminated with compounds such as phenolics or carbohydrates and samples should be between 1.8–2.2 to be free of these organic contamination (Manning, 1991; http://www.nanodrop.com). The kit was not optimal as the gDNA yield was poor and not of good quality. In this regard nucleic acid concentrations ranged from 37.7 to 46.7 ng/µl. The 260/280 absorbance ratios were 1.44 and 1.51 and the 260/230 absorbance ratio were 0.26 and 0.24, respectively. These ratios give an indication that the samples could be contaminated with carbohydrates, phenolic compounds or proteins. The reason for these poor results could be that the kit used was past its expiry date and hence the reagents compromised.

The CTAB DNA extraction protocol adapted from Doyle & Doyle, (1987) and Cullings, (1992) was thus preferred since the reagents are freshly prepared and good quality DNA is expected when using this protocol. Two extractions were performed and the gDNA yield of both samples was high and of good quality. The samples had nucleic acid concentrations of 1200 and 2400 ng/µl respectively. Their absorbance values for both purity indices A_{260}/A_{280} and A_{260}/A_{230} were above 1.8. This indicates that the samples were free of any contamination. The extracted gDNA was analysed using gel electrophoresis and intact gDNA was observed at both samples, as shown in Figure 4.2. An additional band was detected for both samples at the bottom of both lanes 2 and 3, which is partially digested RNA, as both samples were treated with ribonuclease (RNase). If the RNA was completely digested, the band would not have been observed.

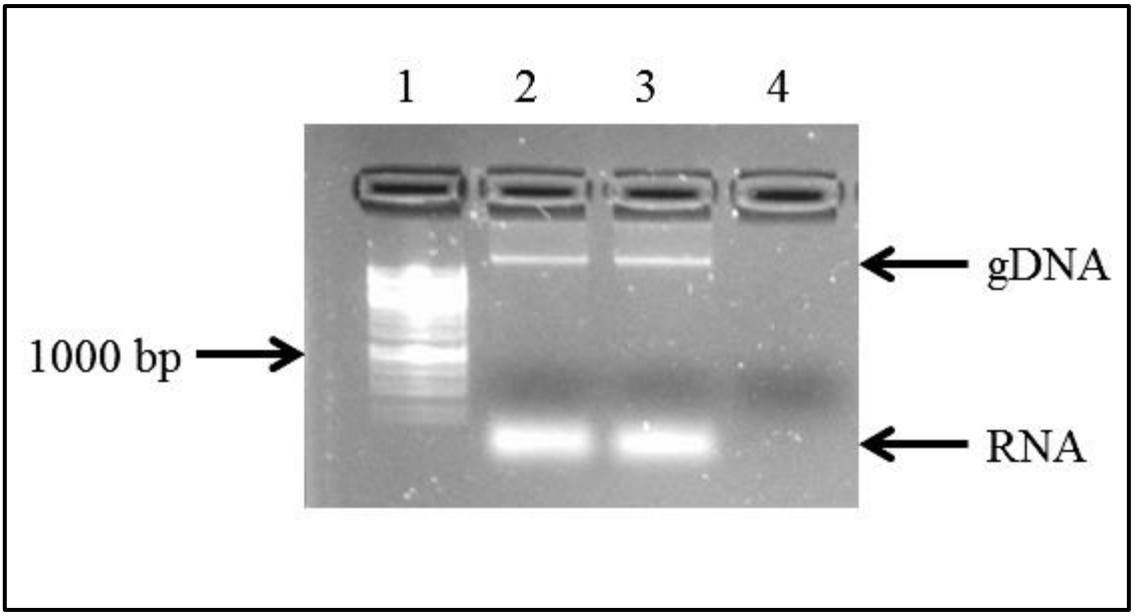


Figure 4.2. Agarose gel electrophoresis of gDNA isolated from untreated cowpea embryonic tissue. Lane 1: 1Kbp DNA ladder (Thermo Scientific); Lane 2 and 3: gDNA from untreated cowpea embryonic tissue.

4.3.3. RNA isolation from cowpea organs and cDNA synthesis

In order to determine if FB_1 had an effect on cowpea ceramide synthase gene expression, a single FB_1 concentration was chosen to determine the effect of the toxin. For the treatment, seeds were imbibed in a 20 mg/L FB_1 solution for 10 hours where after they were removed, sown in seedling trays and incubated at 25 °C for 12 days. The same was done for the control seeds except that the control seeds were imbibed in sterile distilled water for the same time period. Total RNA extractions were done in triplicate for the control and FB_1 treated cowpea seedlings at three different time points with samples from the last time point being divided into two groups consisting of the shoots and roots, respectively. In total 24 total RNA samples were obtained (Table 4.1). Total RNA was first isolated from cowpea samples using RNeasy Plant Mini Kit from QIAGEN, (Hilden, Germany). The root samples yielded low RNA concentrations of poor quality which was due to the roots being rich in secondary metabolites such carbohydrates and phenolics (Appendix, Table 7.1). Kumar *et al*., (2007) reported that potato roots were rich in secondary metabolites such as carbohydrates and phenolic compounds, which could interfere with the RNA extraction. The Qiagen RNeasy Plant Mini Kit was not efficient in removing these secondary metabolites and thus other protocols were considered. The CTAB RNA isolation protocol of Chang *et al*., (1993), was

then tested as the authors reported that it was very efficient in removing RNases and secondary metabolites such as phenolics from pine needles. This protocol yielded higher total RNA concentration of good quality that ranged from 223–544 ng/µl (Appendix. Table 7.2). The CTAB RNA isolation protocol was preferred over the Qiagen RNeasy Plant Mini Kit since it is a more robust protocol that yields higher RNA concentrations of good quality

The RNA extracted from cowpea embryos/seedlings, roots and shoots yielded RNA concentrations of 200 ng/µl and higher for all samples (Appendix. Table 7.2). The only exception was sample 22, which had a RNA concentration of 105.7 ng/µl. Using the Chang *et al*., (1993) method, all of the samples had A_{260}/A_{280} and A_{260}/A_{230} absorbance indice values above the recommended 1.8. The only exception was sample 22, which had an A_{260}/A_{230} absorbance ratio value of 1.58 (Appendix, Table 7.2). The contaminants can be removed by several ethanol precipitation steps, by sedimentation using caesium chloride or by RNA purification columns (Logemann *et al*., 1987).

The integrity of the RNA of the samples was analysed on a non-denaturing agarose gel. Assessing RNA integrity and quality was done by inspection of the 28S rRNA and 18S rRNA bands on a non-denaturing agarose gel. Visible and intact bands were obtained for all samples (Figure 4.3).

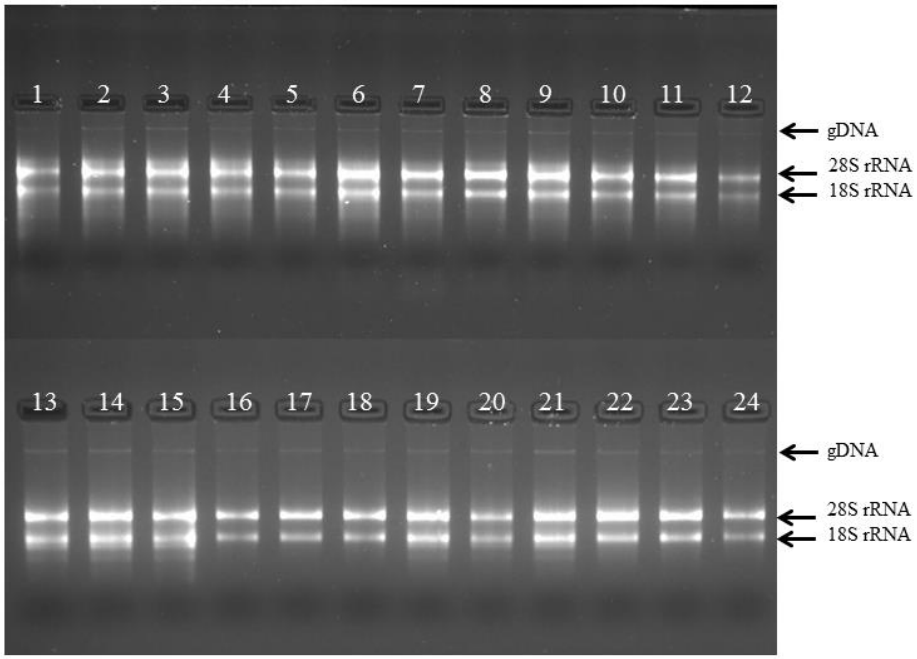


Figure 4.3. Non-denaturing agarose gel electrophoresis of total RNA isolated from the cowpea seedlings. Isolation of total RNA was done in triplicate with four samples per replicate. Lanes 1–12: total RNA from control seedlings; lanes 13–24: total RNA from FB_1 treated seedlings. Lanes 1–4: control replicate 1; lanes 5–8: control replicate 2; lanes 9–12: control replicate 3; lanes 13–16: FB_1 treatment replicate 1; lanes 17–20: FB_1 treatment replicate 2; lanes 21–24: FB_1 treatment replicate 3. Lanes 1, 5, 9, 13, 17 and 21: total RNA from 0 dpi; lanes 2, 6, 10, 14, 18 and 22: total RNA from 3 dpi; lanes 3, 7, 11, 15, 19 and 23: total RNA from shoot samples of 12 dpi; lanes 4, 8, 12, 16, 20 and 24: total RNA from roots samples od 12 dpi

Intact and visible bands at the 28S rRNA and 18S rRNA markers indicated that the RNA was of good quality (Figure 4.3) and could be used to synthesise cDNA. An additional band was visible in all lanes above the 28S rRNA and 18S rRNA markers (Figure 4.3). This additional band indicates that genomic DNA is present in all the samples. This is due to the fact that the RNA samples were not digested with deoxyribonuclease (DNase) to remove the DNA. The samples were subsequently treated with DNAse I, which successfully removed genomic DNA from the RNA samples.

4.3.4. Cloning of cowpea ceramide synthase gene fragments

Sequencing of the cowpea ceramide synthase gene fragments was done to determine if the designed ceramide synthase primers amplified the correct gene fragment in cowpea. Polymerase chain reaction amplification of the ceramide synthase gene fragment from gDNA and cDNA was performed in order to characterise the intronic region in the gene. The ceramide synthase primers successfully amplified a fragment from both gDNA and cDNA (Figure 4.4). These bands were then excised and the DNA fragments were extracted from the gel. Amplication of the gene fragment from cDNA was estimated to yield a 458 bp fragment and a 1000 bp fragment from gDNA, thus the gDNA amplicon was thought to probably contain a intron (Figure 4.4).

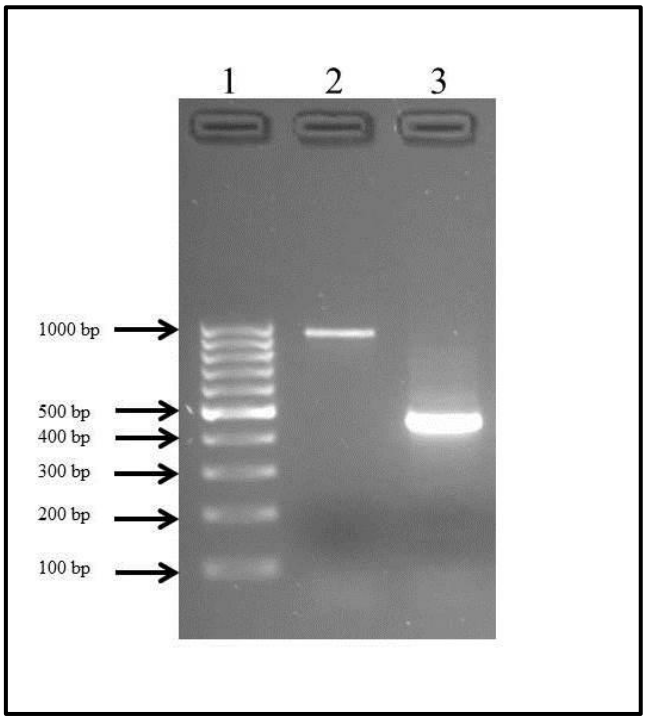


Figure 4.4. Agarose gel electrophoresis of PCR amplification of ceramide synthase gene fragments from cowpea gDNA (lane 2) and cDNA (lane 3). A 100 bp ladder (Thermo Scientific) was included (lane 1).

The purified PCR products were subsequently cloned into a pJET cloning vector. The pJET1.2/blunt cloning vector (Appendix, Figure 7.2) contains a lethal gene *eco47IR* coding for a restriction endonuclease, which is disrupted by the ligation of a DNA insert into its cloning site. As a result only transformed cells with recombinant plasmids are able to grow. The ligation control was used to verify the efficiency of the blunting and ligation steps of the

cloning kit. The recombinant plasmids containing the PCR products were transformed into *E. coli DH5α* competent cells using the calcium chloride transformation method (Lamitina Lab Protocols, 2007). This was done for the ligation and transformation controls as well. The transformation control consisted of the plasmid pUC19. The plasmids containing the two purified PCR products, the ligation control and as well as the transformation control plasmid pUC successfully transformed into the *E. coli DH5α* cells as colonies could be seen on LB ampicillin agar plates for all four transformation templates. If the PCR products as well as the ligation control did not successfully ligate into the pJET1.2/blunt cloning vector, no *E. coli* growth would have been observed, as the lethal gene would not be disrupted by the ligation of DNA into the cloning site.

A colony PCR was performed on the transformed cells to screen for the successful ligation of ceramide synthase gene fragments into the pJET1.2/blunt cloning vector. Both the putative ceramide synthase gDNA gene fragment (lane 3–5) and cDNA gene fragment (lane 7–9) successfully ligated into the cloning vector as well as the ligation control as seen by the amplification of the correct size insert (Figure 4.5). Lane 2 did not contain the correct size insert, whereas lane 6 contained no insert. The colonies represented by these two lanes were thus discarded. A single colony containing the correct size insert was then plated on LB ampicillin agar plate, whereafter the plasmid DNA was extracted and sequenced. This was done for both the gDNA and cDNA insert, but not for the ligation control.

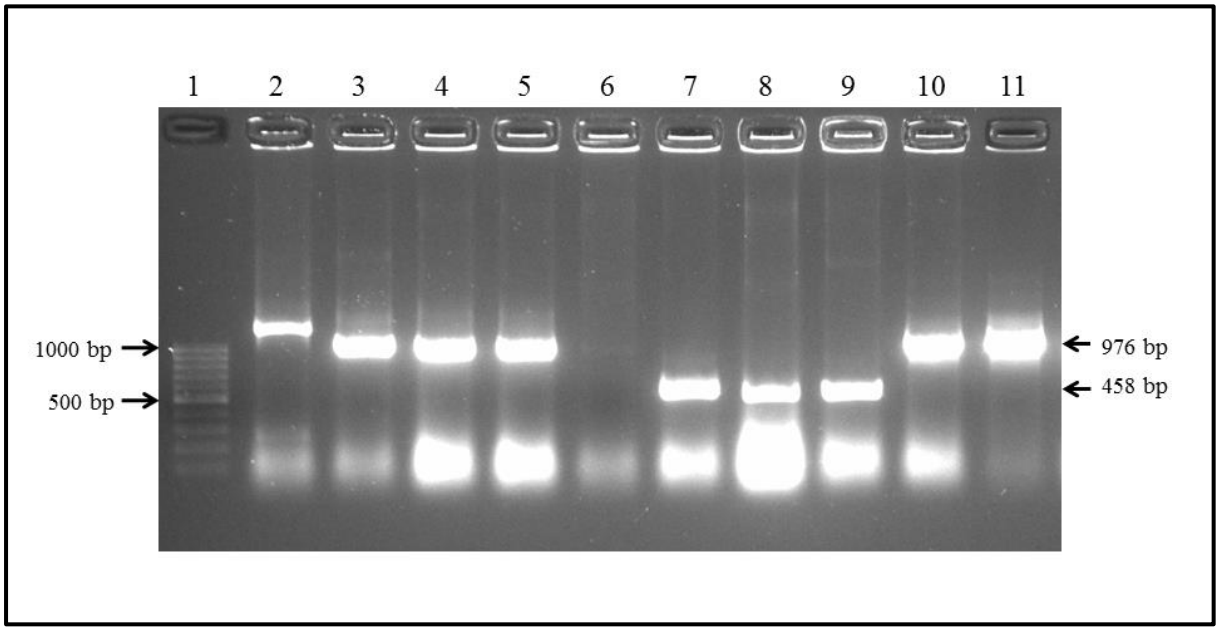


Figure 4.5. PCR amplification of inserts from colonies containing putative ceramide synthase gene fragments. Expected size for ceramide synthase gDNA fragment: 1000bp, ceramide synthase cDNA fragment: 458 bp, control fragment: 976 bp. Lane 1: 100 bp molecular marker (Thermo Scientific); lanes 2–5: vector containing gDNA insert; lanes 6–9: vector containing cDNA insert; lanes 10 and 11: ligation control.

Both the putative gDNA and cDNA ceramide synthase fragments were sequenced to determine if the primers did indeed amplify the correct gene from cowpea, and if the ceramide synthase gene spanned an intron. Sequence analysis was done using the bioinformatics software CLCBio (Aarhus, Denmark) and the databases websites NCBI Blast (http://www.ncbi.nlm.nih.gov/) and PhytozomeV10.2 (http://phytozome.jgi.doe.gov/pz/portal .html). The forward and reverse sequences for both the putative gDNA and cDNA were aligned to each other respectively, using CLCBio to determine the nucleotide sequence for both ceramide synthase fragments. The cDNA sequences is shown in the Appendix, Table 7.3. The ceramide synthase primers were then aligned to both sequences to ensure that they were at the 5’ and 3’ ends of the amplified product. Both the forward and reverse ceramide synthase primers did amplify the cDNA ceramide synthase gene product but not the gDNA ceramide synthase product. The latter sequence revealed that the forward primer annealed at two places on the gDNA, namely, the beginning and at the end of the sequence, resulting in amplification of the incorrect product. This provided valuable information on where the primers annealed when using both gDNA and cDNA as template. The genomic DNA fragment was thus voided as a positive control since only the forward primers annealed to the DNA and the incorrect product was amplified. BLAST analysis of the gDNA fragment

confirmed that it was not part of the ceramide synthase gene. Thus, it could not be determined if whether or not the ceramide synthase gene contained an intron.

The cowpea ceramide synthase cDNA gene fragment nucleotide sequence, together with *G. max*, *P. vulgaris* and *A. thaliana* sequences, which were used to design the cowpea ceramide synthase primers, were aligned using the software CLCBio. Good homology can be seen between the related legume species, but not between the legume species and *A. thaliana*. There were differences between some nucleotides of all four species and thus an amino acid alignment was chosen to compare ceramide synthase between species. This was done since an amino acid sequence is more conserved than a nucleotide sequence since several codons can code for the same amino acid. The amino acid sequences in Figure 4.6 forms part of the conserved region of ceramide synthase protein. The cowpea ceramide synthase sequence was only partially sequenced and thus the amino acid sequence in Figure 4.6 was not the full amino acid sequence of cowpea ceramide synthase gene product. Better homology can be seen between the related legume species than to *A. thaliana* in an amino acid alignment (Figure 4.6).

The cowpea ceramide synthase partial amino acid sequence was used in a reciprocal BLAST on the database Phytozome v10.2 (http://phytozome.jgi.doe.gov) to reconfirm that the correct gene was amplified and cloned from cowpea. A BLASTP analysis of the cowpea ceramide synthase amino acid partial sequence (95 amino acids) was compared to *A. thaliana* ceramide synthase gene sequences, and was found to be more similar to *A. thaliana LOH3* (85.3 %; E-value: 9.47 X 10^{-53}) (At1g13580) than to *LOH1* (At3g25540) (76.8%; E-value: 1.15 X 10^{-48}).

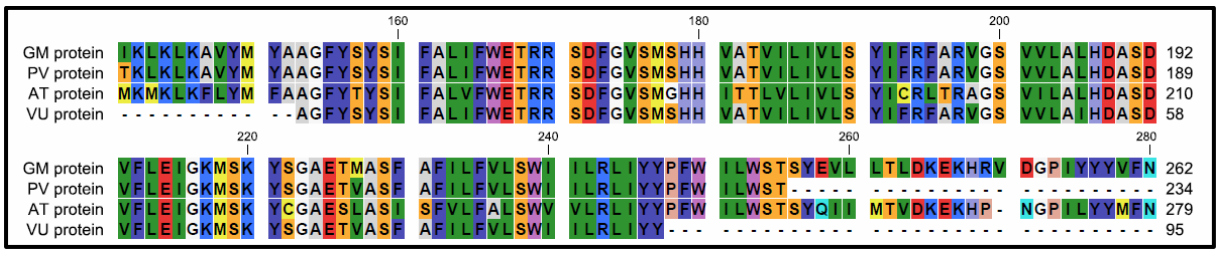


Figure 4.6. The amino acid sequences of the conserved region of the ceramide synthase protein of three closely related legume species and one distant related species (*A. thaliana*). GM- *Glycine max*; PV- *Phaeolus vulgaris*; AT- *Arabidopsis thaliana*; VU- *Vigna unguiculata*.

The cowpea ceramide synthase partial gene fragment amino acid sequence was almost identical to *G. max* (Glyma.16G091200.1; 97.9 %; E-value: 2.04 X 10^{-57}) and *P. vulgaris* (Phvul.003G281100.1; 98.9 %; E-value: 5.49 X 10^{-59}) ceramide synthase gene equivalents over 95 amino acids. Ceramide synthase gene sequences from these two legume relatives were used to design primers for the amplification of the gene from cowpea. Brandwagt *et al*., (2002) did an amino acid alignment of the *LAG*1 gene of various organisms including *G. max, A. thaliana*, *Saccharomyces cerevisiae*, human and mouse. There are a number of amino acid bases that are conserved across all the species including cowpea. Due to the very good homology between cowpea and related legume species, we felt confident that the correct gene had been amplified and cloned from cowpea. As the primers did amplify a fragment of the ceramide synthase gene in cowpea, we went on to use this sequence to analyse the expression levels of the gene following FB_1 treatment.

4.3.5. Cowpea ceramide synthase gene expression

The designed and tested ceramide synthase primers (section 4.3.1) were used to determine whether or not FB_1 had an effect on the gene expression of ceramide synthase in cowpea. The *gapdh* gene was used as a reference gene to normalise for equal loading of the cDNA template. Optimization steps were done to determine the ideal parameters with which to assess the expression levels of cowpea ceramide synthase. In this regard a temperature gradient PCR amplification of both genes was performed to determine the ideal annealing temperatures of the primers sets of both genes, which was found to be 60° C for both genes. The efficacy of cDNA synthesis from cowpea mRNA was also tested using PCR. No amplification would occur if the mRNA was not converted to cDNA as Taq polymerase can only bind and amplify DNA (Joyce & Steitz, 1994). Contamination of RNA with gDNA can also be tested with PCR since the *gapdh* gene contains an intron when amplified from gDNA.

A PCR amplification reaction of both *gapdh* and *LOH1* was performed to determine the number of cycles needed to stop the reaction in the mid exponential phase of the PCR amplification. It is important to select the optimal amount of cycles so that the amplification product is visible on agarose gel and that amplification is in the exponential phase and not yet reached the plateau phase (Marone *et al*., 2001). When a PCR reaction reaches the plateau, cDNA initially present in low concentrations may give products of equal intensity to cDNA of genes present in high concentrations (Marone *et al*., 2001). A single cycle consisted of

three steps: a denaturing step, an annealing step and an elongation step. In determining the optimal number of amplification cycles of PCR reactions, 10 µl of reaction mixture containing PCR products was removed after 15 cycles, and thereafter at every five cycles until 35 cycles were reached. The samples were then visualised following agarose gel electrophoresis (Figure 4.7). Amplification for both genes (*LOH1* & *gapdh*) from control sample cDNA was detected at 30 cycles of amplification. Thirty cycles of amplification was chosen as the optimal amount of cycles required for both *gapdh* and *LOH1*.

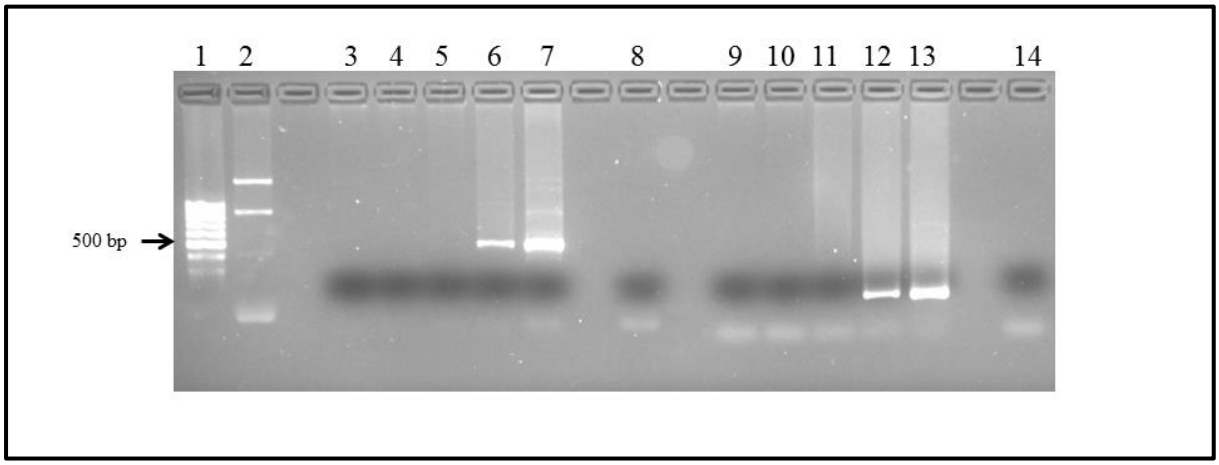


Figure 4.7. Agarose gel electrograph of the ceramide synthase and *gapdh* fragments amplified from cDNA to determine the optimal amount of cycles needed for semi-quantitative PCR reactions. Ceramide synthase lanes 3–8; FB_1 treatment lanes 9–14. Lanes 1 100 bp ladder (Thermo Scientific); lane 2 Low Range FastRuler (Thermo Scientific); lanes 3 & 9: 15 cycles; lanes 4 & 10: 20 cycles; lanes 5 & 11: 25 cycles; lanes 6 & 12: 30 cycles; lanes 7 & 13: 35 cycles; lanes 8 & 14: negative control.

In total 16 samples were used to test the effect of FB_1 on the gene expression in cowpea. Samples 1–8 formed part of the control group whereas samples 13–20 formed part of the FB_1 treated group (Table 4.1). Only samples 1–8 and 13–20 were used for testing the effect of FB_1 on gene expression of ceramide synthase. Both the control and treatment plants were harvested at three different time points: 0 dpi, 3 dpi and 12 dpi, which was split into two groups: shoot and root samples. Only two replicates of both the control and FB_1 treatment were used as the third replicate for both contained degraded cDNA due to possible freeze-thaw of the DNA, and thus no amplification of the two genes occurred during PCR in these mentioned samples.

Equal loading of cDNA was seen for most control and treated samples except when assessing the gene expression levels of *gapdh* (Figure 4.8 A). However, samples 1, 5, 13 and

17 which represented cowpea embryos harvested 0 dpi and showed lower levels of *gapdh* expression (Figure 4.8 A, lanes 3, 7, 11 and 15). Although the intensity of the bands of samples 1, 5, 13 and 17 were less than that of the rest of the samples, the intensity of the *gapdh* bands of these four samples were similar when they were compared to each other. It could be that *gapdh* is developmentally regulated and expressed at different levels in the embryonic and mature tissue. Kim *et al*., (2003) tested a variety of housekeeping genes, which included 18S rRNA, glyceraldehyde-3-phosphate dehydrogenase, actin, and tubulin in rice. They showed that the expression levels of *gapdh* in etiolated rice seedlings started to decrease after five days post germination, whereas 18S rRNA remained constant. The expression levels of *gapdh* varied up to two-fold between different varieties of rice whereas 18S rRNA remained stable, and thus the 18S rRNA gene was found to be the most reliable housekeeping gene in rice. Jain *et al*., (2006) reported that 18S and 25S rRNA housekeeping genes exhibited the most stable expression in rice plants grown under various environmental conditions. Thus the 18S rRNA could possibly have been a better and more stable housekeeping gene than *gapdh* in this study, and in future studies it should be considered when testing of ceramide synthase gene expression levels in cowpea.

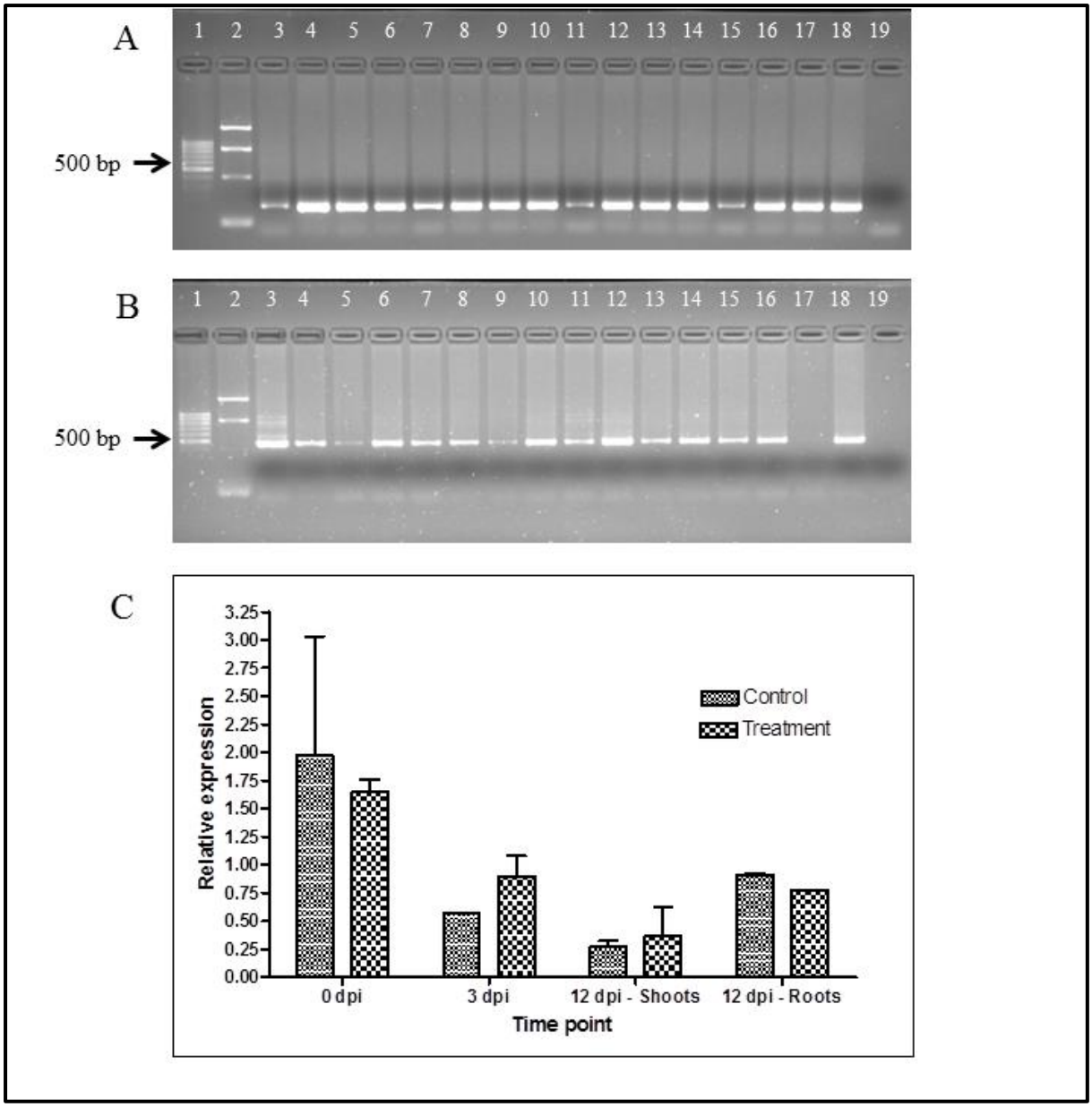


Figure 4.8. Semi-quantitative PCR of ceramide synthase gene relative to *gapdh* in FB_1 treated and non-treated cowpea seedlings. A- reference gene *gapdh*; B- ceramide synthase gene; C- Relative expression (ceramide synthase normalised to *gapdh*) between the control and the FB_1 treatment across the four different samples types. (A and B): Lane 1: 1 kbp ladder (Thermo Scientific); lane 2 Low Range FastRuler (Thermo Scientific); Control samples lanes 3–10; FB_1 treated samples lanes 11–18; lanes 3–6: control replicate 1; lanes 7–10: control replicate 2; lanes 11–14: FB_1 treatment replicate 1; lanes 15–18: FB_1 treatment replicate 2; lane 19: negative control; lanes 3, 7, 11 and 15: 0 dpi; lanes 4, 8, 12, 16: 3 dpi; lanes 5, 9, 13, 17: 12 dpi-shoots; lanes 6, 10, 14 and 18: 12 dpi-roots.

Semi-quantitative PCR amplification was used to determine whether FB_1 had an effect on the gene expression of cowpea ceramide synthase expressed at different growth stages and organs. The gene expression levels of the cowpea ceramide synthase gene were compared between untreated and treated FB_1 samples across three different time points, which included

the embryo stage (0 dpi), three days post imbibition and 12 days post imbibition. The last mentioned time point, was split into shoot and root samples. The ceramide synthase gene fragments were analysed on 1% agarose gel to assess if there was an effect in gene expression between the untreated and treated samples (Figure 4.8-B). It was hypothesised that the amount of ceramide synthase gene expression would decrease with an increase in exposure time to FB_1.

Amplification of the ceramide synthase gene fragment from cDNA yielded a 458 bp fragment and thus the correct size gene fragment was amplified for samples tested (Figure 4.8-B). When the respective times points from both the control and treated samples were compared with each other, the bands of each sample had similar intensity levels. The only exception was for a treated shoot sample that was harvested 12 dpi (lane 17) where no band was detected. This sample did amplify when the reference gene *gapdh* primers were used (Figure 4.8 A) and thus the sample was not degraded. It could be that there were not any copies of the ceramide synthase transcripts in this particular sample.

The expression of ceramide synthase relative to *gapdh* was compared to observe whether or not FB_1 had an influence on the expression of ceramide synthase in cowpea (Figure 4.8 C). No significant difference was observed when the untreated samples were compared to the FB_1 treated samples at each time point. The only significant difference that was noted was that the ceramide synthase gene expression decreased over time, for both the treated and untreated samples (Figure 4.8, C). The gene expression for ceramide synthase was higher for the samples that were harvested 0 dpi when compared to samples that were harvested from both 3 dpi and 12 dpi. This could possibly be attributed to a developmental effect or that *gapdh* was lower and thus dividing it at 0 dpi gave a higher ceramide synthase value.

When considering statistics, there was no significant difference in normalised ceramide synthase expression levels across time points (3 and 12 dpi) or between control and treated samples. There was a significant difference in ceramide synthase expression between 0 dpi (control and treated) and other time points but this is probably due to the developmental expression of *gapdh* to which ceramide synthase was normalised to.

4.4. Conclusion

In conclusion no significant differences in ceramide synthase gene expression were seen between the control and FB_1 treated plants. However, possible developmental effects on gene expression were seen across all time points in both the control and treated samples. From this study we can conclude that FB_1 treatment did not influence cowpea ceramide synthase gene expression whatsoever, and thus FB_1 possibly exerts it effects at some other cellular level such as inhibition of protein syntheses or enzyme activity.

CHAPTER 5

General discussion

Cowpea (*Vigna unguiculata* (L.) Walp) is a nutritious pulse crop that originated from Africa and is cultivated throughout the world in tropical and subtropical regions (Ehlers & Hall, 1997). Cowpea is widely cultivated by subsistence farmers in rural communities due to it being versatile and high in protein content (Ehlers & Hall, 1997; Padulosi & Ng, 1997). As with many legume grain crops, cowpea seeds are susceptible to fungal infestation when subjected to suboptimal storage conditions (Kritzinger *et al*., 2006; McDonald, 1979). Amongst an array of different fungi, *Fusarium verticillioides* and *F. proliferatum*, which produce fumonisins, have been found to be associated with cowpea seed (Kritzinger *et al*., 2004). It is well known that FB_1 is phytotoxic to a wide range of plants and weeds (Abbas *et al*., 1995; Abbas *et al*., 1998; Abbas & Boyette, 1992; Doehlert *et al*., 1994) and the toxin has previously been reported to exhibit phytotoxic effects on cowpea seed (Kritzinger *et al*., 2006). Numerous studies indicate that the possible mode of action of FB_1 is through the inhibition of the enzyme ceramide synthase, which is a key enzyme in the sphingolipid pathway (Abbas *et al*., 1994, 1998; Riley *et al*., 1994; Williams *et al*., 2007).

This study investigated the effects of pure FB_1 and FB_1-producing *Fusarium* spp. on cowpea seedlings under phytotron conditions. Aspects that were evaluated included, seedling emergence, root and shoot length and dry mass, seedling vigour index and total chlorophyll production. A possible mode of action of FB_1 was also investigated in cowpea seedlings by determining whether FB_1 inhibits the gene expression of ceramide synthase.

Previously, Kritzinger *et al*., (2006) investigated the effects of FB_1 on cowpea seed germination using the between paper towel method and in a preliminary study by Kafua, (2011), the effect on emergence under greenhouse conditions was evaluated. It was necessary to investigate and confirm the phytotoxic effects of the toxin on cowpea emergence and growth under more controlled conditions (i.e a phytotron) and to examine other physiological parameters. Cowpea seed was treated with pure FB_1 at concentrations of 5, 20 and 40 mg/L. Furthermore, cowpea seed was artificially inoculated with the conidial suspensions of three *F. verticillioides* strains known to produce FB_1. This was done to simulate what happens under more natural conditions, since the fungi first contaminate the seed and then the toxin is

produced. Cowpea seed emergence was significantly reduced by all three FB_1 concentrations as well as one *F. verticillioides* strain, MRC 8265, when compared to both controls. With regards to the toxin treated seeds, the results correlate well with those of Kritzinger *et al*., (2006) and Kafua, (2011). Similar correlation was also observed by Kritzinger *et al*., (2006) and Zaidi, (2012) who noted that *Fusarium* species also reduced cowpea seed germination. Zaidi, (2012) attributed the reduced emergence rate to either the fungal infestation or the secondary metabolites that the fungi produce, or both factors.

The visual inspection of cowpea seedlings indicated that the majority of seedlings were well developed except for seedlings that were imbibed in 20 and 40 mg/L FB_1. Seedlings whose seeds were imbibed in these two FB_1 concentrations exhibited reduced root development and stunted growth. Williams *et al*., (2007) indicated that maize seedlings that were watered with FB_1 solutions had stunted growth and reduced root development. No significant reduction in the root and shoot length were observed across all treatments except for seeds imbibed in 40 mg/L FB_1 solution. These results compliment the findings by Kritzinger *et al*., (2006) where a reduction in radicle and plumule length in seeds imbibed in 50 and 100 mg/L FB_1 were observed. The root and shoot dry mass of cowpeas in this study was reduced by all three FB_1 concentrations as well as all three *F. verticillioides* strains whereas the non-imbibed control had the highest root and shoot weight.

A useful way to determine if a mycotoxin or a fungal pathogen or both do influence the growth and vigour of a seedling is to determine the seedling vigour index (SVI) (Abdul-Baki & Anderson, 1973). The SVI is a useful measurement, which both scientists and commercial farmers, can use to evaluate more than one physiological factors such as seedling emergence and length (SVI 1) or seedling emergence and mass (SVI 2). While these factors on their own often provide valueble insight on how a seedling reacts to the toxin or the fungal strain, the effects cannot always be seen when only focussing on one factor alone, such as seedling length for instance. Both SVI 1 and SVI 2 were reduced by all three FB_1 concentrations as well as by all three fungal strains. Both indices suggest that FB_1 and the FB_1-producing *F. verticillioides* strains do negatively influence the growth of cowpea seedlings. Total chlorophyll content was lower in the leaves from all the treatments including both the controls after 21 dpi, which was lower than that for 14 dpi. Contrary to what was expected, the leaves from all three FB_1 seed treatments as well as all the fungal strains treatments showed a higher total chlorophyll content when compared to the two controls. Similar

findings were reported by Nadubinska *et al*., (2003), who reported that in FB_1-treated maize seedlings, chlorophyll a and b levels were higher than that of the controls.

The results of this study provide a better understanding on how the pure toxin and the fungal species influence the growth and development of cowpea seedlings. People and animals that consume contaminated cowpea seed can suffer health problems and also experience food insecurity due to the losses of productivity as a result of the phytotoxic effects caused by the toxin and the fungi. It is thus crucial to not only study the effect of FB_1 and FB_1-producing *Fusarium* spp. but as well as other mycotoxins and fungi affecting cowpea production i.e. trichothecenes, zealarenone, aflatoxins etc. The outcomes of this study will assist in better understanding the factors that affect cowpea production losses and will help to develop mechanisms to reduce fungal infestation in cowpea seed and to improve seed health before it is sowed or consumed. Subsistence farmers should be attentive to the conditions in which the seeds are stored. Better storage conditions should also be developed which will aid subsistence farmers in reducing crop losses by minimizing fungal infestation and subsequent mycotoxin contamination.

Future work should include determining the concentrations levels FB_1 of known FB_1-producing *Fusarium* spp. prior to seed treatment and also to determine the levels of the toxin in artificially inoculated cowpea seed after a certain time point. Field studies are also needed to study how the plant reacts to the toxin and the fungal pathogen under natural conditions they are often cultivated in. Since it was noted FB_1 increased the total chlorophyll levels in cowpea leaves, additional chlorophyll studies are necessary to confirm that FB_1 does indeed increase the levels of total chlorophyll. It is also necessary to investigate whether or not the toxin affects the above ground parts such as the leaves and also to determine if the toxin is naturally found in the leaves since the leaves are also used as a vegetable in many cowpea producing regions.

Although it is known that the mechanism behind the toxic effects of FB_1 is linked to the inhibition of ceramide synthase, little information is available with regards to the actual mode of action of FB_1 toxicity in plant cells and tissues. To investigate whether FB_1 does have an effect on the sphingolipid pathway on a molecular level, the effect of FB_1 on the ceramide synthase gene transcript in cowpea was examined. It was hypothesised that the amount of ceramide synthase transcripts would decrease with an increase in exposure time to FB_1.

Cowpea seeds were imbibed in a 20 mg/L FB_1 solution whereas the control seeds were imbibed in water. RNA was isolated from cowpea seedlings after which cDNA were synthesized from the mRNA. The ceramide synthase gene expression was then tested using the cDNA in semi-quantitative PCR reactions.

Cowpea ceramide synthase primers were designed to the conserved regions of the ceramide synthase gene transcript sequence of *Glycine max* (L.) Merr.) and *Phaseolus vulgaris* L.. This was done as both *G. max* and *P. vulgaris* are closely related to cowpea since they are all legume plants. A 458 bp ceramide synthase fragment from cowpea was sequenced and compared to *G. max* and *P. vulgaris* ceramide synthase equivalents. Thus the ceramide synthase gene fragment was successfully amplified and sequenced from cowpea using the designed primers.

Semi-quantitative PCR amplification was used to test if FB_1 did have an effect on the gene expression of ceramide synthase in cowpea at different growth stages and organs. The relative gene expression levels of ceramide synthase were compared between the control and the treated samples to determine whether the toxin did have an effect on the expression of ceramide synthase. No significant differences were seen when the control samples were compared to the FB_1 treated samples at each time point. However, significant developmental expression of the cowpea ceramide synthase gene was observed as expression decreased over time for both the control and treated samples. It thus seems that the toxin did not have an effect on the gene expression of ceramide synthase gene in cowpea. However, possible developmental was seen in both the treated and untreated cowpea samples.

Determining whether FB_1 had an effect on the gene expression of ceramide synthase in cowpea was essential in elucidating a possible mechanism of action of the toxin in cowpea. This was done as ceramide synthase is a key enzyme in the sphingolipid pathway, which is responsible for the formation of sphingolipids and complex sphingolipids. Sphingolipids and complex sphingolipids play an important role in everyday processes such as membrane stability, cell signalling and regulation and programmed cell death (PCD) (Lynch & Dunn, 2003; Sperling & Heinz, 2003). As seen from the results of this study, FB_1 did not have an effect on the expression of the ceramide synthase gene in cowpea and, thus it is possible that it exerts an effect at some other level such as protein inhibition.

Additional studies should be done in determining the mechanism of action of FB_1 in cowpea. Numerous studies have been done in both human and plant tissue that have been treated with FB_1, where the levels of the precursor molecules such as sphinganine and the product such as ceramide have been determined (Abbas *et al*., 1994; Kim *et al*., 2012; Lynch, 1999; Merrill *et al*., 2001; Soriano *et al*., 2005; Ternes *et al*., 2011). These studies did not focus on the enzyme itself and thus an enzyme assay should be considered in determining whether or not FB_1 inhibits the function of ceramide synthase enzyme in cowpea. The amount of free sphingolipids such as sphinganine and the amount of ceramides can also be measured to determine if FB_1 did have an effect on the pathway. Another alternative in determining the mechanism of action of the toxin is to isolate and characterise the entire ceramide synthase gene family from cowpea. It would then be possible to determine if all forms of the ceramide synthase are cowpea is affected by FB_1 or only certain isoforms of the enzyme. It should also be determined if FB_1 affects other metabolic pathways and not only ceramide synthase and the sphingolipid pathway. This can be done by means of doing a DNA microarray to evaluate the expression of cowpea transcripts or doing RNA sequencing of cowpea tissue that was treated with the toxin. It is necessary to understand the mechanism of action of the toxin since it will aid in the development of better control and prevention strategies of not only the toxin but also the fungal pathogens that produces it.

CHAPTER 6

Literature cited

Abbas, H.K., Boyette, C.D., Hoagland, R.E., Vesonder, R.F., 1991. Bioherbicidal potential of *Fusarium moniliforme* and its phytotoxin, fumonisin. Weed Science 39, 673–677.

Abbas, H.K., Boyette, C.D., 1992. Phytotoxicity of fumonisin B_1 on weed and crop species. Weed Technology 6, 548–552.

Abbas, H.K., Tanaka, T., Duke, S.O., Porter, J.K., Wray, E.M., Hodges, L., Sessions, A.E., Wang, E.A.H.M., Riley, R.T., 1994. Fumonisin- and AAL-toxin- induced disruption of sphingolipid metabolism with accumulation of free sphingoid bases. Plant Physiology 106, 1085–1093.

Abbas, H.K., Duke, S.O., Paul, R.N., Riley, R.T., Tanaka, T., 1995. AAL-toxin, a potent natural herbicide which disrupts sphingolipid metabolism of plants. Pesticide Science 43, 181–187.

Abbas, H.K., Riley, R.T., 1996. The presence and phytotoxicity of fumonisins and AAL-toxin in *Alternaria alternata*. Toxixon 34:1, 133–136.

Abbas, H.K., Duke, S.O., Merrill, A.H., Wang, E., Shier, W.T., 1998. Phytotoxicity of australifungin, AAL-toxins and fumonisin B_1 to *Lemna pausicostata*. Phytochemistry 47, 1509–1514.

Abdul-Baki, A.A., Anderson, J.D., 1973. Vigor determination in soybean seed by multiple criteria. Crop Science 13:6, 630–633.

Adam, T., 1990. Diseases affecting cowpea (*Vigna unguiculata* (L. Walpers)) in Niger. AMBIO 19, 358–360.

Adekunle, A.A., Bassir, O., 1973. The effects of aflatoxin B_1 and palmotoxins B_0 and G_0 on the germination and leaf colour of the cowpea (*Vigna sinensis*). Mycopathologia et Mycologia Applicata 61:4, 299–305.

Anjorin, T.S., Salako, E.A., Makun, H.A., 2013. Control of toxigenic fungi and mycotoxins with phytochemicals: potentials and challenges. In: Makun, H.A., (Ed.). InTech, Rijeka, Croatia, 181–202.

Arnon, D.I., 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology 24:1, 1–15.

Arumuganathan, K., Earl, E.D., 1991. Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter 9:3, 208–218.

Asai, T., Stone, L.M., Heard, J.E., Kovtun, Y., Yorgey, P., Sheen, J., Ausubel, F.M., 2000. Fumonisin B_1–induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signalling pathways. The Plant Cell 12, 1823–1835.

Aveling, T.A.S., Powell, A.A., 2005. Effect of seed storage and seed coat pigmentation on susceptibility of cowpeas to pre-emergence damping-off. Seed Science and Technology 33, 461–470.

Barkai-Golan, R., Paster, N., 2008. Mycotoxins in fruits and vegetables. Elsevier, San Diego, CA, USA, 386.

Baudoin, J.P., Maréchal, R., 1985. Cowpea taxonomy, origin and germplasm. In Singh, S.R., Rachie, K.O., (Eds.). Cowpea research, production and utilization. John Wiley & Sons, Chichester, UK, 3–9.

Bennett, J.W., Klich, M., 2003. Mycotoxins. Clinical Microbiology Reviews 16:3, 497–516

Berkey, R., Dendigeri, D., Xiao, S., 2012. Sphingolipids and plant defence/disease: The "death" connection and beyond. Frontiers in Plant Science 3, 1–22.

Bezuidenhout, S.C., Gelderblom, W.C.A., Gorst-Allman, C., Horak, R.M., Marasas, W.F.O., Spiteller, G., Vleggaar, R., 1988. Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*. Journal of the Chemical Society, Chemical Communications 11, 743–745.

Biemond, P.C., Oguntade, O., Lava Kumar, P., Stomph, T.J., Termorshuizen. A.J., Struik, P.C., 2013. Does the informal seed system threaten cowpea seed health? Crop Protection 43, 166–174.

Blount, W.P., 1961. Turkey "X" disease. Turkeys 9:52, 55–58, 61, 77.

Brader, L., 1997. Introduction. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, vi.

Brader, L., 2002. Foreword. In: Fatokun, C.A., Tarawali, S.A., Singh, B.B., Kormawa, P.M., Tamo, M (Eds.). Challenges and Opportunities for Enhancing Sustainable Cowpea Production. Proceedings of the 3rd World Cowpea Conference, 4–8 September 2000, Ibadan, International Institute of Tropical Agriculture (IITA), Ibadan, 351–366.

Brandwagt, B.F., Mesbah, L.M., Laurent, P.L., Takken, F.T., Kneppers, T., Nijkamp, H.J.J., Hille, J., 1998. The interaction of *Alternaria alternata* f. sp. *lycopersici* and its AAL-toxins with tomato. In: Kohmoto, K. & Yoder, O.C., (Eds.). Molecular Genetics of Host-Specific Toxins in Plant Disease, Kluwer, Dordrecht, The Netherlands, 317–330.

Brandwagt, B.F., Mesbah, L.A., Takken, F.L.W., Laurent, P.L., Kneppers, T.J.A., Hille, J., Nijkamp, H.J.J., 2000. A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B_1. Proceedings of the National Academy of Sciences 97:9, 4961–4966.

Caldas, E.D., Sadilkova, K., Ward, B.L., Jones, A.D., Winter, C.K., Gilchrist, D.G., 1998. Biosynthetic studies of fumonisin B_1 and AAL toxins. Journal of Agricultural and Food Chemistry 46, 4734–4743

Chang S., Puryear J., Cairney J. 1993. A Simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113–116.

Chhabra, S.C., Mahunnah, R.L.A, Mshiu, E.N., 1990. Plants used in traditional medicine in eastern Tanzania. IV. Angiosperms (Mimosaceae to Papilionaceae). Journal of Ethnopharmacology 29, 295–323.

Chen, X., Laudeman, T.W., Rushton, P.J., Spraggins, T.A., Timko, M.P., 2007. CGKB: An annotation knowledge base for cowpea (*Vigna unguiculata* L.) methylation filtered genomic genespace sequences. BCM Bioinformatics 8:129.

Coulibaly, O., Lowenberg-De Boer, J., 2002. The economics of cowpea in West Africa. In: Fatokun, C.A., Tarawali, S.A., Singh, B.B., Kormawa, P.M., Tamo, M (Eds.). Challenges and Opportunities for Enhancing Sustainable Cowpea Production. Proceedings of the 3rd World Cowpea Conference, 4–8 September 2000, Ibadan, International Institute of Tropical Agriculture (IITA), Ibadan, 351–366.

Cowpea Genomics Knowledge Base (CGKB). [Online]. Available: < http://cowpeagenomics.med.virginia.edu/CGKB/> [Accessed 29 June 2015].

Craufurd, P.Q., Summerfield, R.J., Ellis, R.H., Roberts, E.H., 1997. Photoperiod, temperature, and the growth and development of cowpea. Walp. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 75–86.

Cullings, K.W., 1992. Design and testing of a plant-specific primer for ecological and evolutionary studies. Molecular Ecology 1, 233–240.

Danielsen, S., Jensen, D.F., 1998. Relationships between seed germination, fumonisin content, and *Fusarium verticillioides* infection in selected maize samples from different regions of Costa Rica. Plant Pathology 47, 609–614.

Davis, D.W., Oelke, E.A., Oplinger, E.S., Doll, J.D., Hanson, C.V., Putnam, D.H., 1991. Cowpea. Alternative Field Crops. [Online]. Available: <https://www.hort.purdue.edu/newcrop/afcm/cowpea.html> [Accessed 29 June 2015].

De Candolle, A. 1886. Origin of cultivated plants. Hafner Publishing Company. New York.

Delgado, A., Casas, J., Llebaria, A., Abad, J.L., Fabrias, G., 2002. Inhibitors of sphingolipid metabolism enzymes. Biochim Biophys Acta 1758, 1957–1977.

Desai, K., Cameron Sullards, M., Allegood, J., Wang, E., Schmelz, E.M., Hartl, M., Humpf, H-U, Liotta, D.C., Peng, Q., Merill, A.H., 2002. Fumonisins and fumonisin analogs as inhibitors of ceramide synthase and inducers of apoptosis. Biochimica et Biophysica Acta 1585, 188–192.

Desjardins, A.N., Hohn, T.M., 1997. Mycotoxins in plant pathogenesis. Molecular Plant-Microbe Interactions 10:2, 147–152.

Directorate Plant Production., 2014. Production guidelines for cowpeas. Department of Agriculture, Forestry and Fisheries, Pretoria. [Online]. Available: <http://www.daff.gov.vza/daffweb3/Portals/0/BrochuresandProductionguidelines/COWPEAPRODUCTION GUIDELINES2014.pdf > [Accessed 29 June 2015].

Doehlert, D.C., Knutson, D.A., Vesonder, R.F., 1994. Phytotoxic effects of fumonisin B_1 on maize seedling growth. Mycopathologia 127, 117–121.

Dombrink-Kurtzman, M.A., 2008. Economic aspects of mycotoxins in fruits and vegetables, In: Mycotoxins in Fruits and Vegetables, Barkai-Golan, R. & Nachman, P., (Eds.). Academic Press, New York, 27–44.

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19, 11–15.

Duke, S.O., Lydon, J., 1987. Herbicides from natural compounds. Weed Technology 1, 122–128.

Edite Bezerra da Rocha, M., Freire, F.C.O., Erlan Feitosa Maia, F., Izabel Florindo Guedes, M., Rondina, D., 2014. Mycotoxins and their effects on human and animal health. Food Control 36, 159–165.

Ehlers, J.D., Hall, A.E., 1997. Cowpea (*Vigna unguiculata* L. Walp.). Field Crops Research 53, 187–204.

Emechebe, A.M., Florini, D.A., 1997. Shoot and pod diseases of cowpea induced by fungi and bacteria. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 176–192.

Emechebe, A.E., McDonald, D., 1979. Seed-borne pathogenic fungi and bacteria of cowpea in Northern Nigeria. Tropical Pest Management 25:4, 401–404.

Fery, R.L., 2002. New opportunities in *Vigna*. In: Janick, J., Whipkey, A., (Eds.). Trends in new crops and new uses. American Society Horticultural Services Press (ASHS Press), Alexandria, USA, 424–428.

Food and Agriculture Organization Corporate Statistical Database (FAOSTAT)., 2015. [Online]. Available: < http://faostat3.fao.org > [Accessed 29 June 2015].

Folic Acid., 2015. [Online]. Available: <http://www.cdc.gov/ncbddd/folicacid/index.html> [Accessed 22 June 2015].

Gazendam, I., 2012. Identification and functional evaluation of a drought-induce “late embryogenesis abundant” gene from cowpea plants. PhD Thesis, University of Pretoria, Pretoria, South Africa.

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

Golzarian, M.R., Frick, R.A., Rajendran, K., Berger, B., Roy, S., Tester, M., Lun, D.S., 2011. Accurate inference of shoot biomass from high-throughput images of cereal plants. Plant Methods 7:2.

Gutierrez-Najera, N., Munoz-Clares, R.A., Palacios-Bahena, S., Ramirez, J., Sanchez-Nieto, S., Plasencia, J., Gavilanes-Ruiz., M., 2005. Fumonisin B_1, a sphingoid toxin, is a potent inhibitor of the plasma membrane H^+-ATPase. Planta 221, 589–596.

Hall, A.E., 2004. Breeding for adaptation to drought and heat in cowpea. European Journal of Agronomy 21, 447–454.

Hampton, R.O., Thottappilly, G., Rossel, H.W., 1997. Viral diseases of cowpea and their control by resistance-conferring genes. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 30–49.

Hedge, D.G., Hiremath, R.V., 1987. Seed mycoflora of cowpea and its control by fungicides. Seed Research 15:1, 60–65.

Hoagland, R.E., Boyette, C.D., Weaver, M.A., Abbas, H.K., 2007. Bioherbicicdes: Research and risks. Toxin Reviews 26, 313–342.

Houssou, P.A., Ahohuendo, B.C., Fandohan, P., Kpodo, K., Hounhouigan, D.J., Jakobsen, M., 2009. Natural infection of cowpea (*Vigna unguiculata* (L.) Walp.) by toxigenic fungi and mycotoxin contamination in Benin, West Africa. Journal of Stored Products Research 45, 40–44.

International Agency for Research of Cancer (IARC), 1993. Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Monograph 56, Lyon, France.

International Seed Testing Association (ISTA), 1999. International Rules for Seed Testing. Seed Science and Technology 27: Supplement, 333.

Jackai, L.E.N., Adalla, C.B., 1997. Pest management practices in cowpea: A review. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 240–258.

Joyce, C.M., Steitz, T.A., 1994. Function and structure relationships in DNA polymerases. Annual Review of Biochemistry 63, 777–822.

Kafua, L.E., 2011. Fumonisin production by and biological control of *Fusarium* species associated with cowpea seed. MSc Dissertation, University of Pretoria, Pretoria, South Africa.

Kenfield, D., Bunkers, G., Strobel, G.A., Sugawara, F., 1988. Potential new herbicides: Phytotoxins from plant pathogens. Weed Technology 2, 519–524.

Kim, B., Nam, H., Kim, S., Kim, S., Chang, Y., 2003. Normalization of reverse transcription quantitative-PCR with housekeeping genes in rice. Biotechnology Letters 25, 1869–1872.

Kim, H.J., Qiao, Q., Toop, H.D., Morris, J.C., Don, A.S., 2012. A fluorescent assay for ceramide synthase activity. Journal of Lipid Research 53:8, 1701–1707.

Knutson, C.A., 1993. Reaction patterns of maize alpha-amylases with soluble starch, granular starch and malto-oligosaccharides. Cereal Chemistry 70, 534–538.

Kotze, R.G., 2012. Phytotoxic effects of fumonisin B_1 on cowpea (*Vigna unguiculata* (L.) Walp). BSc (Hons) Mini-dissertation, University of Pretoria, Pretoria, South Africa.

Kritzinger, Q., 2004. Antimicrobial activity and fumonisins associated with cowpea (*Vigna unguiculata*). PhD Thesis, University of Pretoria, Pretoria, South Africa.

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

Kritzinger, Q., Aveling, T.A.S., Van der Merwe, C., 2006. Phytotoxic effect of fumonisin B_1 on cowpea seed. Phytoparasitica 34:2, 178–186.

Kroschel, J., Elzein, A., 2004. Bioherbicidal effect of fumonisin B_1, a phytotoxic metabolite naturally produced by *Fusarium nygamai*, on parasitic weeds of the genus *Striga*. Biocontrol Science and Technology 14, 117–128.

Kumar, G.N., Iyer, S., Knowles, N.R., 2007. Extraction of RNA from fresh, frozen, and lyophilized tuber and root tissues. Journal Agricultural Food Chemistry 55:5, 1674–1678.

Lamprecht, S.C., Marasas, W.F.O., Alberts, J.F., Cawood, M.E., Gelderblom, W.C.A., Shephard, G.S., Thiel, P.G., Calitz, F.J., 1994. Phytotoxicity of fumonisins and TA-toxin to corn and tomato. The American Phytopathology Society 94:4, 383–391.

Langyintuo, A.S., Lowenberg-DeBoer, J., Faye, M., Lambert, D., Ibro, G., Moussa, B., Kergna, A., Kushwaha, S., Musa, S., Ntoukam, G., 2003. Cowpea supply and demand in West Africa. Field Crops Research 82, 215–231.

Lamitina Lab Protocols 2007. [Online]. Available: < http://www.med.upenn.edu/lamitinalab /documents/BacterialTransformation.pdf> [Accessed 23 April 2015].

Logemann, J., Schell, J., Willmitzer, L., 1987. Improved method for the isolation of RNA from plant tissues. Analytical Biochemistry 163, 16–20.

Lynch, D.V., 1999. Enzymes of sphingolipid metabolism in plants. Methods in Enzymology 311, 130–149.

Lynch, D.V., Dunn, T.M., 2004. An introduction to plant sphingolipids and a review of recent advances in understanding their metabolism and function. New Phytologist 161, 677–702.

Madamba, R., Grubben, G.J.H., Asante, I.K., Akromah, R., 2006. *Vigna*. In: Brink, M., Belay, G., (Eds). Plant Resources of Tropical Africa 1. Cereals and pulses. PROTA foundation, Wageningen, The Netherlands, 221–229.

Mandala, S.M., Frommer, B.R., Thorton, R.A., Kurtz, M.B., Young, N.M., Cabello, M.A., Genilloud, O., Liesch, J.M., Smith, J.L., Horn, W.S., 1994. Inhibition of serine palmitoyl-transferase activity by lipoxamycin. The Journal of Antibiotics 47:3, 376–379.

Manning, K., 1991. Isolation of nucleic acid from plants by differential solvent precipitation. Analytical Biochemistry 195, 45–50.

Marasas, W.F.O., van Rensburg, S.J., Mirocha, C.J., 1979. Incidence of *Fusariu*m species and the mycotoxins, deoxynivalenol and zearalenone, in corn produced in esophageal cancer areas in Transkei. Journal of Agricultural Food Chemistry 27:6, 1108–1112

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

Marion, J., Bach, L., Bellec, Y., Meyer, C., Gissot, L., Faure, J-D., 2008. Systematic analysis of protein subcellular localization and interaction using high-throughput transient transformation of *Arabidopsis* seedlings. Plant Journal 56, 169–179.

Markham, J.E., Molino, D., Gissot, L., Bellec, Y., Hématy, K., Marion, J., Belcram, K., Palauqui, J-C., Satiat-JeuneMaître, B., Faure, J-D., 2011. Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in *Arabidopsis*. The Plant Cell 23, 2362–2378.

Marone, M., Mozetti, S., De Ritis, D., Pierelli, L., Scambia, G., 2001. Semiquantitative RT-PCR analysis to assess the expression levels of multiple transcripts from the same sample. Biological Procedures Online 3:1, 19–25.

Merrill, A.H., Cameron Sullards, M., Wang, E., Voss., K.A., Riley, R.T., 2001. Sphingolipid metabolism: Roles in signal transduction and disruption by Fumonisin. Enviromental Health Perspectives 109:2, 283–289.

Mpiana, P.T., Tshibangu, D.S.T., Shetonde, O.M, Ngbolua. K.N., 2007. In vitro antidrepanocytary actvity (anti-sickle cell anemia) of some congolese plants. Phytomedicine 14, 192–195.

Nadubinska. M., Ritieni, A., Moretti, A., Srobarova, A., 2003. Chlorophyll content in maize plants after treatment with fusariotoxins. Biologia 58:1, 115–119.

National Centre for Biotechnology Information (NCBI). [Online]. Available: <http://www.ncbi.nlm.nih.gov/> [Accsessed 26 May 2015].

Ng, N.Q., Marechal, R., 1985. Cowpea taxonomy, origin and germplasm. In: Singh, S.R., Rachie, K.O., (Eds.). Cowpea Research, Production and Utilization. John Wiley and Sons, Chichester, United Kingdom, 11–21.

Norrad, W.P., Voss, K.A., 1994. Toxicity and role of fumonisins in animal disease and human esopageal cancer. Journal of Food Protection 57, 522–527.

Padulosi. S., 1993. Genetic diversity, taxonomy and ecogeographical survey of the wild relatives of cowpea (*Vigna unguiculata* (L), Walpers. PhD Thesis. Universite Catholique de Louvain-la-Neuve, Belgium.

Padulosi, S., Ng, N.Q., 1997. Origin, taxonomy and morphology of *Vigna unguiculata* (L.) Walp. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 1–12.

Pant, K.C., Chandel, K.P.S., Joshi, B.S., 1982. Analysis of diversity in Indian Cowpea Genetic Resources. SABRO 14, 103–111.

Pata, M.O., Hannun, Y.A., Ng, C.K., 2009. Plant sphingolipids: decoding the enigma of the Sphinx. New Phytologist 185, 611–630.

Phytozome v10.2. [Online]. Available: <http://phytozome.jgi.doe.gov/pz/portal.html> [Accsessed 26 May 2015].

Pitt, J.I., 2000. Toxigenic fungi and mycotoxins. British Medical Bulletin 56:1, 184–192.

Plumlee, K.H., Galey, F.D., 1994. Neurotoxic mycotoxins: a review of fungal toxins that cause neurological disease in large animals. Journal of Veterinary Internal Medicine 8:1, 49–54.

Purity Ratios. [Online]. Available :<http://www.nanodrop.com/TechResCenter-PurityRatios. aspx > [Accessed 26 May 2015].

Quin, F.M., 1997. Introduction. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, xi–xv.

Quinn, J., 1999. Cowpea, a versatile legume for hot, dry conditions. Jefferson Institute. [Online]. Available: <http://www.hort.purdue.edu/newcrop/articles/ji-cowpea.html> [Accessed 29 June 2015].

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

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

Riley, R.T., Voss, K.A., Yoo, H-S., Gelderblom, W.C.A., Merrill, A.H., 1994. Mechanism of fumonisin toxicity and carcinogenesis. Journal of Food Protection 57, 638–645.

Roberts, P.A., Ehlers., J.D., Hall., A.E., Matthews., W.C., 1997. Characetrization of new resistance to root-knot nematodes in cowpea. Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, ix–xii.

Rodrigues, A.A.C., Menezes, M., Identification and pathogenic characterization of endophytic *Fusarium* species from cowpea seeds. Mycopathologia 159, 79–85.

Schmelz, E.M., Dombrink-Kurtzman, M.A., Roberts, P.C., Kozutsumi, Y., Kawasaki, T., Merrill, A.H., 1998. Induction of apoptosis by fumonisin B_1 in HT29 cells is mediated by the accumulation of endogenous free sphingoid bases. Toxicology and Applied Pharmacology 148:2, 252–260

Schultz, C., Gilson, P., Oxley, D., Youl, J., Bacic, A., 1998. GPI-anchors on arabinogalactan-proteins: implications for signalling in plants. Trends in Plant Science 3:11, 426–431.

Seenappa, M., Keswani, C.L., Kundya, T.M., 1983. *Aspergillus* infection and aflatoxin production in some cowpea (*Vigna unguiculata* (L.) Walp) line Tanzania. Mycopathologia 83, 103–106.

Shah, N.H., Khan, M.I., Azam, M.F., 1992. Seed mycoflora of cowpea and its control with extract of *Argemone mexicana*. Bioved 3:2, 167–168.

Shama, S., Raghunathan, A.N., Shetty, H.S., 1988. Seed mycoflora of cowpea (*Vigna unguiculata* L. Walp) and their pathogenic importance. Seed Science and Technology 16:2, 541–548.

Shi, L., Bielawski, J., Mu, J., Dong, H., Teng, C., Chang, J., Yang, X., Tomishige, N., Hanada, K., Hannun, Y.A., Zuo, J., 2007. Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. Cell Research 17, 1030–1040.

Siddhuraju, P., Becker, K., 2007. The antioxidant and free radical scavenging activities of processed cowpea (*Vigna unguiculata* (L.) Walp.) seed extracts. Food Chemistry 101:1, 10–19.

Singh, I, Chohan, J.S., 1974. Seed-borne fungi on cowpea (*Vigna sinensis*). Indian Phytopathology 27, 239–240.

Singh, B.B., Chambliss, O.L., Sharma, B., 1997. Recent advances in cowpea breeding. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 30–49.

Singh, B.B., Emechebe, A.M., 1997. Advances in research on cowpea *Striga* and *Alectra*. In: Singh, B.B., Mohan Raj, D.R., Dashiell, K.E., Jackai, L.E.N., (Eds.). Advances in Cowpea Research. Co-publication of International Institute of Tropical Agriculture (IITA) and Japan International Research Centre for Agricultural Sciences (JIRCAS), IITA, Ibadan, Nigeria, 215–224.

Singh, B.B., Ehlers, J.D., Sharma, B., Freire Filho, F.R., 2002. Recent progress in cowpea breeding. In: Fatokun, C.A., Tarawali, S.A., Singh, B.B., Kormawa, P.M., Tamo, M (Eds.). Challenges and Opportunities for Enhancing Sustainable Cowpea Production. Proceedings of the 3rd World Cowpea Conference, 4–8 September 2000, Ibadan, International Institute of Tropical Agriculture (IITA), Ibadan, 22–40.

Singh, B.B., Ajeigbe, H.A., Tarawali, S.A., Fernandez-Rivera, S., Abubakar, M., 2003. Improving the production and utilization of cowpea as food and fodder. Field Crops Research 84, 169–177.

Singh, B.B., 2005. Cowpea (*Vigna unguiculata* (L.) Walp). In: Singh R.J., Jauhar P.P., (Eds.). Genetic Resources, Chromosome Engineering and Crop Improvement. Volume 1, CRC Press, Boca Raton, FL, USA, 117–162

Singh S.R., Allen D.J., 1979. Cowpea pests and diseases, Manual Series No. 2. International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.

Smith J.E., Korsten L., Aveling T.A.S., 1999. Evaluation of seed treatments for reducing *Colletotrichum dematium* on cowpea seed. Seed Science and Technology 27, 591-598.

Soriano, J.M., González, L., Catalá, A.I., 2005. Mechanism of action of sphingolipids and their metabolites in the toxicity of fumonisin B_1. Progress in Lipid Research 44, 345–356.

Sperling, P., Heinz, E., 2003. Plant sphingolipids: Structural diversity, biosynthesis, first genes and functions. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1632, 1–15.

Sreerama, Y.N., Sashikala, V.B., Pratape, V.M., Singh, V., 2012. Nutrients and antinutrients in cowpea and horse gram flours in comparison to chickpea flour: Evaluation of their flour functionality. Food Chemistry 131:2, 462–468.

Steele, W.M., 1976. Cowpeas, *Vigna unguiculata*. In: Simmonds, N.W., (Ed.). Evolution of Crop Plants. Longmans, London, United Kingdom, 183–185.

Steyn, P.S., 1995. Mycotoxins, general view, chemistry and structure. Toxicology Letters 82–83, 843–851.

Ternes, P., Feussner, K., Werner, S., Lerche, J., Iven, T., Heilmann, I., Riezman, H., Feussner, I., 2011. Disruption of the ceramide synthase *LOH1* causes spontaneous cell death in *Arabidopsis thaliana*. New Phytologist 192:4, 841–854.

Timko, M.P., Ehlers, J.D., Roberts, P.A., 2007. Cowpea. In: Kole, C., (Ed.). Genome Mapping and Molecular Breeding in Plants: Pulses, Sugar and Tuber Crops. Springer Verlag, Berlin, Heidelberg, 49–67.

Timko, M.P., Rushton, P.J., Laudeman, T.W., Bokowiec, M.T., Chipumuro, E., Cheung, F., Town, C.D., Chen, X., 2008. Sequencing and analysis of the gene-rich space of cowpea. BCM Genomics 9:103.

Timko, M.P., Singh, B.B., 2008. Cowpea, a multifunctional legume. In: Moore P.H., Ming, R., (Eds.). Genomics of Tropical Crop Plants, Springer Science and Business Media, LLC, New York, 227–258.

The Plant List. [Online]. Available: <http://www.theplantlist.org/> [Accessed 29 June 2015].

Ueno, Y., Hsieh, D.P.H., 1985. The toxicology of mycotoxins. Critical Reviews in Toxicology 14, 99–132.

Uzogara, S.G., Ofuya, Z.M., 1992. Processing and utilization of cowpeas in developing countries: a review. Journal of Food Processing and Preservation. 16:2, 105–147.

Valenzuela, H., Smith, J., 2002. Cowpea. Cooperative Extension Service, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa.

Van Asch, M.A.J., Rijkenberg, F.H.J., Countinho, T.A., 1992. Phytotoxicity of fumonisin B_1, moniliformin, and T-2 toxin to corn callus cultures. Phytopathology 82, 1330–1332.

Van Duivenbooden, N., Abdoussalam, S., Ben Mohamed, A., 2002. Impact of climate change on agricultural production in the sahel – part 2. Case study for groundnut and cowpea in Niger. Climatic Change 54:3, 349–368.

Van Wyk, B-E., Gericke, N., 2000. People's plants: A guide to useful plants of Southern Africa. Briza Publications, Pretoria, 191–192.

Varga, J., Kocsube, S., Suri, K., Sxigeti, G., Szekeres, A., Varga, M., Toth, B., Bartok, T., Fumonisin contamination and fumonisin producing black *Aspergilli* in dried vine fruits of different origin. International Journal of Food Microbiology 143:3, 143–149.

Vavilov, N.I., 1926. Studies on the origin of cultivated plants. Bulletin of Applied Botany and Plant Breeding 16, 1–248.

Voss, K.A., Chamberlain, W.J., Bacon, C.W., Norred, W.P., 1993. A preliminary investigation on renal and hepatic toxicity in rats fed purified fumonisin B_1. Natural Toxins 1, 222–228.

Voss, K.A., Howard, P.C., Riley, R.T., Sharma, R.P., Bucci, T.J., Lorentzen, R.J., 2002. Carcinogenicity and mechanism of action of fumonisin B_1: A mycotoxin produced by *Fusarium moniliforme* (*=F. verticillioides*). Cancer Detection and Prevention 26, 1–9.

Voss, K.A., Smith, G.W., Haschek, W.M., 2007. Fumonisin: Toxicokinetics, mechanism of action and toxicity. Animal Feed Science and Technology 127, 299–325.

Wang, E., Merrill, A.H., 2000. Ceramide synthase. Methods in Enzymology 311, 15–21.

Wang, S., Lui, S., Yang, L., Shi, R., Sun, G., 2013. Effect of fumonisin B_1 on the cell cycle of normal human liver cells. Molecular Medicine Reports 7:6, 1970–1976.

Warrag, M.O.A., Hall, A.E., 1984. Reproductive responses of cowpea (*Vigna unguiculata* (L.) Walp.) to heat stress. II. Responses to night air temperature. Field Crop Research 8, 17–33.

Williams, L.D., Glenn, A.E., Zimeri, A.M., Bacon, C.W., Smith, M.A., Riley, R.T., 2007. Fumonisin disruption of ceramide biosynthesis in maize roots and the effects on plant development and *Fusarium verticillioides* induced seedling disease. Journal of Agricultural Food Chemistry 55:8, 2937–2946.

Wilson, D., Cooper, J.P., 1969. Effect of light intensity during growth on leaf anatomy and subsequent light-saturated photosynthesis among contrasting *Lolium* genotypes. New Phytologist 68, 1125–1135.

Winter, E., Ponting, C.P., 2002. TRAM, LAG1 and CLN8: Members of a novel family of lipid sensing domains? Trends Biochemical Sciences 27, 381–383.

Zaidi, R.K., 2012. Pathogenic response of seed mycoflora associated with cowpea, *Vigna unguiculata*. Arichives of Phytopathology and Plant Protection 45:15, 1790–1795.

Zhang, W.T., Yang, J.K., Yuan, T.Y., Zhou, J.C., 2007. Genetic diversity and phylogeny of indigenous rhizobia from cowpea (*Vigna unguiculata* (L.) Walp.). Biology and Fertility of Soil 44, 201–210.

Zohri, A.A., 1993. Studies on some cowpea cultivars: II-Suitability for *Aspergillus flavus* growth and aflatoxin production. Qatar University Science Journal 13:1, 54–62.

Zonno, M.C., Vurro, M., 2002. Inhibition of germination of *Orobanche ramosa* seeds by Fusarium toxins. Phytoparasitica 30:5, 519–524.

CHAPTER 7

Appendix

1	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	D-R4-1	D-R4-8	D-R4-15	D-R4-22	B-R2-3	B-R2-10	B-R2-17	B-R2-24	C-R3-5	C-R3-12	C-R3-19		A-R1-7	A-R1-14	A-R1-21
B	D-R4-2	D-R4-9	D-R4-16	D-R4-23	B-R2-4	B-R2-11	B-R2-18	B-R2-25	C-R3-6	C-R3-13	C-R3-20	A-R1-1	A-R1-8	A-R1-15	A-R1-22
C	D-R4-3	D-R4-10	D-R4-17	D-R4-24	B-R2-5	B-R2-12	B-R2-19		C-R3-7	C-R3-14	C-R3-21	A-R1-2	A-R1-9	A-R1-16	A-R1-23
D	D-R4-4	D-R4-11	D-R4-18	D-R4-25	B-R2-6	B-R2-13	B-R2-20	C-R3-1	C-R3-8	C-R3-15	C-R3-22	A-R1-3	A-R1-10	A-R1-17	A-R1-24
E	D-R4-5	D-R4-12	D-R4-19		B-R2-7	B-R2-14	B-R2-21	C-R3-2	C-R3-9	C-R3-16	C-R3-23	A-R1-4	A-R1-11	A-R1-18	A-R1-25
F	D-R4-6	D-R4-13	D-R4-20	B-R2-1	B-R2-8	B-R2-15	B-R2-22	C-R3-3	C-R3-10	C-R3-17	C-R3-24	A-R1-5	A-R1-12	A-R1-19	
G	D-R4-7	D-R4-14	D-R4-21	B-R2-2	B-R2-9	B-R2-16	B-R2-23	C-R3-4	C-R3-11	C-R3-18	C-R3-25	A-R1-6	A-R1-13	A-R1-20	

2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	B-R1-1	B-R1-8	B-R1-15	B-R1-22	E-R4-3	E-R4-10	E-R4-17	E-R4-24	D-R3-5	D-R3-12	D-R3-19		C-R4-7	C-R4-14	C-R4-21
B	B-R1-2	B-R1-9	B-R1-16	B-R1-23	E-R4-4	E-R4-11	E-R4-18	E-R4-25	D-R3-6	D-R3-13	D-R3-20	C-R4-1	C-R4-8	C-R4-15	C-R4-22
C	B-R1-3	B-R1-10	B-R1-17	B-R1-24	E-R4-5	E-R4-12	E-R4-19		D-R3-7	D-R3-14	D-R3-21	C-R4-2	C-R4-9	C-R4-16	C-R4-23
D	B-R1-4	B-R1-11	B-R1-18	B-R1-25	E-R4-6	E-R4-13	E-R4-20	D-R3-1	D-R3-8	D-R3-15	D-R3-22	C-R4-3	C-R4-10	C-R4-17	C-R4-24
E	B-R1-5	B-R1-12	B-R1-19		E-R4-7	E-R4-14	E-R4-21	D-R3-2	D-R3-9	D-R3-16	D-R3-23	C-R4-4	C-R4-11	C-R4-18	C-R4-25
F	B-R1-6	B-R1-13	B-R1-20	E-R4-1	E-R4-8	E-R4-15	E-R4-22	D-R3-3	D-R3-10	D-R3-17	D-R3-24	C-R4-5	C-R4-12	C-R4-19	
G	B-R1-7	B-R1-14	B-R1-21	E-R4-2	E-R4-9	E-R4-16	E-R4-23	D-R3-4	D-R3-11	D-R3-18	D-R3-25	C-R4-6	C-R4-13	C-R4-20	

3	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	E-R3-1	E-R3-8	E-R3-15	E-R3-22	C-R1-3	C-R1-10	C-R1-17	C-R1-24	A-R4-5	A-R4-12	A-R4-19		D-R2-7	D-R2-14	D-R2-21
B	E-R3-2	E-R3-9	E-R3-16	E-R3-23	C-R1-4	C-R1-11	C-R1-18	C-R1-25	A-R4-6	A-R4-13	A-R4-20	D-R2-1	D-R2-8	D-R2-15	D-R2-22
C	E-R3-3	E-R3-10	E-R3-17	E-R3-24	C-R1-5	C-R1-12	C-R1-19		A-R4-7	A-R4-14	A-R4-21	D-R2-2	D-R2-9	D-R2-16	D-R2-23
D	E-R3-4	E-R3-11	E-R3-18	E-R3-25	C-R1-6	C-R1-13	C-R1-20	A-R4-1	A-R4-8	A-R4-15	A-R4-22	D-R2-3	D-R2-10	D-R2-17	D-R2-24
E	E-R3-5	E-R3-12	E-R3-19		C-R1-7	C-R1-14	C-R1-21	A-R4-2	A-R4-9	A-R4-16	A-R4-23	D-R2-4	D-R2-11	D-R2-18	D-R2-25
F	E-R3-6	E-R3-13	E-R3-20	C-R1-1	C-R1-8	C-R1-15	C-R1-22	A-R4-3	A-R4-10	A-R4-17	A-R4-24	D-R2-5	D-R2-12	D-R2-19	
G	E-R3-7	E-R3-14	E-R3-21	C-R1-2	C-R1-9	C-R1-16	C-R1-23	A-R4-4	A-R4-11	A-R4-18	A-R4-25	D-R2-6	D-R2-13	D-R2-20	

4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	E-R2-1	E-R2-8	E-R2-15	E-R2-22	A-R3-3	A-R3-10	A-R3-17	A-R3-24	D-R1-5	D-R1-12	D-R1-19		B-R4-7	B-R4-14	B-R4-21
B	E-R2-2	E-R2-9	E-R2-16	E-R2-23	A-R3-4	A-R3-11	A-R3-18	A-R3-25	D-R1-6	D-R1-13	D-R1-20	B-R4-1	B-R4-8	B-R4-15	B-R4-22
C	E-R2-3	E-R2-10	E-R2-17	E-R2-24	A-R3-5	A-R3-12	A-R3-19		D-R1-7	D-R1-14	D-R1-21	B-R4-2	B-R4-9	B-R4-16	B-R4-23
D	E-R2-4	E-R2-11	E-R2-18	E-R2-25	A-R3-6	A-R3-13	A-R3-20	D-R1-1	D-R1-8	D-R1-15	D-R1-22	B-R4-3	B-R4-10	B-R4-17	B-R4-24
E	E-R2-5	E-R2-12	E-R2-19		A-R3-7	A-R3-14	A-R3-21	D-R1-2	D-R1-9	D-R1-16	D-R1-23	B-R4-4	B-R4-11	B-R4-18	B-R4-25
F	E-R2-6	E-R2-13	E-R2-20	A-R3-1	A-R3-8	A-R3-15	A-R3-22	D-R1-3	D-R1-10	D-R1-17	D-R1-24	B-R4-5	B-R4-12	B-R4-19	
G	E-R2-7	E-R2-14	E-R2-21	A-R3-2	A-R3-9	A-R3-16	A-R3-23	D-R1-4	D-R1-11	D-R1-18	D-R1-25	B-R4-6	B-R4-13	B-R4-20	

5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	A-R2-1	A-R2-8	A-R2-15	A-R2-22	C-R2-3	C-R2-10	C-R2-17	C-R2-24	B-R3-5	B-R3-12	B-R3-19		E-R1-7	E-R1-14	E-R1-21
B	A-R2-2	A-R2-9	A-R2-16	A-R2-23	C-R2-4	C-R2-11	C-R2-18	C-R2-25	B-R3-6	B-R3-13	B-R3-20	E-R1-1	E-R1-8	E-R1-15	E-R1-22
C	A-R2-3	A-R2-10	A-R2-17	A-R2-24	C-R2-5	C-R2-12	C-R2-19		B-R3-7	B-R3-14	B-R3-21	E-R1-2	E-R1-9	E-R1-16	E-R1-23
D	A-R2-4	A-R2-11	A-R2-18	A-R2-25	C-R2-6	C-R2-13	C-R2-20	B-R3-1	B-R3-8	B-R3-15	B-R3-22	E-R1-3	E-R1-10	E-R1-17	E-R1-24
E	A-R2-5	A-R2-12	A-R2-19		C-R2-7	C-R2-14	C-R2-21	B-R3-2	B-R3-9	B-R3-16	B-R3-23	E-R1-4	E-R1-11	E-R1-18	E-R1-25
F	A-R2-6	A-R2-13	A-R2-20	C-R2-1	C-R2-8	C-R2-15	C-R2-22	B-R3-3	B-R3-10	B-R3-17	B-R3-24	E-R1-5	E-R1-12	E-R1-19	
G	A-R2-7	A-R2-14	A-R2-21	C-R2-2	C-R2-9	C-R2-16	C-R2-23	B-R3-4	B-R3-11	B-R3-18	B-R3-25	E-R1-6	E-R1-13	E-R1-20	

Figure 7.1. A randomised block design utilizing five seedling trays were used in total to accommodate four replicates of five treatments. For example A-R1-1: A is treatement, R1 is replicate 1; 1 is the seedling number. Non-imbibed control (A) – red; Imbibed control (B) – yellow; 5 mg/L FB1 (C) – green; 20 mg/L FB1 (D) – blue; 40 mg/L FB1 (E) – purple.

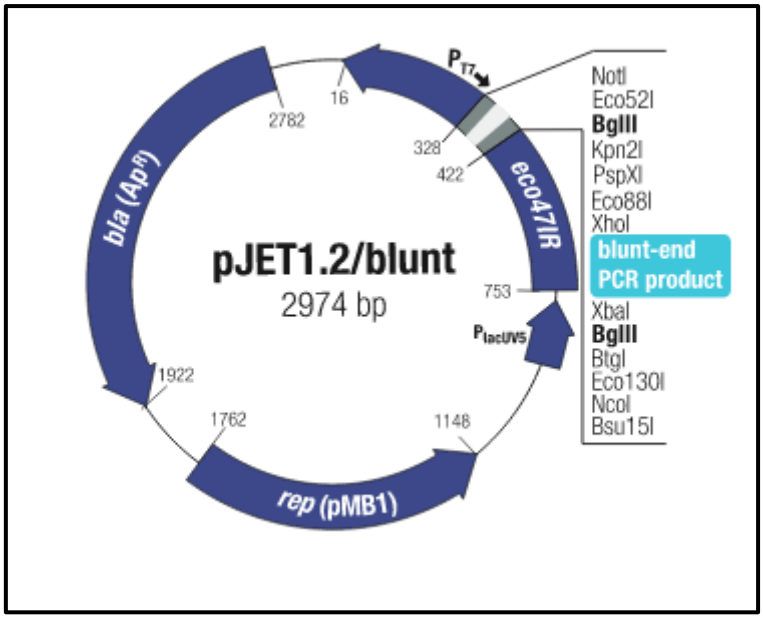


Figure 7.2. pJET1.2/blunt cloning vector map (Thermo Scientific).

Table 7.1. Root samples with poor quality and low concentration RNA isolated using RNeasy Plant Mini Kit, (Qiagen).

Root Sample #	Nucleic Acid Concentration (ng/µl)	260/280	260/230
4	6.9	1.67	0.04
8	41.7	1.71	0.57
12	15.8	1.72	0.48
16	132	1.83	0.75
20	24.2	1.83	0.53
24	165.8	1.56	0.48

Table 7.2. RNA concentration and 260/280 and 260/230 values of control and treated samples.

Sample #	Nucleic Acid Concentration (ng/µl)	260/280	260/230
1	413.1	2.15	2.27
2	223.0	2.23	1.97
3	448.8	2.19	2.14
4	243.5	2.18	2.05
5	473.7	2.13	2.07
6	416.1	2.15	2.04
7	535.4	2.20	2.22
8	324.8	2.20	1.93
9	416.2	2.20	2.04
10	531.9	2.20	2.15

11	484.2	2.22	2.24
12	288.6	2.21	2.04
13	478.6	2.11	1.97
14	351.3	2.16	2.12
15	543.8	2.16	2.11
16	444.9	2.16	2.02
17	353.0	2.23	1.97
18	228.4	2.22	1.89
19	280.4	2.23	1.79
20	386.3	2.16	2.03
21	277.6	2.18	1.86
22	105.7	2.29	1.58
23	472.8	2.14	1.95
24	298.1	2.17	2.00

Table 7.3. The partial nucleotide and amino acid sequences of cowpea ceramide synthase gene fragment.

Nucleotide sequence
5'-GGAATCAGCTTGGAAATGTGTTTATTATCTTTCTGCTGAGGTTCTGGCTCTCTA TGTTACGTATGACGAACCCTGGTTTACCAACACGAGAAACTTTTGGGTGGGGCCAGGGTCTCAGGTCTGGCCAGATCAAAAGACCAAGTTGAAATTGAAGGCGGTGTATATGTATGCTGCTGGGTTTTACTCATACTCCATTTTTGCTTTAATATTTTGGGAAACCAGGCGCTCCGACTTTGGGGTCTCCATGAGTCATCACGTTGCTACTGTAATTCTCATTGTGCTGTCTTACATTTTTAGGTTTGCTCGTGTTGGATCGGTTGTGTTAGCAATTCATGATGCTAGCGATGTGTTTCTGGAGATAGGGAAAATGTCCAAATACAGTGGTGCTGAAACTGTGGCTAGCTTTGCTTTTATTCTATTTGTTTTATCCTGGATCATATTGCGCCTCATTTACTACC-3'
Amino acid sequence
N-AGFYSYSIFALIFWETRRSDFGVSMSHHVATVILIVLSYIFRFARVGSVVLAIHDAS DVFLEIGKMSKYSGAETVASFAFILFVLSWIILRLIYY-C