Fungal diversity of *Tylosema esculentum* ((Burch.) A.Schreib.) and *T. fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.) seeds, leaves and tubers from South Africa

by

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Declaration

I, Taryn Jean Armfield declare that the dissertation, which I hereby submit for the degree Master of Science: Plant Science 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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Summary

The marama bean, encompassing both *Tylosema esculentum* ((Burch.) A.Schreib.) and *Tylosema fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.), has been identified as an orphan crop of southern Africa with a great potential for commercialisation. The potential of the marama bean to be developed into a valuable crop poses a question regarding possible biotic constraints, including fungal pathogens and mycotoxigenic fungi. This study investigates the mycoflora and mycotoxins associated with marama bean in South Africa, as well as the potential pathogenicity and mycotoxigenicity of various fungal species on the marama bean. Various fungal isolates were obtained from marama bean leaves, seeds and tubers collected from eight locations in South Africa, ranging from the Gauteng and North West to Limpopo and Mpumalanga provinces. The various fungal isolates were identified using a dual morphological and molecular approach. A total of 116 species spanning 27 families were molecularly identified, several of which have been previously reported on marama beans in Namibia, including various *Alternaria*, *Epicoccum*, *Fusarium*, *Penicillium*, *Phoma* and *Rhizopus* species. Other notable fungal genera isolated from the South African marama bean include *Aspergillus*, *Lasiodiplodia*, *Neofusicoccum*, *Botryosphaeria*, *Chaetomium*, *Diaporthe*, *Bipolaris*, *Pestalotiopsis*, *Neopestalotiopsis*, and *Talaromyces*. Multi-mycotoxin analysis was performed on the plant material as well as on isolated fungal cultures *in vitro* using maize patty cultures, focusing on *Aspergillus*, *Fusarium* and *Penicillium*. Several of the species analysed indicated the potential to produce aflatoxin

B_1 and fumonisins B_1, B_2 and B_3 *in vitro*, however, no mycotoxins were detected in any of the collected plant material. Twenty of the fungal isolates (spanning thirteen different species) were selected to investigate the potential of the fungal species to be pathogenic to the marama bean, and subjected to detached leaf assays and further pathogenicity analysis. A disease symptom rating system was established to account for the various symptoms produced by the different fungal species. Black leaf spots, leaf lesions, stem lesions and leaf discoloration were the most prevalent symptoms observed. *Lasiodiplodia pseudotheobromae, Botryosphaeria dothidea, Neofusicoccum parvum*, *Bipolaris* sp., and *Diaporthe rhusicola* were the top five fungal species with the highest disease rating scores after the five-week period. However, *Pestalotiopsis maculans* was predicted to be pathogenic over a longer time period. Koch's postulates confirmed that eleven of the thirteen fungal species were re-isolated after the pathogenicity trial, with the exceptions being *Colletotrichum fructicola* and the *Fusarium solani* species complex isolates. Multi-mycotoxin analysis of the seedlings indicated that zearalenone was detected in the *Alternaria alternata*, *F. fujikuroi* and *F. oxysporum* species complex treatments. In conclusion, an unexpectedly large diversity of fungal species was found to be associated with the marama bean in South Africa, several of which have the potential to be pathogenic to the marama bean and produce mycotoxins *in vitro* and *in vivo*. Further studies would be required to determine the extent of the pathogenicity of the various fungal species, as well as investigating under what conditions the pathogens would thrive and produce mycotoxins on the plants, thereby identifying future obstacles for marama bean cultivation and commercialisation.

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List of Abbreviations

Organisations:

ARC	-	Agricultural Research Council, South Africa
BCA	-	Botswana College of Agriculture
BODATSA	-	Botanical Database of Southern Africa
BUAN	-	Botswana University of Agricultural and Natural Sciences
CAF	-	Central Analytical Facility, Stellenbosch, South Africa
CSIR	-	The Council for Scientific and Industrial Research
DAFF	-	Department of Agriculture, Forestry and Fisheries, South Africa
DFFE	-	Department of Forestry, Fisheries and the Environment, South Africa
DST	-	Department of Science and Technology, South Africa
EFSA	-	European Food Safety Authority
FABI	-	Forestry and Agricultural Biotechnology Institute, South Africa
FAO	-	Food and Agriculture Organisation of the United Nations
GBIF	-	Global Biodiversity Information Facility
MARAMA II	-	European Union funded project for development of marama bean products
NCBI	-	National Centre for Biotechnology Information
NRF	-	National Research Foundation, South Africa
POSA	-	Plant of Southern Africa
SANBI	-	South African National Biodiversity Institute
SARChI	-	South African Research Chairs Initiative
SIDA	-	Swedish International Development Agency
SLU	-	Swedish University of Agricultural Sciences
UP	-	University of Pretoria, South Africa
WHO	-	World Health Organisation

General:

AA	-	Amino acid
ACC	-	Aminocyclopropane-1-carboxylate
ADP-glucose	-	Adenine-diphosphate glucose
AFLP	-	Amplified fragment length polymorphism
AHL	-	N-acylhemoserine lactones

ANOVA - Analysis of variance

BenA - B-tubulin [gene region]

BLAST - Basic Local Alignment Search Tool

BOX-PCR - Polymerase chain reaction using BOX elements

CLA - Carnation leaf agar

COVID-19 - Coronavirus disease 2019

CS - Cassava starch

CYA - Czapak yeast extract agar

CYA20S - CYA with 20% sucrose

CV % - Coefficient of variation

DIC - Differential interference contrast

DLA - Detached leaf assay

DMF - Defatted marama flour

DMF-CS - Defatted marama bean-Cassava starch composite

DNA - Deoxyribonucleotide acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EST - Expressed sequence tags

EtOH - Ethanol

FA - Fatty acid

FT-IR - Fourier transform infrared spectroscopy

FT-Raman - Fourier transform Raman spectroscopy

G25N - 25% glycerol nitrate agar

GC-MS - Gas chromatography-mass spectrometry

HIV - Human Immunodeficiency Virus

IAA - Indole 3-acetic acid

IC_{50} - Half-maximal inhibitory concentration

IGS - Intergenic spacers [DNA]

ITS - Internal Transcribed Spacer [gene region]

LSD - Least significant difference

MAFFT - Multiple alignment program for amino acid or nucleotide sequences

MEA - Malt extract agar

MLST - Multilocus sequence typing

NaOAc - Sodium acetate

NaOCl - Sodium hypochlorite

NIR - Near infrared spectroscopy

NMR - Nuclear magnetic resonance

PCA - Potato carrot agar

PCR - Polymerase chain reaction

PDA - Potato dextrose agar

PGP - Plant growth promoting

RAPD - Randomly amplified polymorphic DNA

rDNA - Ribosomal DNA

RNA - Ribonucleotide acid

rRNA - Ribosomal RNA

SC - Species complex

SSR - Simple sequence repeats

TB - Tuberculosis

TDI - Tolerable daily intake

TEFα - Translation Elongation Factor 1α [gene region]

TIU/mg - Trypsin Inhibitor Units/milligram

UDP-glucose - Uracil-diphosphate glucose

UPLC - Ultra-performance liquid chromatography

UPLC-ESI-MS/MS - Ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry

USA - United States of America

WA - Water agar

Experiment treatments:

C - Compost

CT - Clay topsoil

H - Hygromix

L - Loam

LT - Loam topsoil

MC - Malanseuns compost

NSC - No seed coat

NSS - Non-surface sterilised

P - Perlite

R - River sand (unfiltered)

S - Filtered sand

SC - Seed coat

SS - Surface sterilised

V - Vermiculite

Mycotoxins:

AFB$_1$ - Aflatoxin B$_1$

ALT - Alterneune

AME - Alternariol monomethyl ether

AoH - Alternariol

ATX - Altertoxin

CPA - Cyclopiazonic acid

DAS - Diacetoxyscripenol

DON - Deoxynivalenol

FB$_{1,}$ FB$_{2,}$ FB$_3$ - Fumonisins B$_1$, B$_2$ and B$_3$

LOD - Limit of Detection

NIV - Nivalenol

OTA - Ochratoxins

TeA - Tenuazonic acid

ZEA - Zearalenone

Chapter 1 - General introduction

Image 1. *Tylosema esculentum* observed in the wild population in Laudium, Centurion, South Africa. Photo by T. Armfield

1.1 Background and motivation for the study

Tylosema esculentum ((Burch.) A.Schreib.) and *Tylosema fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.), both commonly referred to as the marama bean (Image 1, pg 16), are currently classified as orphan legume crops (Cullis et al., 2019; Cullis & Kunert, 2017). Orphan crops are regarded as crops that have become staple food crops in their respective countries, but are underutilized for one or a number of reasons, such as minimal economic importance as an agricultural crop (Cullis et al., 2018, 2019; Cullis & Kunert, 2017). Orphan crops offer a wide range of benefits, including novel ingredients, compounds or components that can be harvested from or produced by the crop and used for a wide variety of purposes ranging from food to cosmetics to breeding (Dawson et al., 2018). This has resulted in a growing interest in investigating native orphan crops.

The two *Tylosema* species are naturally distributed throughout Africa. *Tylosema esculentum* is generally found within southern Africa, including South Africa, Namibia, Botswana, and a few other regions throughout Africa (National Research Council, 2006); whereas *T. fassoglense* is generally found throughout eastern and central Africa, spreading south towards the northern parts of South Africa (Coetzer et al., 2011). As a result of the vastly different geographical locations, the environmental conditions required for the growth of the two species are also quite different. Within South Africa, marama bean is generally found in the wild and is restricted to the North West, Gauteng, Mpumalanga, and Limpopo provinces (Department of Agriculture, Forestry and Fisheries, 2014; Jackson et al., 2010). There are currently no records regarding marama bean cultivation by smallholder farmers within South Africa.

Nutrient analysis of the marama bean seeds revealed high protein, as well as high lipid levels consisting of linoleic acid, oleic acid, and palmitic acid (Bower et al., 1988; Dubois et al., 1995; Holse et al., 2010, 2011; Mosele et al., 2011; Müseler & Schonfeldt, 2006). Marama beans contain numerous important nutritional components such as various vitamins, minerals, carbohydrates, antioxidants, tuber starch and enzyme inhibitors, among many others (Amonsou et al., 2012; Bower et al., 1988; Holse et al., 2010; Kuvare et al., 2015; Müseler & Schonfeldt, 2006; Nadaraja et al., 2010; Nepolo, 2014; Shelembe et al., 2012). Various studies have reported medicinal properties of marama bean, including anti-tumour (lithospermoside), anti-diarrhoeal, anti-viral, anti-HIV, antibacterial and anticandidal activity (Chingwaru et al., 2011; Chingwaru et al., 2015; Fort et al., 2001; Kapewangolo et al., 2011; Mazimba et al., 2011).

Uses of the marama bean range from roasting and eating the seeds, or grinding the roasted seeds in order to make coffee, porridge, or processed further into products such as flour, milk and ice-cream

(Adeboye & Emmambux, 2017; Cole et al., 2014; Maruatona et al., 2010; Mattana et al., 2022; Nyembwe et al., 2018). The seed's oil could potentially be used for biodiesel fuel (Gandure et al., 2014). Tubers can be boiled, roasted or baked, or used for water in times of drought (Batanani, 2020; National Academy of Sciences, 1979). The tubers of the marama bean provide a valuable water source in arid areas and during drought periods due to their water storage ability, as the marama bean naturally grows in areas of high temperatures and low rainfall, with regular droughts and poor soil conditions (Batanani, 2020; National Academy of Sciences, 1979; Nepolo et al., 2010). Conflicting reports regarding the use of the marama bean leaves and whether the leaves are suitable for animal consumption (Dakora, 2013; National Academy of Sciences, 1979; Powell, 1987; Von Koenen, 2001; Watt & Breyer-Brandwijk, 1962), indicates a need for further research and investigation into the physiology and chemical composition of the leaves which may affect their usage for human or animal consumption, as well as mycoflora of the leaves which may have an additional impact on the usage of the leaves.

The marama bean is currently a locally important wild-harvested plant widely used by the Bantu and Khoisan people in southern Africa and Namibia that has the potential to become an economically important crop (Cole et al., 2014). The marama bean seeds and tuber have the potential to address malnutrition and hunger issues within many arid countries where conventional and agricultural crops often cannot survive due to the climatic conditions and underdeveloped agricultural infrastructure (Cullis et al., 2018; Jackson, 2017; National Academy of Sciences, 1979). A combination of responses and traits possessed by the marama bean has enabled the survival of the marama bean under harsh climatic conditions in drought-stricken areas (Cullis et al., 2018). The high protein, oil and nutrient content, as well as the potential health and medicinal benefits contribute to the potential value of the plant as a commercial crop in areas lacking crops and food sources with suitable nutrient content (Cole et al., 2014; Jackson, 2017; National Academy of Sciences, 1979; Smýkal et al., 2018).

There are various constraints facing the commercialisation and production of the marama bean as a crop. The constraints include the environmental conditions in which the two species grow, a general lack of information regarding the production requirements, as well as a lack of information regarding symbiotic associations, such as insect pests, and fungal and bacterial pathogens (Cullis et al., 2018; National Academy of Sciences, 1979; National Research Council, 2006). To date, there are no records of insect pests on the marama bean. Previous reports of bacterial associations indicate potential plant growth promoting (PGP) bacteria in the soil (Kandjimi et al., 2015), as well as seed-associated bacterial endophytes (Chimwamurombe et al., 2016). Reports on studies of fungal associations with *T. esculentum* performed in Namibia indicate the presence of several fungal species isolated from the pods and seeds, the leaves and as leaf endophytes (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013).

To date, there have been few studies regarding the fungal associations with marama beans, with the only reports of fungal associations stemming from Namibian plants. There have also been no reports of pathogens or diseases on the marama bean related to the fungal species previously isolated and identified. Furthermore, there have been no reports of either fungal species, pathogens or diseases on South African marama beans. In addition to the presence of various fungal species and their ability to cause diseases, comes the threat of fungal mycotoxin production. There are currently no reports of mycotoxin presence on marama beans. However, if the seeds are contaminated with mycotoxins, it raises health concerns for those communities that utilize the seeds for food and medicinal purposes. The identification of fungal species and the presence or absence of mycotoxins on the seeds will form a crucial part of determining whether the marama is a suitable crop for commercial production and utilization, considering the potential of the plant.

1.2 Hypotheses, aim and objectives

1.2.1 Hypotheses

A wide variety of fungal species are expected to occur on and in seeds, leaves and tubers of marama bean species in different localities within South Africa. Among these, it is expected that a portion of the fungi will have the potential to be mycotoxigenic or phytopathogenic. It is expected that some mycotoxins will be naturally produced and therefore detectable on the plant material obtained from the various localities within South Africa. It is expected that the levels of mycotoxins present in the fungal cultures will be greater than the levels of mycotoxins present on the plant parts. It is expected that some of the fungal species isolated throughout the study will be able to cause disease symptoms on marama beans.

1.2.2 Aim

The primary aim of this study was to study and identify the mycoflora and mycotoxins associated with *Tylosema esculentum* and *T. fassoglense* (marama bean) seeds, leaves and tubers collected from different localities within South Africa, as well as to investigate the potential pathogenicity of the isolated fungal species.

1.2.3 Objectives

The specific objectives of the study were to:

1. Isolate fungal species on *Tylosema esculentum* and *Tylosema fassoglense* seeds, leaves and tubers from various localities in South Africa.
2. Identify fungal species by morphological characteristics, and phylogenetic analyses of single and multigene DNA sequences.
3. Investigate the natural occurrence of mycotoxins associated with marama bean seeds, leaves and tubers.
4. Investigate potential of selected isolated mycotoxigenic species to produce mycotoxins *in vitro* (maize patty cultures) and *in vivo* (pot trial plants).
5. Determine the optimal growth conditions for the marama beans through various soil and drainage trials.
6. Investigate potential pathogenicity of selected isolated fungal species associated with marama bean seeds, leaves and tubers.

1.3 Structure of dissertation

Chapter 1 includes a brief introduction and background to the project, as well as the motivation, hypotheses, aim, and objectives of the study.

Chapter 2 provides an in-depth review of literature about the marama bean, ranging from the morphological and nutritional characteristics to the constraints in cultivation, including potential fungal pathogens. A brief introduction to mycotoxins is also given.

Chapter 3 focuses on the isolation and identification of mycoflora associated with marama bean plants sampled from eight locations in South Africa through morphological, molecular and phylogenetic analysis, as well as the mycotoxins associated with the plants *in vitro* and *in vivo.*

Chapter 4 investigates the potential of various fungal species to cause disease symptoms on the marama bean plant, thus providing an insight as to which fungal species may be pathogenic to marama beans.

Chapter 5 includes a general discussion and conclusion, along with shortcomings of the study, the significance of the study, and recommendations for future research.

Chapter 6, the appendices, provides additional material relevant to the various chapters and the interpretation of their respective results.

The working chapters (Chapter 3 and 4) of this dissertation have been prepared as stand-alone chapters, each with its own introduction, materials and methods, and results and discussion. Subsequently, some elements of the dissertation may be repetitive, particularly the background information regarding the marama bean.

1.4 Conference contributions

As a part of this study, the following conference contribution was made:

Armfield, T.J., Kritzinger, Q., Truter, M. and Barker, N.P. 2020. Fungal diversity of *Tylosema esculentum* and *Tylosema fassoglense* seed in South Africa. 1st Seed Congress of Argentina, 3 – 4 November (online) (poster presentation).

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Chapter 2 - Literature review: Introduction to the marama bean and the constraints facing its production

Image 2. A two month old *Tylosema esculentum* seedling, displaying the typical growth of marama beans. Photo by T. Armfield

2.1 Introduction to the marama bean

2.2.1 Classification

The genus *Tylosema* forms part of the third largest flowering plant family, Fabaceae (Fig. 2.1), classified under the order Fabales (Lewis, 2005). The leguminous family is well-known for the characteristic ability to fix atmospheric nitrogen (N_2) into ammonia (NH_3), which plants then utilize as nutrients (Sørensen & Sessitsch, 2007). However, this characteristic is absent in all of the *Tylosema* species (Coetzer et al., 2011). Fabaceae was recently re-classified into six sub-families, namely Caesalpinioidaea, Dialioideae, Detarioideae, Duparquetioideae, Papilionoideae and Cercidoideae (Azani et al., 2017). The sub-family Cercidoideae can be further classified into a single tribe, Cercideae. Tribe Cercideae can be further divided into three sub-tribes: Bauhiniinae, Cercidinae and Lasiobema, based on floral, fruit and seed characteristics (Fig. 2.1) (Azani et al., 2017). *Tylosema* and *Bauhinia* form part of Bauhiniinae (Azani et al., 2017). Until recently, *Tylosema* was classified under *Bauhinia* due to morphological similarities, making *Bauhinia* the closest known relative to *Tylosema* (Castro et al., 2005; Sinou et al., 2009). *Bauhinia* and *Tylosema* share common diagnostic characteristics such as simple, bilobed, palmately veined leaves. The division of *Tylosema* from *Bauhinia* was based on several significantly different morphological features, such as the large underground tuber, heterostylous flowers, and trailing stems with tendrils that are present in *Tylosema*, but absent in *Bauhinia* (Coetzer et al., 2011).

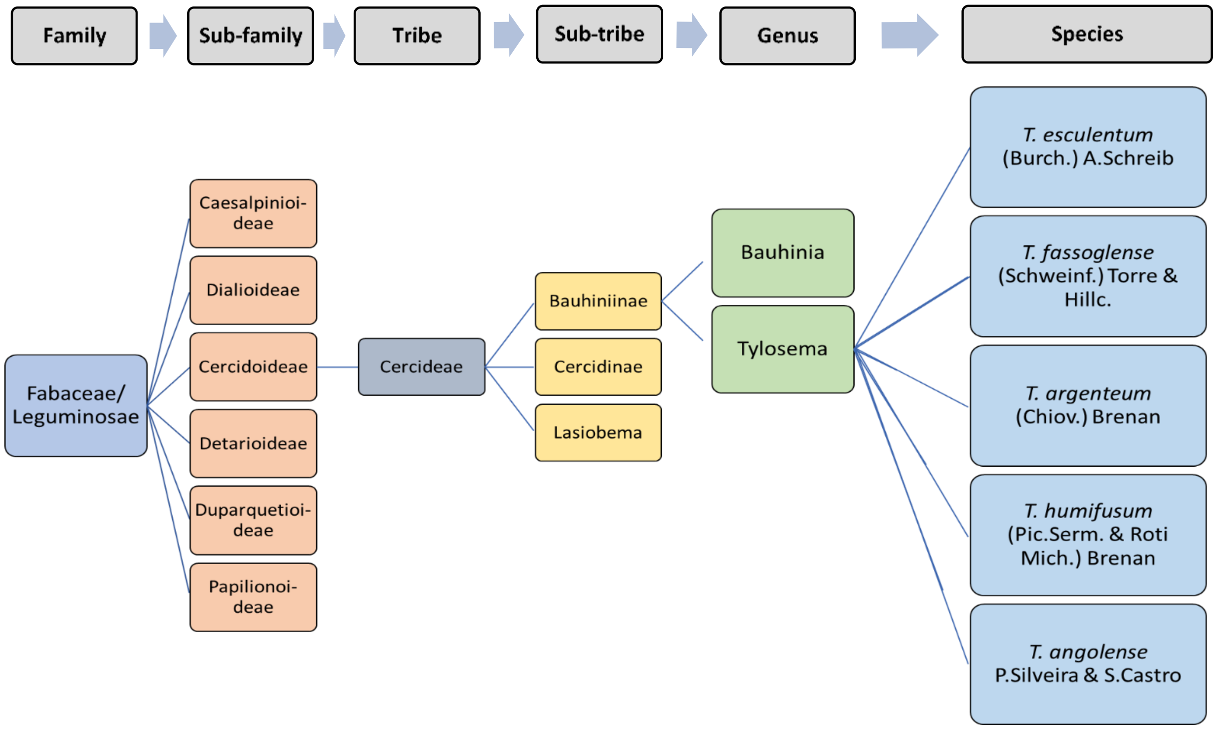

Figure 2.1. A summary of the classification of the *Tylosema* species.

There are currently five classified *Tylosema* species: *Tylosema argenteum* (Chiov.) Brenan; *T. esculentum* (Burch.) A.Schreib.; *T. fassoglense* (Schweinf.) Torre & Hillc.; *T. humifusum* (Pic.Serm. & Roti Mich.) Brenan; and *T. angolense* P.Silveira & S.Castro (Castro et al., 2005). *Tylosema esculentum* and *T. fassoglense* are both commonly referred to as the marama bean (Jackson et al., 2010), therefore, use of the term marama bean throughout this study will refer to both *T. esculentum* and *T. fassoglense* unless stated otherwise.

The marama bean is currently classified as an orphan legume crop (Cullis et al., 2018, 2019; Cullis & Kunert, 2017). Orphan crops are regarded as crops that have become staple food crops in their respective countries but are underutilized for one or a number of reasons (Cullis et al., 2018, 2019; Cullis & Kunert, 2017). These reasons can include lack of interest by breeders to improve the crop, or minimal economic importance to encourage development of the crop as an agricultural crop (Cullis et al., 2018). Due to survival strategies that have been developed by the plant to withstand harsh growing conditions, orphan crops are better suited to their environments than conventional crops that require alteration of the environment to survive. As such, the orphan crops can outcompete other agricultural crops within the specific environment with regards to general plant growth and productivity (Cullis et al., 2018; National Research Council, 2006). Orphan crops offer a wide range of benefits, including novel ingredients, compounds or components that can be harvested from or produced by the crop and used for a wide variety of purposes ranging from food to cosmetics to breeding to plant pathology (Dawson et al., 2018).

2.2.2 Distribution and growth conditions

Tylosema esculentum and *T. fassoglense* have significantly different distribution patterns (Fig. 2.2), and as a result have vastly different environmental condition requirements for optimal growth (Coetzer et al., 2011). *Tylosema esculentum* is predominantly distributed throughout arid and semi-arid areas of South Africa, Namibia, and Botswana, as well as trial populations located in America and Australia (National Research Council, 2006). The distribution of *T. fassoglense* ranges from Sudan, southwards through eastern and central Africa towards South Africa (Coetzer et al., 2011) (Fig 2.2). Figure 2.3 illustrates a grid distribution map of the *T. esculentum* and *T. fassoglense* specimens collected in South Africa and Namibia as recorded in the HGWJ Schweikerdt Herbarium, Department of Plant and Soil Sciences, University of Pretoria, Pretoria. It is worth noting that some of the data points in the *T. esculentum* GBIF map (Fig 2.2) far north and near Mozambique within South Africa may be misrepresented or misidentified, and may instead be *T. fassoglense* specimens, based on the general distribution patterns and growing conditions of each species and the respective localities. Furthermore, some of the *T. fassoglense* points indicated in the *T. fassoglense* GBIF map (Fig. 2.2) may have been misidentified specimens stored in various herbaria, such as the point in the Northern Cape, South Africa.

Available records indicate that marama bean is limited to being a wild growing plant at present (DAFF, 2014; Jackson et al., 2010). Within South Africa, the marama bean is naturally occurring in open woodlands and grasslands (Mogotsi & Ulian, 2013; Nepolo et al., 2010b) and is restricted to the North West, Gauteng, Mpumalanga, and Limpopo provinces (Department of Agriculture, Forestry and Fisheries (DAFF), 2014). The sporadic and patchy nature of the marama bean population distribution may be attributed to the migration of indigenous people and traditional plant users recorded throughout history (Nepolo et al., 2010b). There are currently no records on the Department of Forestry, Fisheries and the Environment (DFFE, previously known as DAFF) or the Food and Agriculture Organisation of the United Nations (FAO) websites regarding marama bean cultivation by smallholder farmers within South Africa. There are indications of isolated experimental marama bean cultivation projects in Namibia, Botswana, Australia, and Texas (USA), however the use of the cultivation projects was generally restricted to experimental purposes or propagation (Chimwamurombe, 2010; Jackson et al., 2010; Powell, 1987). Domestication attempts were made in Namibia, including an important aspect of starting the process of plant domestication by creating well-characterized germplasm resources (Chimwamurombe, 2010, 2011; Magadza, 2010; Nepolo et al., 2010a). Efforts to compile a comprehensive germplasm resource have been started in Namibia (Chimwamurombe, 2010). Many of the experimental projects were performed many years ago to little result – the attempts at marama bean cultivation were generally unsuccessful and produced very poor yields, if any at all (Chimwamurombe, 2010; Jackson et al., 2010; Powell, 1987).

Tylosema esculentum naturally grows in arid to temperate climatic conditions with high temperatures, low annual rainfall, prolonged drought periods, and grassland-type biomes (Coetzer et al., 2011; Mitchell et al., 2005). *Tylosema fassoglense* prefers subtropical to tropical climatic conditions with comparatively lower temperatures, higher annual rainfall levels, and savanna to woodland-type biomes (Coetzer et al., 2011). A combination of responses and traits possessed by the marama bean has enabled the survival of the marama bean under harsh climatic conditions in drought-stricken areas (Cullis et al., 2018). These responses include various drought avoidance mechanisms such as stomatal closure and leaflet movement to limit transpiration and stomatal conductance, osmotic adjustment to maintain leaf turgor and water potential gradient for soil water extraction, as well as using the tuber as a water reservoir which helps maintain leaf water potential and relative water content (Karamanos & Travlos, 2012). Travlos and Karamanos (2008) reported a significant decrease in stem and leaf growth when the plants were placed under drought conditions, indicating a clear shift in allocation of dry matter from the shoots to the roots – a valuable mechanism for a crop to grow under harsh water-stressed conditions.

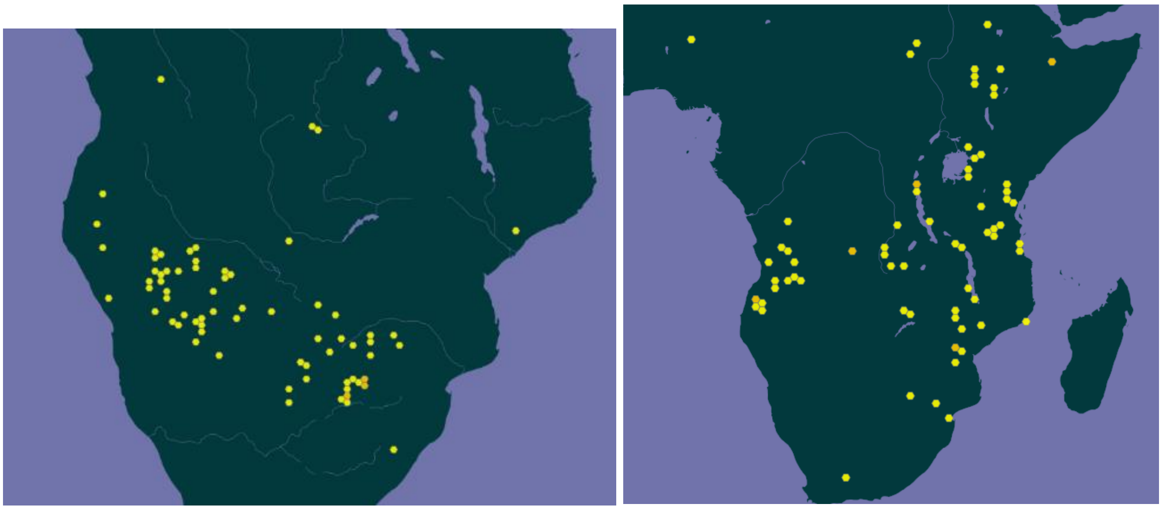

Figure 2.2. The general distribution maps of *Tylosema esculentum* (left) and *Tylosema fassoglense* (right) recorded in southern Africa, neighbouring countries Namibia and Botswana, and central African countries, as generated by the Global Biodiversity Information Facility (GBIF) website (GBIF Backbone Taxonomy, 2017a, 2017b).

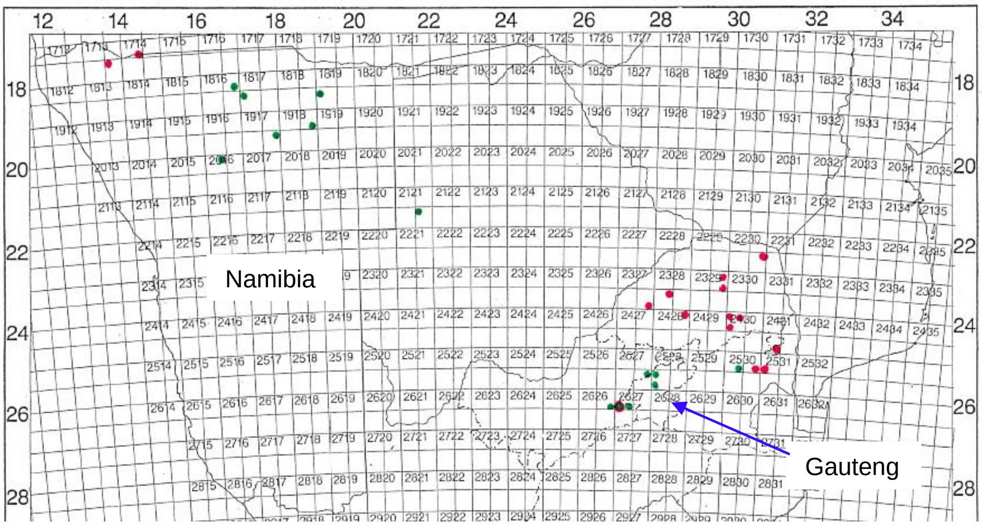

Figure 2.3. A grid distribution map of the *Tylosema esculentum* (green) and *Tylosema fassoglense* (red) specimens as collected from locations within South Africa and from neighbouring countries, recorded in the HGWJ Schweikerdt Herbarium located on the Hatfield campus, University of Pretoria, Pretoria, South Africa.

Data regarding *T. esculentum* and *T. fassoglense* specimens collected and recorded by the National Herbariums throughout southern Africa can be found in a database collection, the South African National Biodiversity Institute (SANBI) Plants of Southern Africa (POSA) database (South African National Biodiversity Institute (SANBI), 2016). The database provides access to the South African plant taxa, herbarium sheets and botanical records of plants collected and recorded throughout southern Africa, obtained from the Botanical Database of Southern Africa (BODATSA), containing records from the Compton Herbarium in Cape Town (NBG & SAM), the National Herbarium in Pretoria (PRE), and the KwaZulu-Natal Herbarium in Durban (NH) (SANBI, 2016). The distribution maps for

T. esculentum and *T. fassoglense* obtained from the POSA database have been recorded in Appendix 6.1, Figures 6.1 and 6.2.

2.2.3 Morphological characteristics

Within South Africa *T. esculentum* and *T. fassoglense* are often confused on the basis of similar morphological features as seen in Fig. 2.4. As a result, the two species could be considered as one single species with a number of infraspecific taxa (Coetzer et al., 2011).

The marama bean (Images 1, pg 16; and Image 2, pg 26) consists of a large underground tuber [or storage root] and reddish-brown stems that originate from the tuber and branch off into Y-shaped tendrils and prostrate vines (Figure 2.4 A+B) (Cole et al., 2014). The leaves are simple, alternately-arranged and bilobed (Fig. 2.4 A) (Coetzer et al., 2011). *Tylosema esculentum* leaves are smaller (30-75 mm long) with an apical division of half the lobe length along the central main vein and a shorter petiole of 15-35 mm (Coetzer et al., 2011). The leaves mature from young red-brown leaves into leathery grey-green leaves (DAFF, 2014). In comparison, *T. fassoglense* leaves are also bilobed but are larger, with lobes 60-200 mm long. The division along the central main vein spans 1/10 to 1/3 of the lobe length while the petiole is relatively long compared to *T. esculentum*, ranging between 30 and 70 mm (Coetzer et al., 2011).

Tylosema flowers are small to medium in size and yellow to red in colour. The flowers are bisexual and irregular, arranged in a raceme pattern, and show heterostyly in which the style length differ considerably between flowers of the same species. The presence of heterostyly promotes cross-pollination by honeybees and other pollinator insects (Coetzer et al., 2011). Diagnostic flower characteristics of the genus *Tylosema* include the presence of a lobed non-spathaceous calyx-limb as well as nine or ten stamens, two of which are fertile and the remaining stamens sterile (Castro et al., 2005). The pods develop from the flowers, beginning as rounded pink-green soft pods and developing into hard, brown, flattened pods (Fig. 2.4 C). *Tylosema fassoglense* pods are woody and can contain either one seed or up to three seeds, resulting in oval or oblong-ovate pods, respectively (Image 5, pg 176). The seeds are large and oblate, with a chestnut-brown to black seed coat and a U-shaped line that extends a short way from the hilum (Fig. 2.4 D-E) (Coetzer et al., 2011). The *Tylosema* seeds have a smaller white-cream fleshy inner seed (Fig. 2.4 F) and are roughly the size of a South African R5 coin (Fig. 2.4 G). The *T. esculentum* pods are structurally similar, with slight variation in the degree of oblateness compared to the *T. fassoglense* seeds. The *T. esculentum* seeds (Fig. 2.4 D) are slightly smaller in diameter but thicker than *T. fassoglense* seeds (Fig. 2.4 E). The *T. esculentum* pods typically contain two seeds, one of which often aborts. However, they have been noted to contain up to four seeds in a single pod (Cole et al., 2014).

Seed germination occurs within eight to 21 days, after which the tuber develops during a period of vegetative growth that lasts between five and six months. Following the vegetative growth stage, the tuber remains dormant while runners develop which produce the flowers and pods that die back once the flowering season has concluded (Coetzer et al., 2011; Cole et al., 2014; DAFF, 2014). Marama bean flower production generally begins two to three years after germination, and every year thereafter (Powell, 1987; A. Frisby, personal communication, February, 2019). The tuber continues to grow and develop over the lifespan of the plant as the branches grow and die back seasonally (Coetzer et al., 2011; Cole et al., 2014; DAFF, 2014).

Figure 2.4. *Tylosema* bilobed leaves and reddish-brown vines [A]. *Tylosema* mature prostrate vines [B]. *Tylosema* pink-green young pods next to hard brown older pods [C]. *Tylosema esculentum* seeds indicating the smaller diameter, larger width, and U-shaped line extending from the hilum [D]. *Tylosema fassoglense* seeds indicating the larger diameter, smaller width, and U-shaped line extending from the hilum [E]. *Tylosema esculentum* seed with and without seed coat, indicating the smaller white-cream fleshy inner seed and hard brown outer seed coat [F]. *Tylosema esculentum* seed compared to a South African R5 coin [G]. All photos by T. Armfield.

Growth of the plant following seed propagation results in low flower and seed production. The low seed set often occurs due to high fruit abortion levels (Coetzer et al., 2011). Seed propagation requires scarification of the seed to enable seed germination, making seed propagation difficult for crop domestication and production (Coetzer et al., 2011).

Several engineering properties related to the processing capabilities of the marama bean have been tested, including size and shape, density, volume, static coefficient of friction, porosity, grain mass and texture. The investigation of such properties can aid in the production of more efficient means for seed production as the tough seed coat makes processing the seeds difficult and tedious, thereby deterring domestication attempts (Emesu & Mabuza, 2014).

2.2.4 Genetic analysis

Various studies have been performed regarding the genetic composition of the marama bean. Table 2.1 summarizes some of the studies performed on the marama bean to date.

Table 2.1. A summary of some of the genetic studies performed on *Tylosema esculentum* and *Tylosema fassoglense* to date.

Year	**Study performed**	**Brief description**
1977	Chromosome numbers in legumes.	*Tylosema fassoglense* was determined to be a tetraploid with $x = 13$ as a base number, different to *Bauhinia*'s $x = 14$ base number, supporting the separation of the two species. *Tylosema fassoglense* chromosome number $2n = 52$ (Goldblatt & Davidse, 1977).
1996	RAPD variation within and between natural populations of morama *[Tylosema esculentum* (Burchell) Schreiber] in southern Africa.	Randomly amplified polymorphic DNA (RAPD) was used to determine genetic variability in three marama populations found in Botswana. Considerable variation was found in the three populations, with 85% of the variation being within rather than between the populations. No population-specific RAPD markers were identifiable (Monaghan & Halloran, 1996).
2004	Assessment of genetic variation in natural populations of marama bean (*Tylosema esculentum*) using molecular markers.	Amplified fragment length polymorphism (AFLP) analysis was conducted to determine the genetic variation of a population of marama bean. The results indicated a 0.154 genetic diversity when considering the whole Namibian population, indicating a high genetic variation (Naomab, 2004).
2010	Determining genetic diversity based on ribosomal intergenic spacer length variation in marama bean (*Tylosema esculentum*) from the Omipanda area, Eastern Namibia.	Length variation in the marama bean was investigated using the large ribosomal DNA gene intergenic spacers (IGS) region. A primer pair amplifying the IGS region between repeat units within tandem arrays of rDNA genes yielded 79 scorable bands, of which seven alleles were polymorphic (Nepolo et al., 2010a).
2010	Development and use of microsatellites markers for genetic variation analysis, in the Namibian germplasm, both within and between populations of marama bean (*Tylosema esculentum*).	Molecular markers are a requirement for assessing genetic variation within and between a plant population. Eighty (80) primer pairs were designed to amplify identified microsatellite repeats. Of these, 76% detected polymorphisms, 21% monomorphisms, and 3% were inconsistent. Four polymorphic primer pairs were used to determine genetic variability and were very informative for inter- and intra-specific variability in marama beans (Takundwa et al., 2010).

2010	Domestication of *Tylosema esculentum* (marama bean) as a crop for southern Africa: Genetic diversity of the Omitara marama subpopulation of Namibia. A low genetic diversity between sub-populations was found.	Microsatellite markers were used to assess the genetic variation within a marama bean population, resulting in 86% similarity, indicating low genetic diversity. Shannon's genetic diversity measure was also used, resulting in 2.6041 genetic diversity per population, a value higher than 0.95 indicating the population was monomorphic and had low genetic diversity (Chimwamurombe, 2010).
2010	Isolation and characterization of microsatellite repeats in marama bean (*Tylosema esculentum*).	Microsatellites, or simple sequence repeats (SSRs) are one of the more effective tools for molecular identification. Nine marama bean microsatellite libraries were created to aid in developing SSRs for the detection of polymorphisms in marama beans. Eighty primer pairs were designed, with four proving the most effective. The marama bean germplasm appears to have abundant polymorphic microsatellites, of the 30 loci initially screened, 77% were polymorphic (Takundwa et al., 2010; Takundwa et al., 2012c).
2012	Development and use of microsatellite markers in marama bean.	
2012	A chromosome count in marama bean (*Tylosema esculentum*) by Feulgen staining using garden pea (*Pisum sativum* L.) as a standard	Feulgen or Schiff's staining technique was used in conjunction with oil immersion microscopy using 100X magnification to determine chromosome number. *Tylosema esculentum* presented a chromosome number of n=22 (2n=44) compared to the n=7 (2n=14) chromosome number of *Pisum sativum*. However, the 44 chromosomes in *T. esculentum* were found to occupy less cell space than the 14 chromosomes of P. sativum, indicating *T. esculentum* may have a smaller genome size than garden peas. Genome wide next generation sequencing is being used to determine the *T. esculentum* genome size (Takundwa et al, 2012a).
2012	Developing DNA barcoding (*matK*) primers for marama bean [*Tylosema esculentum* (Burchell) Schreiber].	The *matK* gene (formerly *orfK*), possibly encoding a maturase-related protein, has a potential to contribute to plant molecular systematics and evolution. It also has the potential to be used as a marama bean barcode. Comparison on Genbank indicated similarity to the *T. fassoglense trnK* gene and *Pisum sativum matK* gene. Results indicate the *matK* gene could be used for barcoding and genetic variation determination (Takundwa et al., 2012b).
2015	Gene mining a marama bean expressed sequence tags (ESTs) database: Embryonic seed development genes and microsatellite marker identification.	Expressed sequence tags (ESTs) have become the economic choice for obtaining specimen gene sequences in large numbers. The study aimed to identify genes that associated with valuable agronomic traits and microsatellite sequences. These were identified as genes associated with embryonic development and microsatellite sequences (Sheehama & Chimwamurombe, 2015).
2017	A novel inversion in the chloroplast genome of marama (*Tylosema esculentum*).	Next-generation sequencing was used to assemble the marama bean chloroplast genome which covers 161537 bp and includes a large single-copy region, a small single-copy region separated by a pair of inverted repeats, and an inversion specific to the genus, and two complete *ycf1* gene copies (Kim & Cullis, 2017).

2.2.5 Chemical and nutritional composition

Numerous studies investigating the chemical properties and nutritional value of marama beans have been performed. Table 2.2 includes some of the recent publications involving the marama bean as a potential food and medicinal crop.

Table 2.2. A summary of the chemical and nutritional composition studies performed on *Tylosema esculentum* as a potential food crop to date.

Year	**Study performed**	**Brief description**
1988	Nutritional evaluation of marama bean (*Tylosema esculentum*, Fabaceae): Analysis of the seed.	The marama bean was analysed for various nutrient contents, including amino acids, proteins, fatty acids, oil, caloric values, fibres, minerals and trypsin inhibitor content. Protein, mineral, lipid and carbohydrate levels are high and satisfactory for human diets; however, trypsin inhibitor activity should be reduced before consumption, especially through heating. Protein constituents include globulins, albumins, prolamins, alkali soluble glutelins, and acid-soluble glutelins (Bower et al., 1988).
1995	Chemical characterization of *Tylosema fassoglense* (Kotschy) Torre & Hillc oilseed.	*Tylosema fassoglense* seeds have high lipid (240-300 g/kg) and protein (446 g/kg dry weight) levels. The fatty acid content includes linoleic acid 36-42% of total fatty acids; oleic acid 32-35% and palmitic acid 11.5-15.7%. High levels of lysine, proline, tyrosine and low levels of methionine and cysteine are present. Defatted meal contains 295 TIU/mg trypsin inhibitors and 35 g/kg dry weight phytates (Dubois et al., 1995).
2001	Lithospermoside from *Bauhinia fassoglense* (Fabaceae).	*Bauhinia fassoglense* root extracts were examined. Lithospermoside, a cyanoglucoside, was observed in the root extracts (Fort et al., 2001). Lithospermoside is known to have antitumor properties (Ito et al., 1999).
2005	Evaluation of the quality characteristics of the marama bean (*Tylosema esculentum)*, an underutilized grain and tuber producing legume in southern Africa.	Sensory attributes and nutrient content, as well as potential for cattle fodder, were determined for the marama bean (Müseler, 2005).
2006	The nutrient content of the marama bean (*Tylosema esculentum*), an underutilized legume from southern Africa.	Marama beans from Namibia and Botswana were analysed for nutrient content. After roasting, beans showed high protein levels. Unsaturated fatty acids of which 87% was linoleic, oleic and palmitic acids. Significant amounts of vitamins (A, B_3, B_6, B_{12}, E and folic acid) and minerals (iodine, iron and zinc) were present (Müseler & Schonfeldt, 2006).
2009	Profiling of phytosterols, tocopherols and tocotrienols in selected seed oils from Botswana by GC-MS and HPLC	Five seed oil plants found in Botswana, including *Tylosema esculentum*, were analysed for their respective phytosterol, tocopherol and tocotrienol content. It was found a total tocol content in marama bean of 199.10 µg/g; and a total 4-desmethylsterol content of 149.15 µg/g (75% content), as well as 15.72% 4,4-dimethylsterol and 4-monomethylsterol content (Mitei et al., 2009).

2010	Chemical composition of marama bean (*Tylosema esculentum*) — A wild African bean with unexploited potential.	The chemical content of sixteen samples of marama beans from South Africa, Namibia, and Botswana was determined. The following results were obtained: protein 29-38%; lipids 32-42%; dietary fibre 19-27%; ash 2.5-3.7%; vitamin E isomers α-, β-, γ-tocopherol levels 14-48, 1.1-3.3, 59-234 µg/g, respectively. Mineral content similar to peanuts, good source for zinc and iron. Secoisolariciresinol, lariciresinol and pinoresinol lignins present. Absence of potent allergens and cyanogenic compounds (Holse et al., 2010).
2010	Isolation and partial sequence of a Kunitz-type elastase specific inhibitor from marama bean (*Tylosema esculentum*).	High levels of the elastase specific inhibitor were found in *T. esculentum*. Protease inhibitors are important for growth and development regulation as well as insect and pathogen defence. Inhibitor levels were observed to be higher than in soybean or other beans tested to date (Nadaraja et al., 2010).
2011	Characterization of marama bean (*Tylosema esculentum*) by comparative spectroscopy: NMR, FT-Raman, FT-IR and NIR.	In this study, marama beans from different geographical sites and harvest years were analysed by use of infrared, near infrared, Raman, and 1H as well as 13C nuclear magnetic resonance spectroscopy. Results included: large amounts of protein, dietary fibre and unsaturated fat; carbohydrate fraction largely pectin, smaller fraction raffinose; high tyrosine content; no starch or β-glycans (Holse et al., 2011).
2011	Characterization of the arabinose-rich carbohydrate composition of immature and mature marama beans (*Tylosema esculentum*).	The carbohydrate fraction of immature and mature seeds was characterized. Starch and soluble sugar levels were negligible. High arabinose content in the cell walls, containing pectin arabinan. High mannose sugar content was observed (Mosele et al., 2011a).
2011	Microstructure of protein bodies in marama bean species.	The protein body structures of *T. esculentum* and *T. fassoglense* were determined and compared to those of *Glycine max* (soybean). The protein bodies surrounded by lipid bodies of marama beans were similar to that of soybeans, but marama beans have spherical globoid and druse crystal inclusions which soybeans do not (Amonsou et al., 2011).
2011	Proximate composition, histochemical analysis and microstructural localization of nutrients in immature and mature seeds of marama bean (*Tylosema esculentum*) – An underutilized food legume.	The mature and immature seed nutrient levels were compared. Immature seeds: 67% moisture; 21% protein; 1.5% lipid content. Mature seeds: 32% protein; 40% lipids; no starch; increased pectin and cellulose levels (Mosele et al., 2011b).
2012	Characterization of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean [*Tylosema esculentum*] – An underutilized food legume.	The major flavonoids were methyl (epi)afzelechin-3-O-gallate (40%) and methyl (epi)catechin-3-O-gallate (28%), and the major phenolic acid was gallic acid (10%). Proanthocyanidins were predominantly epicatechin-3-O-gallate and epigallocatechin, and epigallocatechin-3-O-gallate and epicatechin (Shelembe et al., 2012).
2012	Chemical and structural characterization of marama bean (*Tylosema esculentum*) carbohydrates.	The chemical and structural composition of carbohydrates in the marama bean was determined. The study revealed that the main carbohydrate was pectin, made up of homogalacturonan and arabinan. Alpha-anomeric carbohydrates including pectin

		dominate the carbohydrate fraction from carbonyl groups in esters and acids (Mosele, 2012).
2012	Composition of marama bean protein.	The marama bean protein composition was determined in comparison to that of soybean protein. Marama bean protein has higher tyrosine and proline levels; potential absence of disulphide bonds, vicilin and acidic 11S subunits (Amonsou et al., 2012a).
2012	Headspace volatile composition and oxidative storage stability of pressed marama bean (*Tylosema esculentum*) oil.	Freshly pressed marama bean oil's headspace volatile composition was explored and studied over seven months of storage under different temperature and light conditions using dynamic headspace GC-MS. Peroxide value, FT-IR spectra and vitamin E content were used to measure the oxidative stability of the oil. Results included: marama oil has good natural antioxidant properties; the oil is highly stable; enzymatic lipid oxidation of the oil does not occur; light affects lipid oxidation more than temperature – oil should be stored in darkness between 25-35°C to have a shelf life of 7 months (Holse et al., 2012a).
2012	Oxidative storage stability of roasted marama beans (*Tylosema esculentum*).	The oxidative storage stability of roasted marama beans was investigated under different storage conditions such as presence or absence of light and oxygen for various storage periods. Results showed that seeds should be stored in bags in darkness with little oxygen contact. Hexanal is a limiting storage factor (Holse et al., 2012b).
2013	Adhesive potential of marama bean protein.	The marama bean protein was examined for adhesive potential. Marama bean proteins contain more β-sheet structures than soybean, therefore have a higher adhesive potential (Amonsou et al., 2013).
2014	Isolation and characterization of starch, starch biosynthetic genes and protease inhibitors from marama bean (*Tylosema esculentum*).	The physicochemical and pasting properties of native marama bean starch were determined in this study by isolating and characterizing the starch biosynthesis genes and detecting serine protease inhibitor activities in green and mature marama beans (Nepolo, 2014).
2015	Sensory and enzymatic factors associated with defects in low fat UHT milk	Protease inhibitors extracted from marama beans, cowpeas and soybeans were analysed for their ability to inhibit native milk enzymes (plasmin) and bacterial enzymes from *Pseudomonas fluorescens*, *Bacillus licheniformis*, and *B. lentus*. These enzymes are involved in the deterioration of milk over time. The protease inhibitors extracted from the three legumes proved successful at reducing or preventing *Bacillus* protease enzymes as well as milk plasmin enzymes (Dennill, 2015).
2015	Comparative study of antioxidant properties, polyphenols and flavonoid contents of the tuber and seed extracts of marama bean (*Tylosema esculentum*).	Phenolic compound rich marama tuber extracts exhibited higher antioxidant activity than those of marama seeds. DPPH free radical scavenging IC_{50} values were 95.62 ± 7.08 µg/ml and >1000 µg/ml for marama tuber and seed extracts, respectively. A positive correlation exists between the phenolic content and antioxidant activity of marama bean tuber and seed samples (Kuvare et al., 2015).
2015	Physicochemical and functional characteristics of starch extracted from	In this study the functional and physiochemical properties of marama bean tuber starch were analysed. These properties were

	marama bean tuber (*Tylosema esculentum* Burchell *A.* Schreiber).	compared to those of commercial potato (*Solanum tuberosum* L.) starch. A relatively high proportion of amylose was present in the tuber starch. The pasting profile was similar to potato starch but with higher pasting gelatinization and peak viscosity temperature. (Nepolo et al., 2015).
2015	Sensory and physicochemical analyses of roasted marama beans [*Tylosema esculentum* (Burchell) *A.* Schreiber] with specific focus on compounds that may contribute to bitterness.	This study investigated the role of saponins and phenolics in the perceived bitterness of marama beans. Roasting for more than 20 min resulted in bitterness. Gallic and protocatechuic acid levels increased with longer roasting times, along with saponins and unidentified compounds could contribute to bitterness (Nyembwe et al., 2015).
2016	Identification and manipulation of starch synthases from marama bean and potato.	Starch synthesis genes were identified via transcriptome analysis, as well as random mutagenesis of a potato SSI gene for the utilization of UDP-glucose instead of ADP-glucose for starch synthesis (Hartzenberg, 2016).
2016	The effect of processing on the antinutrients in selected legumes in Botswana.	Antinutritional factors such as enzyme inhibitors and condensed tannins can restrict the availability of proteins. Two local varieties of cowpea (*Vigna unguiculata*) as well as processed and unprocessed marama bean samples were analysed for trypsin inhibitors and condensed tannins. The marama bean samples were roasted. The butanol-HCl methods was used to analyse condensed tannin content. Unprocessed marama content was 0.381% and 0.223% after processing (41% reduction). Benzoyl-DL-arginine-p-nitroanilide hydrochloride method was used to analyse trypsin inhibitor activity. Activity for unprocessed marama was 27.8 TIU/g dry matter, and for processed marama was 26.4 TIU/g dry matter (5% reduction). Processing therefore reduces antinutrient content and improves digestibility of the protein (Tibe et al., 2016).
2018	Characterization of starch accumulation in storage root of marama (*Tylosema esculentum*).	Marama bean root and starch quality was determined at different times to determine the most suitable time for harvesting by investigating the thermal properties, physicochemical and size properties of starch. With increased harvesting time, starch content increased, amylose content decreased, and crude protein content decreased. Freeze-dried marama bean root thermal properties indicated an endothermic peak of 73.4-93.0˚C (Hamunyela, 2018).
2020	Proximate and starch composition of marama (*Tylosema esculentum*) storage roots during an annual growth period.	Tuber harvesting time can impact the starch content and general quality of marama bean tubers. Tubers were planted in September (Spring) and harvested at 2-, 4-, 8-, and 12-month intervals after planting. Proximate starch and amylose contents, as well as thermal properties were analysed. Starch content and starch granule size increased significantly between the 2- and 12-month intervals, while crude protein and amylose content decreased significantly over the same period. To conclude, marama beans should be planted in early summer and harvested between 4 and 8 months for optimal starch content (Hamunyela et al., 2020).

2.2.6 Uses

The marama bean is a very useful plant due to the high protein and oil content of the seeds, and the water content of the tuber. The tuber can be boiled, roasted or baked and used as a sweet vegetable dish additive (National Academy of Sciences, 1979). The water storage ability of the tuber also contributes to human and animal survival in arid and semi-arid areas and especially during periods of drought (National Academy of Sciences, 1979). The protein content of the marama bean seed is comparable to that of soybeans (*Glycine max* (L.) Merr.) and is lysine rich, while the oil content of the marama seed is linoleic acid rich and comparable to that of peanuts (*Arachis hypogaea* L.) (National Academy of Sciences, 1979). The raw seeds are often stored in the seed coats for extended time periods for use as propagation material. Parratt (1994) reported that fresh marama bean material was available between March and May in Botswana, while dried material was available during the remaining nine months of the year. Storage helps during times of drought when plant yield decreases, and food becomes more difficult to find (Campbell, 1986). Storage of the seeds at room temperature in a dry place can increase the shelf-life of the seeds for later consumption (Mahgoub et al., 2013). The seed coats are removed before roasting, after which they can be eaten as nuts or ground into powder for use in making porridge or coffee (Lewis, 2005), or as a substitute to almonds (*Prunus dulcis* (Mill.) D.A. Webb) (National Academy of Sciences, 1979). Several innovative marama bean products have been developed by the European Union-funded project (MARAMA II) aimed at niche markets in southern Africa, including South Africa, as a first step in the commercialisation of the marama bean (Jackson et al., 2010). Examples of these products include: marama milk as a dairy substitute; marama oil for use in cosmetics; salads and cooking; and marama flour in defatted and full fat variants to be added to staple cereals such as sorghum (*Sorghum bicolor* (L.) Moench) to improve the nutritional quality.

Various products can be made from the seed after processing, including cooking oil, butter, flour, sorghum-marama composite porridge, ice-cream and canned marama beans in tomato sauce (Cole et al., 2014; Jackson et al., 2010; Mattana et al., 2022). Due to the high protein content, the seeds can also be used as a substitute for meat (Cole et al., 2014; Kayitesi et al., 2010). The Department of Consumer and Food Sciences at the University of Pretoria along with other universities both local and abroad have investigated the potential of marama bean food products such as oil, milk, roasted marama beans and protein-rich flours for niche markets (Kgosikoma et al., 2020; Nyembwe et al., 2015, 2018). The marama bean is a good source of antioxidants, thus useful for preventing oxidative stress related diseases such as neurodegenerative diseases and certain cancers (Anderson & Wolf, 1995).

Another common use of the seeds and tubers of the marama includes the treatment of diarrhoea in domestic animals, immunocompromised people, infants and small children (Chingwaru et al., 2015b;

Kapewangolo et al., 2011). Chingwaru et al. (2015b) investigated the anti-HIV (Human Immunodeficiency Virus) activity of *T. esculentum* and other sub-Saharan African plants, and various compounds were found to have anti-HIV and antiviral activity. These compounds included: gallic acid and vanillic acid (anti-HIV); hesperidin (anti-influenza virus); fisetin (anti-dengue fever); quercetin (anti-Mayaro fever virus); and tannins (anti-influenza and coxsackie virus) (Chingwaru et al., 2015b). The roots of *T. fassoglense* produce a cyanoglucoside, lithospermoside, a compound which could be important as a potentially strong anti-tumour promoter (Ito et al., 1999). Considering the vast range of compounds present in the marama bean and their respective anti-pathogen activities, there is a potential for the plant to be used to combat a wide variety of current and novel pathogens, such as the novel coronavirus, COVID-19.

A brown dye can be made from the roots, while the stems can be used for basket weaving and rope making. Branches and pods can be used for animal fodder, while the tuber can be used as a decoction which acts as a galactagogue (a substance used to induce or increase milk production in humans and animals) to cows before calving (Coetzer et al., 2011). The seeds can be used as an alternative to soybean meal for feeding Tswana hens without compromising the nutritional value and chemical composition of the meat that is provided by the soybean meal (Manyeula et al., 2019), and as additional supplements fed to pigs to aid in fattening pigs (Elfant et al., 1985). Reports suggest conflicting uses for the marama bean leaves. Dakora (2013) and National Academy of Sciences (1979) report that the leaves are highly palatable and often consumed by both livestock and wild animals, while Powell (1987) and Watt and Breyer-Brandwijk (1962) stated that the foliage is not palatable and therefore not browsed. Reports suggest that the leaves could contain phenolic compounds that are used to deter grazers and insects, but they could be useful as wound and arthritis treatments when crushed into a thick paste by the people of the Kalahari (Von Koenen, 2001).

A summary of studies performed on the marama bean for chemical and physical properties related to the usage of the marama bean as a food crop and medicinal plant is provided in Table 2.3.

Table 2.3. A summary of the studies regarding the chemical and physical properties of the marama bean related to the usage of the marama bean seeds and tuber as a potential food and medicinal crop to date.

Year	**Study performed**	**Brief description**
1999	Anti-tumour promoting activity of polyphenols from *Cowania mexicana* and *Coleogyne ramosissima*.	The roots of *T. fassoglense* produce a cyanoglucoside, lithospermoside, a compound which could be important as a potentially strong anti-tumour promoter (Ito et al., 1999).
2007	Quality evaluation of morama milk.	The chemical and nutritional composition of marama bean milk was investigated. Total solids were 6%, consisting of 1.5% protein, 3.1% fat, 1.1% carbohydrates, and 0.2% ash. It has a sodium content of 47.9 mg/100 g, iron content of 3.7 mg/100 g

		and calcium content of 6.8 mg/1000 g, as well as a high level of unsaturated fatty acids (Mpotokwane et al., 2007).
2009	Physical properties of *Tylosema esculentum* and the effect of roasting on the functional properties of its flour.	Roasting was determined to significantly improve the ash and protein content, water absorption capability, oil absorption ability, and emulsifying activity. Overall, roasting the seeds before processing into flour increases the nutritional properties of the flour (Jideani et al., 2009).
2010	Physicochemical, nutritional and functional properties of marama bean flour.	Physicochemical and protein-based functional properties for defatted flour made from unheated and dry-heated whole marama beans were analysed. Heating significantly increased protein digestibility *in vitro* and water absorption capacity, reduced protein solubility and emulsifying capability (Maruatona et al., 2010).
2011	Antibacterial and anticandidal activity of *Tylosema esculentum* (marama) extracts	*Tylosema* extracts made using ethanol, methanol and water were tested for antibacterial and anticandidal activity against methicillin-resistant *Staphylococcus aureus*, *Mycobacterium terrae*, *Corynebacterium diphtheriae* and *Candida albicans*. Marama bean ethanol and methanol extracts, and crude seed coat extracts proved the most effective in antibacterial and anticandidal activity at various concentrations (Chingwaru et al., 2011).
2011	*Tylosema esculentum* (marama) tuber and bean extracts are strong antiviral agents against Rotavirus infection	Extracts obtained from marama bean seeds and seed coats indicated antiviral activity against Rotaviruses. Rotaviruses are known to cause diarrhoea in domestic animals, immunocompromised people, infants and small children (Kapewangolo et al., 2011).
2011	Nutritional quality and antioxidant activity of marama–composite flours and porridges	Various properties of marama, sorghum, and marama-sorghum flours were analysed, including proximate and amino acid composition, energy value, phenolic content, fatty acid content, and antioxidant activity. Flours with marama added had increased protein and fat levels, lysine content, energy levels, oleic acid levels, and phenolic content. Phenolic content and antioxidant activity were positively correlated (Kayitesi et al., 2011).
2012	Highly viscous dough-forming properties of marama protein	The rheological properties of the marama bean protein were compared to those of soya and gluten. Marama protein formed a highly extensible and viscous dough when compared to dough made from soya or gluten. Marama protein was found to be more hydrophobic with more β-sheet structures than found in soya (Amonsou et al., 2012b).
2013	Challenges of designing a cracker for Morama bean – Short communication	The capability of macadamia nut crackers for use for marama beans was tested. Results indicated various physical properties of the marama bean seed presented challenges for cracking seeds that cannot be solved by conventional methods (Tjiparuro, 2013).
2013	Effect of pre-processing on the physicochemical properties of morama bean *(Tylosema esculentum)* milk	The effect of pre-processing treatments including salt-water-blanching, sodium-bicarbonate-blanching, hot-water-blanching on the proximate composition, total solids non-fat, colour, viscosity, sediments, pH and titratable acidity of morama milk was assessed. Consumer preference of the morama milk was also studied, using a soymilk control. Pre-processing sodium-

		bicarbonate-blanched samples produced significant differences in protein and carbohydrate composition, as well as increased viscosity. Salt-water blanched milk has higher levels of sediment. Baking-soda blanched marama milk was preferred over other marama milk samples and over soya milk. (Jackson et al., 2013).
2014	Fuel properties of biodiesel produced from selected plant kernel oils indigenous to Botswana: A comparative analysis	Oils from *T. esculentum* and other seeds were investigated for their biodiesel fuel properties compared to petroleum diesel. Some of the tested fuel properties of *T. esculentum* and other seed oils were similar or better than those of petroleum fuel, indicating feasibility of biodiesel fuel production from indigenous seeds (Gandure et al., 2014).
2015	The potential of Sub-Saharan African plants in the management of Human Immunodeficiency Virus Infections: A review	The anti-HIV activity of *T. esculentum* and other sub-Saharan African plants was investigated. Various compounds were found to have anti-HIV and antiviral activity. These compounds included: gallic acid and vanillic acid (anti-HIV); hesperidin (anti-influenza virus); fisetin (anti-dengue fever); quercetin (anti-Mayaro fever virus); tannins (anti-influenza and coxsackie virus) (Chingwaru et al., 2015b).
2015	Therapeutic and prophylactic potential of morama (*Tylosema esculentum*): A review	*Tylosema esculentum* has been observed to have various chemicals that inhibit infectious organisms such as bacteria, viruses, fungi, and drug-resistant strains. Extracts have also been seen to promote wound healing, gut health, and protect against certain human non-communicable diseases such as tuberculosis (TB), rotavirus gastroenteritis, immunodeficiency, heart diseases, cancer, and neurodegenerative diseases, among others. Various antinutrients were observed, such as allergens and protease inhibitors (Chingwaru et al., 2015a).
2017	Influence of extraction pH on the foaming, emulsification, oil-binding and visco-elastic properties of marama protein	Protein extraction under slightly acidic conditions was investigated to determine the effect pH has on functional properties of the protein extract. Marama protein extracted at pH 6 lacked legumin polypeptides present at pH 8, and contained acidic high molecular weight polypeptides that disulphide crosslinked in order to produce larger proteins. The emulsification properties of the pH 6 sample were similar to that of soya protein isolates, while the foaming capacity and elasticity was higher than the pH 8 marama sample (Gulzar et al., 2017).
2017	Physicochemical, morphological, thermal and pasting properties of marama (*Tylosema esculentum*) storage root starch	The physicochemical, microstructure and molecular structure, thermal and pasting properties of *T. esculentum* storage roots were characterized and compared to that of maize (*Zea mays* L.), cassava (*Manihot esculenta* Crantz), and potato starches. Marama amylose content was similar to cassava but lower than maize and higher than potato. The marama peak viscosity was double that of maize and cassava. Marama gel firmness is higher than cassava but half that of maize and potato. Marama starch has the potential to be used as thickeners in food (Adeboye & Emmambux, 2017).
2018	Potential of defatted marama flour-cassava starch composites to produce functional gluten-free bread-type dough	Defatted marama flour (DMF) and cassava starch (CS) composite (DMF-CS) dough properties were compared with that of wheat (*Triticum aestivum* L.) flour dough to determine the potential for DMF as a gluten-free ingredient in bread. Results indicated a shorter Mixolab development time and stability, that the dough

	can inflate into a bubble, and that they could hold gas produced by yeast fermentation. Defatted marama flour appears to be a good candidate for functional gluten replacement in protein- and fibre-rich gluten-free breads (Nyembwe et al., 2018).

Jackson et al. (2010) provides an extensive list of potential health benefits and negative effects related to the different chemical and nutritional components of the marama beans, a summary of which is available in Table 2.4.

Table 2.4. A summary of the potential health benefits and negative effects related to the different chemical and nutritional components of the marama beans, as detailed by Jackson et al. (2010).

Positive effects	
Component*	**Effect**
Alpha-linoleic acid (FA)	Protects against coronary heart disease, inhibits breast cancer cell growth and reduces platelet stickiness. Deficiency can result in abnormal eye and brain function.
Arachidonic acid (FA)	Phospholipid membrane component. Needed for skeletal muscle tissue regrowth and repair as well as neuron repair and brain protection.
Arginine (AA)	Protein synthesis, wound healing and alcohol toxicity reduction, antioxidant, inhibits platelet aggregation, and reduces toxic compound accumulation.
Aspartic acid (AA)	Induces apoptosis, assists brain and liver functioning.
Cysteine (AA)	Precursor for N-acetyl-cysteine, which is important for various processes, including reducing the risk of breast cancer.
Dietary fibre	Aids in absorption of nutrients.
Erucic acid (FA)	Protects against coronary heart disease and reduces platelet stickiness.
Gallic acid	Anticancer and antiviral (Herpes) properties.
Histidine (AA)	Growth and repair, modulates physiological processes and clears necrotic material.
Lignans	Anticancer properties.
Linoleic acid (FA)	Aids children with cystic fibrosis and attenuates complications related to diabetes.
Lysine (AA)	Lowers cholesterol.
Methionine (AA)	Parkinson’s disease treatment, aid chronic fatigue and pain, antioxidant, and breaks down fatty acids preventing accumulation.
Myristic acid (FA)	Stabilizes immune system proteins and promotes membrane binding. Deficiency can lead to immune system dysfunction and cancer.
Oleic acid (FA)	Hinders adrenoleukodystrophy progression.
Palmitoleic acid (FA)	Fights weight gain.
Phenylalanine (AA)	Pain and inflammation reduction, Parkinson’s disease and vitiligo treatments.
Phytoestrogens	Anticarcinogenic and lowers cholesterol.
Stearic acid (FA)	Reduction of low-density blood lipoprotein.
Threonine (AA)	Growth and repair, glucose-sparing effect, and promotes thymus activity and growth.
Tryptophan (AA)	Enhance sleep and relaxation, sooth anxiety and stress and reduce carbohydrate cravings.
Tyrosine (AA)	Improves cognitive function and reduces stress.

Vitamins	Improves immune status.
	Negative effects
Erucic acid (FA)	Cause thrombocytopenia (few blood platelets).
Palmitic acid (FA)	Increases the risk of cardiovascular disease.
Glutamic acid (AA)	Causes numbness and palpitations.
Protease inhibitors	Potent allergen (Kunitz-type elastase inhibitor)

*FA – fatty acid; AA – amino acid

2.3 Insect and microbial associations with marama bean

Marama bean production can be benefitted or constrained by various factors, including the environmental conditions under which the plant grows, information surrounding the plant, as well as insect and microbial associations with marama bean. For example, humid conditions and tropical areas are not suited for *T. esculentum* production, potentially limiting the areas where *T. esculentum* can be cultivated, while *T. fassoglense* prefers subtropical and tropical conditions restricting growth in arid areas (National Research Council, 2006). Potential production constraints include poor soil quality, water and lan availability, slow growth cycles with low yield, labour inputs, and availability of instant cash provided by wild marama bean populations in comparison to domesticated plants (Jackson et al., 2010). Lack of information and understanding surrounding the production and growing conditions, nutrients, benefits and potential of the marama bean also places constraints on the production of the marama bean as a commercial crop (National Research Council, 2006). Understanding the different growth parameters of the marama bean regarding the genetic, environmental, and symbiotic relationships of the marama bean's growth will aid in the cultivation of the marama bean (Cullis et al., 2018; National Academy of Sciences, 1979). Symbiotic relationships include fungal and insect pathogens that attack the plant, specifically the pods and seeds. Insects feed on the plant, creating opportunities for secondary infections by various plant pathogens, including bacteria and fungi. The insects may also bore through the pods into the seeds, introducing fungal pathogens which attack the seeds (Agrios, 2005). Fungal pathogens can affect plant growth and crop yield, as well as provide opportunity for other pathogens to infect the plant and cause disease. The potential of certain fungal pathogens to produce mycotoxins, which are harmful to human and animal health (Bennett & Klich, 2003), is of great concern regarding the cultivation and commercialisation of potentially valuable crops such as the marama bean.

2.3.1 Insect associations

Insect associations can be beneficial to plants, or act as constraints, or alternatively they can be both beneficial or detrimental depending on certain conditions. Bees have been observed on the marama bean flowers and implicated to be pollinators, thus beneficial to marama bean production (Jackson et al., 2010). Ants have been observed on marama bean flowers, however there is debate whether the ants are potential pollinators or merely nectar robbers (Beattie et al., 1984; Mbewe, 1992).

Limited literature regarding insect pests of *Tylosema* species is available. Although Jackson et al. (2010) noted insect damage on a wild population that resulted in seed damage, no insects were identified. Bagai (2020) isolated and identified several insect pests in field populations of *T. esculentum* collected in two different regions within Botswana. These pests included *Afropachylepta nigrotibialis* (Jacoby) of the family Coleoptera; *Afrosteles* sp. (Theron), *Callidea dregii* (Germ.), *Bemisia tabaci* (Gennadius), and *Aphis gossypii* (Glover) of the family Hemiptera; Macrotermitinae of the Isoptera family; and Lepidoptera insects. Insect damage to the marama bean included dried and dead leaves due to sap sucking, leaf- and vine dieback due to termite attacks, and holes bored into fresh pods and seeds by moths. The confused flour beetle, *Tribolium confusum* (Jacquelin du Val) of the family Coleoptera, and *Plodia interpunctella* (Hubner) of the family Lepidoptera were identified as storage pests of marama bean seeds stored in the BUAN Food Science and Technology laboratory storeroom (Bagai, 2020).

A well-known insect pest of most leguminous plants are beetles commonly known as seed weevils, of the Bruchinae sub-family, and part of the Chrysomelidae family. Bruchinae can be divided into six tribes, including the Acanthoscelidini, Bruchini and Pachymerini tribes (Delobel et al., 2013). The borer beetle *Caryedon serratus* (Olivier) and Caryedon gonagra (Fabricius), both part of the Pachymerini tribe (Delobel et al., 2013), is a well-known pest of *Bauhinia* (Sembène et al., 2011) and has been described on *Bauhinia variegata* L. (Nilsson & Johnson, 1992) and *Bauhinia rufescens* Lam. (Pierre & Huignard, 1990). Various insects have been reported on *Bauhinia*, including various borers, mites and insect larvae; *Psylla simlae* Crawford nymphs (Hemiptera) and Malaysian locusts (*Valanga nifricornis* (Burmeister)) (Orwa et al., 2009); Lewis spider mite (*Eotetranychus lewisi* (McGregor) (Doucette, 1962); False codling moth (*Thaumatotibia leucotreta* (Meyrick) (Venette et al., 2003); and giant whitefly (*Aleurodicus dugesii* Cockerell) (Dreistadt et al., 2001). The genus *Bauhinia* has been described as a close relative of *Tylosema* (Sinou et al., 2009), therefore, an insect pest of *Bauhinia* could potentially affect *Tylosema* as well.

Tylosema seeds collected from Centurion, South Africa by the author were found to have holes bored through the seed pods and into the seeds, potentially introducing disease to the seeds. Pathogen infected leaves (Fig. 2.5 A – as indicated by the red circle) and seed pods (Fig 2.5 B) which presented signs of insect damage were also observed in the Centurion *Tylosema* population. Other potential pests could include *Acanthoscelides obtectus*, the bean weevil of the Tribe Bruchini (NCBI Taxonomy Browser, n.d.-a), and the genus *Caryedes* Hummel of the Tribe Acanthoscelidini (NCBI Taxonomy Browser, n.d.-b). However, further studies into the presence of insect pests on the marama bean would need to be performed to definitively conclude such a relationship. Insect pest control on the marama bean could be achieved naturally through the presence of high levels of elastase specific inhibitors which are known to be natural defences against plant insect pests due to toxicity to insects (Nadaraja et al., 2010). However, due to lack of knowledge of the *Tylosema* pests, identification of the insect pests as well as the extent of the damage caused by the insect pests need to be investigated before control options can be considered.

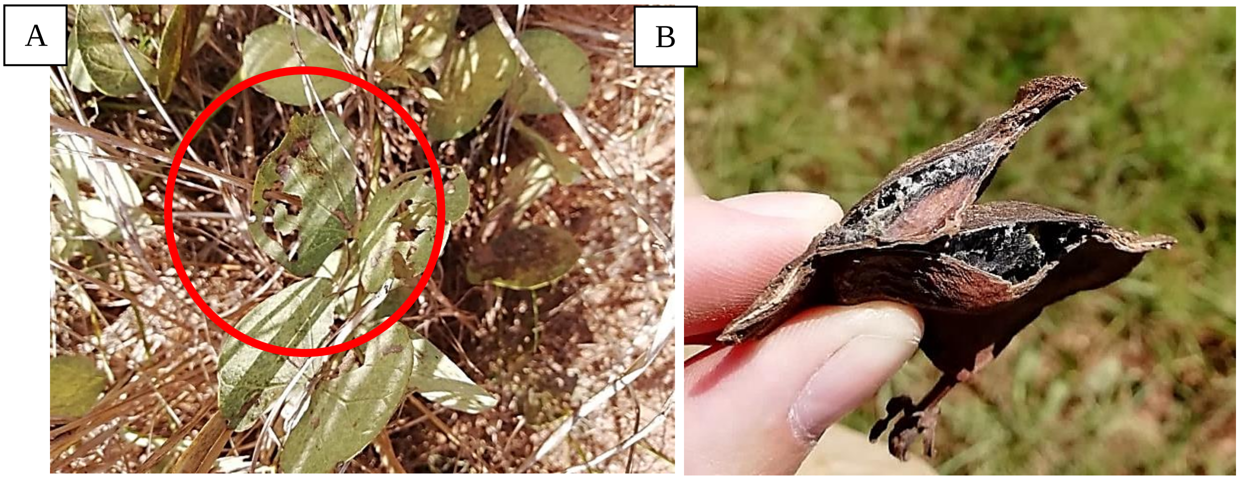

Figure 2.5. Signs of insect damage on *Tylosema fassoglense* leaves observed on plants growing in the Centurion sampling area [A], and the pathogen infection within the seed pods [B]. Photos by T. Armfield.

2.3.2 Bacterial associations

Plants are often subjected to pathogenic attacks from various plant pathogens including fungi, bacteria, nematodes and viruses. Plant-microbe relationships have historically been described in very simplistic terms as either parasitic, where one organism benefits while the other organism is harmed; or as mutualistic, where both organisms benefit from the association; or as pathogenic, where one organism causes disease in another (Newton et al., 2010). However, these descriptions can be regarded as extremes, and microbes often migrate between the different relationships depending on the host and situation (Newton et al., 2010). In the case of mutualistic associations, certain bacteria may be beneficial to their host plants, providing various benefits such as nitrogen fixation, anti-fungal activity, nematodical activity, and anti-bacterial activity against other bacterial genera (Hayat et al., 2010; Kandjimi et al., 2015). Therefore, the lack of information and understanding of indigenous wild crops such as the marama bean and the microorganisms associated with them, results in the inability for the crop to successfully be utilized for its potential as a commercially produced crop.

2.3.2.1 Plant growth promoting (PGP) bacteria in the rhizosphere and soil

Although many groups of bacteria can be considered pathogenic or parasitic, some bacterial groups such as plant growth promoting (PGP) bacteria live in association with plants and stimulate plant development and growth through a variety of mechanisms that can be either direct or indirect, and simultaneously or independently active at different plant growth stages (Kandjimi et al., 2015). These mechanisms aid the plant in various functions such as photosynthesis, energy transfer, and sugar and starch transformation, among many others (Kandjimi et al., 2015).

Kandjimi et al. (2015) isolated bacteria from the rhizosphere and bulk soil of *T. esculentum* plants and screened for various activities typically considered beneficial to plants. These activities included: phosphate solubilization activity (using the phosphate solubilization index); aminocyclopropane-1-carboxylate (ACC) deaminase activity; catalase and protease activity; and ammonia and hydrogen cyanide production. The bacterial cultures were used for DNA extraction to obtain 16S rDNA amplicons, used to molecularly identify the bacterial species. Eight bacterial strains that exhibited potential plant growth promoting traits were isolated from the marama bean rhizosphere, while 19 strains were isolated from the bulk soil. The isolated bacterial strains included *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Kosakonia*, and *Raoultella* genera. All 27 isolates indicated phosphate solubilization activity, with eight having a solubilization index of at least 6. Aminocyclopropane-1-carboxylate (ACC) deaminase activity was evident in 23 of the isolates, catalase activity in 26 isolates, hydrogen cyanide production in sixteen isolates, and protease activity in 23 isolates. All 27 isolates were observed to produce ammonia (Kandjimi et al., 2015).

2.3.2.2 Seed-associated bacterial endophytes

Chimwamurombe et al. (2016) collected *T. esculentum* seeds from an experimental cultivation plot in eastern Namibia, surface sterilized the seeds and grew them gnotobiotically (in a controlled environment with few organisms) until the seedlings were two weeks old. Using three types of growth media, 123 distinct bacterial isolates were cultured from the surface-sterilized shoots and roots. BOX-PCR (Polymerase Chain Reaction using BOX elements) fingerprinting and sequence analyses of *nifH* genes and 16S rRNA were used to identify the strains. Of the 123 isolates, 73 were determined to be putative endophytes and subjected to phylogenetic analysis, yielding fourteen genera in four phyla: Proteobacteria: *Burkholderia*, *Caulobacter*, *Kosakonia*, *Massilia*, *Methylobacterium*, *Pantoea*, *Pseudorhodoferax*, *Rhizobium*, and *Shingomonas*; Firmicutes: *Bacillus*; Actinobacteria: *Curtobacterium* and *Microbacterium*; and Bacteroidetes: *Chitinophaga* and *Mucilaginibacter*. PGP activity screening indicated that the isolates produced ACC deaminase, endoglucanase, siderophores, indole 3-acetic acid (IAA), protease, and N-acylhomoserine lactones (AHLs), as well as had the ability to fix atmospheric nitrogen and solubilize phosphates.

2.3.3 Fungal associations

2.3.3.1 Fungi associated with the pods and seeds

Uzabakiriho et al. (2013) collected *T. esculentum* pods, which presented necrotic spots with densely sporulated centres, in Eastern Namibia in the Omaheke region. The seed pods were used for fungal isolation using direct plating methods resulting in two endophytic fungal isolates. The sequences from the internal transcribed spacer (ITS) region obtained from the two fungal isolates presented 99% homology based on BLAST (Basic Local Alignment Search Tool) analysis in each case to a *Phoma* species and *Alternaria tenuissima* (Kunze) Wiltshire (now *A. alternata* (Fr.) Keissler), respectively. *Alternaria* and *Phoma* species are both highly dependent on environmental conditions such as humidity for spore dispersal. Bouts of increased rainfall uncommon for the areas where marama beans grow can affect spore dispersal, as well as the distancing between plants (Uzabakiriho et al., 2013).

2.3.3.2 Fungi associated with leaves

Takundwa et al. (2015) observed marama bean seedlings grown in a University of Namibia greenhouse experiment to have signs of necrosis and spotting on the newly developed seedling leaves. The necrotic leaves were used with direct plating techniques to obtain pure cultures of all present fungal species, yielding eight fungal isolates. The fungal isolates were identified based on BLAST analysis using the ITS region as *Alternaria solani* Sorauer, *Epicoccum sorghi* (Sacc.) Aveskamp, Gruyter & Verkley (now *E. sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley), *Fusarium equiseti* (Corda) Sacc., *F. chlamydosporum* Wollenw. & Reinking, *F. incarnatum* (Desm.) Sacc., *Penicillium brevicompactum* Dierckx, *P. olsonii* Bainier & Sartory, and *Rhizopus stolonifer* (Ehrenb.) Vuill (Takundwa et al., 2015). These fungi are all known plant pathogens causing various plant diseases on various other plants (Kwon et al., 2001; Spletzer & Enyedi, 1999; Wagner et al., 2000), presenting a potential problem for the crop domestication of the marama bean (Takundwa et al., 2015).

Surveys of fungal diseases on plants performed in the Otjiwarongo region of Namibia included *T. esculentum*. *Phoma sorghina* (Sacc.) Boerema, Dorenb. & Kesteren (now *E. sorghinum*), *A. tenuissima* and *A. alternata* were isolated and identified on the *T. esculentum* leaf samples collected during the survey (Chimwamurombe, 2016).

2.3.3.3 Leaf endophytic fungi associated with the marama bean

Fungal species, though sometimes pathogenic, can survive endophytically within the host plant in the absence of disease symptoms or apparent damage to the host, gaining nutrients from the host plant while often conferring benefits to the host plant through biotic and abiotic stress resistance and tolerance, such as drought tolerance, high salt and low pH tolerance, and enhanced photosynthetic rates. Herbivores can also be deterred due to the inedible taste produced due to the presence of the endophytic fungi (Fischer et al., 2005; Lewis, 2004; Saikkonen et al., 1998; Wilson, 1995; Zhang et al., 2006).

Martin et al. (2012) isolated and identified several endophytic fungi from apparently healthy-looking *T. esculentum* leaves collected in the Otjiwarongo region of Namibia. Following identification based on BLAST analysis using the ITS region, the fungal species were identified as *P. sorghina*, *A. alternata*, *A. tenuissima*, and *Penicillium commune* Thom (Martin et al., 2012)

2.3.4 Summary of the known fungi associated with *Tylosema esculentum*

Fungal species cause a wide variety of symptoms and diseases on a wide variety of plants throughout the world. The specific effect that a particular fungal species has on a host plant is dependent on the type of host plant, thus different host plants will be affected in different ways by the same fungal species. In line with that, different fungal species will also affect the same host plant in different ways (Kwon et al., 2001; Spletzer & Enyedi, 1999; Wagner et al., 2000). This creates a need to study the individual pathogen-plant interactions, in order to understand the relationship between pathogen and host, and subsequently prevent or manage detrimental interactions. The following summaries focus on several fungal species that have previously been identified from marama beans, and some of the known pathogen-plant interactions that have been reported.

2.3.4.1 *Alternaria* spp.

Alternaria diseases affect every part of the plant and generally affect most ornamental and vegetable crops and some tree crops (Pinto & Patriarca, 2017; Uzabakiriho et al., 2013). *Alternaria* spp. mycelia are dark in colour and produce distinctly identifiable air-borne conidia with transverse and longitudinal cross walls (Pinto & Patriarca, 2017). General symptoms include: tan to light brown, circular or irregular lesions surrounded by a dark red border on lower leaves, leading to eventual defoliation; fruit decay and mould; and necrotic leaf lesions (Martin et al., 2012; Pinto & Patriarca, 2017; Uzabakiriho et al., 2013).

Alternaria alternata is a seasonal human and plant pathogenic endophyte, is highly adaptable to various environmental conditions, and can produce various host specific toxins (Martin et al., 2012). *Alternaria alternata* has been shown to cause various diseases on a wide range of crops, including leaf blight of onion (*Allium cepa* L.) (Ramjegathe & Ebenezar, 2012), brown spot disease of citrus (Kohmoto et al., 1991), leaf spot and black rot of citrus (Timmer et al., 2003), tomato (*Solanum lycopersicum* L.) stem canker (Gilchrist & Grogan, 1975), and sunflower (*Helianthus annuus* L.) leaf spot (Lagopodi & Thanassoulopoulos, 1998). Similarly, reports of *A. tenuissima* (now *A. alternata*) include a wide range of diseases and symptoms such as late blight and rot of fruits (Uzabakiriho et al., 2013), leaf spot disease of Highbush blueberry (*Vaccinium corymbosum* L.) (Milholland, 1973), and tomato leaf spot (Agamy et al., 2013), among many others. *Alternaria solani* is a necrotrophic plant pathogen that causes early blight in tomatoes and potatoes (*S. tuberosum* L.), and can affect almost every part of the plants (Chaerani & Voorrips, 2006; Fritz et al., 2006; Pasche et al., 2004; van der Waals et al., 2004). This fungus can also cause collar rot, stem lesions and fruit rot of tomato plants (Chaerani & Voorrips, 2006). .

2.3.4.2 Didymellaceae spp.

Didymellaceae is a highly species-rich family that can inhabit a vast range of ecosystems (Chen et al., 2017). Due to the wide variety of species within the Didymellaceae family, classification of genera and species within the family can be difficult without multi-locus DNA sequencing data, whereby multiple genes are used in DNA sequence analysis in order too obtain an accurate molecular identification (Chen et al., 2017). Didymellaceae has been redefined and reclassified over the years to include *Didymella*, *Phoma*, *Ascochyta*, *Epicoccum*, *Peyronellaea*, *Stagonosporopsis*, *Boeremia*, *Leptoshaerulina*, and *Macroventuria* (Chen et al., 2017; Crous et al., 2004; de Gruyter et al., 2009; Aveskamp et al., 2010). Several new genera have also been added to the family over the last few years (Chen et al., 2017).

Phoma spp. are ubiquitous saprophytic and plant pathogenic fungi that affect a wide variety of plants worldwide, especially vegetables, of which common symptoms include necrotic spots and lesions that develop during cool, wet conditions (Uzabakiriho et al., 2013). Examples of diseases caused by *Phoma* species include cabbage (*Brassica oleracea* var. *capitate* L.) root rot and black leg, tomato rot, sunflower black stem, leaf spot, leaf scorch, dying-off of seedlings, potato rot, and various human diseases often of a subcutaneous nature (Bennett et al., 2018). *Phoma sorghina* (now *E. sorghinum*) is a non-specific soil- and seed-borne parasitic fungus that attacks a variety of plants (Uzabakiriho et al., 2013), most notably bananas (*Musa* L.) and sorghum (*Sorghum bicolor* (L.) Moench) (Bennett et al., 2018), generally, as a secondary invader of plants already infected by another pathogen. It is also a known producer of the tenuazonic acid mycotoxin (Bennett et al., 2018; Martin et al., 2012).

Epicoccum sorghinum [formerly *E. sorghi*] is an Ascomycete that produces tenuazonic acid. Common diseases associated with *E. sorghinum* include sorghum grain mould (Davenport et al., 2017), taro (*Colocasia esculenta* (L.) Schott) leaf spot (Liu et al., 2018) and *Bletilla striata* leaf spot (Zhou et al., 2018).

Didymella species can range from severe pathogens to endophytes and saprophytes, all of which are able to colonise a wide variety of plants and crops (Aveskamp et al., 2010). Diseases associated with various *Didymella* species include gummy stem blight of cucurbits (Grube et al., 2011), Ascochyta blight of chickpea (*Cicer arietinum* L.) and pea (Barilli et al., 2016), tan spot of pyrethrum daisy (*Tanacetum cinerariifolium* (Trevir.) Sch.Bip.) (Pearce et al., 2015), and leaf and pod spot (Ascochyta blight) of cool-season pulses such as fababeans (*Vicia faba* L.) (Davidson & Kimber, 2007).

2.3.4.3 *Penicillium* spp.

Penicillium species are commonly known for the production of blue and green mould rots, postharvest diseases that affect a wide range of fruits and vegetables that can occur in the field (such as in citrus fields), but most commonly occur during storage and on processed foods such as bread as well (Dupont et al., 1999). General symptoms of *Penicillium* infections include soft rots of the tissue before the mould forms on the surface while the fruit decays (Agrios, 2005).

Penicillium brevicompactum has been identified as a leaf endophyte in coffee plants (*Coffea* L.) (Vega et al., 2006), as well as causing ginger (*Zingiber officinale* Roscoe) postharvest storage rot (Overy & Frisvad, 2005). *Penicillium commune* is a pathogenic green mould with airborne spores that can cause allergic reactions in sensitive individuals (Rao et al., 2014). Examples of plant diseases caused by *P. commune* include pear (*Pyrus* L.) and apple (*Malus domestica* Borkh.) postharvest decay (Sanderson & Spotts, 1995), and apple pre- and postharvest diseases (Amiri & Bompeix, 2005). *Penicillium olsonii* to date have been identified only on a few plant hosts (Chatterton et al., 2012), including the marama bean (Takundwa et al., 2015). Another example of disease associated with *P. olsonii* is eucalyptus (*Eucalyptus* L'Hér.) damping-off (Mwanza & Kellas, 1987). *Penicillium olsonii* has been known to survive as an endophyte in coffee plants (Vega et al., 2006) as well as infect *Arabidopsis thaliana* leaves without causing severe disease (Wagner et al., 2000).

2.3.4.4 *Fusarium* spp.

The *Fusarium* genus includes necrotrophic soil-borne pathogens, known to produce white to cream-coloured mycelia that develop into pink, red, orange, purple or salmon colours, one of the characteristics used for identifying or grouping *Fusarium* spp. (Nelson et al., 1983). *Fusarium* spp. are well known for causing vascular wilts, a complete dysfunction of water translocation through the xylem in various shade trees, vegetable, flower and field crops; as well as root, stem and seed rots of bean plants and potatoes, among others, often accompanied by mycotoxin production (Agrios, 2005).

Fusarium equiseti is a soil-borne plant pathogen that affects the tubers, seeds, fruit and roots of various crops, causing diseases such as root rot and damping-off of various crops (Goswami et al., 2008), vascular wilt of cumin (*Cuminum cyminum* L.) (Ramchandra & Bhatt, 2012), damping-off of pine (*Pinus* sp. L.) (Lazreg et al., 2014), maize (*Zea mays* L.) sheath rot (Li et al., 2014), and cauliflower (*Brassica oleracea* var. *botrytis* L.) *Fusarium* wilt (Shi et al., 2017), among many other diseases. *Fusarium incarnatum* causes ginseng (*Panax ginseng* C.A.Mey.) root rot (Song et al., 2014), walnut (*Juglans* L.) canker (Seta et al., 2004) and bell pepper fruit rot (*Capsicum annuum* L.) (Ramdial et al., 2015). *Fusarium incarnatum* is morphologically similar to *F. equiseti*, together with over 30 other species, and these closely related species are grouped within the *F. incarnatum-equiseti* species complex (Wang et al., 2019). Species within the *F. incarnatum-equiseti* species complex can affect a range of crops worldwide (Jacobs et al., 2018). Some examples of diseases caused by *F. chlamydosporum* include wilt disease on guava (*Psidium guajava* L.) (Gupta & Misra, 2012), mango (*Mangifera indica* L.) leaf spot (Omar et al., 2018), and tomato fruit rot (Oladiran & Iwu, 1993).

2.3.4.5 *Rhizopus* spp.

Rhizopus forms part of the order Mucorales which affects fruits and vegetables, and stored grains postharvest, and causes diseases such as peach (*Prunus persica* L.) and strawberry (*Fragaria* spp. L.) rot (Oliveira et al., 2019). *Rhizopus stolonifer* is known for causing postharvest diseases such as stone fruit rot (Bonaterra et al., 2003), nectarine (*Prunus persica* var. *nucipersixca* (Suckow) C.K.Schneid) rot (Qing & Shiping, 2000), and rot of table grapes (Vitaceae Juss.) (Sarig et al., 1996).

2.4 Mycotoxins: An introduction

Several of the previously discussed fungal genera (section 2.3.4) are able to produce various secondary metabolites, the most notable of which are mycotoxins. Mycotoxins are secondary metabolites produced by various fungal species primarily to aid in disease development and pathogenesis, and to aid in fungal growth albeit seldomly (Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003).

Mycotoxins can be classified in a variety of ways depending on who classifies them - clinicians classify them by the organ they affect; organic chemists by the chemical structure; biochemists by the biosynthetic origins; and mycologists by the fungus that produces them; therefore, making standard classification of mycotoxins difficult (Klich & Bennett, 2003). The concentration and target of the mycotoxins can also play a role in their classification. Mycotoxins can be toxic to bacteria (as antibiotics), plants (as phytotoxins), and animals and humans (through mycotoxicosis); and can be host-specific, host-selective, or non-host-specific (Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003). While *Fusarium*, *Penicillium, Aspergillus* and *Alternaria* are the main genera known to produce mycotoxins, various species within other genera are also capable of producing mycotoxins to a lesser extent, for example, *Trichoderma*, *Stachybotrys*, *Trichothecium*, and *Myrothecium* (Boysen et al., 2002; Goswami et al., 2008; Ismaiel & Papenbrock, 2015; Martins et al., 2020; Minervini et al., 2004; Pinto & Patriarca, 2017; Siciliano et al., 2015; Visagie et al., 2014).

The effects of mycotoxins in humans and animals depends on various conditions such as dosage, host age, time of exposure, type and breed, as well as presence of other diseases (Escrivá et al., 2017; Iheshiulor et al., 2011; Klich & Bennett, 2003; Zain, 2011). Mycotoxicosis is the result of mycotoxin poisoning through respiratory, dietary, dermal or other exposure to mycotoxins (Klich & Bennett, 2003). Intestinal health and immune function can also be affected by mycotoxins (Antonissen et al., 2014; Iheshiulor et al., 2011; Zain, 2011). General symptoms of exposure to mycotoxins include diarrhoea, vomiting, loss of appetite and weight, immune system suppressions, as well as reduced milk production in cows (Agrios, 2005). The effects of mycotoxins in plants can be seen in poorer seed viability, quality and germination, seedling vigour, as well as reduced root and coleoptile growth (Ismaiel & Papenbrock, 2015). Accumulation of the mycotoxins within plant tissues can also be an identifying factor as many mycotoxigenic fungi are endophytes (Ismaiel & Papenbrock, 2015).

Mycotoxins are produced under a variety of conditions that are often species specific, and include among others, the moisture content where the fungi are situated, aeration and temperature, the microbial population, the susceptibility of the host plant, and the variation within fungal species (Ciegler, 1978; Fernández-Cruz et al., 2010; Ismaiel & Papenbrock, 2015). Various conditions in nature that cannot be replicated in a laboratory setting – such as microbial flora interaction, herbicide and pesticide presence, oxygen levels, weather conditions, irrigation, insect presence, host species and age, and the presence of other mycotoxins – also contribute to the variations in mycotoxin production conditions (Ciegler, 1978).

2.4.1 Common mycotoxins

The largest group of commonly produced *Aspergillus* mycotoxins includes the aflatoxin group, produced primarily by *A. flavus*, *A. parasiticus* and *A. nomius* Kurtzman, B.W. Horn & Hesselt., as well as a few other species such *A. pseudocaelatus* Varga, Samson & Frisvad and *A. pseudonomius* Varga, Samson & Frisvad (Varga et al., 2011). Aflatoxins are primarily found on maize and legumes in low concentrations, and on cottonseed (*Gossypium* L.), Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.), and peanuts (*Arachis hypogaea* L.) in high concentrations. Aflatoxin exposure in humans is generally a consequence of contaminated foods and crops pre- or postharvest, such as nuts, grains, and their associated products (EFSA Panel on Contaminants in the Food Chain (CONTAM) et al., 2018). However, some aflatoxins retain their toxicity after processing in animal digestive systems causing downstream effects. An example is the case of aflatoxins in cow feed, which is then secreted in the milk they produce and passed to humans upon consumption of the milk, which can cause acute liver toxicity (aflatoxicosis), liver cancer, oedema, haemorrhages or death (Bbosa et al., 2013; Fakruddin et al., 2015). Symptoms of aflatoxin poisoning can vary depending on age, dosage, animal species, toxin derivative, among other factors (Fakruddin et al., 2015). Phytotoxic effects include reduced germination, reduced root and shoot growth, reduced plant biomass accumulation, and reduced leaf mass regeneration (McLean, 1994).

Citrinin, luteoskyrin, penicillic acid and citreoviridin mycotoxins are among many mycotoxins produced by various *Penicillium* species. The mycotoxins are often produced on barley (*Hordeum vulgare* L.), rice (*Oryza sativa* L.), and maize in storage, as well as dried fish (Udagawa & Tatsuno, 2004). Citrinin, known to be associated with wheat (*Triticum aestivum* L.), maize, barley, rye (*Secale cereale* L.) and oats (*Avena sativa* L.), has been identified as a nephrotoxin that destroys kidney tissue (Ismaiel & Papenbrock, 2015). Phytotoxic effects of citrinin include seed germination and seedling growth inhibition, wilting, and reduced radicle growth (Ismaiel & Papenbrock, 2015).

Ochratoxins are commonly produced by both *Aspergillus* and *Penicillium* spp., and can occur on a variety of plants including: cereals, coffee plants, oats, grains such as rye, barley and wheat (Bui-Klimke & Wu, 2015; Ciegler, 1978). Ochratoxins can affect domestic animals and cause liver and kidney necrosis and deterioration, among other diseases. Phytotoxic effects include cell death, necrosis and subsequent plant growth inhibition (Ismaiel & Papenbrock, 2015). Other toxins produced by both genera include tremorgenic toxins found on stored and refrigerated foods and cause body tremors, seizures and eventual death in dogs (Boysen et al., 2002), and patulin which is produced by several fungi including *P. expansum* and *A. clavatus* (Lopez-diaz & Flannigan, 1997) and can be found in infected food stuffs and various fruits such as apples, pears, stone fruit and grapes. Patulin can cause oedemas, paralysis and cancer in humans due to long-term exposure (Koutchma, 2016). Phytotoxic effects include reduced seed number and weight, reduced number of flowers and biomass accumulation (Ismaiel & Papenbrock, 2015).

Fusarium is one of several genera capable of producing trichothecenes, along with *Trichoderma*, *Stachybotrys*, *Trichothecium*, and other genera in the Hypocreales order (Ismaiel & Papenbrock, 2015). Trichothecenes consist of a tetracyclic ring system which has been arranged or modified in different ways to create each of the 120 trichothecene mycotoxins known to date. Nivalenol (NIV), 4-deoxynivalenol (DON), zearalenone (ZEA), and fumonisins fall within the trichothecene group, all of which are known to be produced by various *Fusarium* species (Antonissen et al., 2014; Ismaiel & Papenbrock, 2015). *Fusarium* trichothecenes are known to cause chlorosis, necrosis and mortality in plants, leading to various diseases including wilts, and root, leaf, and stalk rots (Antonissen et al., 2014; Ismaiel & Papenbrock, 2015).

Deoxynivalenol (DON, also known as vomitoxin) is commonly produced by *Fusarium culmorum*, *F. incarnatum* and *F. graminearum* (Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003; Marasas et al., 1977; Minervini et al., 2004; Pestka, 2007; Sweeney & Dobson, 1998; Vesonder & Hesseltine, 1980). DON often results in the inhibition of seedling, root and plant growth (Ismaiel & Papenbrock, 2015), as well as toxicity, vomiting, and reduced feeding in swine and domestic animals (Klich & Bennett, 2003; Pestka, 2007). DON has been reported on various plants and crops including tomatoes, wheat, rye, maize, and onion, among many others (Ismaiel & Papenbrock, 2015; Marasas et al., 1977; Sweeney & Dobson, 1998; Vesonder & Hesseltine, 1980). DON is also a DNA and protein synthesis inhibitor, inhibiting the production defence enzymes in the host plant which allows *F. graminearum* to spread throughout the plant (Ismaiel & Papenbrock, 2015; Minervini et al., 2004; Snijders & Krechting, 1992).

Nivalenol (NIV) is produced by *Fusarium equiseti*, *F. graminearum*, *F. chlamydosporum*, *F. sporotrichoides*, *F. crookwellwense*, and *F. nivale* (Goswami et al., 2008; Ismaiel & Papenbrock, 2015; Minervini et al., 2004; Sweeney & Dobson, 1998). Nivalenol production results in reduced shoot and root growth, chlorosis, necrosis and mortality (Ismaiel & Papenbrock, 2015) in plants such as cereals and legume plants (Goswami et al., 2008; Sweeney & Dobson, 1998), among many others. Nivalenol exposure in humans and animals results in gastrointestinal distress, as well as decreased levels of cell proliferation due to inhibition of protein and DNA synthesis (Minervini et al., 2004).

Fusarium cerealis, F. semitectum, F. roseum, F. crookwellense, F. culmorum, F. equiseti and *F. graminearum* are known producers of zearalenone (ZEA, also known as the F-2 toxin) (Antonissen et al., 2014; EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011; Goswami et al., 2008; Ismaiel & Papenbrock, 2015; Marasas et al., 1977; Martins et al., 2020; Mirocha & Christensen, 1974; Sweeney & Dobson, 1998; Zain, 2011). ZEA production has been reported in cereals, legumes, sorghum, rice, maize, millet (*Panicum miliaceum* L.), wheat, oats, and barley (Goswami et al., 2008; Ismaiel & Papenbrock, 2015; Sweeney & Dobson, 1998), and cause necrosis, shoot and root inhibition, chlorosis, inhibition of biomass accumulation, and mortality in plants (Ismaiel & Papenbrock, 2015). Infertility in dairy cows were suspected to be caused by zearalenone, as well as toxicity in swine causing hyperestrogenism, among others (Marasas et al., 1977; Mirocha & Christensen, 1974).

Several fumonisin structures have been identified to date, namely FB_1, FB_2, FB_3 and FB_4, FA_1 and FA_2, as well as FC_1 and FC_3 (Sweeney & Dobson, 1998). Fumonisin B_1 is commonly produced by *Fusarium proliferatum, F. nygamai, F. monoliforme, F. incarnatum, F. verticilloides*, as well as *Alternaria alternata* (Abbas & Boyette, 1992; Alberts et al., 1993; Chen et al., 1992; Doehlert et al., 1994; Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003; Kritzinger et al., 2006; Sweeney & Dobson, 1998). In plants, FB1 is known to cause reduced height and biomass accumulation, necrosis and chlorosis, wilting, root and shoot growth inhibition, inhibition of seedling emergence and radicle elongation, and eventual death (Abbas & Boyette, 1992; Doehlert et al., 1994; Ismaiel & Papenbrock, 2015). Host plants include jimsonweed (*Datura stramonium* L.), tomatoes, legumes such as soybeans and cowpeas (*Vigna unguiculata* (L.) Walp.), as well as cereals such as sorghum and maize (Abbas & Boyette, 1992; Doehlert et al., 1994; Ismaiel & Papenbrock, 2015; Kritzinger et al., 2006; Sweeney & Dobson, 1998). Fumonisin B_1 has been identified as a potential cause of oesophageal cancer in humans, liver cancer in rats, equine leukoencephalomalacia in horses, donkeys, and mules, as well as hydrothorax and pulmonary oedema in pigs (Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003; Marasas, 1995).

Alternaria sp. has been reported to have more than 70 metabolites, many of which are used primarily for disease development, including alternuene, alternariol, altertoxins, fumonisins, tenuazonic acid, and cyclopiazonic acid, to name a few (Bashyal et al., 2014; Burdock & Flamm, 2000; Chen et al., 1992; Davis et al., 1977; Escrivá et al., 2017; Martins et al., 2020; Ostry, 2008; Pinto & Patriarca, 2017; Pollock et al., 1982; Siciliano et al., 2015; Sweeney & Dobson, 1998; Troncoso-Rojas & Tiznado-Hernández, 2014). However, there are records of various of the *Alternaria* toxins that are toxic to humans and animals through carcinogenicity, DNA strand breakage, mutagenicity, disruption of sphingolipid metabolism, phosphorylation, or enzyme activity inhibition (Escrivá et al., 2017;

Martins et al., 2020; Pollock et al., 1982). Common mycotoxins produced by *Alternaria* sp. belong to five chemical classes: pyranones or benzopyrones, amine/amide metabolites, perylenequinones, and anthraquinones (Escrivá et al., 2017).

Several of the fungal species previously isolated from *T. esculentum* seeds and leaves are capable of producing mycotoxins. To date there is no literature available regarding mycotoxins associated with *T. esculentum* or *T. fassoglense* seeds, leaves or tubers. However, there is a possibility that the fungal species isolated from the plants could have the potential to produce mycotoxins. As such, there is also a possibility for the natural occurrence of mycotoxins on the plants as a result of the association with the fungal species. Appendix 6.2 Table 6.1 details some of the common mycotoxins produced as well as their common fungal producers, common plant hosts, toxicity to humans and animals, and toxicity to plants (phytotoxicity).

Throughout the years there has been a focus of research on commercial crops and their associated pests and pathogens, however, research into lesser known crops and orphan crops is beginning to gain momentum due to the various benefits that indigenous orphan crops offer, such as optimal growth in their respective environment and climate (Cullis et al., 2018, 2019; Cullis & Kunert, 2017; National Research Council, 2006). Regarding the marama bean, there has been a large amount of research on the nutritional and medicinal value when compared to the research related to the cultivation and potential constraints of the marama bean. To date, there are few reports of insect, bacterial or fungal associations from populations found in Namibia, Botswana and Kenya, but no reports from South African populations. However, the identification of the fungal species is largely related to the association with the marama bean rather than the potential of the fungal species to cause disease on the marama bean. There are currently no records of mycotoxins associated with the marama bean plant material or the fungal species associated with the marama bean. The lack of information surrounding the marama bean, particularly in South Africa, provides an opportunity for novel research in order to fill the information gap.

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Chapter 3 - Exploring the mycoflora and mycotoxins associated with the marama bean

Image 3. Several of the fungal isolates isolated from the various marama bean plant materials. From top left to bottom right: *Epicoccum* sp.; *Colletotrichum* sp.; *Pestalotiopsis* sp.; Unknown sp.; *Purpureocillium lilacinum*; *Acremonium*-like sp.; *Bipolaris* sp.; *Trichoderma* sp.; *Curvularia akaiiensis*; *Talaromyces* sp.; *Fusarium solani* SC; *Fusicolla acetilerea*; *Botryoderma lateritium*; *Aspergillus* sp. Photos by T. Armfield

Abstract

The marama bean, encompassing both *Tylosema esculentum* ((Burch.) A.Schreib.) and *Tylosema fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.), has been identified as an orphan crop of southern Africa with a great potential for commercialisation. However, various biotic constraints require further research and understanding, including fungal pathogens and mycotoxigenic fungi. This study delves into the mycoflora and mycotoxins associated with marama bean in South Africa. Various fungal isolates were obtained from marama bean leaves, seeds and tubers collected from eight locations in South Africa, and identified by single- or multigene Sanger sequencing and phylogenetic analysis. Multi-mycotoxin analysis was performed on the plant material as well as on isolated fungal cultures *in vitro* using maize patty cultures, focusing on *Aspergillus*, *Fusarium* and *Penicillium* species. A total of 116 species spanning 27 families were molecularly identified, several of which have been previously reported on marama beans in Namibia, including various *Alternaria*, *Epicoccum*, *Fusarium*, *Penicillium*, *Phoma* and *Rhizopus* species. Other notable fungal genera isolated from the South African marama bean material, include *Aspergillus*, *Lasiodiplodia*, *Neofusicoccum*, *Botryosphaeria*, *Chaetomium*, *Diaporthe*, *Trichoderma*, *Bipolaris*, *Pestalotiopsis*, *Neopestalotiopsis*, and *Talaromyces*. Mycotoxins were not detected in any plant material, however, several *Aspergillus*, *Fusarium* and *Penicillium* species indicated the potential to produce aflatoxin B_1 and fumonisins B_1, B_2 and B_3 *in vitro*. This is the first study of the mycoflora associated with the marama bean in South Africa, and the first report of mycotoxin producing fungi associated with the marama bean.

3.1 Introduction

Fungi play an important role in plant survival and adaptation to the surrounding environment, whether it be as root associated fungi that aid in nitrogen production and absorption by the plants, or deterring herbivores through a variety of secondary metabolites produced by the fungal species which make the plants undesirable for consumption (Ciegler, 1978). Fungi also play a role in plant decomposition, as saprophytes help to degrade plant material, providing nutrients for the soil and for future plants to thrive on (Ostry, 2008). Many fungal species obtain their nutrients through the environmental and by non-pathogenic means, however, a large number of fungal species obtain their nutrients through parasitizing and often killing their host plants in the pursuit of nutrients and ultimately, fungal species survival. While fungal pathogens are not always the most successful in infecting plants and causing disease, they are responsible for a vast amount of the damage incurred on plants, often through secondary infections after other more successful pathogens (McDonald et al., 2014). Aiding disease development in plants and helping the fungi to obtain nutrients from the plant are not the only reason for the production of mycotoxins. Antimicrobial mycotoxins and secondary metabolites can also give the fungus a competitive advantage in colonising substrates by suppressing the growth of other fungi or bacteria, regardless of whetherthis is in a pathogenic interaction with a plant host (Ciegler, 1978; Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003). While some mycotoxins are only phytotoxic and effective in harming the plant host, many mycotoxins also create hazards for the humans and animals consuming the mycotoxin-infested plant material (Ciegler, 1978; Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003).

Tylosema esculentum ((Burch.) A.Schreib.) and *Tylosema fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.) are both commonly referred to as the marama bean (Chimwamurombe, 2010; Cullis et al., 2019). The marama bean is currently classified as an orphan crop, a typically underutilized plant that has become an important food crop of the local communities and indigenous people, predominantly in South Africa, Botswana and Namibia (Chimwamurombe, 2010; Cullis et al., 2019; Cullis & Kunert, 2017). Within South Africa, marama bean is generally found in the wild and is restricted to the North West, Gauteng, Mpumalanga, and Limpopo provinces (Department of Agriculture, Forestry and Fisheries, 2014; Jackson et al., 2010). The sporadic and patchy nature of the marama bean population distribution may be attributed to the migration of indigenous people and traditional plant users recorded throughout history (Nepolo et al., 2010). Several domestication and experimental cultivation projects have been attempted in Namibia, Botswana, Australia and Texas, United States of America (Chimwamurombe, 2010, 2011; Jackson et al., 2010; Magadza, 2010; Nepolo et al., 2010; Powell, 1987). There are currently no records on the Department of Forestry, Fisheries and the Environment (DFFE) or the Food and Agriculture Organisation of the United Nations (FAO) websites regarding marama bean cultivation by smallholder farmers within South Africa.

Analyses of marama bean (*T. esculentum*) seeds have revealed a high protein, lipid, vitamin, and mineral content, as well as high levels of starch in the tubers. Medicinal properties of the marama bean seeds and tubers include antioxidant levels, anti-tumour, anti-HIV, anti-viral, antibacterial, anticandidal and ant-diarrhoeal (Bower et al., 1988; Chingwaru et al., 2011; Chingwaru 2015b; Dubois et al., 1995; Fort et al., 2001; Holse et al., 2010, 2011; Jackson et al., 2010; Kuvare et al., 2015; Mazimba et al., 2011; Müseler & Schonfeldt, 2006; Shelembe et al., 2012). The seeds and tubers of the marama bean are predominantly used for human consumption. The seeds must be roasted, and can be eaten as is, ground into powder for porridge or coffee, or processed further into various products such as flour, milk and oil (Adeboye & Emmambux, 2017; Cole et al., 2014; Kayitesi et al., 2011; Maruatona et al., 2010; Mattana et al., 2022; Nyembwe et al., 2018). The tubers can be used as a sweet vegetable by roasting, boiling or baking the tuber, or they can be used as a water source during times of drought due to the ability of the tubers to store water to compensate for the arid environments in which the plants grow (Batanani, 2020; National Academy of Sciences, 1979; Nepolo et al., 2010).

Though a detailed mycoflora profile of the marama bean has not yet been completed, studies have reported on the isolation and identification of various fungal species associated with the marama bean, mainly from Namibia (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013). Several species were identified associated with the marama bean leaves, seeds or pods, namely *Alternaria alternata* (Fr.) Keissler, *A. solani* Sorauer, *A. tenuissima* (Kunze) Wiltshire (now *A. alternata*), *Epicoccum sorghi* (Sacc.) Aveskamp, Gruyter & Verkley (now *E. sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley), *Fusarium equiseti* (Corda) Sacc., *F. chlamydosporum* Wollenw. & Reinking, *F. incarnatum* (Roberge ex Desm.) Sacc., *Penicillium brevicompactum* Dierckx, *P. olsonii* Bainier & Sartory, *P. commune* Thom, *Rhizopus stolonifer* (Ehrenb.) Vuill, and *Phoma sorghina* (Sacc.) Boerema, Dorenb. & Kesteren (now *E. sorghinum*), (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013). To date, there are currently no records from South Africa identifying fungal species associated with the marama bean. The marama bean has been identified as a potential crop worth commercialisation (Cullis et al., 2019; Cullis & Kunert, 2017; Jackson et al., 2010). However, the mycoflora and mycotoxins associated with the marama bean, their ability to cause diseases on the plants, and their potential as a hazard for human and animal consumption must be understood before commercialisation can become a reality.

The primary aim of this chapter was to identify the mycoflora associated with *Tylosema esculentum* and *T. fassoglense* (marama bean) seeds, leaves and tubers collected from different localities within South Africa, using morphological characteristics and phylogenetic analyses of single and multigene DNA sequences. The plant material collected was analysed for natural mycotoxin presence, while the potential of selected isolated mycotoxigenic species to produce mycotoxins *in vitro* was also determined using maize patty cultures.

3.2 Materials and Methods

3.2.1 Plant material

Marama bean seeds were collected from natural populations at various locations in South Africa during periods when the seeds were abundant during the course of 2018 (Table 3.1). The seeds were stored in labelled brown bags and sealable plastic bags until use. Per locality, three marama bean tubers were collected at random from plants corresponding to the locations of the previously collected seeds, between 2020 and 2021 (Table 3.1). A minimum of 15 symptomatic and asymptomatic leaves were also collected per locality, from which five leaves were randomly selected for plating out. The tuber and leaf samples were stored in separate labelled brown paper bags at 4°C until use. The localities included two in the North West province (*T. esculentum*), three in Gauteng (*T. esculentum*), one in the Limpopo province (*T. fassoglense*) and one in Mpumalanga (*T. fassoglense*).

Table 3.1. Sample number, species names, locality, and climatic regions of the *Tylosema esculentum* and *Tylosema fassoglense* seeds, leaves and tubers collected for this study.

Sample number	**Species**	**Province**	**Locality**	**GPS co-ordinates**	**Climatic region**[d]
1	*T. esculentum*	North West	Roadside R501 near Boskop Dam	26°30'43.0"S 27°08'66.2"E	Cold semi-arid/ steppe (Bsk)
2	*T. esculentum*	North West	N14, Klerkskraal Dam	26°15'04.0"S 27°10'15.9"E	Cold semi-arid/ steppe (Bsk)
3	*T. esculentum*	Gauteng	Centurion	25°52'33.0"S 28°04'48.3"E	Warm temperate, dry winter, warm summer (Cwb)
4	*T. fassoglense*	Limpopo	Roadside R40	24°33'39.4"S 31°02'16.3"E	Hot semi-arid/ steppe (Bsh)
5[a]	*T. fassoglense*	Mpumalanga	Roadside Uitkyk Road	25°31'44.5"S 31°06'32.0"E	Warm temperate, dry winter, hot summer (Cwa)
6	*T. esculentum*	Gauteng	Innovation Africa, Hillcrest campus, University of Pretoria[c]	25°44'50.0"S 28°15'30.6"E	Warm temperate, dry winter, hot summer (Cwa)
7[b]	*T. esculentum* seedlings	Gauteng	Innovation Africa, Hillcrest campus, University of Pretoria[c]	25°44'50.0"S 28°15'30.6"E	Warm temperate, dry winter, hot summer (Cwa)
8	*T. esculentum*	Gauteng	Laudium, Centurion	25°49'43.6"S 28°03'16.9"E	Warm temperate, dry winter, warm summer (Cwb)

[a] Tubers were not collected from the Mpumalanga province due to tuber unavailability.

[b] Seeds were not collected from marama bean seedlings as the seedlings were too young to produce seed pods.

[c] Original population from Wierdapark, Centurion, South Africa.

[d] Climatic regions classified according to the Köppen-Geiger climate classification map of South Africa (The Council for Scientific and Industrial Research, 2015).

The seeds, leaves and tubers of the marama bean were collected from each locality where available. Plant material availability is highly season dependent, as such the collection trips were planned according to the spring and summer months. However, due to the COVID-19 pandemic and South African national lockdown several of the collection trips were postponed, therefore affecting plant material availability.

3.2.2 Direct plating technique

3.2.2.1 Direct plating of seed samples

Eighty seeds per locality were plated individually onto half concentration potato dextrose agar (1/2 PDA) amended with chloramphenicol antibiotic (10 mg/L) to reduce bacterial contamination in 90 mm diameter Petri dishes. The seeds were plated under four treatment conditions consisting of 20 seeds each (four replicates of five seeds): surface sterilized seeds plated with (1) and without seed coat (2); non-surface sterilized seeds plated with (3) and without seed coats (4). Surface sterilized seeds were soaked in 1% sodium hypochlorite (NaOCl) solution for 3 min followed by rinsing three times with sterile distilled water. The sterilized seeds were dried under sterile conditions on sterile paper towels placed on the biosafety cabinet bench for several minutes until dry. The seeds used in the treatments without seed coats were prepared by removing the seed coats by means of a desk vice. The plates were incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles, using cool white light, before being examined for fungal growth.

3.2.2.2 Direct plating of tuber samples

Three tuber samples per locality were used under three treatment conditions consisting of 20 tuber pieces (5 x 5 mm x 3 mm) each (20 tuber pieces x 3 treatments x 3 tuber samples per locality). The three treatments included tuber pieces with non-surface sterilized outer layer of the tuber (‘skin’) (1), tuber pieces with surface sterilized outer layer (2) and tuber pieces from the inside of the tubers (3). The tubers in the surface sterilized treatment were soaked in 1% NaOCl solution for 1.5 min, rinsed three times with sterile distilled water and dried under sterile conditions. The tuber pieces from each treatment were directly plated onto 1/2 PDA 90 mm plates with chloramphenicol, using four tuber pieces per plate (4 x 5 plates = 20 pieces). The plates were incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles, using cool white light, before being examined for fungal growth.

3.2.2.3 Direct plating of leaf samples

Five leaves per locality were used under two treatment conditions consisting of 10 leaf pieces (5 x 5 mm) each (10 leaf pieces x 5 leaves per locality x 2 treatments). The two treatments included non-surface sterilized and surface sterilized leaves. The leaf pieces in the surface sterilized treatment were soaked in 1% NaOCl solution for 1 min, rinsed three times with sterile distilled water and dried under sterile conditions. The leaf pieces from each treatment were directly plated onto 1/2 PDA 90 mm plates with chloramphenicol, using five leaf pieces per plate. The plates were incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles, using cool white light, before being examined for fungal growth.

3.2.3 Fungal isolation

Following incubation, fungi were isolated from each agar plate by cutting a 2 x 2 or 3 x 3 mm squares from each area of desired fungal growth or obtaining small amounts of fungal spores through scrapings. The isolated fungal growth was transferred to two 65 mm Petri dishes containing Malt Extract Agar (MEA) and PDA respectively. The agar plates were then incubated at ±25°C with 12-hour light/dark cycles using cool white light until sporulation was observed.

Due to the number of isolates obtained from the plant samples, representative cultures with differing morphological features were selected for identification and single spore isolates were prepared. Spore suspensions with low spore numbers were prepared using Water Agar (WA) plates. The plates were incubated upside down for one day after which they were observed for spore germination using a stereoscope microscope (Nikon Eclipse E200, Japan). A single spore or micro-colony originating from a single spore was then transferred to a clean MEA plate and incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles using cool white light. Duplicate cultures were made from the pure cultures for preservation and use in identification.

3.2.4 Morphological identification and growth study

Representative cultures of all fungal isolates were morphologically identified using microscopy techniques. The culture morphology and sporulation patterns were observed using the Zeiss Axio Zoom stereo-microscope (Zeiss, Germany) and the sporulating elements were observed through microscope slide preparation with lactoglycerol mounting liquid and using the Zeiss Axio Imager compound microscope with differential interference contrast (DIC) (Zeiss, Germany). Morphological identification was completed to species level where possible, using “A laboratory guide to common *Aspergillus* species and their telomorphs” (Klich & Pitt, 1988); “*Fusarium* species, An illustrated manual for identification” (Nelson et al., 1983); and “A laboratory guide to common *Penicillium* species” (Pitt, 2000).

Representative cultures based on preliminary morphological groupings were used for growth studies involving various media types to emphasize different aspects of fungal growth. The media and growth conditions used were dependent on the taxa retrieved. For *Aspergillus* and *Penicillium* species, this included MEA incubated at 25°C, and Czapak yeast extract agar (CYA) in triplicate, incubated for seven days in the dark at -4°C, 25°C and 37°C, respectively. In addition, CYA with 20% sucrose (CY20S) and 25% glycerol nitrate agar (G25N) were used for *Aspergillus* and *Penicillium* species respectively, and incubated at 25°C under 12-hour light/dark cycles for seven days. The *Aspergillus* and *Penicillium* species were inoculated on the respective agar plates using three spore suspension droplets placed equidistantly apart. *Fusarium* species were cultivated on full strength PDA and carnation leaf agar (CLA), inoculated using mycelial scrapings, and incubated at 25°C under 12-hour light/dark cycles, using cool white light. Pleosporaceae taxa were cultivated on potato carrot agar (PCA) incubated at 25°C under 12-hour light/dark cycles, using cool white light. Initial unknown fungi were cultivated on MEA and PCA incubated at 25°C under 12-hour light/dark cycles, using cool white light.

The diversity of fungal genera was represented by isolation frequencies (%). Following plant material sample collection and fungal isolation, the number of isolates obtained from different seed, tuber or leaf samples were counted. Isolates originating from the same seed, tuber or leaf pieces were excluded from the isolate count. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location. The isolation frequency (%) provides an overview of the fungal genera isolated in each sample location, treatment type and plant material type.

3.2.5 Molecular identification

Deoxyribonucleic acid (DNA) from the selected representative fungal isolates was extracted using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Inqaba Biotec, South Africa) according to the manufacturer’s instructions (Appendix 6.3 Protocol 1). The extracted DNA was used as a template for polymerase chain reactions (PCR) to amplify desired gene regions for each fungal genus using the appropriate primer set (Appendix 6.3 Protocol 2). DNA-based identification of the fungal species present was based on the use of the Internal Transcribed Spacer (ITS) region [all selected isolates], the Translation Elongation Factor 1-alpha (*TEFα*) gene region [*Fusarium* species specifically], as well as β-tubulin (*BenA*) gene region [*Aspergillus* and *Penicillium* species specifically] (Table 3.2) (Dupont et al., 1999; O’Donnell et al., 2013; Schoch et al., 2012; Zhang et al., 2013). The ITS1F and ITS4 primers used for gene amplification span the ITS1, 5.8S rDNA (ribosomal RNA-encoding genes (rDNA)) and ITS2 regions in the fungal DNA, covering an ITS region of between 550 and 600 base pairs in length (Raja et al., 2017).

The resulting amplicons were purified by ExoSAP reactions using ExoSAP-IT PCR product clean-up (Thermofischer Scientific, Johannesburg, South Africa). The clean PCR amplicons were then used for cycle sequencing reactions (Appendix 6.3 Protocols 3 and 4) containing the PCR amplicon, the respective primers reported above, and BigDye Terminator v3.1 Ready Reaction Mix with 5X sequencing buffer (Thermofischer Scientific, Johannesburg, South Africa). The cycle sequencing products were then subjected to sodium acetate/ethanol precipitation (NaOAc/EtOH) (Appendix 6.3 Protocol 5) and stored at 25°C overnight to dry before submitting to the DNA Sanger Sequencing Facility of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria for sequencing.

Table 3.2. The primer sets and respective amplified gene regions and primer sequences used during polymerase chain reaction (PCR) amplification of the desired gene region for fungal molecular identification.

Primer set	**Gene region amplified**	
ITS1F	CTT GGT CAT TTA GAG GAA GTA A	**Internal Transcribed Spacer (ITS)**
ITS4	TCC TCC GCT TAT TGA TAT GC	(White et al., 1990)
Bt$_2$a	GGT AAC CAA ATC GGT GCT GCT TTC	**β-tubulin (*BenA*)**
Bt$_2$b	ACC CTC AGT GTA GTG ACC CTT GGC	(Glass & Donaldson, 1995)
EF1	ATG GGT AAG GAR GAC AAG AC	**Translation elongation factor 1-alpha (*TEFα*)**
EF2	GGA RGT ACC AGT SAT CAT GTT	(O’Donnell et al., 1998)

The sequencing results were analysed using the software CLCBio (Aarhus, Denmark) to obtain full length nucleotide consensus sequences of the amplicons using the sequences of the respective forward and reverse primers. The molecular identities of all the samples were obtained using Basic Local Alignment Search Tool (BLAST) and the fungal databases at MycoBank and GenBank at the National Centre for Biotechnology Information (NCBI). The respective consensus sequences in each of the datasets were aligned using MAFFT Online service (Katoh et al., 2018) with all options listed as ‘same as input’ and using default settings. The aligned datasets were trimmed and edited using CLCBio, before analysing the datasets using IQTree version 2.1.2 to obtain the maximum likelihood, optimal model, and the phylogenetic trees with bootstrap values. Several phylogenies were prepared per dataset – single gene phylogeny with ITS, single gene phylogeny with *BenA* or *TEF*α, and multigene phylogenies using the concatenated ITS and secondary gene data.

3.2.6 Multi-mycotoxin analysis

3.2.6.1 Natural presence of mycotoxins on plant material

The marama bean seeds collected from eight localities were physically separated into seed (internal) and seed coat (external) samples using a desk vice. The leaf and tuber samples from each locality were freeze-dried and stored at -80°C until use. The dry seed and seed coat, as well as the freeze-dried leaf and tuber samples were ground to a fine powder under sterile conditions using a laboratory blender that was thoroughly washed with detergent, sprayed with ethanol and dried between samples. Five grams (5 g) of each sample was placed in labelled sealable plastic bags in duplicate. The processed samples were submitted to the Central Analytical Facility (CAF) at Stellenbosch University for multi-mycotoxin analysis to detect the natural presence of mycotoxins on the respective samples using ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). Briefly, sample preparation involved placing 5 g of the sample into a 50 mL tube, adding 20 mL of extraction solvent (50% water: 25% methanol: 25% acetonitrile) and sonicating the samples for 1 h. One mL of the sample was aliquoted into a 2 mL Eppendorf tube, double diluted with 75% water: 25% methanol solvent and centrifuged at 13 000 rpm for 5 min. One mL of diluted sample was aliquoted into an analysis vial. A Waters UPLC BEH C18 (2.1 x 100 mm) column was used with two mobile phase solvents – solvent A containing 0.1% formic acid and solvent B containing 49% acetonitrile: 49% methanol: 2% isopropanol – with a run time of 14 min. Multi-mycotoxin analysis was used to detect the presence of aflatoxin B_1 (AFB_1), deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), ochratoxin (OTA), and fumonisin B_1, B_2 and B_3 (FB_1, FB_2, FB_3, respectively) mycotoxins.

3.2.6.2 *In vitro* analysis of mycotoxin production by potential mycotoxigenic isolates

Maize patty cultures were prepared by adding finely ground maize kernel meal (20 g) and distilled water (30 mL) to Pyrex Petri dishes (100 mm x 18 mm) and autoclaving twice over two consecutive days at 121°C and 120 kPa for 1 h each time. Selected fungal isolates from the marama bean seed, leaf and tuber samples that were molecularly identified and determined to be potentially mycotoxigenic were used to inoculate the maize patty media in duplicate using a 3 x 3 mm block cut from the original subculture, or by producing a spore suspension of the desired subculture and applying 1 mL of the suspension to inoculate the medium (Alberts et al., 1993). The cultures were incubated in the dark at 25°C for ~21 days or until the fungus had completely colonized the medium.

The maize patty cultures were dried for four days at 40°C and ground to a fine powder under sterile conditions using a laboratory blender that was thoroughly washed with detergent, sprayed with ethanol and dried between samples. Five grams (5 g) of each sample was placed in labelled sealable plastic bags in duplicate. The ground maize patty culture samples were submitted to the CAF at Stellenbosch University for multi-mycotoxin analysis to detect the presence of AFB_1, DON, NIV, ZEA, OTA, FB_1, FB_2 and FB_3 mycotoxins.

3.3 Results

3.3.1 Fungal association with marama bean – morphological identification

The fungal associations per locality, treatment, and type of plant material were investigated to determine the effect of these factors on the fungi identified from marama bean. Eight localities were selected for plant material collection (Table 3.1) ranging from cold or hot semi-arid to warm temperate with dry winters and hot or warm summer climatic regions. Figure 3.1 indicates the fungal association per locality, specifically referring to the isolation frequencies observed. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location. The isolation frequency (%) provides an overview of the fungal genera isolated in each sample location, treatment type and plant material type. Due to the highly variable nature of fungal species, several fungal species have been included into one category of 'like' fungi with common morphological similarities when examining the fungal isolate cultures visually and microscopically. These groups were further classified and regrouped based on the molecular identification. Samples 1 and 2 were affected by *Rhizopus* sp. (Ehrenb) contamination during plating out of the seed samples, resulting in an inability to determine the morphological identity of other fungal isolates that were present on the plates due to the overgrowth by the *Rhizopus* sp. Due to the unavailability of the seeds collected from Samples 1 and 2, the seeds could not be replated under improved aseptic conditions.

As a result of the contamination in samples 1 and 2, the genera groupings *Rhizopus*, *Trichoderma* Pers., and unknown genera presented the highest isolation frequencies (Figure 3.1), followed by *Chaetomium* Kunze, *Penicillium* Link, *Alternaria*-like Nees, *Mucor*-like Fresen., and *Epicoccum* Link. The remaining fungal genera were observed in small percentages in samples 1 and 2, with the exception of *Colletotrichum*-like Corda and *Rhizoctonia*-like DC. specimens which were absent in both samples. When considering the remaining five samples (Samples 3 to 8), samples 5 and 7 indicated a low presence of *Chaetomium* and *Rhizopus* (Figure 3.1). *Aspergillus* P. Micheli ex Haller, *Cladosporium* Link, *Epicoccum*, *Fusarium* Link, *Lasiodiplodia*-like species Ellis & Everh., and *Phoma* Sacc. occurred in fairly equal ratios throughout all of the localities. Sample 4 had higher incidences of *Chaetomium*, while sample 8 had higher incidences of *Colletotrichum*-like species.

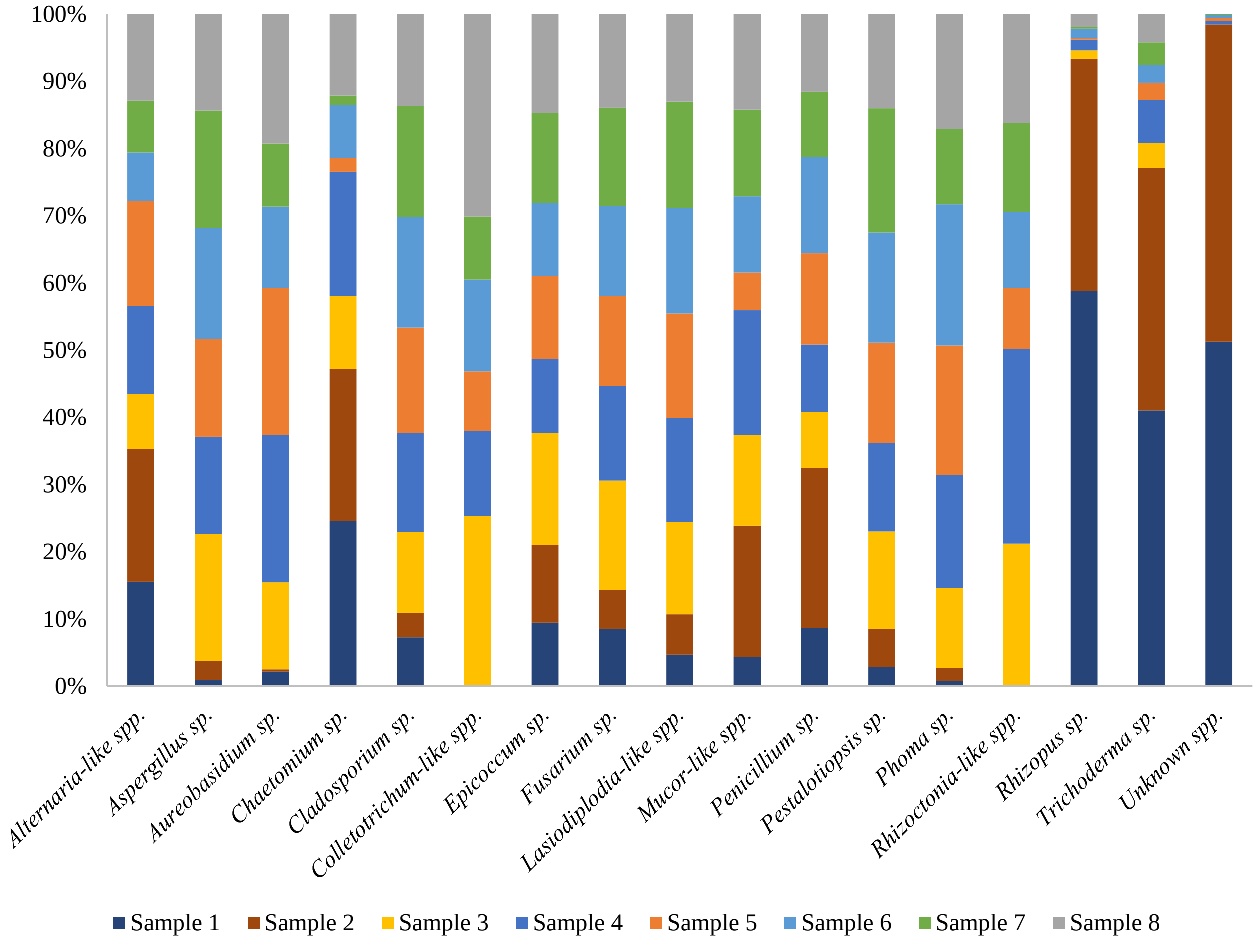

Figure 3.1. The isolation frequencies (%) of fungal genera per locality where plant material was collected, spanning all treatments and plant material. Nomenclature was based on primary morphological identification of the fungal cultures.

Figures 3.2 and 3.3 indicate the fungal occurrence for the surface sterilized and non-surface sterilized treatments, respectively, averaging all eight localities and three plant material types. When considering the genera that had the highest isolation frequencies between the surface sterilised and non-surface sterilised treatments, *Epicoccum*, *Fusarium,* and *Trichoderma* had the highest frequencies in both the surface sterilised and non-surface sterilised treatments. However, *Penicillium* and *Alternaria* presented slightly higher isolation frequencies in the surface sterilised treatments compared to *Aspergillus* and *Pestalotiopsis,* while the opposite was observed in the non-surface sterilised treatments with *Aspergillus* and *Pestalotiopsis* presenting slightly higher isolation frequencies than *Alternaria* and *Penicillium*. In contrast, *Colletotrichum*, *Aureobasidium* Viala & G. Boyer, and *Rhizoctonia* presented the lowest occurrence rate for both treatments.

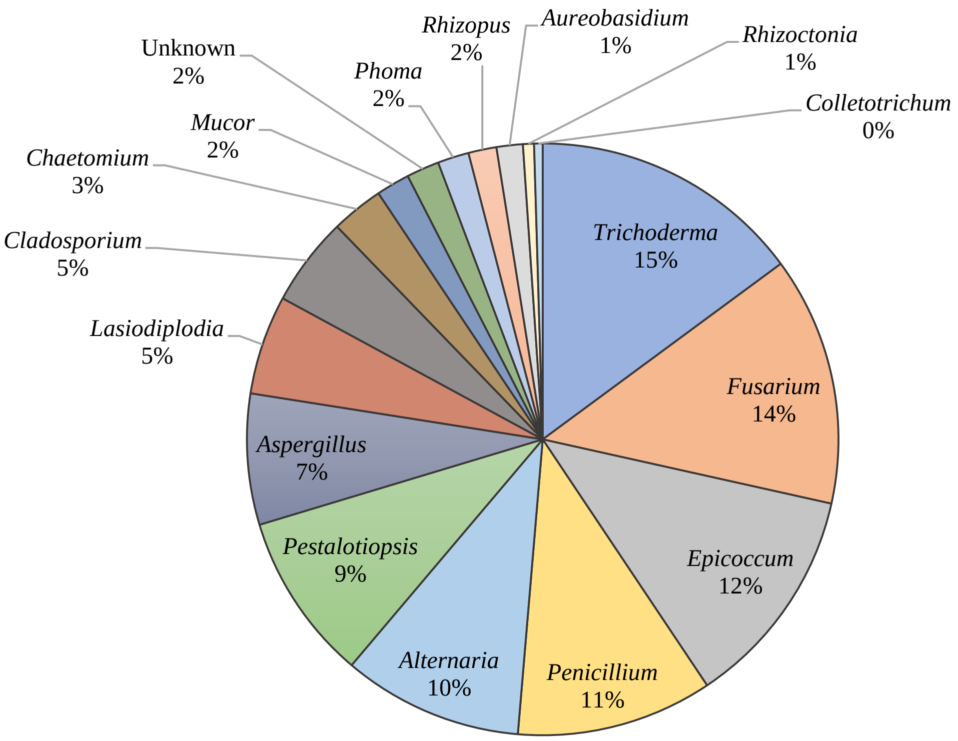

Figure 3.2. The isolation frequencies (%) of fungal genera from the surface sterilized treatments spanning all locations and plant material collected. Nomenclature was based on primary morphological identification of the fungal cultures. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location.

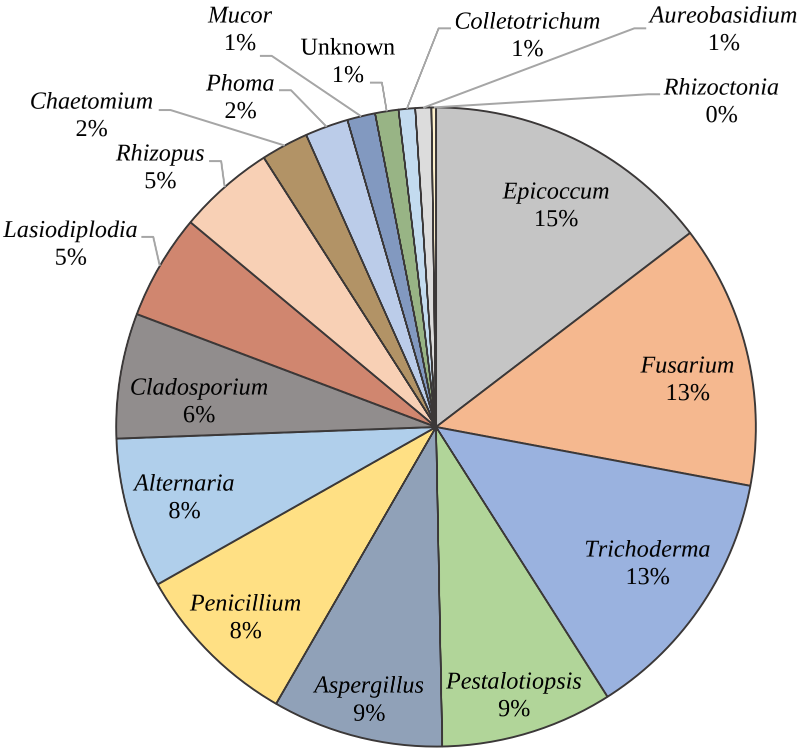

Figure 3.3. The isolation frequencies (%) of fungal genera from the non-surface sterilized treatments spanning all locations and plant material collected. Nomenclature was based on primary morphological identification of the fungal cultures. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location.

Figures 3.4 to 3.6 provide the fungal frequency rates per marama bean plant material investigated – the leaves, seeds and tubers, respectively, averaging all eight localities where the plant material was collected. The leaf samples were generally dominated by *Epicoccum*, *Alternaria*, and *Cladosporium*, with lower isolation frequencies of *Aspergillus*, *Fusarium* and *Penicillium* when compared to both the seed and tuber samples. Similarly, *Pestalotiopsis* also presented higher isolation frequencies in the seed and tuber samples when compared to the leaf sample. In contrast, *Trichoderma* was more prevalent in the tuber samples when compared to the low isolation frequencies in the seed and leaf samples. *Mucor*-like species, including *Rhizopus*, had low isolation frequencies in all three of the plant material samples, with the exemption of the seed sample due to the *Rhizopus* contamination.

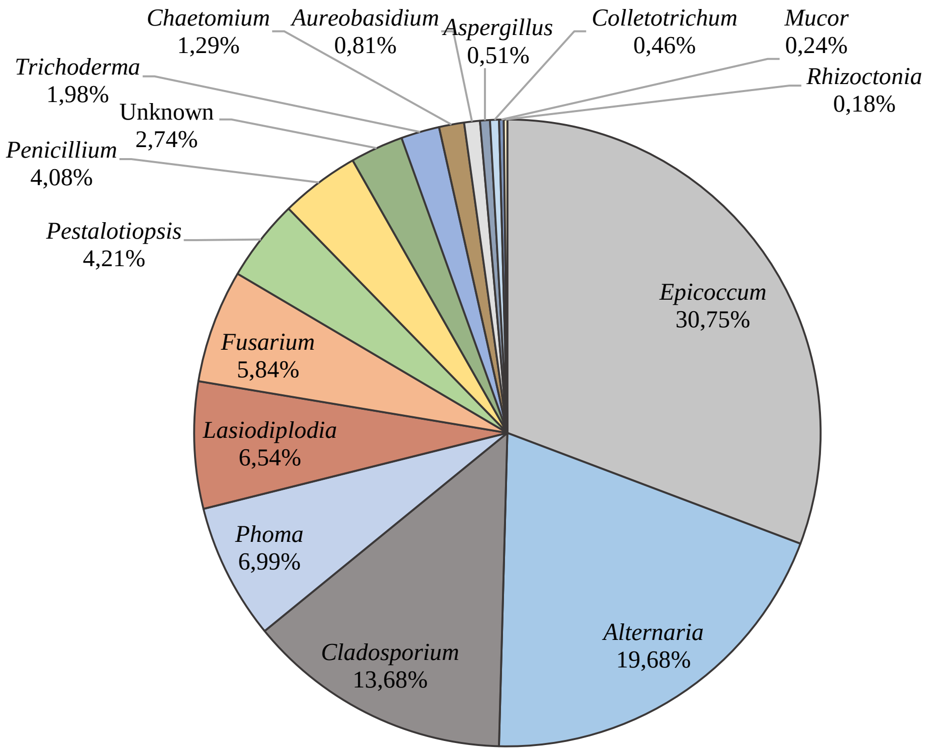

Figure 3.4. The isolation frequencies (%) of fungal genera for the marama bean leaves, comprising of the fungal genera isolated from marama bean leaves collected across all of localities and treatments (surface disinfected and non-surface disinfected). Nomenclature was based on primary morphological identification of the fungal cultures. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location.

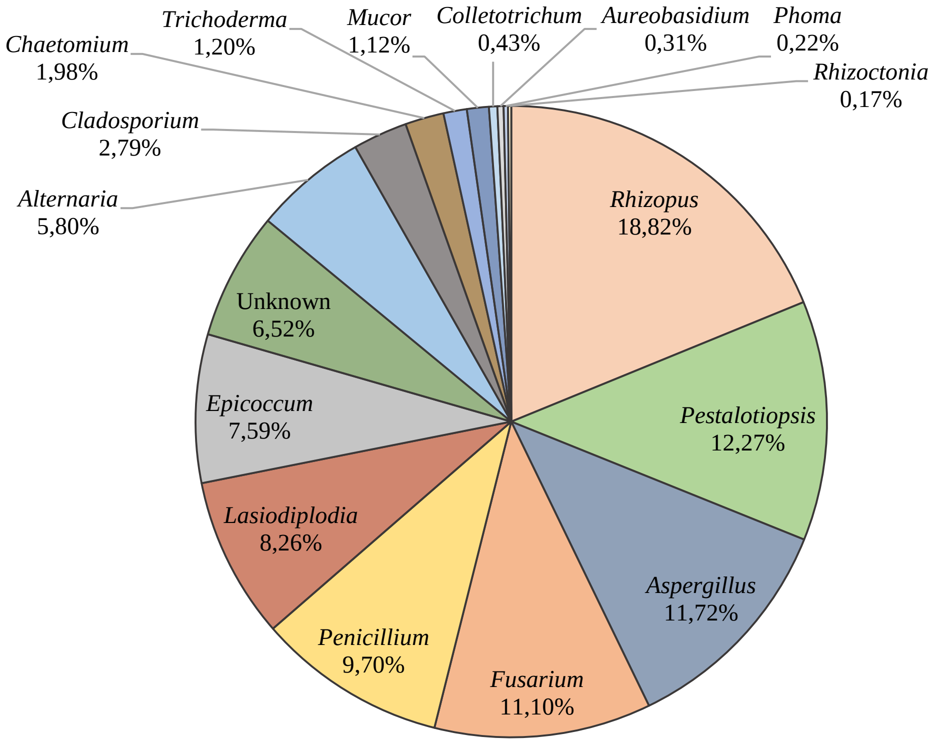

Figure 3.5.The isolation frequencies (%) of fungal genera for the marama bean seeds, comprising of the fungal genera isolated from marama bean seeds collected across all of localities and treatments (surface disinfected, non-surface disinfected, with and without seed coat). Nomenclature was based on primary morphological identification of the fungal cultures. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location.

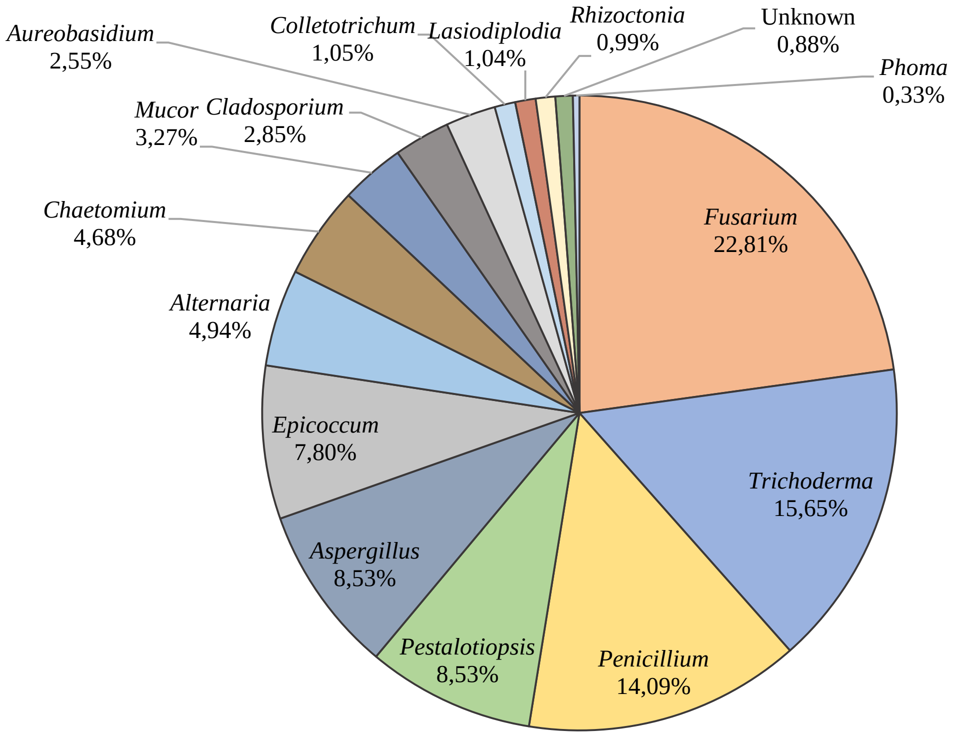

Figure 3.6. The isolation frequencies (%) of fungal genera for the marama bean tubers, comprising of the fungal genera isolated from marama bean tubers collected across all of localities and treatments (surface disinfected; non-surface disinfected; tuber surface and flesh). Nomenclature was based on primary morphological identification of the fungal cultures. The isolation frequency percentage was obtained using the number of isolates per genus / the total number of isolates obtained per sample location.

3.3.2 Fungal association with marama bean – molecular identification

Representative cultures isolated from the different localities and plant material were used for molecular identification using Sanger sequencing. Figure 3.7 includes all fungal isolates identified using molecular identification based solely on the ITS region, with the different isolates presented by family name. The *Aspergillus*, *Penicillium* and *Fusarium* species were selected for further analysis using a secondary gene (multigene analysis) based on their interest as mycotoxigenic genera. The representative isolates that were molecularly identified, represent only a fraction of the number of isolates obtained from the various plant materials collected from the eight localities.

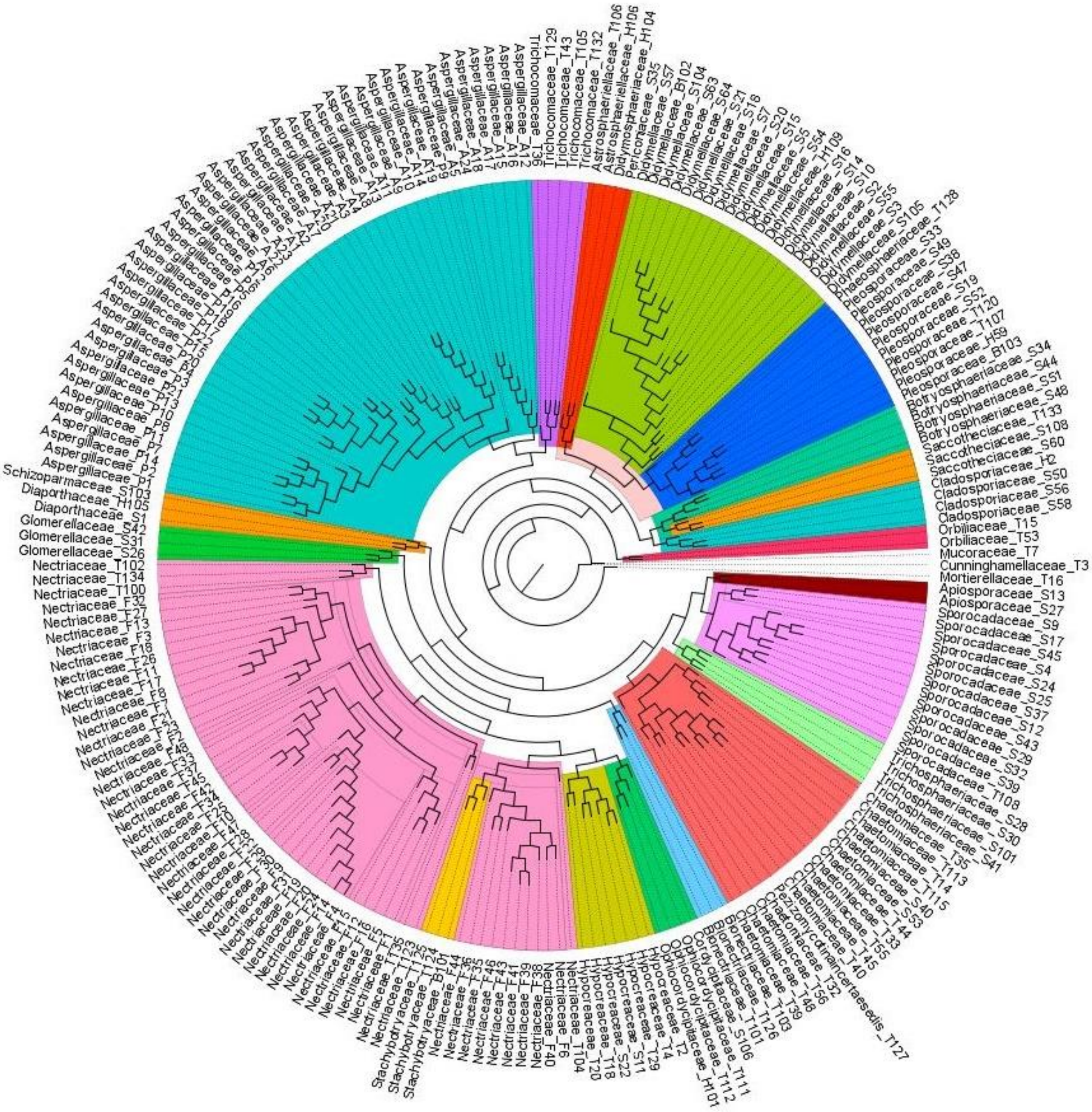

Figure 3.7. Single gene phylogenies (midpoint rooted Maximum Likelihood tree) based on the ITS1 and ITS2 regions of the various fungal isolates isolated and molecularly identified from the marama bean plant material. Isolates are reported to family level classification, followed by the unique isolate number [isolate numbers: A- *Aspergillus*, F- *Fusarium*, P- *Penicillium*, S- Seed, T- Tuber, L/H- Leaf samples]

A total of 215 isolates were molecularly identified, yielding a total of 116 species spanning 27 families, as well as species from the class Dothideomycetes (Figure 3.7). The two largest families were Nectriaceae and Aspergilliaceae due to the large number of *Aspergillus, Fusarium* and *Penicillium* isolates identified, followed by the families Didymellaceae, Sporocadaceae and Chaetomiaceae (Figure 3.7).

Figures 3.8 to 3.16 provide the multigene and single gene phylogenetic trees for *Aspergillus*, *Fusarium* and *Penicillium*, ITS and *BenA* [*Aspergillus* and *Penicillium*] or *TEF*α [*Fusarium*] gene regions, respectively. A minimum of four reference strains per fungal species were included where available, including the species type strain in bold followed by "T", while the respective isolates obtained per genus in this study have been included in red text. A total of 24 *Aspergillus* and 22 *Penicillium* isolates were selected for sequencing using the ITS and *BenA* regions; and 48 *Fusarium* isolates were selected using the ITS and *TEF*α gene regions (Appendix 6.4, Table 6.2). Two *Penicillium* species were used as outgroups to root both the *Aspergillus* and *Penicillium* phylogenetic trees, whilst two *Fusarium* species were used as the *Fusarium* tree outgroups to root the tree. In order to simplify the presentation of the trees, not all the closely related species within the genus were included. The complete reference strain list can be found in Table 6.3, Appendix 6.5.

Based on the results of the combined trees (Figures 3.8, 3.11, 3.14), the following species were identified: *Aspergillus alabamensis* Balajee, Baddley, Frisvad & Samson*; A. awamori* Nakaz.*; A. japonicus* Saito*; A. luchuensis* Inui*; A. magaliesburgensis* Visagie*; A. nidulans* (Eidam) G. Winter*; A. nidulans var dentatus* D.K. Sandhu & R.S. Sandhu*; A. ochraceus* K. Wilh.*; A. pseudodeflectus* Samson & Mouch.*; A. quadralineatus* Thom & Raper*; A. sojae* Sakaguchi & K. Yamada ex Murak.*; A. sydowii* (Bainier & Sartory) Thom & Church*; A. udagawae* Y. Horie, Miyaji & Nishim.*; A. welwitschiae* (Bres.) Henn.*; Fusarium chlamydosporum* Wollenw. & Reinking*; F. chlamydosporum* species complex*; F. citri* M.M. Wang, Qian Chen & L. Cai*; F. fujikuroi* (Nirenberg) species complex*; F. incarnatum-equiseti* species complex*; F. lacertarum* Subrahm.*; F. oxysporum* Schltdl.*; F. oxysporum* species complex*; F. sambucinum* (Fuckel) species complex*; F. scirpi* Lambotte & Fautrey*; F. solani* (Mart.) Sacc.*; F. solani* species complex*; Penicillium abidjanum* Stolk*; P. annulatum* Visagie & K. Jacobs*; P. brevicompactum* Dierckx*; P. caperatum* Udagawa & Y.Horie*; P. chrysogenum* Thom*; P. citrinum* Thom*; P. cluniae* Quintan.*; P. momoi* Visagie & K. Jacobs*; P. onobense* C. Ramírez & A.T. Martínez*; P. ortum* Visagie & K. Jacobs*; P. raperi* G. Sm.*; P. rubens* Biourge; and *P. striatisporum* Stolk.

Figure 3.8. Multigene Maximum Likelihood phylogeny of *Aspergillus* based on a combined ITS and *BenA* dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

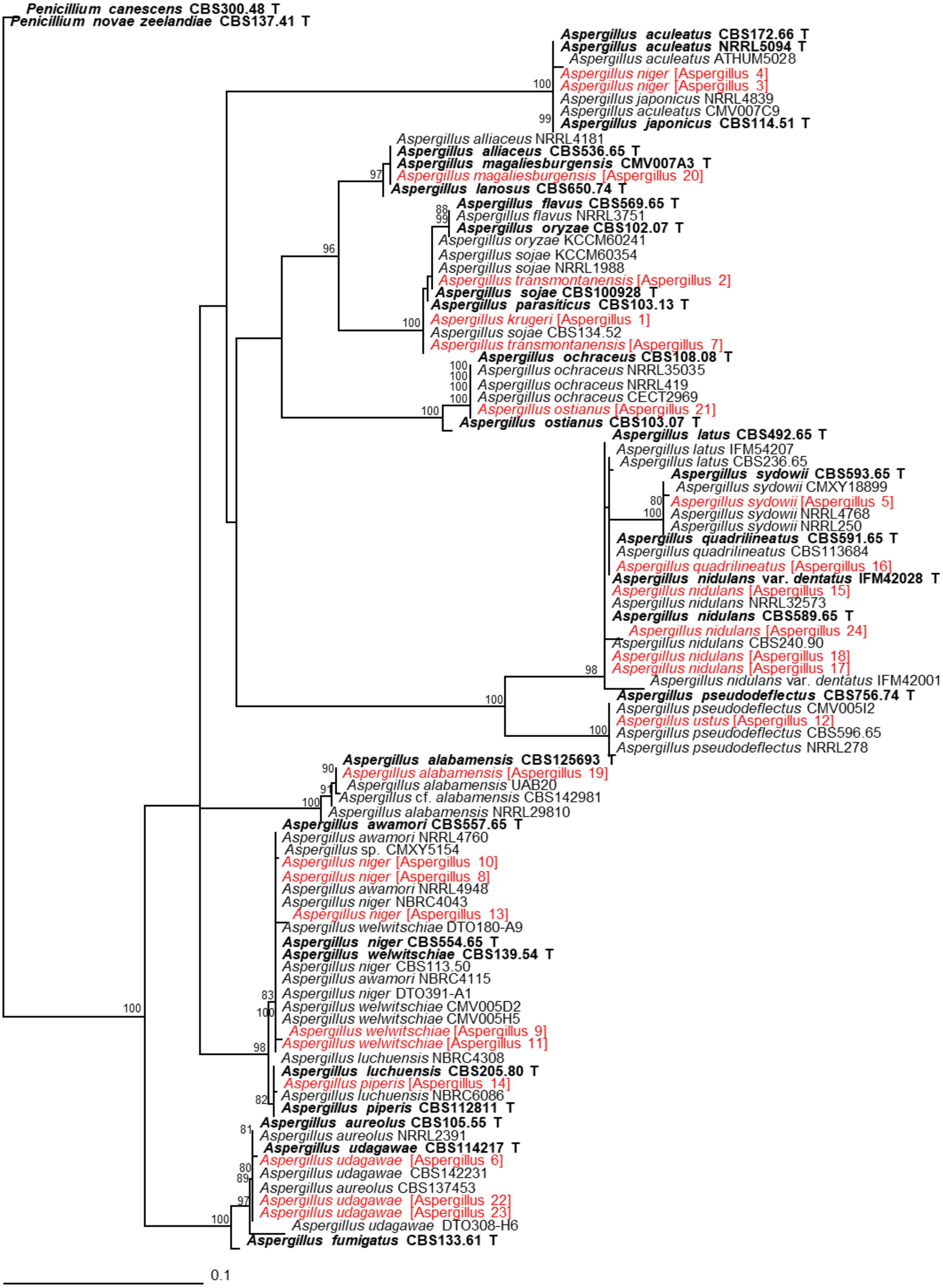

Figure 3.9. Single gene Maximum Likelihood phylogeny of *Aspergillus* based on the ITS dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

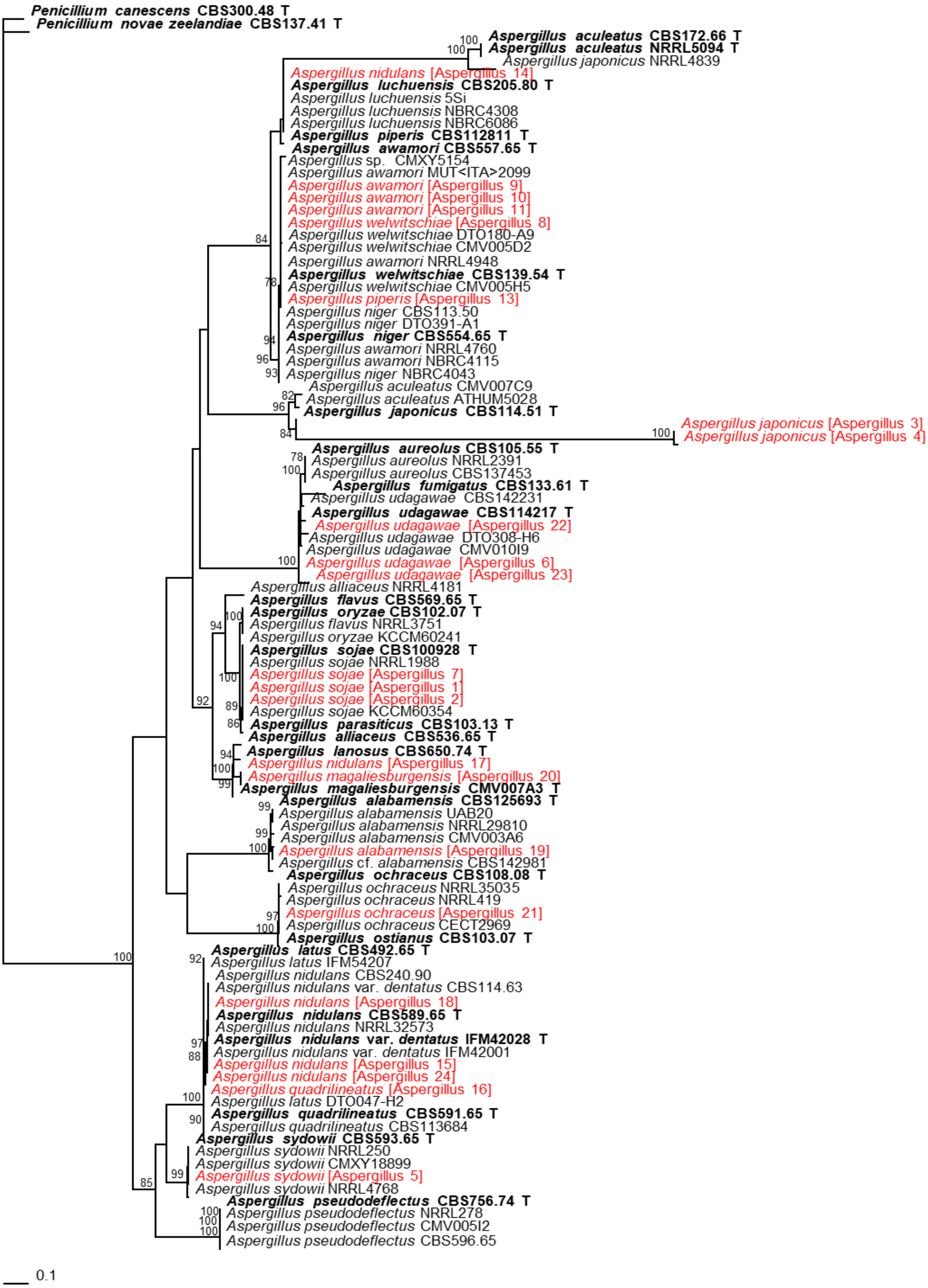

Figure 3.10. Single gene Maximum Likelihood phylogeny of *Aspergillus* based on the *BenA* dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

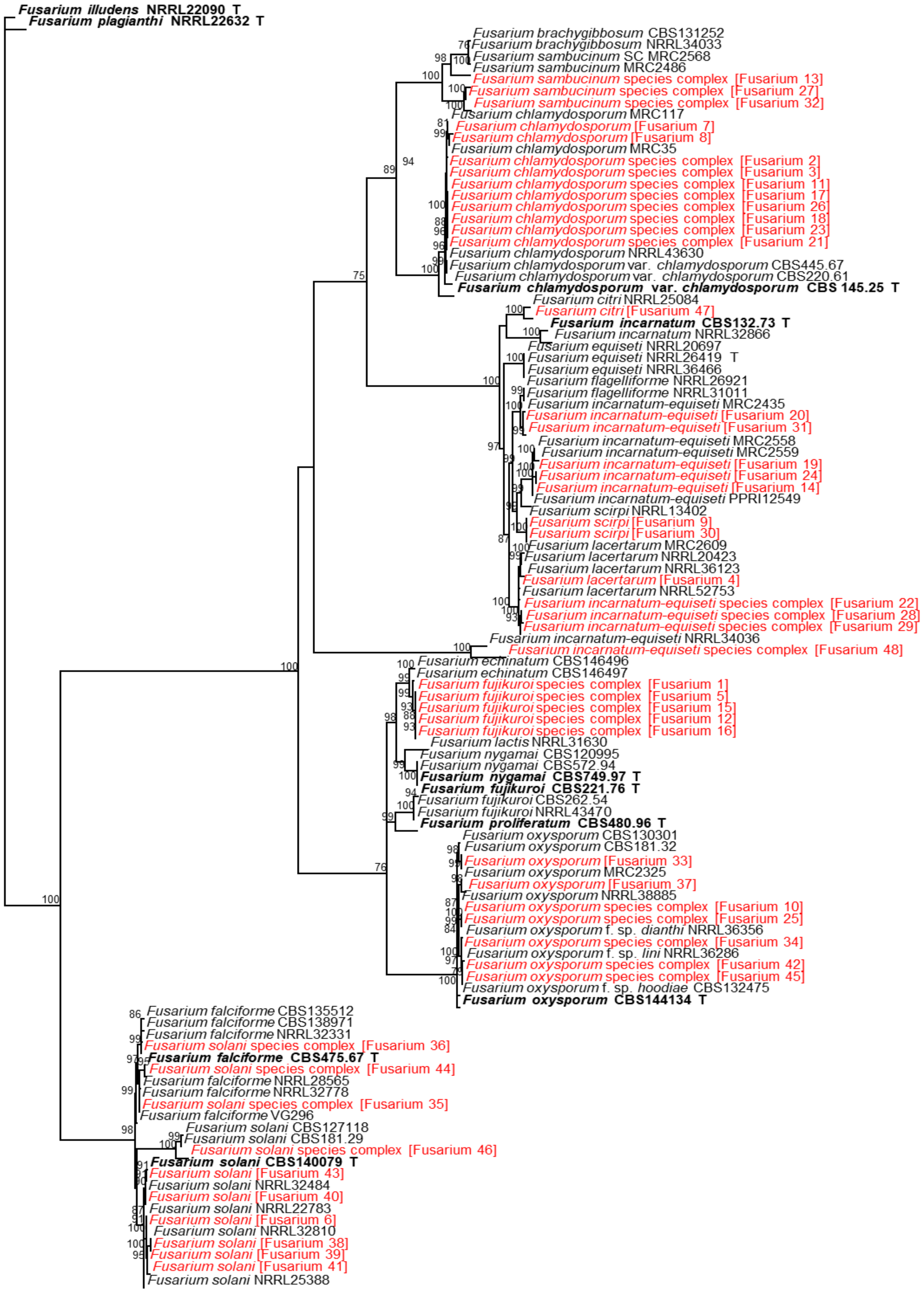

Figure 3.11. Multigene Maximum Likelihood phylogeny of *Fusarium* based on a combined ITS and *TEF*α dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

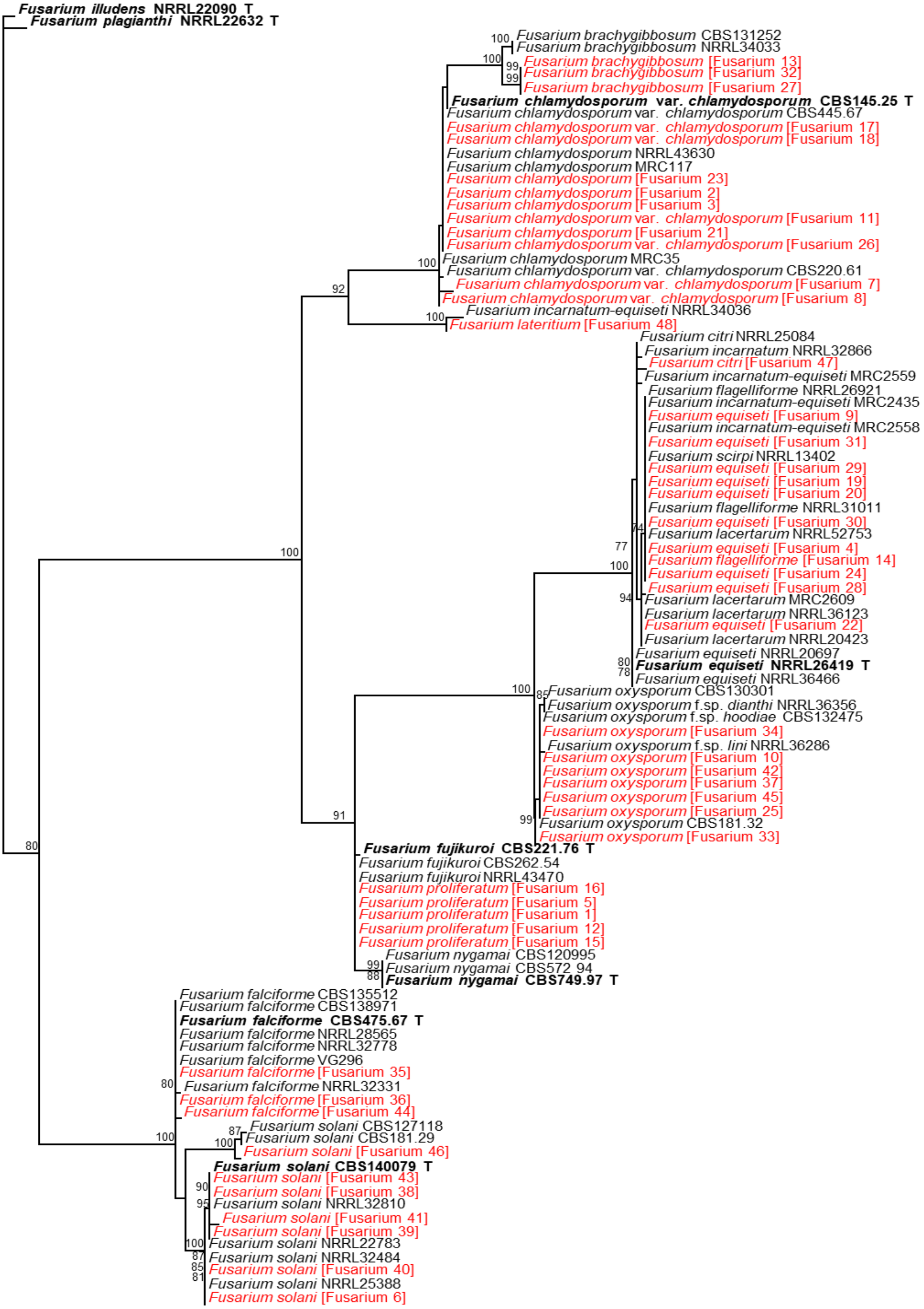

Figure 3.12. Single gene Maximum Likelihood phylogeny of *Fusarium* based on the ITS dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

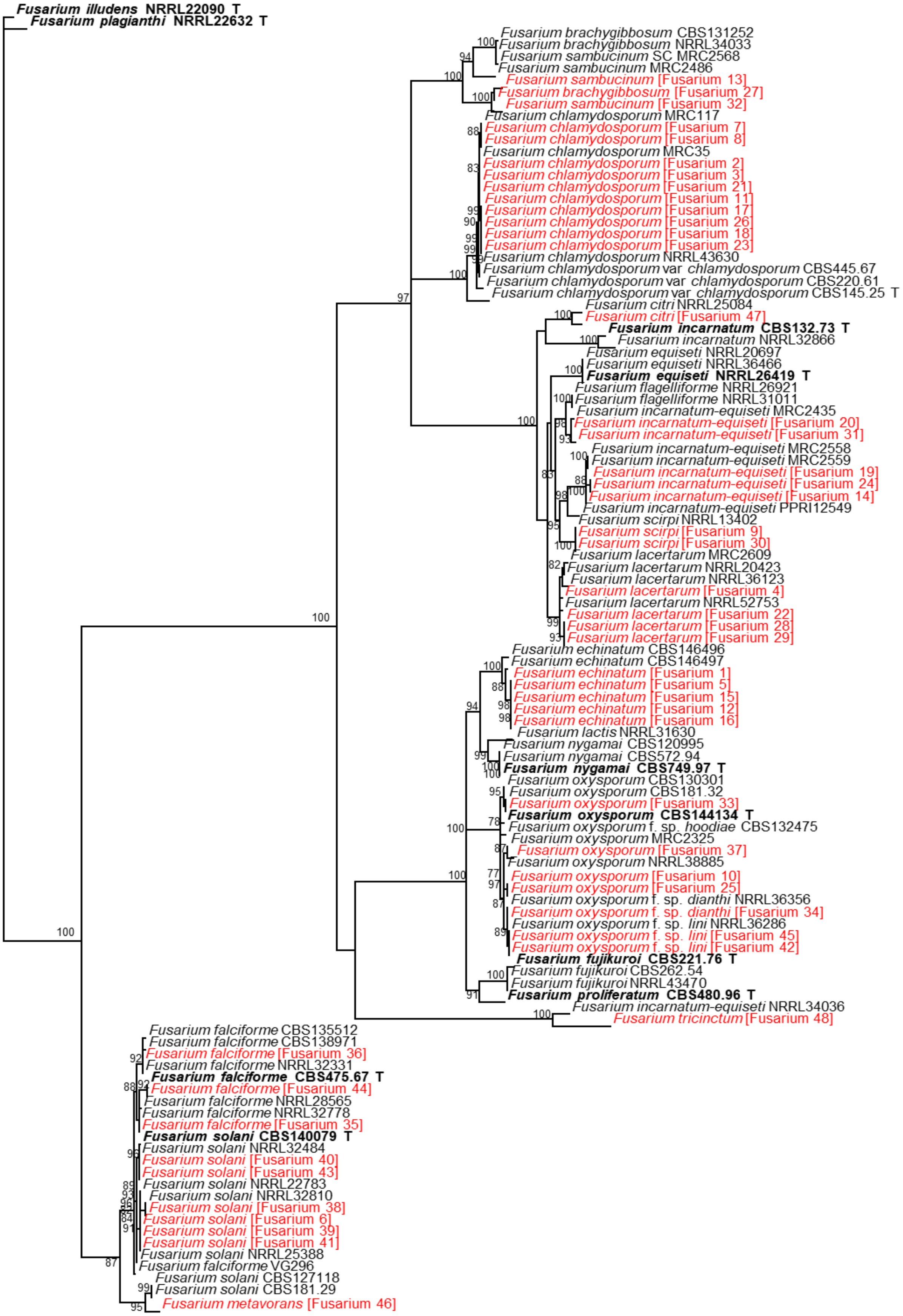

Figure 3.13. Single gene Maximum Likelihood phylogeny of *Fusarium* based on the *TEF*α dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

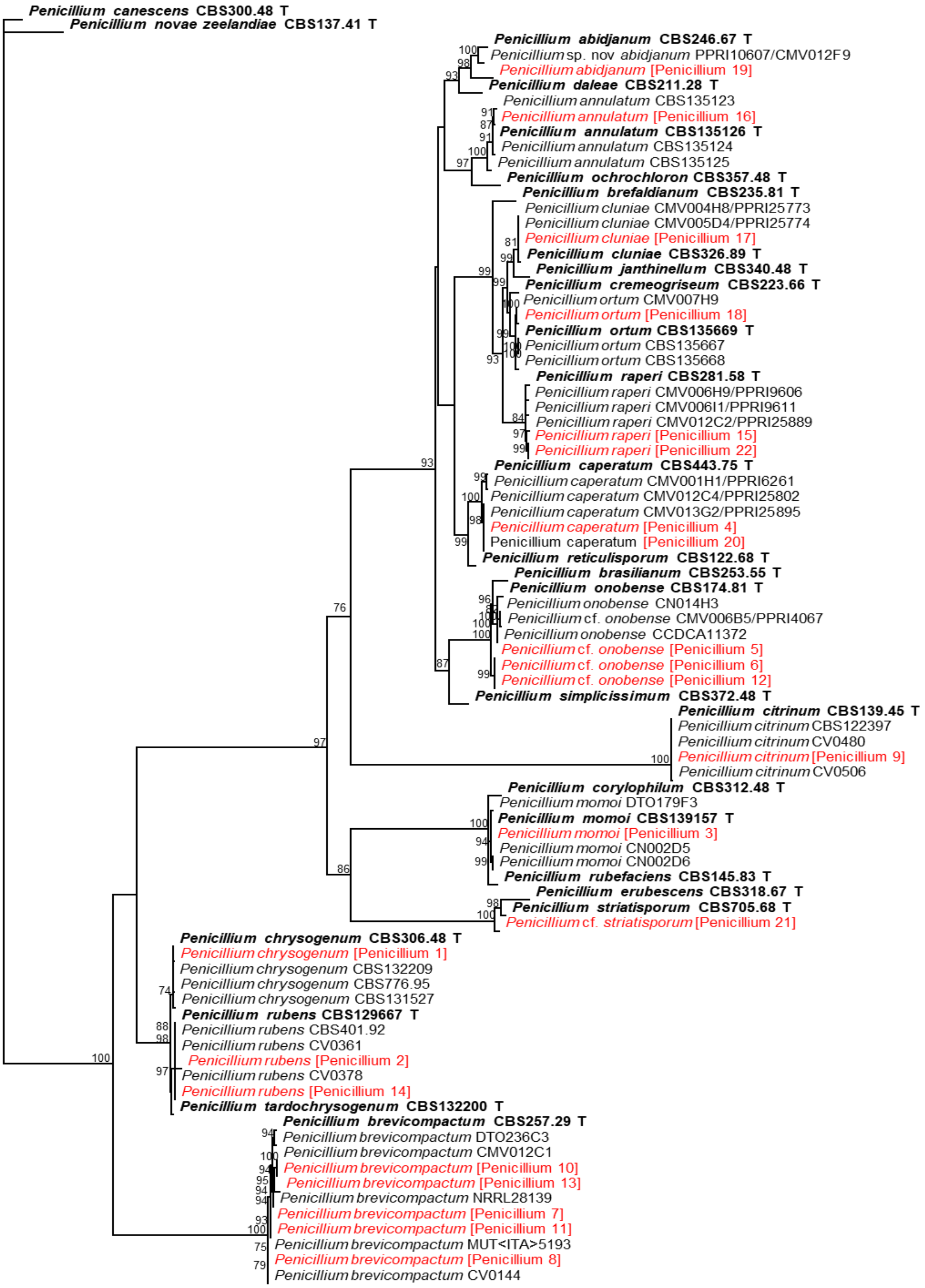

Figure 3.14. Multigene Maximum Likelihood phylogeny of *Penicillium* based on a combined ITS and *BenA* dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

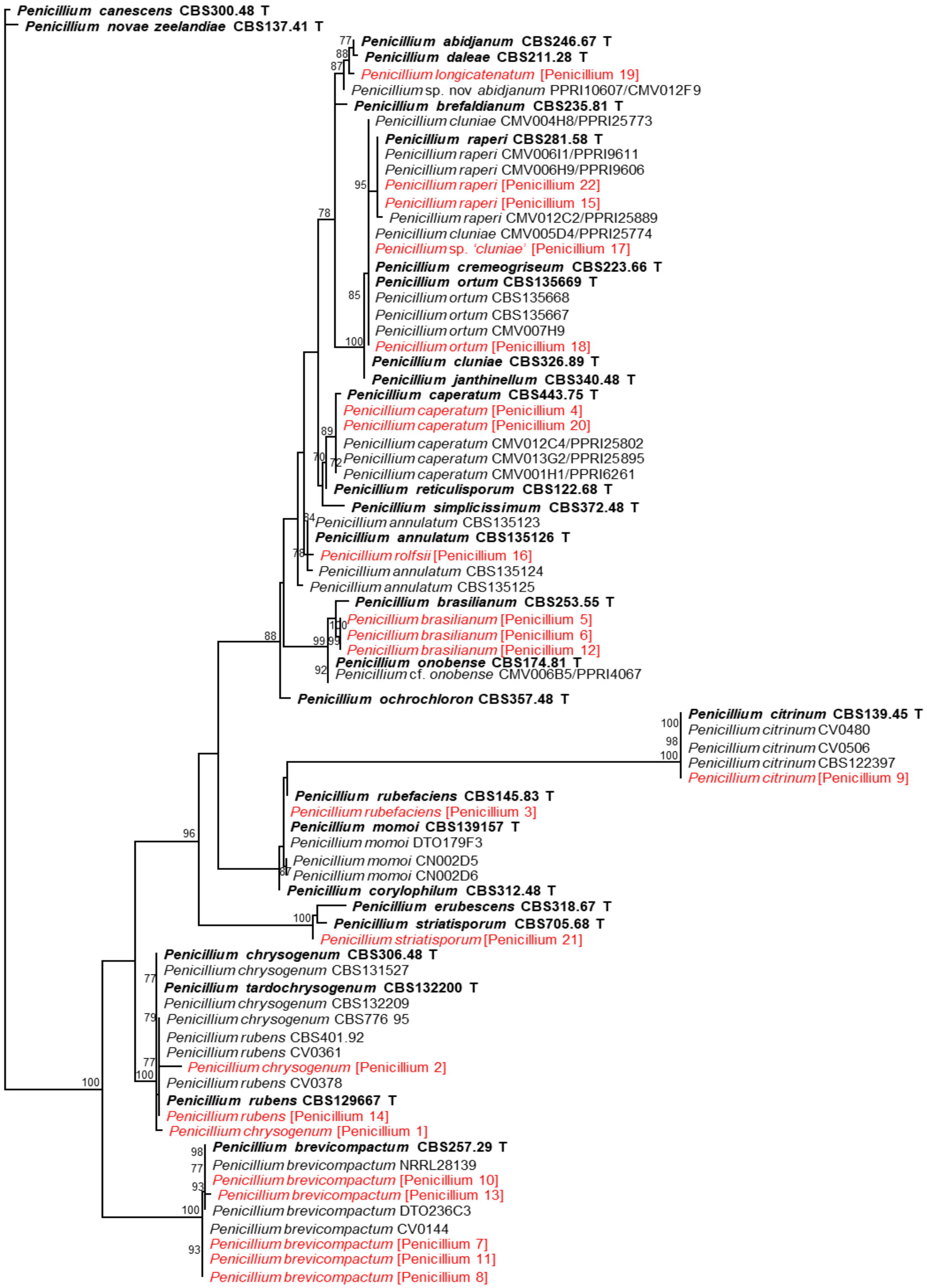

Figure 3.15. Single gene Maximum Likelihood phylogeny of *Penicillium* based on the ITS dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

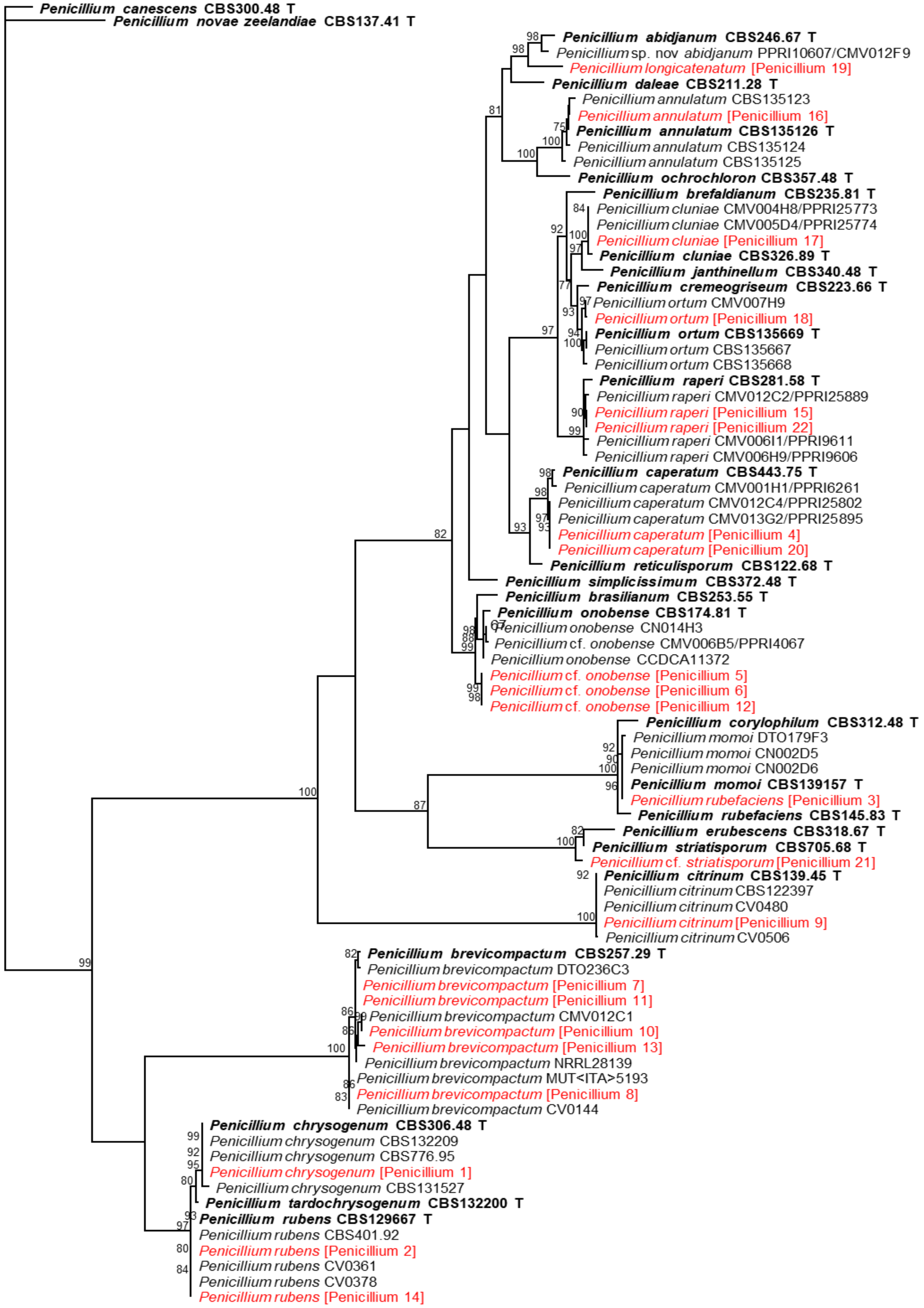

Figure 3.16. Single gene Maximum Likelihood phylogeny of *Penicillium* based on the *BenA* dataset. Strains identified during this study are shown in red text, references in black text and type strains in bold black text. GenBank strain numbers are given behind the reference strains. Branch support in nodes higher than 75% bootstrap are indicated.

Table 3.3 contains the isolate identities of the remaining families that were sequenced using the ITS region only, sorted according to the families to which the respective isolates are classified. Amongst the 116 species identified molecularly were *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Chaetomium globosum* Kunze, three *Cladosporium* species, *Cunninghamella echinulata* (Thaxt.) Thaxt. ex Blakeslee, *Diaporthe rhusicola* Crous and *D. ueckerae* Udayanga & Castl, *Epicoccum nigrum* Link and *E. sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley, *Beauveria bassiana* (Bals.-Criv.) Vuill., *Botryoderma lateritium* Papendorf & H.P. Upadhyay, *Colletotrichum fructicola* Prihastuti, L. Cai & K.D. Hyde, four *Trichoderma* species, *Arthrobotrys amerospora* S. Schenck, W.B. Kendrick & Pramer, *Periconia macrospinosa* Lefebvre & Aar.G. Johnson, *Alternaria alternata* (Fr.) Keissl., *Bipolaris zeae* Sivan. *and B. variabilis* Y. Marin, Y.P. Tan & Crous, *Curvularia akaiiensis* Sivan. and *C. mebaldsii* Y.P. Tan & R.G. Shivas, two *Aureobasidium* species, *Bartalinia pondoensis* Marinc., Gryzenh. & M.J. Wingf., *Neopestalotiopsis clavispora* (G.F. Atk.) Maharachch., K.D. Hyde & Crous and *N. saprophytica* (Maharachch. & K.D. Hyde) Maharachch., K.D. Hyde & Crous, as well as *Pestalotiopsis maculans* (Corda) Nag Raj and *P. mangiferae* (Henn.) Steyaert.

3.3.3 Multi-mycotoxin analyses

Eight mycotoxin standard concentrations were used per mycotoxin during the analyses. These standards consisted of 0.001 mg/kg, 0.005 mg/kg, 0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1 mg/kg, and 5 mg/kg concentrations. Mycotoxin detection within a sample was determined according to the Limit of Detection (LOD) (mg/kg) of each mycotoxin analysed, which is the minimum concentration of mycotoxin detected in a sample that can be reliably distinguished from zero (Armbruster & Pry, 2008). The LOD (mg/kg) of each mycotoxin was as follows: 0.005 mg/kg AFB_1; 0.001 mg/kg FB_1, FB_2, and FB_3; and 0.01 mg/kg DON, NIV, ZEA, and OTA. Samples where the mycotoxin concentration was below the LOD of the specific mycotoxin were considered as Below LOD or ‘Not detected’. No mycotoxins were detected in any of the marama bean leaf, seed or tuber material analysed for multi-mycotoxin presence.

The results of the *in vitro* mycotoxin production by selected potential mycotoxigenic isolates can be found in Table 3.4. The mycotoxins DON, NIV, OTA and ZEA were not detected in any of the fungal isolates analysed. Many isolates throughout the *Aspergillus*, *Fusarium* and *Penicillium* sp. indicated the production of FB_1, FB_2 and FB_3 in very low concentrations, however these concentrations were below the LOD of the respective mycotoxins.

Table 3.3. The fungal isolates excluding *Aspergillus*, *Penicillium* and *Fusarium* spp. isolated from marama bean plant material and molecularly identified using the ITS gene region.

Family	**Isolate number**	**Species names inferred by study**	**Closest similarity identity**				
			% Similarity MLST	**% Query cover**	**Accession number**	**Host**	**Country**
Apiosporaceae	S27	*Arthrinium phaeospermum*	98.73	94	MK120545	Digestive tract of *Phylloicus amazonas*	Brazil
Apiosporaceae	S13	*Arthrinium taeanense*	100	100	MH498513	Seaweed	South Korea
Astrosphaeriellaceae	T106	*Pseudopithomyces palmicola*	100	100	MN788110	Cocoa	Nigeria
Astrosphaeriellaceae	H106	*Pseudopithomyces palmicola*	100	100	MN788110	Cocoa	Nigeria
Bionectriaceae	T103	*Clonostachys rosea*	99.65	100	MT945263		USA
Bionectriaceae	T126	*Clonostachys* sp.	100	100	MH550499		
Bionectriaceae	T101	*Clonostachys* sp.	100	100	MH550499		
Botryosphaeriaceae	T34	*Botryosphaeria dothidea*	100	100	LC317472		
Botryosphaeriaceae	T44	*Botryosphaeria dothidea*	100	100	LC317472		
Botryosphaeriaceae	T48	*Lasiodiplodia pseudotheobromae*	100	100	MN223982		
Botryosphaeriaceae	T51	*Neofusicoccum parvum*	100	100	MN904795	Tea leaf	
Chaetomiaceae	T40	*Chaetomium globosum*	100	100	JX501299		
Chaetomiaceae	S53	*Chaetomium globosum*	100	100	MG664777	*Brachiaria*	Uganda
Chaetomiaceae	T44	*Chaetomium globosum*	100	100	JX981455	Plant	Poland
Chaetomiaceae	T33	*Chaetomium grande*	100	99	MT529959		
Chaetomiaceae	T55	*Chaetomium grande*	100	99	MT529959		
Chaetomiaceae	T35	*Chaetomium puliforum*	99.83	100	MH861633		Spain
Chaetomiaceae	T45	*Chaetomium* sp.	99.83	100	JX160051		UK
Chaetomiaceae	T40	*Chaetomium* sp.	99.83	100	JX160051		UK
Chaetomiaceae	T32	*Dichotomopilus indicus*	100	100	MH864199		Iran
Chaetomiaceae	T56	*Dichotomopilus indicus*	100	100	MH864199		Iran
Chaetomiaceae	T14	*Humicola fuscoatra*	98.97	100	MH911407	Loktak Lake, Manipur	India
Chaetomiaceae	T113	*Humicola* sp.	99.48	100	MK775948	Rhizosphere soil	China
Chaetomiaceae	T115	*Humicola* sp.	99.48	100	MK775948	Rhizosphere soil	China
Chaetomiaceae	T48	*Retroconis fusiformis*sp.	95.73	100	EU040239	Gossypium sp.	Pakistan

Chaetomiaceae	T39	*Thielavia* sp.	99.83	99	KM268653	Tobacco	China
Cladosporiaceae	S50	*Cladosporium delicatulum*	100	100	MT548673	*Danthonia_californica*	USA
Cladosporiaceae	H2	*Cladosporium halotolerans*	100	100	MN859971	*Dactylospongia* sp.	Indonesia
Cladosporiaceae	S56	*Cladosporium pseudocladosporioides*	100	100	MT582794		
Cladosporiaceae	S58	*Cladosporium pseudocladosporioides*	100	100	MT582794		
Cordycipitaceae	S106	*Beauveria bassiana*	99.83	100	KF937310		Columbia
Cunninghamellaceae	T3	*Cunninghamella* sp.	96.27	99	MZ325951	Soil	New Zealand
Diaporthaceae	S1	*Diaporthe rhusicola*	98.18	100	MT304006	*Prunus dulcis*	Spain
Diaporthaceae	H105	*Diaporthe ueckerae*	99.83%	99	MK942678		
Didymellaceae	S3	*Calophoma* sp.	97.61	100	MH858338		Netherlands
Didymellaceae	B102	*Didymella musae*	99.27	100	LT592917		
Didymellaceae	S57	*Didymella* sp.	99.82	100	MG967669	*Eriobotrya japonica*	China
Didymellaceae	S5	*Epicoccum nigrum*	99.81	100	FJ904918	*Grevillea robusta* seeds	Kenya
Didymellaceae	S54	*Epicoccum nigrum*	99.82	100	MT548679	*Festuca roemeri* seeds	USA
Didymellaceae	H109	*Epicoccum nigrum*	100	100	MH645206	*Citrus reticulata*	Pakistan
Didymellaceae	S104	*Epicoccum sorghinum*	99.45	100	MG976431	*Stryphnodendron adstringens* leaf	Brazil
Didymellaceae	S18	*Epicoccum sorghinum*	100	100	MG969883	Sorghum grains	Brazil
Didymellaceae	S20	*Epicoccum sorghinum*	100	100	MN215621	*Saccharum officinarum*	China
Didymellaceae	S7	*Epicoccum sorghinum*	99.82	100	MN215627	*Saccharum officinarum*	China
Didymellaceae	S63	*Epicoccum* sp.	99.45	100	MG976431	*Stryphnodendron adstringens* leaf	Brazil
Didymellaceae	S64	*Epicoccum* sp.	99.45	100	MG976431	*Stryphnodendron adstringens* leaf	Brazil
Didymellaceae	S105	*Juxtiphoma* sp.	97.28	100	MG098275	*Pinus sylvestris* twig	Germany
Didymellaceae	S21	*Leptosphaeria spegazzinii*	99.27	100	MG664743	*Brachiaria*	Uganda
Didymellaceae	S15	*Phoma* sp.	99.47	99	JN207257	*Chloris barbata*	Venezuela
Didymellaceae	S55	*Phoma* sp.	99.1	100	MT251173	Sorghum	Nigeria
Didymellaceae	S2	*Stagonosporopsis* sp.	97.94	100	EU167573		
Didymellaceae	S10	Uncultured *Phoma*	99.48	100	KF493958	Rhizosphere of Heinz 2401 tomato	Canada
Dothideomycetes[a]	T127	*Botryoderma lateritium*	98.07	98	MH858889	*Acacia karroo*, leaf litter	South Africa
Glomerellaceae	S26	*Colletotrichum fructicola*	99.5	100	KU642470		

Glomerellaceae	S31	*Colletotrichum fructicola*	99.64	100	MK874586	*Liquidambar formosana*	China
Glomerellaceae	S42	*Colletotrichum fructicola*	99.28	100	MK874588	*Liquidambar formosana*	China
Hypocreaceae	T2	*Trichoderma asperellum*	100	100	MK086064	Soil	Brazil
Hypocreaceae	T29	*Trichoderma asperellum*	100	100	MG171157	Tobacco floating seedling matrix	China
Hypocreaceae	S11	*Trichoderma atroviride*	100	100	MH260267	Deep-Sea Sediment	China
Hypocreaceae	S22	*Trichoderma atroviride*	99.82	100	KY225682	Soil	China
Hypocreaceae	T4	*Trichoderma hamatum*	98.7	100	MT355443	Rhizosphere of mung bean	India
Hypocreaceae	T18	*Trichoderma harzianum*	99.82	100	MK348218		China
Hypocreaceae	T20	*Trichoderma harzianum*	99.82	100	MK348218		China
Mortierellaceae	T16	*Mortierella alpina*	99.85	100	KT699142	*Padina pavonica*	Italy
Mucoraceae	T7	*Actinomucor* sp.	95.51	100	JN943000		China
Nectriaceae	T104	*Campylocarpon pseudofasciculare*	99.34	100	KF447564	Grape vine	Brazil
Nectriaceae	T123	*Fusicolla acetilerea*	100	99	NR_111603		
Nectriaceae	T135	*Fusicolla acetilerea*	100	99	NR_111603		
Nectriaceae	T100	*Sarcopodium circinosetiferum*	100	100	KM231781	Soil	Argentina
Nectriaceae	T102	*Sarcopodium circinosetiferum*	100	100	KM231781	Soil	Argentina
Nectriaceae	T134	*Sarcopodium circinosetiferum*	100	100	KM231781	Soil	Argentina
Ophiocordycipitaceae	T111	*Purpureocillium lilacinum*	100	100	MH860675	*Chamaeleo dilepsi*, intestine	Netherlands
Ophiocordycipitaceae	T112	*Purpureocillium lilacinum*	99.82	100	MT446064	*Stipa purpurea*	China
Ophiocordycipitaceae	H101	*Purpureocillium lilacinum*	100	100	MG857645	Poultry Feed	Nigeria
Orbiliaceae	T15	*Arthrobotrys amerospora*	99.82	100	AF106533		
Orbiliaceae	T53	*Arthrobotrys amerospora*	99.82	100	AF106533		
Periconiaceae	S35	*Periconia* sp.	94.88	100	MT446142	*Stipa purpurea* root	China
Pleosporaceae	T128	*Alternaria* sp.	100	100	HQ630996	*Miscanthus giganteus*	USA
Pleosporaceae	S33	*Alternaria alternata*	100	100	MT453271	Grassland soil	Germany
Pleosporaceae	S38	*Alternaria alternata*	100	100	MT453271	Grassland soil	Germany
Pleosporaceae	S49	*Alternaria alternata*	100	100	MK968038	*Panax ginseng* root	China
Pleosporaceae	S47	*Alternaria alternata*	100	100	MH141243	*Vitex rotundifolia*	Taiwan
Pleosporaceae	S19	*Bipolaris* sp.	100	100	MK247742		

Pleosporaceae	T120	*Bipolaris variabilis*	99.83	100	NR_151855	*Pennisetum clandestinum* leaf spots	Argentina
Pleosporaceae	S52	*Bipolaris zeae*	100	100	MT505870	*Helianthus annuus*	South Africa
Pleosporaceae	T107	*Curvularia akaiiensis*	98.86	100	LT631342	Unknown	Unknown
Pleosporaceae	H59	*Curvularia mebaldsii*	100	100	MH414903	*Cynodon dactylon transvaalensis*	Australia
Pleosporaceae	B103	*Setosphaeria rostrata*	100	100	LT837845		
Saccotheciaceae	S108	*Aureobasidium leucospermi*	99.32	100	KY294713	Wood	South Korea
Saccotheciaceae	S60	*Aureobasidium leucospermi*	99.32	100	KY294713	Wood	South Korea
Saccotheciaceae	T133	*Aureobasidium pullulans*	100	100	MN700641	Children with *Tinea capitis*	Tanzania
Schizoparmaceae	S103	*Coniella heterospora*	99.83	100	LT800501	Herbivorous animal dung	Spain
Sporocadaceae	S17	*Bartalinia pondoensis*	99.82	100	JQ425386	Air of citrus plantation	Egypt
Sporocadaceae	S45	*Bartalinia pondoensis*	99.68	100	NR_153599		South Africa
Sporocadaceae	S45	*Diversimediispora humicola*	99.78	79	MH554028	Soil	USA
Sporocadaceae	S4	*Neopestalotiopsis clavispora*	99.83	100	MT151848	Deciduous deadwood	Germany
Sporocadaceae	S37	*Neopestalotiopsis clavispora*	99.83	100	MT151848	Deciduous deadwood	Germany
Sporocadaceae	S43	*Neopestalotiopsis saprophytica*	100	100	MT576586	*Psidium guajava* fruit	China
Sporocadaceae	S12	*Pestalotiopsis maculans*	99.83	100	KX610327	Epilithic biofilms	Mexico
Sporocadaceae	S24	*Pestalotiopsis maculans*	99.83	99	KX610327	Epilithic biofilms	Mexico
Sporocadaceae	S29	*Pestalotiopsis mangiferae*	99.66	100	MN888956	Loquat fruit	Pakistan
Sporocadaceae	S25	*Pestalotiopsis* sp.	99.83	100	JX436803	Citrus limon leaf	Cameron
Sporocadaceae	S32	*Pestalotiopsis* sp.	99.83	100	EF451804		
Sporocadaceae	S39	*Pestalotiopsis* sp.	99.83	100	EF451804		
Sporocadaceae	T108	*Robillarda sessilis*	100	100	KR873255	*Heterodera glycines* cyst	USA
Stachybotryaceae	B101	*Alfaria terrestris*	99.16	99	MH864516		USA
Stachybotryaceae	T124	*Alfaria terrestris*	99.49	100	MH864516		USA
Stachybotryaceae	T125	*Alfaria terrestris*	99.49	100	MH864516		USA
Trichocomaceae	T105	*Talaromyces flavus* var. *flavus*	99.49	100	MH857785		Netherlands
Trichocomaceae	T36	*Talaromyces macrosporus*	98.99	100	MH860463		South Africa
Trichocomaceae	T132	*Talaromyces oumae-annae*	99.32	100	MT530252		
Trichocomaceae	T129	*Talaromyces pinophilus*	100	100	MT093464	Soil	

Trichocomaceae	T43	*Talaromyces pinophilus*	100	100	MT093464	Soil	
Trichosphaeriaceae	S28	*Nigrospora oryzae*	99.83	100	KC954151	*Sporobolus fertilis*	Australia
Trichosphaeriaceae	S101	*Nigrospora oryzae*	100	100	HQ607943	*Cyphomyrmex wheeleri* nest	USA
Trichosphaeriaceae	S41	*Nigrospora osmanthi*	99.83	99	MH645207	*Citrus reticulata*	Pakistan
Trichosphaeriaceae	S30	*Nigrospora* sp.	99.83	97	FJ478134	*Castanea mollissima* fruit	China

[a] Class of fungi

Several mycotoxin concentrations have been highlighted in blue in Table 3.4. The concentration of the mycotoxins detected in each sample were compared to the concentrations reported using the eight mycotoxin standard concentrations (0.001 mg/kg to 5 mg/kg). The concentrations highlighted in blue represent the mycotoxin levels that were higher than the concentrations recorded using the 0.01 mg/kg mycotoxin standard in the case of each mycotoxin analysed. Three samples recorded AFB$_1$ concentrations above the 0.01 mg/kg standard, namely: a single isolate of *Aspergillus sojae*, *Fusarium chlamydosporum* SC, and *Penicillium onobense*. *Aspergillus sydowii* reported an FB$_1$, FB$_2$ and FB$_3$ concentration higher than that of the 0.01 mg/kg standard, while *F. scirpi* presented a high concentration of FB$_2$ that was greater than that of the 5 mg/kg standard.

Table 3.4. Fungal species, the sample number, locality of the plant sample, treatment, seed coat presence, and the respective concentrations of aflatoxin B$_1$, and fumonisins B$_1$, B$_2$, and B$_3$ present.

Fungal species	**Sample #**	**Locality**	**Surface sterilization**	**Seed coat**	**Concentration (ppm (mg/kg))**			
					AFB$_1$	**FB$_1$**	**FB$_2$**	**FB$_3$**
Aspergillus japonicus	5	Mpumalanga	SS	SC		0.0225		
Aspergillus sojae	2	North West	SS	NSC	0.687	0.0895	0.0198	0.0084
Aspergillus sojae	5	Mpumalanga	NSS	NSC				
Aspergillus sydowii	2	North West	NSS	NSC		0.1354	0.1867	0.1363
Aspergillus udagawae	4	Limpopo	SS	NSC				
Aspergillus welwitchiae	4	Limpopo	NSS	NSC				
Fusarium chlamydosporum	3	Gauteng	NSS	SC		0.0232	0.0077	
Fusarium chlamydosporum	3	Gauteng	NSS	SC		0.0093	0.0097	
Fusarium chlamydosporum SC	3	Gauteng	SS	NSC		0.0272	0.0155	0.0113
Fusarium chlamydosporum SC	3	Gauteng	NSS	NSC	0.6131	0.0097	0.0043	
Fusarium chlamydosporum SC	3	Gauteng	SS	NSC		0.0075	0.0026	
Fusarium fujikuroi SC	3	Gauteng	SS	SC		0.0197	0.0066	
Fusarium lacertarum	6	Gauteng	SS	SC		0.0264	0.0251	0.0171
Fusarium oxysporum SC	3	Gauteng	NSS	NSC		0.0163	0.0048	
Fusarium scirpi	3	Gauteng	NSS	SC		0.0179	11.5876	
Fusarium solani	3	Gauteng	SS	NSC		0.0112	0.0034	
Penicillium brevicompactum	6	Gauteng	NSS	SC		0.0378	0.0066	0.0032
Penicillium brevicompactum	6	Gauteng	NSS	SC				
Penicillium brevicompactum	5	Mpumalanga	NSS	NSC		0.0324	0.0057	
Penicillium caperatum	5	Mpumalanga	NSS	NSC		0.0309	0.0062	0.0036
Penicillium chrysogenum	2	North West	SS	NSC		0.0202	0.0051	
Penicillium momoi	6	Gauteng	NSS	SC		0.0101		
Penicillium onobense	3	Gauteng	SS	NSC		0.0271	0.0037	
Penicillium onobense	3	Gauteng	SS	NSC		0.0172		
Penicillium onobense	3	Gauteng	SS	NSC	0.8504	0.031	0.0055	
Penicillium rubens	6	Gauteng	NSS	SC				

*SS – Surface sterilized; NSS – Non-surface sterilized; SC – With seed coat; NSC – Without seed coat
**Values highlighted in blue represent high concentrations of the mycotoxin analysed.

3.4 Discussion

The identification of fungal species has progressed over time from a morphological identification-based approach, to a dual morphological and molecular based approach, and more recently to a system more reliant solely on molecular identification. This is due to the fact that there is often extreme variation in morphological features – between different fungal species, within any one given fungal species, as well as between and within isolated cultures of a single species that have experienced a mutation. This high level of variation in fungi can complicate the identification of fungal isolates when using a solely morphological based identification system (Tibpromma et al., 2018). Single gene molecular identification soon became a dominant tool in aiding morphological identification (Visagie et al., 2014), creating a dual morphological and molecular identification system. The Internal Transcribed Spacer region (ITS) is often considered the preferred barcoding gene for fungal species as the ITS region is a conserved DNA region which is found in all fungal species (Bellemain et al., 2010). However, due to the extensive number of fungal genera and species already identified and still to be identified, the DNA sequence within the ITS region is observed to have extensive variation between and within species. Along with additional species-specific variations and fungal mutations over time (Bellemain et al., 2010; Stielow et al., 2015), the ITS region has become increasingly unreliable as the sole DNA region used for molecular identification, largely due to the high probability of misidentification of various genera and species. The more genes that are sequenced, the higher the probability of receiving an accurate identification through molecular means, leading to the development of an identification system more reliant on multigene identification of species (Bellemain et al., 2010; Stielow et al., 2015; Visagie et al., 2014; Visagie & Houbraken, 2020). Using a multigene molecular identification system is more accurate; however, the merits of morphological identification should not be overlooked when considering the maintenance and preservation of specific fungal isolates in order to avoid unintentional errors when working with fungal species, such as contamination or mutations within a working fungal culture which may impact the research.

For the purpose of this study, a two gene system was used for the identification of the three main mycotoxin producing genera within the study, using the ITS region as the primary identification region and the secondary beta-tubulin (*BenA*) and transcription elongation factor 1-alpha (*TEF*α) gene regions to identify the various *Aspergillus* and *Penicillium*, and *Fusarium* spp., respectively (Figures 3.8 to 3.16). Beta-tubulin (*BenA*) was proposed as a secondary identification marker gene region for *Penicillium* spp. (Visagie et al., 2014), while the calmodulin (*CaM*) gene region has been recommended for the identification of *Aspergillus* spp. due to the higher specificity offered by the *CaM* gene compared to the *BenA* gene (Visagie & Houbraken, 2020), however, *BenA* was selected for the identification of both the *Aspergillus* and *Penicillium* species in this study. The *TEFα* gene region offers a higher specificity on species level when identifying *Fusarium* spp. (Karlsson et al., 2016), and with many *Fusarium* specific databases such as Mycobank available, the *TEFα* gene is the ideal secondary gene for *Fusarium* identification. The specific groupings of each isolate within a species tended to vary between the ITS and *BenA/TEFα* gene regions. A good example of this is the *Aspergillus* 3 and 4 isolates – the ITS region resulted in the two species being grouped between *Aspergillus aculeatus* and *A. japonicus*, while the *BenA* gene grouped the two isolates within the *A. aculeatus* and *A. japonicus* group, but with a much longer branch. However, the combined tree based on the ITS and *BenA* data, placed the two isolates within the *A. aculeatus* and *A. japonicus* group but separated with a shorter branch than the *BenA* gene tree alone. Numerous isolates presented a challenge in identification due to the conflicting BLAST results obtained per fungal isolate using the different gene regions, however, the phylogenetic trees aided in clarifying the results. In majority of the cases, the combined trees were used to confirm the BLAST results of the various isolates according to the two genes used for the specific genus. In several cases, especially within the *Aspergillus* genus, the ITS region aided in the identification more than the *BenA* tree, largely due to the conflicting results presented by the *BenA* tree, as in the case of *Aspergillus* isolates 3 and 4. For the remaining fungal isolates that were not classified within the *Aspergillus*, *Fusarium* or *Penicillium* genera, single gene identification using the ITS region was performed (Table 3.3).

A total of 116 species from 27 families and the Dothideomycete class, spanning Ascomycota and Mucoromycota, were identified as being associated with the marama bean leaves, seeds and tubers collected from the eight localities within South Africa, indicating an incredibly wide biodiversity of fungi associated with the marama beans within South Africa (Image 3, pg74).

Among the 116 species identified were several plant pathogenic species such as *Alternaria alternata* (Uzabakiriho et al., 2013), *Bipolaris zeae* and *B. variabilis* (Manamgoda et al., 2014), *Diaporthe rhusicola* and *D. ueckerae* (Agustí-Brisach et al., 2019), *Epicoccum nigrum* and *E. sorghinum* (Davenport et al., 2017), *Lasiodiplodia pseudotheobromae* (L. Li et al., 2020), *Neofusicoccum parvum* (Massonnet et al., 2017), as well as several *Aspergillus* (Pechanova & Pechan, 2015), *Penicillium* (Dupont et al., 1999), *Fusarium* (Goswami et al., 2008; Nelson et al., 1983), *Neopestalotiopsis* and *Pestalotiopsis* species (Maharachchikumbura et al., 2014), *Trichoderma* (Pfordt et al., 2020) and *Talaromyces* species (Stošić et al., 2020), among many others. Several of the genera are also comprised of known mycotoxigenic species, including *Alternaria* (Escrivá et al., 2017), *Aspergillus* (Sweeney & Dobson, 1998; Varga et al., 2011), *Fusarium* (Ismaiel & Papenbrock, 2015) and *Penicillium* (Ciegler, 1978; Ismaiel & Papenbrock, 2015). Along with the known pathogenic and mycotoxigenic species isolated, there were numerous fungal species of interest that were isolated from the various marama bean parts and localities, including: *Aspergillus alabamensis*, *A. magaliesburgensis*, *A. welwitschiae*, *Bartalinia pondoensis*, *Beauveria bassiana*, *Botryoderma lateritium*, *Calophoma clematidis-rectae*, *Fusicolla acetilerea*, *Fusarium fujikuroi*, *F. lacertarum*, *F scirpi*, *Juxtiphoma eupyrena*, *Leptosphaeria spegazzinii*, *Mortierella alpina*, *Penicillium abidjanum*, *P. momoi*, *P. onobense* and *P. ortum*, *Purpureocillium lilacinum*, *Periconia macrospinosa*, *Retroconis fusiformis*, *Stagonosporopsis cucurbitacearum*, and *Thielavia* sp., .

Studies previously performed on *Tylosema esculentum* in Namibia yielded several fungal species, including: *Alternaria alternata, A. solani, A. tenuissima* (now *A. alternata*), *Epicoccum sorghi* (now *E. sorghinum*), *Fusarium chlamydosporum, F. equiseti, F. incarnatum, Penicillium brevicompactum, P. commune, P. olsonii, Phoma sorghina* (now *E. sorghinum*), an unknown *Phoma* sp., and *Rhizopus stolonifer* (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013). From the thirteen previously isolated fungal species, six of the species were not isolated during this study on South African marama beans, namely: *A. solani, P. commune, P. olsonii,* and *R. stolonifer*. Despite the high isolation frequency of the *Rhizopus* species contaminating or present on the plant material samples, the molecular identity of the *Rhizopus* species was not determined and cannot be confirmed to be *R. stolonifer* as isolated in the Namibian studies. Uzabakiriho et al. (2013) reported *A. tenuissima* (now *A. alternata*) and a *Phoma* sp. on marama bean seeds and pods collected from the Omaheke region of Namibia, while all thirteen of the fungal species were reported to be associated with marama bean leaves found in the Otjiwarongo region of Namibia, or with marama bean seedlings that were part of a greenhouse trial at the University of Namibia (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015). The isolation frequency of both *Alternaria* and *Phoma* species were considerably low on the SA marama bean seed samples, likely due to the large number of overall isolates obtained and species identified. When considering the leaf samples, a large portion of the species isolated from the SA marama bean leaves were identified as *Alternaria* or *Epicoccum* species, while the *Fusarium*, *Penicillium* and *Phoma* species tended to have a lower isolation frequency, again likely attributed to the larger number of overall isolates obtained and identified. However, it could be extrapolated that the fungal species associated with the marama bean leaves followed the same trend in the Namibian and South African populations.

Alternaria and *Epicoccum*, along with *Cladosporium*, *Lasiodiplodia*, *Pestalotiopsis*, *Diaporthe* and *Colletotrichum*, are common examples of endophytic fungal genera (Tibpromma et al., 2018). Several species within these genera have been identified on the marama bean leaves in Namibia and South Africa, with high isolation frequencies of the *Epiccocum*, *Alternaria*, and *Cladosporium* species on the

South African leaf samples. Endophytic fungal species, even known pathogenic species, have the potential to survive and thrive on plants without causing disease, while providing the host plant with a variety of benefits including resistance or tolerance to biotic and abiotic stress (Fischer et al., 2005; Lewis, 2004; Saikkonen et al., 1998; Wilson, 1995; Zhang et al., 2006). The endophytic nature of the various fungal species may provide and explanation for the high isolation frequencies of the *Alternaria*, *Epicoccum* and *Cladosporium* species on the leaves compared to that of the seeds and tubers collected from the various localities, as well as the high isolation frequencies of *Alternaria* and *Epicoccum* species even under surface sterilised conditions. However, endophytes are not limited to the leaves. Fungi can be endophytic in the branches, stem, fruits, tubers and roots (Bamisile et al., 2018). The records of the seed and tuber associated fungi also presented a large number of known endophytic fungi, such as *Lasiodiplodia*, *Pestalotiopsis*, *Epicoccum* and *Trichoderma* species. Due to the nature of the marama bean consisting of prostrate vines along the ground from which the leaves, flowers and seed pods develop (Coetzer et al., 2011; Cole et al., 2014), it is possible for endophytic fungi common on the leaves to become associated with the seeds and pods, both during development and after development once the pods split open and the seeds are released.

Once the marama bean pods split open and the seeds are released, they remain on the ground until germination (Coetzer et al., 2011). As such, there will also be a possibility for various soil-borne fungal species to develop on or infect the seeds. Similarly, the tubers presented a dominant presence of soil-borne fungal species when compared to the other plant material. The most studied soil-borne plant pathogens include various *Fusarium*, *Pythium*, *Rhizoctonia*, and *Phytophthora* species, all of which can be detrimental to many conventional crops, including various legume crops (Deshwal et al., 2003; Ramírez-Suero et al., 2010). However, several other genera pose a threat as potential soil-borne pathogens, including various *Pestalotiopsis* and *Neopestalotiopsis* spp., as well as various *Penicillium*, *Trichoderma*, *Alternaria*, *Chaetomium* and *Aspergillus* species (Babu et al., 2015; Guo et al., 2015; Jiang et al., 2017, 2019; Maharachchikumbura et al., 2014; Mousavi et al., 2016; Pfordt et al., 2020). *Aspergillus*, *Penicillium*, *Pestalotiopsis* and *Fusarium* had high isolation frequencies in both the seed and tuber samples, while *Trichoderma* was also highly prevalent in the tuber samples. The severity of the diseases caused by the different species can differ vastly, from the highly pathogenic *Fusarium* species that cause *Fusarium* wilt, to *Trichoderma* species which are opportunistic and often grow in a plant's rhizosphere without causing disease (Li et al., 2019; Shi et al., 2017; Vinale et al., 2008). As this is the first report of fungal species associated with marama bean tubers, the general trend of fungal species associated with the tubers from different regions cannot yet be explored. However, one could assume that the fungal species associated with the tubers could follow the same trend as the seed associated species, since both the tubers and seeds would be exposed to similar fungal species, particularly the soil-borne fungal species.

When looking at the trends of fungal groups associated with the marama bean in different localities within South Africa (Figure 3.1), the relative percentages of the different fungal genera or groups were relatively similar between the different localities, with the exception of Samples 1 and 2 that were contaminated with *Rhizopus*. Sample 3 and 8, located in Gauteng (warm temperate zone with warm summers) indicated higher isolation frequencies of *Colletotrichum*-like species, while *Rhizoctonia*-like species were prevalent only in sample 3. Sample 5 and 7 located in Mpumalanga and Gauteng, respectively (warm temperate zone with hot summer), both indicated the lowest isolation frequencies of *Chaetomium* species, while sample 5 indicated a higher frequency of *Aureobasidium* and *Phoma* spp., compared to the higher frequency of *Aspergillus* and *Pestalotiopsis* spp. in sample 7. Despite the similar climatic zones and respective conditions, there was some variation in diversity and incidence of fungal species between the localities. This could be due to the immediate environment in which the plants occur, or several other biological conditions related to the plants or fungal species specifically that could result in the differences in fungal diversity between each location.

During the multi-mycotoxin analysis of the plant material or fungal isolate samples, the limit of detection (LOD, mg/kg) of each mycotoxin was determined – the LOD being the minimum mycotoxin concentration detected in a sample that can be reliably distinguished from zero (Armbruster & Pry, 2008). In the cases of the aforementioned mycotoxins that were not detected, there may have been mycotoxins present in concentrations lower than the LOD, which are subsequently labelled as ‘Not detected’. Despite the incredible diversity of fungi on the marama bean, and many of the species being known mycotoxin producers, mycotoxins were not detected on any of the marama bean leaves, seeds and tubers collected from each of the eight localities. As well, the mycotoxins DON, NIV, OTA and ZEA were not detected in the fungal isolates that were analysed.

Ochratoxin A (OTA) is commonly produced by both *Aspergillus* and *Penicillium* spp., including *A. ochraceus, A. carbonarius, A. niger*, and *P. verrucosum*. (Bui-Klimke & Wu, 2015; Ciegler, 1978). Phytotoxic effects include cell death, necrosis and subsequent plant growth inhibition (Ismaiel & Papenbrock, 2015). *Fusarium*, *Trichoderma*, *Stachybotrys*, and several other genera in the Hypocreales order are able to produce trichothecene mycotoxins, including Nivalenol (NIV), 4-deoxynivalenol (DON), zearalenone (ZEA), and fumonisins (Antonissen et al., 2014; Ismaiel & Papenbrock, 2015). *Fusarium* trichothecenes are known to produce chlorosis, necrosis and mortality in plants, leading to various diseases including wilts, and root, leaf, and stalk rots (Antonissen et al., 2014; Ismaiel & Papenbrock, 2015). Deoxynivalenol (DON, also known as vomitoxin) is commonly produced by *Fusarium culmorum*, *F. incarnatum* and *F. graminearum* (Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003). Nivalenol (NIV) is produced by *Fusarium equiseti*, *F. graminearum*, *F. chlamydosporum*, *F. sporotrichoides*, *F. crookwellwense*, and *F. nivale*, and has been reported in cereal and legume crops (Ismaiel & Papenbrock, 2015; Sweeney & Dobson, 1998). *Fusarium cerealis* (syn. *F. crookwellense*), *F. semitectum*, *F. roseum*, *F. culmorum*, *F. equiseti* and *F. graminearum* are known producers of zearalenone (ZEA, also known as the F-2 toxin) (Ismaiel & Papenbrock, 2015; Sweeney & Dobson, 1998; Zain, 2011).

Aflatoxin B_1 was detected in high concentrations in three of the fungal isolate samples – *A. sojae*, *F. chlamydosporum* and *P. onobense*. The production and detection of high levels of aflatoxins is unexpected for all three fungal species, as neither are known aflatoxin producers. *Aspergillus sojae* in particular is unusual, however, *A. sojae* is a domesticated form of *A. parasiticus* (Frisvad et al., 2019), which is able to produce aflatoxins, thus a misidentification of the sample could explain the production of aflatoxins. Aflatoxins, particularly aflatoxin B_1 (AFB_1), are primarily produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* Kurtzman, B.W. Horn & Hesselt. (Kumar et al., 2017), as well as a few other species such *A. pseudocaelatus* Varga, Samson & Frisvad and *A. pseudonomius* Varga, Samson & Frisvad (Varga et al., 2011). Possible reasons for the detection of aflatoxins in the three uncommon species could be that the production of aflatoxin in these species has not been studied, or that the conditions for aflatoxin production were not considered when studying the species previously, or contamination of the fungal samples or maize patty sent for mycotoxin analysis, or possibly due to fungal species misidentification. The findings would need to be confirmed through more rigorous investigation. Aflatoxins are primarily found on maize and legumes, cottonseed (*Gossypium* L.), Brazil nuts (*Bertholletia excelsa* Humb. & Bonpl.), and peanuts (*Arachis hypogaea* L.). Phytotoxic effects of aflatoxin exposure include reduced germination, reduced plant biomass accumulation, reduced root and shoot growth, and reduced leaf mass regeneration (McLean, 1994).

Fumonisins B_1, B_2 and B_3 were detected in low concentrations in a majority of the fungal isolates analysed, however, high concentrations of all three fumonisins were only detected in the *A. sydowii* sample, while only FB_2 was detected in the *F. scirpi* sample. However, neither of the two species are commonly known fumonisin producing species. Fumonisins are commonly produced by *Fusarium proliferatum*, *F. nygamai*, *F. incarnatum*, *F. verticillioides*, as well as *Alternaria alternata* (Abbas & Boyette, 1992; Alberts et al., 1993; Chen et al., 1992; Doehlert et al., 1994; Ismaiel & Papenbrock, 2015; Klich & Bennett, 2003; Kritzinger et al., 2006; Sweeney & Dobson, 1998). In plants, FB_1 is known to cause necrosis and chlorosis, wilting, root and shoot growth inhibition, reduced height and biomass accumulation, inhibition of seedling emergence and radicle elongation, and eventual death on plants such as tomatoes, legumes such as soybeans (*Glycine max* (L.) Merr.) and cowpeas (*Vigna unguiculata* (L.) Walp.), as well as cereals such as sorghum and maize (Abbas & Boyette, 1992; Doehlert et al., 1994; Ismaiel & Papenbrock, 2015; Kritzinger et al., 2006; Sweeney & Dobson, 1998).

Despite many of the fungal isolates being able to produce AFB$_1$ and FB$_{1,}$ FB$_2$ and FB$_3$ mycotoxins *in vitro* using maize patty cultures (Table 3.4), the absence of mycotoxins present on any of the marama bean plant material indicates that mycotoxins were not naturally being produced on the plants. This may be due to the absence of optimal environmental conditions necessary for fungi to produce mycotoxins on the plants, such as optimal temperature, moisture content, aeration, or susceptibility of the host plant to the fungal species (Ciegler, 1978; Fernández-Cruz et al., 2010; Ismaiel & Papenbrock, 2015). Many of the conditions required for mycotoxin production are not easily replicated in a laboratory setting, such as *in vitro* maize patty cultures, which could result in mycotoxin production *in vivo* but not *in vitro*. Further investigation would be required to determine under which conditions the fungi would naturally produce mycotoxins on the plants, which would impact the use and consumption of the plants.

In conclusion, the South African marama bean has an unexpectedly large diversity of fungal species associated with the various plant material, ranging from endophytes and saprophytes, to potential pathogenic and mycotoxigenic species. Though the number of species identified is impressive in itself, it represents only a portion of the total number of fungal species that may be associated with the marama bean. Despite the extensive diversity of fungal species, whether the fungal species would be capable of being pathogenic or mycotoxigenic to the marama bean under cultivated conditions would require further research. Further research is recommended regarding the potential of pathogenic and mycotoxigenic species to produce diseases and/or mycotoxins, as well as the production of mycotoxins by non-typical mycotoxin producing species, as these aspects pose a threat to crop yield, human and animal consumption, and food safety and security in general. This is the first comphrehensive report of fungal associations with the marama bean in South Africa.

3.5 References

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Chapter 4 - Investigating fungal pathogenicity on marama beans

Image 4. The pathogenicity trial set up in the shade cloth tunnel at Innovation Africa @UP, Hillcrest campus. Photo by T. Armfield

Abstract

A wide range of fungal species were previously identified on the orphan crop, marama bean (*Tylosema esculentum* ((Burch.) A.Schreib.) and *Tylosema fassoglense* ((Kotschy ex Schweinf.) Torre & Hillc.)), from South Africa, indicating the need for further investigation regarding the fungal pathogenicity amd mycotoxigenicity on the marama bean. Twenty fungal isolates spanning thirteen species with the greatest potential for pathogenicity on the marama bean were identified using detached leaf assays and were further analysed for pathogenicity on marama bean seedlings. A disease symptom rating system was established to account for the various symptoms produced by the different fungal species. Black leaf spots, leaf lesions, stem lesions and leaf discoloration were the most prevalent symptoms observed. *Lasiodiplodia pseudotheobromae*, *Botryosphaeria dothidea*, *Neofusicoccum parvum*, *Bipolaris* sp., and *Diaporthe rhusicola* were the top five fungal species with the highest disease rating scores after the five-week period. However, *Pestalotiopsis maculans* was predicted to be pathogenic over a longer time period. *Lasiodiplodia pseudotheobromae*, *N. parvum*, and *B. dothidea* consistently caused the largest negative difference in above and below ground dry weight compared to the negative control. Koch's postulates confirmed that eleven of the thirteen fungal species were re-isolated after the pathogenicity trial, with the exceptions being *Colletotrichum fructicola* and the *Fusarium solani* species complex isolates. Multi-mycotoxin analysis of the seedlings indicated that zearalenone was detected in the *Alternaria alternata*, *F. fujikuroi* and *F. oxysporum* species complex treatments. In conclusion, several fungal species have the potential to be pathogenic to the marama bean, however further studies are recommended.

4.1 Introduction

When evaluating the potential pathogenicity of fungal species, one must keep in mind how plants and pathogens interact. Disease development in plants caused by fungal pathogens depends on the environmental conditions the plant occurs in, how susceptible the plant is to fungal infection, and how virulent the fungal species is (Abdullah et al., 2017; Velasquez et al., 2015). These factors can act in the favour of the plant or the pathogen. If the environmental conditions favour the plant, the plant will operate at optimal level, reducing the ability of the pathogen to infect the plant and causing disease (Abdullah et al., 2017; Velasquez et al., 2015). However, if the environmental factors favour the pathogen more, the plant may not be operating at optimal level and the pathogen will have a greater chance of successfully infecting the plant and causing disease. Susceptibility of the plant to the pathogen can be affected by many factors, including but not limited to the environmental conditions, internal factors such as the genetics of the plants and mutations, and external factors such as pests and pesticides, weeds, and herbicides (Velasquez et al., 2015). These factors can favour the plant or the pathogen – such as genetically altered crop lines that are resistant to certain fungal pathogens, or fungicides applied to prevent and reduce fungal associations; or plants that have genetic mutations resulting in sub-optimal or non-existent functions relating to disease management (Velasquez et al., 2015).

The detached leaf assay provides a 'sneak peek' at how different fungal species will affect a plant, by using a single leaf from the plant of interest rather than the entire plant. This helps to eliminate fungal species from the eventual pathogenicity trial that do not have an effect or do not have a severe effect on the plants, helping to narrow down the focus of the pathogenicity trial to fungal species that may be pathogenic and may cause severe disease symptoms (Mushin et al., 1959). Pathogenicity trials provide an opportunity to determine whether pathogens can be pathogenic to certain plants or crops, and whether the pathogen can cause a disease on the plant, as well as investigate the severity of the diseases caused (Mushin et al., 1959). The pathogenicity trial is conducted under extreme pathogen conditions. This means that the amount of fungal isolate applied to the plants is at a much higher rate than may naturally occur; and the inoculation of the fungal species is isolated to a single species, ensuring that the inoculated species is the only fungus present, in order to reduce the possibility of other microbes deterring or preventing the fungus from infecting the plant (Mushin et al., 1959). The extreme pathogen conditions used during the pathogenicity trial provide an indication of whether the specific fungal species has the ability to infect the plant and cause disease, and how severe the disease may become – whether the fungus has the possibility of being a pathogen to the plant of interest under farming conditions. Under general farming conditions, the amount of fungus present is generally lower than that used during pathogenicity trials. However, the pathogenicity trial provides an indicator for which fungal species to be aware of during the growing season, giving farmers a head-start on possible disease mitigation actions while farming the plant.

Tylosema esculentum (marama bean) has the potential to become an economically important crop, as it is currently a locally important plant that widely used by local communities in southern Africa and Namibia, although only as a wild-harvested plant (Cole et al., 2014). Climatic conditions and underdeveloped agricultural infrastructure in many arid countries result in inadequate yield production and availability of conventional and agricultural crops, while local indigenous plants such as the marama bean have the potential to solve these problems while addressing the malnutrition and hunger issues prevalent in many of these arid countries (Cullis et al., 2018; Jackson, 2017; National Academy of Sciences, 1979). The high protein, oil and nutrient content, the potential health and medicinal benefits that the plants offer, as well as their ability to survive and thrive in arid areas where it is acclimatised to the environmental conditions enhance the potential of the marama bean to provide relief in these areas (Cole et al., 2014; Jackson, 2017; National Academy of Sciences, 1979; Smýkal et al., 2018).

Unfortunately, there is a general lack of information regarding the marama bean production requirements, such as the environmental conditions required for growth, as well as symbiotic associations with insects, bacteria, and fungi (Cullis et al., 2018; National Academy of Sciences, 1979; National Research Council, 2006). *Tylosema esculentum* naturally grows in arid climatic conditions prefering high temperatures and dry, drought-like conditions (Coetzer et al., 2011; Mitchell et al., 2005). In the Kalahari, the marama bean typically grows in low quality sandy soils due to the lack of organic matter and nutrients (Chimwamurombe, 2010; Coetzer et al., 2011; Mitchell et al., 2005; National Research Council, 2006). In Namibia and Botswana, marama beans grew in soils with “Brown fine aeolian sand” with or without “limestone/dolomite concretions” (Thomas, 2004). In Texas, USA , marama beans were reported to have a 100% germination rate in alluvial soil (sand, clay, gravel, silt and organic material deposited by rivers (Hosch, 2009)) (Powell, 1987). Takundwa (2014) and Travlos & Karamanos (2006) indicated that potting soil, clay and clay loam soils were unsuitable for marama bean growth, while sandy soils were ideal due to the high drainage rate (Travlos & Karamanos, 2006). Odhiambo (2020) investigated seed germination conditions, concluding that 30-35ºC was optimal for seed germination, while 10ºC and 40ºC inhibited seed germination. Investigation into the cultivation requirements and practices for marama bean has begun. However, currently, there is no concensus for marama bean cultivation and production.

Fungal associations have not been highly reported on, with the only reports being on marama beans in Namibia. These reports indicate the presence of several fungal species isolated from the pods and seeds, the leaves and as leaf endophytes (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013). However, there have been no reports of the fungal species being pathogens on the marama bean, nor reports on diseases produced by the fungal species on the marama bean. The fungal species identified as associated with the marama bean in Namibia have not been listed as known pathogens of the marama bean according to the Genera of phytopathogenic fungi: GOPHY 1, 2, 3, 4 (Chen et al., 2022; Marin-Felix et al., 2019a, 2019b, 2022). To date, there have there have been no reports of either fungal species, pathogens or diseases on *Tylosema esculentum* in South Africa.

Therefore, the aim of this chapter was to investigate whether any of the fungal species previously isolated and identified from marama beans (Chapter 3) located in several areas of South Africa, have the potential to be pathogenic to the marama bean. The potential of isolated fungal species to be pathogenic and to possibly cause disease on the plants will negatively affect the growth, nutritional aspects, and thus the possibility of commercialising the marama bean for farming purposes. Unfortunately, there is currently limited information regarding the propagation and cultivation of marama beans, therefore the ideal growth conditions of the plants had to be determined through various soil and drainage trials prior to commencing the pathogenicity trials.

4.2 Materials and Methods

4.2.1 Fungal selection

Using the list of molecularly identified fungal isolates previously isolated from the various marama bean samples, the fungal isolates were grouped into four categories (Figure 4.1), namely: potential pathogens (red group), weak or postharvest pathogens (yellow group), saprophytes (black group), and fungi that are only of interest under certain conditions (white group). Sixty-nine (69) cultures of representative fungal isolates from the red, yellow and white groups were selected for analysis using detached leaf assays. The samples from the black group (saprophytes) were not considered for further analysis. The specific fungal species included in each of the groupings are shown in Table 4.1.

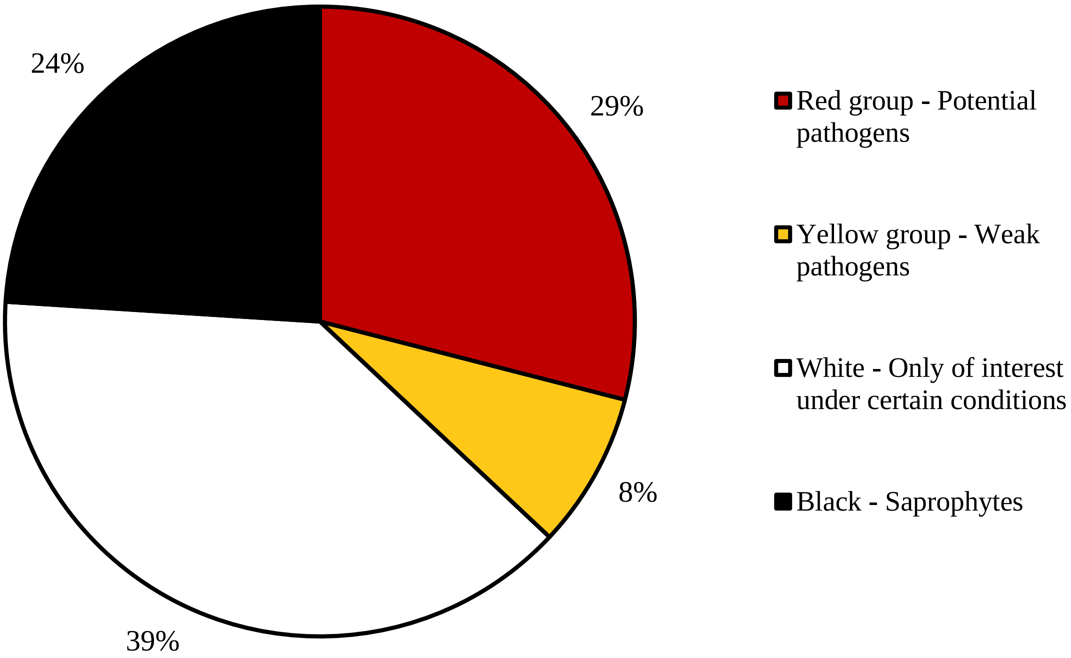

Figure 4.1. The groupings of all molecularly identified fungal isolates according to four groups: red group [potential pathogens], yellow group [weak-or postharvest pathogens], white group [fungi only of interest under certain conditions], and black group [saprophytic fungi]. The percentage of fungi categorized into each group is also presented. The fungal isolates in the red, yellow and white groups were considered for detached leaf assays and pathogenicity trials.

Table 4.1. The groupings of all molecularly identified fungal isolates according to four groups: red group [potential pathogens], yellow group [weak-or postharvest pathogens], white group [fungi only of interest under certain conditions], and black group [saprophytic fungi]. The fungal isolates in the red, yellow and white groups were considered for detached leaf assays and pathogenicity trials.

Red group - Potential pathogens

🟥

- *Alternaria alternata*
- *Alternaria* sp.
- *Bipolaris* sp.
- *Bipolaris variabilis*
- *Bipolaris zeae*
- *Colletotrichum fructicola*
- *Curvularia akaiiensis*
- *Curvularia mebaldsii*
- *Diaporthe rhusicola*
- *Diaporthe ueckerae*
- *Fusarium chlamydosporum*
- *Fusarium chlamydosporum* SC
- *Fusarium citri*
- *Fusarium fujikuroi* SC
- *Fusarium incarnatum-equiseti* SC
- *Fusarium lacertarum*
- *Fusarium oxysporum*
- *Fusarium oxysporum* SC
- *Fusarium sambucinum* SC
- *Fusarium scirpi*
- *Fusarium solani*
- *Fusarium solani* SC
- *Neofusicoccum parvum*
- *Neopestalotiopsis clavispora*
- *Neopestalotiopsis saprophytica*
- *Pestalotiopsis maculans*
- *Pestalotiopsis mangiferae*
- *Pestalotiopsis* sp.

Yellow Group - Weak or post-harvest pathogens

🟨

- *Actinomucor* sp.
- *Botryosphaeria dothidea*
- *Cunninghamella* sp.
- *Didymella* sp.
- *Lasiodiplodia pseudotheobromae*
- *Phoma* sp.
- Uncultured *Phoma*

White group - Pathogens only of interest under certain circumstances

⬜

- *Aspergillus alabamensis*
- *Aspergillus awamori*
- *Aspergillus japonicus*
- *Aspergillus luchuensis*
- *Aspergillus magaliesburgensis*
- *Aspergillus nidulans*
- *Aspergillus nidulans* var. *dentatus*
- *Aspergillus ochraceus*
- *Aspergillus pseudoflectus*
- *Aspergillus quadralineatus*
- *Aspergillus sojae*
- *Aspergillus sydowii*
- *Aspergillus udagaeae*
- *Aspergllus welwitschiae*
- *Cladosporium delicatulum*
- *Cladosporium halotolerans*
- *Cladosporium pseudocladosporioides*
- *Epicoccum nigrum*
- *Epicoccum sorghinum*
- *Penicillium abidjanum*
- *Penicillium annulatum*
- *Penicillium brevicompactum*
- *Penicillium caperatum*
- *Penicillium chrysogenum*
- *Penicillium citrinum*
- *Penicillium cluniae*
- *Penicillium momoi*
- *Penicillium* cf. *onobense*
- *Penicillium ortum*
- *Penicillium raperi*
- *Penicillium rubens*
- *Penicillium striatisporum*
- *Stagonosporopsis* sp.
- *Talaromyces flavus* var. *flavus*
- *Talaromyces macrosporus*
- *Talaromyces oumae-annae*
- *Talaromyces pinophilus*
- *Thielavia* sp.

Black group - Saprophytes

⬛

- *Arthrinium phaeospermum*
- *Arthrinium taeanense*
- *Arthrobotrys amerospora*
- *Aureobasidium leucospermi*
- *Aureobasidium pullulans*
- *Bartalinia pondoensis*
- *Chaetomium globosum*
- *Chaetomium grande*
- *Chaetomium piluliferum*
- *Chaetomium* sp.
- *Dichotomopilus indicus*
- *Diversimediispora humicola*
- *Humicola fuscoatra*
- *Humicola* sp.
- *Mortierella alpina*
- *Nigrospora oryzae*
- *Nigrospora osmanthi*
- *Nigrospora* sp.
- *Periconia* sp.
- *Retroconis fusiformis*
- *Trichoderma asperellum*
- *Trichoderma atroviride*
- *Trichoderma hamatum*
- *Trichoderma harzianum*

4.1.1 Detached leaf assay (DLA)

A pilot trial of the DLA was conducted to determine the best mode of fungal inoculation. Four inoculation methods were examined – a single agar plug placed on the centre of the leaf; three agar plugs placed equidistantly on the leaf; a single agar plug placed on the petiole of the leaf; and three drops of spore suspension placed equidistantly on the leaf. The spore suspensions were created by pipetting 1 mL of sterile distilled water onto the agar plate, scraping the surface to dislodge spores, and pipetting the spore suspension onto the leaves. Two fungal isolates were selected for the pilot trial, *Aspergillus sojae* Sakaguchi & K. Yamada ex Murak. and *Penicillium rubens* Biourge. Four pieces of sterile circular tissue paper were placed in each of eight 90 cm Petri plates (two fungal isolates with one replicate per inoculation method). Five mL sterile distilled water was used to wet the tissue paper in each Petri plate. Marama bean leaves were surface disinfected using 1% sodium hypochlorite (NaOCl) solution for 1 min, rinsed three times with sterile distilled water, and placed on sterile tissue paper to air dry for a few minutes. The fungal isolates were used to inoculate the leaves using the respective inoculation method. Each plate was sealed with Parafilm® to maintain moisture levels in the plates. Petri plates were then incubated for 14 days at ±25°C with 12-hour light/dark cycles, using cool white light. Observations of the progress of each plate were made daily from the third day after inoculation until day 14. The results were recorded in terms of the number of plates per fungal isolate presenting disease symptoms, such as areas of necrosis or severe discolouration. Three agar plugs placed equidistantly on the leaf presented the best results in terms of visible disease symptoms, thus this inoculation method was used for future DLA inoculations.

Kimwipes™ tissue paper was cut to the size of a plastic 90 mm Petri plate, wrapped in aluminium foil and autoclaved at 121°C for 15 min to sterilize the tissue paper. Four pieces of circular tissue paper were placed in each of 201 Petri plates (69 fungal isolates with three replicates). Five mL sterile distilled water was used to wet the tissue paper in each Petri plate. Marama bean leaves were surface disinfected using 1% NaOCl solution for 1 min, rinsed three times with sterile distilled water, and placed on sterile tissue paper in a biosafety cabinet to air dry for a few minutes. Due to the size of the leaves used for the assay, the petiole was removed from each bilobed leaf and the leaves were separated into two halves. Each half was used for a separate fungal isolate. Each half was placed in the centre of a Petri plate. From each fungal isolate of interest, three 5 x 5 mm agar plugs were removed from the one-week old PDA (potato dextrose agar) culture and placed upside down and equidistantly spaced on the leaf surface to allow direct contact between the fungus and the leaf. Each plate was sealed with Parafilm® to maintain moisture levels in the plates. Petri plates were then incubated for 14 days at

±25°C with 12-hour light/dark cycles, using cool white light. Observations of the progress of each plate were made daily from the third day after inoculation until day 14. The results were recorded in terms of the number of plates per fungal isolate presenting disease symptoms, such as areas of necrosis or severe discolouration. Twenty of the fungal isolates presenting the most severe symptoms or disease development on the leaves after 14 days were selected for further analysis in the pathogenicity trial.

4.1.2 Soil trials for optimal growth condition determination

Prior to the pathogenicity trial, ideal growth conditions for marama bean needed to be determined, including the optimal location, pot size, soil combinations and respective ratios, and seed scarification method. Four separate soil trials, as well as a fifth soil drainage trial were conducted in different locations between October 2020 and November 2020 – Trial 1 in a greenhouse at the Agricultural Research Council (ARC) Roodeplaat campus; Trials 2 and 3 at the Cycad and indigenous plant nursery and shade cloth tunnel, respectively, both at Innovation Africa @UP, Hillcrest campus; and Trials 4 and 5 in Pretoria East. The respective soil combinations and ratios are indicated in Table 4.2 according to the different locations in which the trials were conducted.

4.1.2.1 Trials 1 to 4 – Soil components

Ten different soil components were used in various combinations and ratios. Trial 1 consisted of filtered sand (0.6 – 1.5 mm diameter grains), loam soil, and Garden Master Organic compost purchased from Builders Warehouse, Pretoria; Hygromix growing medium (Hygrovent) purchased from Hygrotech, Pretoria; and Mondoval Vermiculite innovation purchased from Mondoval, Alberton. The components used in Trials 2 to 4 included unfiltered and unwashed river sand purchased from C&M Landscaping Supplies, Pretoria; clay topsoil collected from Hillcrest campus, University of Pretoria; Malanseuns compost purchased from Plantland Atterbury, Pretoria; perlite purchased from Leroy Merlin, Johannesburg; and loam topsoil purchased from street vendors in Johannesburg.

4.1.2.2 Trials 1 to 4 – Site specific climatic conditions

Trial 1 was conducted at a controlled temperature of 32°C in a greenhouse. Trial 2 was conducted in an enclosed area covered by 60% black shade cloth, while Trial 3 was conducted in a 30% white shade cloth tunnel located in an open area. Due to the location of Trial 2, the trial received roughly four to six hours of sunlight daily, whereas Trial 3 received full day sunlight due to the open area. The temperature, rainfall, and air movement could not be controlled in either trial. Trial 4 was conducted in a fairly enclosed area without shade cloth, where the pots received about four to six hours of sunlight in the afternoons after midday. The temperature, rainfall and air movement could not be controlled.

Table 4.2. Four separate marama bean soil trials were conducted at different locations using different soil combinations and ratios. All seeds were scarified in the same manner using a wood file, with the exception of Trial 3 (Hillcrest campus) that were scarified with secateurs.

		Soil combination				
		1	2	3	4	5
Trial 1: ARC Roodeplaat campus	Soil combination	Sand : Compost	Sand : Compost : Vermiculite	Loam : Sand : Compost	Loam : Sand : Compost : Vermiculite	Loam : Sand : Compost : Hygromix
	Ratio	3:1	4:4:1	2:2:1	2:2:1:1	2:2:1:1
Trial 2: Cycad nursery, Innovation Africa @UP, Hillcrest campus	Soil combination	River sand : Clay topsoil	River sand : Clay topsoil : Malanseuns compost	River sand : Clay topsoil *		
	Ratio	7:5	3:6:1	7:5		
Trial 3: Shade cloth tunnel, Innovation Africa @UP, Hillcrest campus	Soil combination	River sand : Clay topsoil	River sand : Clay topsoil : Malanseuns compost	River sand : Clay topsoil *		
	Ratio	7:5	3:6:1	7:5		
Trial 4: Pretoria East	Soil combination	River sand : Loam topsoil	River sand : Perlite : Malanseuns compost	River sand : Perlite	River sand only	
	Ratio	1:1	4:4:1	1:1	1	

* Soil combination 3 consisted of the same soil combination and ratios as combination 1 in Trials 2 and 3, only the seed preparation method differed.

4.1.2.3 Trials 1 to 4 – Trial set-up

Trial 1 consisted of five soil combinations, each combination with five pots (15 cm) per combination and one seed per pot; a total of 25 seeds. Trials 2 and 3 each consisted of three soil combinations, each with one pot of each size (12.5 cm, 15 cm, and 20 cm), and one seed per pot; a total of nine seeds in each location's trial. Trial 4 consisted of four soil combinations, each trial with one of each pot sizes (12.5 cm, 15 cm, 20 cm). One seed was planted in each of the 12.5 and 15 cm pots (nine seeds), while three seeds were planted in each 20 cm pot (nine seeds); a total of eighteen seeds.

Tylosema esculentum ((Burch.) A. Schreib.) seeds previously collected from the Centurion, Gauteng population (Sample 3 collected by Lizo Masters, 2018, Chapter 3 section 3.2.1) were prepared for each trial by scarifying the seeds and creating a hole to allow water to pass through the seed coat. Scarification was achieved by one of two methods – using a wood file to create a groove and hole in the seed coat, or using secateurs to manually remove a small portion of seed coat, creating a hole for the water to enter. In both cases, care needed to be taken to not damage the cotyledons. Wood file scarified seeds were used in Trial 1, and Trial 2 and 3 soil combinations 1 and 2. Secateur scarified seeds were used in Trial 2 and 3 soil combination 3, and Trial 4. After scarifying the seeds, the seeds were soaked in just boiled water overnight, allowing the seeds to swell to nearly double their size.

A volume of 600 mL, 1 L and 3 L of each soil combination was placed in each of the 12.5, 15 and 20 cm pots, respectively. The seeds were planted in each pot at a depth of 2 cm and covered with soil. The pots were watered until the water flowed freely through the drainage holes in the base of the pot. The pots of Trial 1 were placed on benches within the greenhouse with drips trays, while all trial pots in Trials 2 to 4 were placed on the ground without drip trays. The pots were watered once a week unless rain occurred. Results of Trials 1 and 4 were recorded over a four-week period after planting, while those of Trials 2 and 3 were recorded over a three-week period. Observations were recorded in terms of the climatic conditions of each selected area (temperature, rainfall, amount of sunlight the plants received); the number of seedlings that emerged in each soil trial; and the overall growth of the seedlings – the time taken to emerge, rate at which the seedlings grow and the overall health of the seedlings.

4.1.2.4 Trial 5 – Soil drainage trial

A drainage trial was conducted simultaneous to the soil trials (4.2.3.1-3), in the same location in Pretoria East as Trial 4. The soil trials were conducted to determine which soil components would likely be best for propagating and growing the marama beans, while the drainage trial confirmed which soil combinations had the highest drainage rate.

Eleven soil combinations consisting of different soil components were used to determine the water drainage capability of the different soil combinations. The same components used in Trials 2 to 4 were used for the drainage trials – unwashed river sand, perlite, clay topsoil, Malanseuns compost, and loam topsoil. The different soil compositions were as follows: perlite only; river sand only; clay topsoil only; perlite to compost ratio 3:1; perlite to loam topsoil ratio 1:1; river sand to perlite ratio 1:1; clay topsoil to perlite ratio 1:1; river sand to perlite to compost ratio 4:4:1; river sand to perlite to compost ratio 8:8:1; clay topsoil to river sand ratio 1.5:2.5; and clay topsoil to river sand to perlite ratio 2:3:5.

Three litres of each soil combination was placed in 20 cm terracotta plastic pots and soaked with water until water ran freely through the drainage holes in the base of the pot. The pots were moved into an enclosed and covered area where the shade and moisture levels for the trial could be controlled. The area was covered to prevent accidental watering or rainfall affecting the pots' moisture levels; and the area was constantly shaded to prevent uneven sunlight and heating of the pots. The temperature could not be controlled and varied daily between 23°C and 29°C. On the first day of the trial, the pH value for each soil combination was recorded using a standard pH meter (Kirchhoffs, South Africa). Daily soil moisture readings were recorded for a 7-d period using a soil moisture meter (Kirchhoffs, South Africa). The readings on the meter ranged from 0 (very dry) to 10 (very wet).

4.1.3 Pathogenicity trial

Based on the results obtained through the aforementioned soil and drainage trials, the pathogenicity trial was completed in the shade cloth tunnel at the Innovation Africa @UP on Hillcrest campus (Image 4, pg 122) (section 4.2.3 Trial 4). The seedlings were grown in summer over a three-month period from mid-December 2020 to mid-March 2021after planting. The pathogenicity trial was performed over a ten-week period, from mid-March 2021 to the end of May 2021. Due to the nature of the marama bean to die back for winter, both pathogenicity Trials 1 and 2 were terminated prematurely after a six-week period.

The pathogenicity trial consisted of two trials temporally separated by seven days. Twenty fungal isolates were used with three replicates per isolate for inoculations. Two sets of three replicate negative control plants were used, with one set used per trial. The negative control plants were not inoculated with any fungal isolate. A total of 126 seedlings were used for the pathogenicity trial.

4.1.3.1 Soil preparation

Based on the soil experiments, the soil combination selected for the pathogenicity trial was river sand to perlite to compost in an 8:8:1 ratio. The soil combination was selected based on the high drainage ability of the soil, the number of seeds that emerged when compared to the other soil combinations, and the presence of compost as natural nutrients (Table 4.5 and Figure 4.2). Each component of the soil combination was sterilized separately before mixing into the required soil combination ratio. To achieve sterilization, each soil component was placed into a sterilizer bin and steam sterilized at 91°C for 5 min. The compost component was sterilized at 91°C for 1 min to prevent potential development of toxicity caused by the high temperature. The components were removed from the sterilizer bin and placed on top of clean bags overnight to air out. The components were then placed in a bin in the desired ratio and mixed thoroughly. To accommodate 126 seedlings, 140 pots were used for planting seeds. Three litres of soil combination was placed in each of the 20 cm terracotta-coloured plastic pots.

4.1.3.2 Seed preparation

A total of 240 marama bean seeds were prepared by scarifying the seed coat using secateurs and soaking the seeds in just boiled water overnight to allow the seeds to swell. The following day, one to two seeds were randomly planted in each pot at a depth of 2 cm and covered with soil. Due to the low germination rate of the marama bean seeds observed in the soil and drainage trials (Table 4.5 and Figure 4.2), additional seeds were prepared and planted to ensure the required number of seedlings were obtained for the pathogenicity trial. The pots were watered until the water flowed freely through the drainage holes in the base of the pot.

4.1.3.3 Germination

The duration of the germination and seedling growth was three months. During this time, the pots were monitored every second day for germination. The pots were watered once a week unless rainfall occurred, which was monitored closely to prevent over watering. No additional soil or fertilizers were added to the pots. Due to the low germination rate, two seeds were planted in certain pots. In the pots where both seedlings had emerged, one of the seedlings was selected at random and removed from the pot so that each pot had only a single seedling. The seeds that had not emerged or germinated were removed from each of the pots. These seeds were observed to have signs of fungal infection with extensive fungal growth on the seed coats, and were selected to be processed for multi-mycotoxin analysis (section 4.2.5).

4.1.3.4 Inoculation

a. Fungal concentration

Multiple pure cultures of each selected fungal isolate were obtained on full strength (100%) potato dextrose agar (PDA), quarter strength (25%) PDA, and full strength potato carrot agar (PCA). A 200 mL spore suspension was made for each fungus. Approximately 5 drops of Tween were added to 1 L of distilled water before sterilizing in the autoclave at 121°C for 20 min. Sufficient sterile Tween water was obtained to make all twenty 200 mL spore suspensions. Fresh sterile Tween water was obtained for the second trial to prepare fresh spore suspensions. For each suspension, 200 mL sterile Tween water was placed in a sterile beaker. A sterile cotton bud was dipped in the Tween water and used to gently rub the surface of the fungal plate, collecting spores on the cotton bud. The cotton bud was dipped back into the Tween water and swirled around to release the spores, before repeating the process to collect more spores. The concentration of each spore suspension was determined using a haemocytometer (Neubauer-improved counting chamber, Marienfeld Laboratory Glassware, Germany) whereby spores were placed on the counting chamber of the haemocytometer. The concentration was determined using:

$$\frac{number\ of\ spores}{number\ of\ blocks\ in\ grid}\ x\ 4x10^6\ spores/mL$$

The target concentration of each fungus was 5 x10^5 spores/mL. If the concentration was too low, more spores were added to the suspension to achieve the desired concentration. If the concentration was too high, the equation $C_1V_1 = C_2V_2$ was used to determine the dilution ratio for each fungal isolate to obtain a concentration of 5 x10^5 spores/mL. Once the correct concentration was obtained, the spore suspension was transferred from the beaker into a 500 mL plastic spray bottle. This was repeated for each fungus, making the spore suspensions fresh before spraying the plants.

b. Inoculation

The three-month old seedlings were inoculated over two days, with seven days between the inoculation days of trial 1 and 2. The fungal suspension was used as a foliar spray to inoculate the respective seedlings. Each seedling was sprayed until there was a visible run-off from the leaves, and each pot was covered in a clear plastic butcher bag for two days to increase humidity and ensure fungal infection by preventing disturbances by the wind or rain that could hamper infection. After two days, the bags were removed. Seedlings within the control treatment were not inoculated with fungal suspension.

c. Observation of the pathogenicity trial

The intended duration of each of the two pathogenicity trials was ten weeks, with Trial 1 being inoculated one week before Trial 2. However, due to the nature of the marama bean to die back for winter, the pathogenicity trial was terminated prematurely after six weeks, resulting in the duration of Trial 1 being six weeks while Trial 2 was five weeks. The seedlings were watered once a week unless rainfall occurred. For the duration of the trial, any disease symptoms present on the seedling leaves and stems were recorded weekly. A disease rating system was established to record the symptom severity on each of the seedlings for each of the fungal treatments (Table 4.3). Once the trials had been terminated, the seedlings were harvested and processed for above and below ground wet and dry mass measurements and a single leaf was removed from each seedling for Koch’s postulates. Per seedling, the above ground parts (stem and leaves) were separated from the below ground parts (tubers and roots), and wet mass weight measurements for each were recorded. The harvested plant material was then placed in a drying oven for three weeks to ensure that the tubers had dried completely. The above and below ground dry mass measurements were then recorded.

Table 4.3. The disease rating score system for recording disease severity on marama bean plants inoculated with selected fungal isolates.

Symptom	Symptom severity	Score	Total score achievable
No symptoms	No symptoms	0	0 /20
Black leaf spots	A few spots on one leaf	1	4 /20
	A few spots on multiple leaves	2	
	Multiple spots on one leaf	3	
	Multiple spots on multiple leaves	4	
Leaf lesions	Small brown lesions <5 mm on one leaf	1	6 /20
	Small brown lesions <5 mm on multiple leaves	2	
	Medium brown lesions >5 mm on one leaf	3	
	Medium brown lesions >5 mm on multiple leaves	4	
	Large brown lesions >>5 mm on one leaf	5	
	Large brown lesions >>5 mm on multiple leaves	6	
Vein symptoms	Turning brown or lesioned	1	1 /20
Stem lesions	One small lesion <5 mm	1	4 /20
	One medium lesion >5 mm	2	
	One large lesion >>5 mm	3	
	Multiple lesions	4	
Discolouration	Leaves or stem displaying yellow discolouration	1	2 /20
	Leaves or stem displaying brown discolouration	2	
Wilting	One leaf appears to have wilted	1	2 /20
	Multiple leaves or stem appears to have wilted	2	
Total score achievable calculated from above symptom conditions			**19 /20**
Above ground parts	Above ground parts have wilted completely	20	**20 /20**

Due to the wide range of symptoms observed as a result of the various of fungal species used for inoculation, the point in the trial at which the seedlings developed symptoms and could be considered as infected was challenging to identify. As such, a calculative approach was used to determine at which point in the trial period the seedlings could be considered as infected and showing expression of symptoms. The respective mean and median of the Week 1 disease ratings were calculated and averaged for Trials 1, 2 and the combined results, respectively. The averaged mean and median score assumed the role of the minimum disease rating (MDR) threshold. The individual ratings of each fungal isolate for each week were subsequently compared to the MDR of Week 1, and ratings from each week that were below the MDR threshold were considered below the threshold and the symptoms considered ‘underdeveloped’. The MDR served as an indicator for the amount of time taken for seedlings to develop significant symptoms in each fungal treatment – the week in which the specific fungal treatment's rating surpassed the MDR for the trial, was considered as the week in which the specific fungal treatment started to develop considerable symptom severity.

d. Statistical analysis

Statistical analysis was performed on the pathogenicity trial disease rating and weight measurement data using one-way analysis of variation (ANOVA) and Fisher's least significant difference (LSD) post hoc test, where $P = 0.05$. Analysis was performed using the SAS® version 9.4 software program (SAS Institute Inc).

e. Koch's postulates

One leaf per seedling was removed from each of the inoculated and control seedlings from Trials 1 and 2. Half concentration PDA (50% PDA) amended with 10 mg per L chloramphenicol antibiotic to reduce bacterial contamination in 90 mm diameter Petri plates was used for leaf plating. The leaves were surface sterilized by soaking in 1% NaOCl solution for 1 min, rinsing three times with sterile distilled water and drying under sterile conditions on sterile paper towels placed on a biosafety cabinet bench for several minutes until dry. Ten leaf pieces (5 x 5 mm) were cut from each surface sterilized leaf and directly plated onto two agar plates, with five leaf pieces per plate. The plates were incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles, using cool white light, after which the leaves were examined for fungal growth. The fungal growth present on each agar plate was recorded and fungi that were identified as potentially identical to the fungus used to inoculate the pathogenicity trial, were isolated. Each identified fungal growth was isolated as a 3 x 3 mm square and transferred to a clean full concentration PDA plate. The plates were incubated for 5 to 7 days at ±25°C with 12-hour light/dark cycles, using cool white light, or until sporulation occurred.

The fungal isolates were morphologically identified based on culture morphology and using microscopy techniques in order to fulfil Koch's postulates. The culture morphology and sporulation patterns were observed using the Zeiss Axio Zoom stereo-microscope (Zeiss, Germany) and the sporulating elements were observed through microscope slide preparation with lactoglycerol mounting liquid and using the Zeiss Axio Imager compound microscope with differential interference contrast (DIC) (Zeiss, Germany).

4.1.4 Multi-mycotoxin analysis of pathogenicity trial seedlings

The 85 ungerminated seeds with visible fungal infection removed from the seedling pots (section 4.2.4.3) were separated into smaller portions to ensure all seeds dried entirely, and dried in a drying oven at 65°C for two weeks. The dried seeds were then ground together into a fine powder under sterile conditions using a laboratory blender that was thoroughly washed with detergent, sprayed with ethanol and dried between samples. Four 5 g samples were obtained and placed in labelled sealable plastic bags.

Several of the fungal isolates used for the pathogenicity trial are known mycotoxin producers, for example, *Alternaria alternata* (Fr.) Keissler, *Fusarium incarnatum-equiseti* species complex (SC), *F. fujikuroi* SC, *F. solani* SC, and *F. oxysporum* SC. Once the dry weight measurements were determined for all of the above and below ground plant material, the material was ground for mycotoxin analysis. For each fungal isolate, all six seedlings (combined Trial 1 and 2 above and below ground material) were ground to a fine powder under sterile conditions using a laboratory blender that was thoroughly washed with detergent, sprayed with ethanol and dried between samples. Five grams (5 g) of each sample was placed in labelled sealable plastic bags.

The processed samples were submitted to the Central Analytical Facility (CAF) at Stellenbosch University for multi-mycotoxin analysis to detect the natural presence of mycotoxins on the respective samples using ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) (see Chapter 3 section 2.6 for full UPLC-ESI-MS/MS method). Multi-mycotoxin analysis was used to detect the presence of aflatoxin B_1 (AFB_1), deoxynivalenol (DON), nivalenol (NIV), zearalenone (ZEA), ochratoxin (OTA), and fumonisin B_1, B_2 and B_3 (FB_1, FB_2, FB_3 respectively) mycotoxins.

4.2 Results

4.2.1 Detached leaf assay (DLA)

The fungal isolates categorized under the red, yellow, and white groups (Figure 4.1; Table 4.1) were selected to conduct detached leaf assays with the aim of determining which of the fungal isolates may be pathogenic to marama bean seedlings. A summary of the DLA results recorded over a 14-day period for the 20 fungal isolates selected for further analysis during the pathogenicity trial is presented in Table 4.2. Days 1 to 3 form part of the incubation period therefore results were not recorded. Several fungal species had repeat isolates due to the varying nature of their culture morphology or if they are known to be highly pathogenic.

A total of 69 isolates were selected for analysis during the DLA. The various disease symptoms and respective severities observed included small to large lesions at the site of fungal inoculation with the agar plug, discolouration and lesions of the veins extending from the site of inoculation to the petiole, discolouration of the leaf, small to large lesions spots over the leaf surface, and mycelial growth across the surface of the leaves (Appendix 6.6 Table 6.4). Table 6.4 of Appendix 6.6 provides the visual results of each fungal isolates' three replicate plates, as recorded on the final day of the DLA trial (Day 14). Of the 69 isolates, 60 isolates indicated disease symptoms on all three replicate plates; five isolates indicated disease symptoms on only two of the replicate plates, while four isolates indicated disease symptoms on only one replicate plate. Twenty nine (29) of the isolates indicating disease symptoms on all three replicate plates were in the red pathogen group, eight isolates in the yellow pathogen group, and 21 isolates in the white pathogen group. Three of the isolates that indicated symptoms on only two replicate plates were in the red groups, and two isolates were in the white group. Two of the isolates with symptoms only on one replicate plate were in the red group, and two isolates were in the white group. The 60 isolates indicating disease symptoms on all three replicate plates were narrowed down to twenty isolates (Table 4.4) according to the following factors: time and trial size constraints; prevalence of the pathogen on other crops; general severity of the diseases caused by the pathogen; and pre- or postharvest disease conditions.

By the end of Day 4 of the DLA, the first day of recording results after the three-day incubation period, *Actinomucor elegans* (Eidam) C.R. Benj. & Hesselt., *Aspergillus awamori* Nakaz., *A. japonicus* Saito, *Bipolaris zeae* Sivan., *Cladosporium halotolerans* Zalar, de Hoog & Gunde-Cim., *C. pseudocladosporioides* Bensch, Crous & U. Braun, *Diaporthe rhusicola* Crous, *Epicoccum sorghinum* (Sacc.) Aveskamp, Gruyter & Verkley, *Fusarium solani* isolate 1, *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous, *Penicillium brevicompactum* Dierckx, *P. cluniae* Quintan., *P. onobense* C. Ramírez & A.T. Martínez, *P. raperi* G. Sm., *P. rubens* Biourge isolate 2, and *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg already presented disease symptoms on all three replicate plates. One week after the day of inoculation, 41 of the 69 isolates presented symptoms on all three plates, seventeen of which were in the red group, six in the yellow group, and eighteen in the white group. After the full two-week trial period, 58 of the 69 isolates selected for the DLA trial indicated disease symptoms on all three of the replicate plates, five isolates with symptoms on two replicate plates, and four isolates with symptoms on only one replicate plate (Table 4.4 and Appendix 6.7 Table 6.5). Within the red group, *F. lacertarum* Subrahm. isolate 1, *F. sambucinum* Fuckel SC isolate 2, and *Neopestalotiopsis clavispora* (G.F. Atk.) Maharachch., K.D. Hyde & Crous presented symptoms on only two replicate plates, while *F. lacertarum* isolate 2 and *F. oxysporum* SC isolate 1 presented symptoms on only one replicate plate. Within the white group, *Talaromyces pinophilus* (Hedgc.) Samson, Yilmaz, Frisvad & Seifert and *Trichoderma harzianum* Rifai presented symptoms on two replicate plates, while *P. annulatum* Visagie & K. Jacobs and *P. momoi* Visagie & K. Jacobs presented symptoms on only one replicate plate (Table 4.4).

Table 4.4. A visual representation of 20 fungal isolates, indicating disease symptoms during the detached leaf assay (DLA) trial conducted over a two-week period. These isolates were selected for the pathogenicity trial.

		Day*										
	Fungal isolate	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Red group	*Alternaria alternata* isolate 1	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Alternaria alternata isolate 2	🟩	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Bipolaris sp.	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Bipolaris zeae	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Colletotrichum fructicola isolate 1	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥
	Colletotrichum fructicola isolate 2	🟩	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Diaporthe rhusicola	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC[1] isolate 1	⬜	⬜	🟩	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC isolate 2	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC isolate 3	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium incarnatum-equiseti SC	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium oxysporum SC isolate 2	🟩	🟩	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium oxysporum SC isolate 3	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium solani SC isolate 1	⬜	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium solani SC isolate 2	⬜	⬜	⬜	⬜	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Neofusicoccum parvum	⬜	⬜	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Pestalotiopsis maculans	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Yellow group	*Botryosphaeria dothidea*	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Didymella sp. isolate 3	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Lasiodiplodia pseudotheobromae	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥

*Colours were assigned according to the number of plates (total three) that indicated disease symptoms. No plates = grey; One plate = green; Two plates = yellow; Three plates = red.
[1]SC = species complex

4.2.2 Soil trials for optimal growth condition determination

4.2.2.1 Trials 1 to 4 – Soil trials

The locations of the different soil trials appeared to have little effect on the soil trials when compared to the different soil combinations themselves (Table 4.5). Throughout the duration of each soil trial, the climatic conditions and respective growth of the plants was observed. The greenhouse trial at the ARC Roodeplaat campus on average had a higher humidity level throughout the trial, with a controlled temperature of 32°C. The temperature ranges recorded for Hatfield, Pretoria, South Africa over period of the Trials 2 and 3 conducted on Hillcrest campus included a minimum temperature range of 12 to 26°C, and maximum temperature range of 19 to 36°C. The more open conditions of the Cycad nursery (Trial 2) and shade tunnel (Trial 3) promoted more air movement around the pots. Soil moisture readings recorded randomly throughout the three-week period generally presented lower moisture readings from the pots in Trial 3 (the shade tunnel) compared to the readings of those in Trial 2 (the Cycad nursery).

Table 4.5. Four soil trials were conducted over a three- to four-week period to investigate various conditions required for optimal marama bean seedling growth.

	Soil combination[b]		Ratio	Pot size (cm)	Scarification method	Total seeds planted	# Seedlings emerged per week			
							1	2	3	4
Trial 1	1	S : C	3:1	15	Wood file	5	0	0	1	1
	2	S : C : V	4:4:1	15	Wood file	5	0	1	2	2
	3	L : S : C	2:2:1	15	Wood file	5	0	0	0	0
	4	L : S : C : V	2:2:1:1	15	Wood file	5	0	0	1	2
	5	L : S : C : H	2:2:1:1	15	Wood file	5	0	0	1	1
Trial 2	1 – A[a]	R : CT	7:5	12.5	Wood file	1	0.5[c]	0[d]	0	-
	1 – B	R : CT	7:5	15	Wood file	1	0.5	0	0	-
	1 – C	R : CT	7:5	20	Wood file	1	0	0	0	-
	2 – A	R : CT : MC	3:6:1	12.5	Wood file	1	0.5	0	0	-
	2 – B	R : CT : MC	3:6:1	15	Wood file	1	0.5	1	1	-
	2 – C	R : CT : MC	3:6:1	20	Wood file	1	0.5	1	1	-
	3 – A	R : CT	7:5	12.5	Secateurs	1	0	0.5	1	-
	3 – B	R : CT	7:5	15	Secateurs	1	0.5	1	1	-
	3 – C	R : CT	7:5	20	Secateurs	1	0.5	0	0	-
Trial 3	1 – A	R : CT	7:5	12.5	Wood file	1	0.5	0.5	0	-
	1 – B	R : CT	7:5	15	Wood file	1	0.5	1	1	-
	1 – C	R : CT	7:5	20	Wood file	1	0.5	0.5	0	-
	2 – A	R : CT : MC	3:6:1	12.5	Wood file	1	0.5	0.5	0	-
	2 – B	R : CT : MC	3:6:1	15	Wood file	1	0	0	0	-
	2 – C	R : CT : MC	3:6:1	20	Wood file	1	0.5	1	1	-
	3 – A	R : CT	7:5	12.5	Secateurs	1	0	0	0	-
	3 – B	R : CT	7:5	15	Secateurs	1	0.5	0	0	-
	3 – C	R : CT	7:5	20	Secateurs	1	0.5	0.5	0	-
Trial 4	1 – A	R : LT	1:1	12.5	Secateurs	1	0	0	0	0
	1 – B	R : LT	1:1	15	Secateurs	1	0	0	0	0
	1 – C	R : LT	1:1	20	Secateurs	3	0	0	0	0
	2 – A	R : P : MC	4:4:1	12.5	Secateurs	1	1	1	1	1
	2 – B	R : P : MC	4:4:1	15	Secateurs	1	0	1	1	1
	2 – C	R : P : MC	4:4:1	20	Secateurs	3	1	2	2	2
	3 – A	R : P	1:1	12.5	Secateurs	1	1	1	1	1
	3 – B	R : P	1:1	15	Secateurs	1	1	1	1	1
	3 – C	R : P	1:1	20	Secateurs	3	2	3	3	3
	4 – A	R only	1	12.5	Secateurs	1	1	1	1	1
	4 – B	R only	1	15	Secateurs	1	1	1	1	1
	4 - C	R only	1	20	Secateurs	3	2	2	2	2

[a] Trials consisted of the soil type (numbers 1-4) and pot size in cm (letters A to C).

[b] Various soil combination components were used. S = sand (filtered); C = compost (Garden Master); L = loam; V = vermiculite; H = Hygromix (Hygrovent); R = river sand (unfiltered); CT = clay topsoil; LT = loam topsoil; MC = Malanseuns compost; P = perlite.

[c] Regarding number of shoots visible, 0.5 indicates the presence of a root without a shoot.

[d] Instances where the number of emerged seedlings decreased from 0,5 to 0 indicate the root had died off and failed to emerge as a seedling.

The minimum and maximum temperature ranges recorded for the location of Trial 4 were 11 to 25°C and 18 to 34°C, respectively. The location of Trial 4 (Pretoria East) seemed to have the greatest effect on the trial's success, however the specific location and space availability made the location unsuitable for the pathogenicity trial, and the specific soil combinations used may have had a greater effect on trial success compared to the specific location and respective climatic conditions. Based on the previously mentioned climatic conditions (section 4.2.3.2) and lower average soil moisture readings observed, the location of Trial 3 – the shade tunnel, Innovation Africa @UP on Hillcrest campus – was selected for the pathogenicity trial.

When comparing the seedling emergence success rate according to the scarification method, little to no difference in the success of the wood file scarified and secateur scarified seedling emergence was observed (Table 4.5). Only 27% of the seeds scarified by the wood file emerged, while 54% of the seeds scarified by secateurs emerged. Statistical analysis using a two-tailed t-test with Welch's correction (equal variances are not assumed, $\alpha = 0.05$) indicated a non-significant difference between the means of wood file vs secateur scarified seed germination (P value = 0.5948). However, the F test performed simultaneously indicated that the variances of the wood file and secateur scarified germination means were significantly different (P value = 0.0367). The overall conclusion, based on the F-test analysis, indicated that the wood file and secateur methods of seed scarification were significantly different. It was observed throughout the preparations of the different soil trials and the pathogenicity trial, that the scarified seeds that were successful in creating a hole in the seed coat absorbed a large amount of water allowing the seeds to swell to nearly double their original seed size.

When comparing the effect of pot sizes (cm) on seedling emergence, focus was placed on Trials 2 to 4. Four out of a possible ten seedlings emerged in the 12.5 cm pots; six out of ten seedlings emerged from the 15 cm pots; and nine out of eighteen seedlings emerged in the 20 cm pots (Table 4.5). At the conclusion of the four soil trials, several of the seedlings from the various soil Trial 2, 3, and 4 pots were removed at random from each pot size and soil combination type for observation. The seedlings from the 12.5 and 15 cm pots tended to have development larger and more prominent above ground components (stems and leaves) compared to the 20 cm pot seedlings. However, the opposite was true for the below ground material (roots and tubers). The 20 cm pot seedlings developed larger cylindrical tubers with long tap roots that extended to the base of the pot, often curling around the base of the pot (Image 2, pg 26), whereas the 12.5 and 15 cm pots developed shorter tap roots that tended to stop developing at the base of the pots, with short, round tubers and short semi-cylindrical tubers, respectively. Tuber and root development were also affected by the soil combination, observed by thinner and longer tubers in the compact clay topsoil containing combinations compared to the fatter tubers of the looser soil combinations. Based on the success rate and greater volume of space available, as well as the observed differences in tuber and root development, the 20 cm pots were selected for the pathogenicity trial. It should be noted, however, that the effect of the scarification method, pot size, and trial location variables may be attributed to the specific soil combinations rather than the variables themselves, thus these results were used as a guideline for this specific pathogenicity trial rather than definite propagation methods.

As seen in Table 4.5, Trial 2 was successful in terms of seedling emergence in the R : CT : MC (3:6:1 soil ratio; wood file scarification) 15 and 20 cm pots, as well as the R : CT (7:5; secateurs) 12.5 and 15 cm pots. Trial 3's R : CT (7:5; wood file) 15 cm pot and R : CT : MC (3:6:1; wood file) 20 cm pot were successful in seedling emergence. Trial 4 had successful seedling emergence in the R: P: MC (4:4:1; secateurs) 12.5, 15 and 20 cm pots, in the R : P (1:1; secateurs) 12.5, 15 and 20 cm pots, as well as the R only (1; secateurs) 12.5, 15 and 20 cm pots. No seedlings emerged in the Trial 4 soil combinations containing loam topsoil. Trial 1 was fairly unsuccessful when considering the number of seedlings that emerged compared to the number of seedlings planted in each soil combination (maximum two seedlings emerged when five seeds were planted for each soil combination), and as such the soil combinations from Trial 1 were not considered further. Although Trials 2 and 3 with the clay topsoil were fairly successful in terms of seedling emergence, the emergence rate of 33% in the soil combinations containing clay topsoil was very low compared to the 87% seedling emergence rate of the soil combinations in Trial 4 containing either river sand, perlite, and/or compost. The three soil combinations from Trial 4 – R: P: MC (4:4:1), R: P (1:1) and R only (1) – were identified as the most suitable soil combinations.

4.2.2.2 Trial 5 – Soil drainage trial

Simultaneously to the four soil trials conducted (Table 4.5), Trial 5 was conducted to determine the soil drainage abilities of each soil combination, and identify which combination would be optimal for the pathogenicity trial. A soil combination with a high drainage rate would be preferable for plants that establish and thrive in dry, drought-like conditions (Travlos et al., 2007a; Travlos & Karamanos, 2006). In line with the drainage ability of the various soil combinations, the optimal pH of the different soil combinations needed to be determined for optimal seedling growth. Jackson et al. (2010) stated that marama beans grow best in sandy loamy soils with a pH around 7, as found in the Namibian *T. esculentum* population locations. Figure 4.2 presents the results of the soil drainage trial conducted. The drainage rate is inversely proportional to the soil moisture. A high soil moisture meter reading indicates high soil moisture content and therefore a low drainage rate. Whereas a low soil moisture meter reading indicates a high drainage rate and a low soil moisture content.

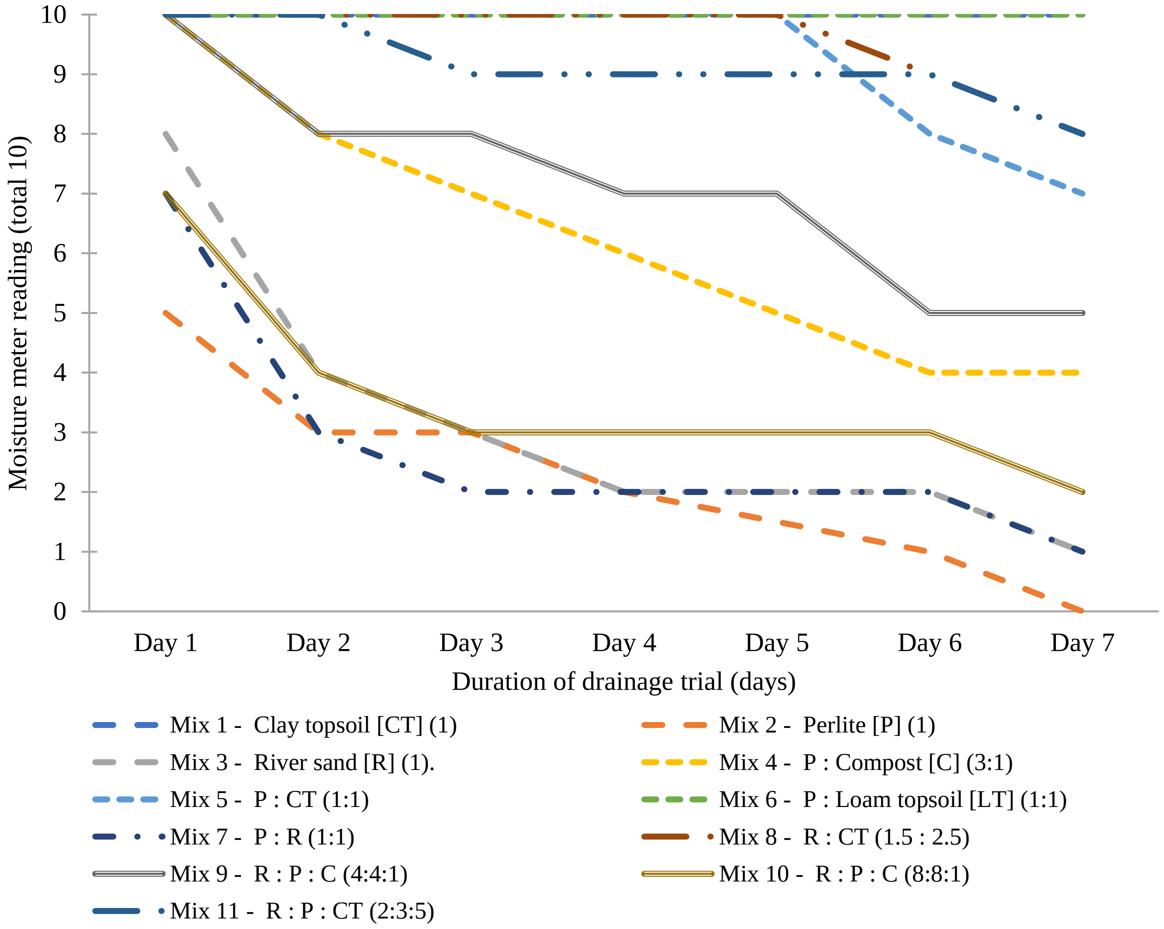

Figure 4.2. A drainage trial was performed to determine the optimal soil composition for marama bean propagation, consisting of various soil components and ratios. The drainage rate was determined by obtaining soil moisture readings daily for 7 days using a soil moisture meter.

The pH values of each soil combination were measured on the first day of the trial: Mix 1 (clay topsoil) measured pH 5.5. Mix 5 (perlite: clay topsoil), 6 (perlite: loam topsoil) and 11 (river sand: perlite: clay topsoil) measured pH 6. Mix 8 (river sand: clay topsoil) measured pH 6.5. Mix 3 (river sand), 4 (perlite: compost), 7 (perlite: river sand), 9 (perlite: river sand: compost 4:4:1) and 10 (perlite: river sand: compost 8:8:1) measured pH 7, which has been reported as the optimal soil pH for marama beans. Mix 2 (perlite) measured pH 8.

Mix 1, 5, 6, 8, and 11 including either the clay or loam topsoil indicated the lowest drainage rates, as seen by the higher moisture meter readings between seven and ten on day 7 of the trial, one week after being watered (Figure 4.2). The presence of clay or loam topsoil in each of these soil combinations likely contributed to the lower pH readings recorded. Mix 3 (R – 1), 7 (R:P – 1:1), and 9 (R:P:MC – 4:4:1) were identified during the four soil trials as the most promising soil combinations. Based on the results from Trials 1 to 4, and the preference of marama beans to grow in sandy soils (Coetzer et al., 2011; National Research Council, 2006), an additional soil combination of R:P:C in a ratio of 8:8:1 (Mix 10) was added to Trial 5. Three of the combinations (Mix 3, 7, and 10) indicated a similar drainage pattern over the seven-day trial, each with a rapid decrease in soil moisture over the first two days, followed by a plateau-like period and further reduction of soil moisture over the last two days of the trial. In comparison, Mix 9 indicated a more staggered pattern of alternating reduction and plateau periods regarding the soil moisture levels. Mix 2, 3, 7 and 10 indicated the lowest soil moisture meter readings and subsequently the highest drainage rates on day 7 (Figure 4.2), measuring 0, 1, 1, and 2, respectively. Based on the high drainage rate due to the river sand and perlite portions, the neutral pH of 7 that concurs with Jackson et al. (2010) and the presence of compost for nutrients, Mix 10 was selected as the soil combination to be used during the marama bean seedling pathogenicity trial.

4.2.3 Pathogenicity trial

4.2.3.1 Disease rating and weight measurements

In order to temporally separate the two trials, Trial 2 was inoculated one week after Trial 1. Due to the wide variety of fungal species and isolates used during the pathogenicity trial, there was a subsequent wide variety of symptoms observed throughout the trial (Figure 4.3). A disease rating score system was established (Table 4.3), and the various symptoms per fungal treatment for both Trials 1 and 2 were recorded. The total number of seedlings per fungal treatment displaying each symptom was recorded at the conclusion of the trials 1 and 2, with a maximum value of three per symptom and treatment, as there were three seedlings per treatment (Tables 4.6 and 4.7). The total number of seedlings displaying a particular symptom (symptom observation) was calculated, and a percentage of symptom observation was calculated using the overall total number of seedlings displaying all of the symptoms.

The most prevalent symptom at the conclusion of pathogenicity trial 1 (Table 4.6) was black leaf spots with a 24.19% prevalence, followed by stem lesions with 22.33% prevalence, leaf lesions with 18.60% prevalence, and discolouration of the leaves with 14.88% prevalence. Trial 2 (Table 4.7) displayed similar symptom prevalence trends, with leaf spots being most prevalent at 23.71%, followed by leaf lesions at 20.26%, stem lesions at 17.67%, and leaf discolouration at 15.95%. In both trials, complete wilting of the above ground parts and no symptom development were the least prevalent symptoms observed, with above ground wilting at 3.72% and 3.02% for trials 1 and 2, respectively; and no symptoms at 0.47% and 0.00% for trials 1 and 2, respectively.

An overall disease rating was calculated for each individual seedling within each fungal treatment and the average disease ratings per fungal treatment (Appendix 6.8, Tables 6.6 and 6.7), as well as the average disease rating scores per fungal treatment for pathogenicity Trials 1 and 2 (Tables 4.8 and 4.9, respectively).

Figure 4.3. Visual representation of the various symptoms observed throughout the pathogenicity trial. Left to right, top to bottom: Black leaf spots; Multiple small brown leaf lesions; Medium brown leaf lesion; Large brown leaf lesion; Vein lesions; Vein discolouration and lesions; Small stem lesion; Large stem lesion; Leaf yellow discolouration; Leaf yellow/ brown discolouration; Seedling above ground parts lesioned and wilted; Seedling above ground parts wilted and dried.

Table 4.10 provides the overall summarized average disease rating scores for Trials 1 and 2, as well as for duplicate fungal treatments per species recorded over the five-week period in which both trials had data for all fungal treatments. According to the disease rating score system, the symptoms observed on the marama bean seedlings in each fungal treatment could be rated with a minimum rating of 0 indicating no disease symptoms were observed, and the maximum rating of 20 indicating that the entire plant above ground had wilted. The various symptoms observed on each seedling were rated according to the symptom severity scale (Table 4.3) in order to receive a possible total rating of 19 /20, unless the above ground parts wilted completely, earning a rating of 20 /20. After the six-week pathogenicity trial period, the disease rating scores for Trial 1 (Table 4.8) ranged from 3.17 (negative control) to 20.00 (*F. oxysporum* SC), while the disease rating scores for Trial 2 (Table 4.9) ranged from 3.17 (negative control) to 14.67 (*Bipolaris* sp. Shoemaker) after the five-week trial period.

Table 4.6. The number of seedlings per fungal species treatment (maximum 3 seedlings per treatment) that displayed various symptoms for Trial 1, the total number of seedlings displaying each of the identified symptoms, and the percentage of seedlings indicating each of the identified symptoms.

Treatment	**Symptoms observed**							
	No symptoms	**Black leaf spots**	**Leaf lesions**	**Vein lesions**	**Stem lesions**	**Discolouration of leaves**	**Wilting of leaves**	**Above ground parts wilted**
Negative control	1	2	0	0	0	0	0	0
*Alternaria alternata*_1	0	3	2	1	3	1	0	0
*Alternaria alternata*_2	0	3	2	2	3	1	2	0
Bipolaris sp.	0	3	2	0	3	1	1	0
Bipolaris zeae	0	3	3	1	3	2	1	0
Botryosphaeria dothidea	0	2	2	0	2	1	0	1
*Colletotrichum fructicola*_1	0	1	1	0	0	2	1	1
*Colletotrichum fructicola*_2	0	3	3	1	3	1	2	0
Diaporthe rhusicola	0	3	2	0	3	2	2	0
Didymella sp.	0	3	3	1	3	3	0	0
Fusarium fujikuroi SC_1	0	3	2	2	3	2	0	0
Fusarium fujikuroi SC_2	0	3	0	0	3	2	1	0
Fusarium fujikuroi SC_3	0	3	3	1	3	2	1	0
Fusarium incarnatum-equiseti SC	0	3	2	1	3	3	2	0
Fusarium oxysporum SC_1	0	0	0	0	0	0	0	3
Fusarium oxysporum SC_2	0	2	1	1	2	2	1	1
Fusarium solani SC_1	0	3	3	0	3	2	2	0
Fusarium solani SC_2	0	3	2	1	3	2	2	0
Lasiodiplodia pseudotheobromae	0	2	2	2	2	1	0	1
Neofusicoccum parvum	0	2	2	2	2	0	0	1
Pestalotiopsis maculans	0	2	3	0	1	2	0	0
Total (overall total of 215 counts)	1	52	40	16	48	32	18	8
Percentage (%)	0.47	24.19	18.60	7.44	22.33	14.88	8.37	3.72

Table 4.7. The number of seedlings per fungal species treatment (maximum 3 seedlings per treatment) that displayed various symptoms for Trial 2, the total number of seedlings displaying each of the identified symptoms, and the percentage of seedlings indicating each of the identified symptoms.

Treatment	**Symptoms observed**							
	No symptoms	**Black leaf spots**	**Leaf lesions**	**Vein lesions**	**Stem lesions**	**Discolouration of leaves**	**Wilting of leaves**	**Above ground parts wilted**
Negative control	0	3	1	0	3	2	0	0
*Alternaria alternata*_1	0	3	3	2	2	2	2	0
*Alternaria alternata*_2	0	2	2	1	2	2	1	1
Bipolaris sp.	0	2	2	1	2	1	1	1
Bipolaris zeae	0	3	3	2	2	2	1	0
Botryosphaeria dothidea	0	2	2	0	1	2	0	1
*Colletotrichum fructicola*_1	0	2	3	1	1	2	2	0
*Colletotrichum fructicola*_2	0	3	3	1	2	3	1	0
Diaporthe rhusicola	0	3	3	0	3	2	2	0
Didymella sp.	0	3	3	1	3	3	0	0
Fusarium fujikuroi SC_1	0	2	0	2	2	0	2	1
Fusarium fujikuroi SC_2	0	3	2	0	2	2	1	0
Fusarium fujikuroi SC_3	0	3	3	2	2	0	3	0
Fusarium incarnatum-equiseti SC	0	2	2	0	1	0	1	1
Fusarium oxysporum SC_1	0	3	3	2	2	2	0	0
Fusarium oxysporum SC_2	0	3	1	2	3	3	1	0
Fusarium solani SC_1	0	3	2	0	2	3	2	0
Fusarium solani SC_2	0	3	3	1	3	3	1	0
Lasiodiplodia pseudotheobromae	0	2	2	0	2	2	1	1
Neofusicoccum parvum	0	2	1	2	1	1	2	1
Pestalotiopsis maculans	0	3	3	1	0	0	0	0
Total (overall total of 232 counts)	0	55	47	21	41	37	24	7
Percentage (%)	0.00	23.71	20.26	9.05	17.67	15.95	10.34	3.02

Table 4.8. The average disease rating scores (maximum 20) for marama bean pathogenicity Trial 1.

Treatment	**Isolate**	**Week**						**LSD $^{(v-z)}$**	**CV (%)**
		1	**2**	**3**	**4**	**5**	**6**		
Negative control		0.50[a] de, y	0.84 d, y	0.84 e, y	1.50 f, xy	2.34 e, wx	3.17 e, w	4.555	55.526
Alternaria alternata	1[b]	3.67 abcd, w	3.67 abcd, w	4.00 bcde, w	4.00 cdef, w	5.67 cde, w	6.67 cde, w	5.271	49.379
Alternaria alternata	2	3.00 abcde, x	4.00 abcd, wx	5.33 abcd, wx	7.00 abc, wx	7.67 abcde, wx	9.00 bcd, w	9.225	62.225
Bipolaris sp.	1	4.00 abcd, y	4.33 abcd, xy	5.33 abcd, wxy	6.67 abcd, wx	7.00 abcde, w	7.67 bcd, w	2.445	23.561
Bipolaris zeae	1	3.67 abcd, z	4.67 abc, yz	5.33 abcd, xyz	6.67 abcd, wxy	7.33 abcde, wx	8.00 bcd, w	2.481	23.458
Botryosphaeria dothidea	1	3.00 abcde, x	4.67 abc, wx	7.33 ab, wx	8.33 a, wx	13.00 a, w	13.67 ab, w	1.326	48.787
Colletotrichum fructicola	1	0.00 e, w	1.00 d, w	2.67 cde, w	4.00 cdef, w	9.00 abcde, w	9.00 bcd, w	10.558	138.736
Colletotrichum fructicola	2	4.00 abcd, x	4.67 abc, x	6.33 abc, wx	7.33 abc, wx	8.67 abcde, w	9.67 bcd, w	3.607	29.915
Diaporthe rhusicola	1	2.00 cde, y	4.67 abc, xy	5.00 abcde, xy	6.67 abcd, wx	9.00 abcde, w	9.33 bcd, w	3.911	35.975
Didymella sp.	1	5.67 a, y	5.67 ab, y	7.00 ab, xy	7.33 abc, xy	8.67 abcde, wx	10.67 bcd, w	2.258	16.924
Fusarium fujikuroi SC	1	5.00 abc, z	5.67 ab, yz	7.00 ab, xy	8.00 ab, wx	8.67 abcde, vw	9.67 bcd, v	1.453	11.134
Fusarium fujikuroi SC	2	5.00 abc, y	5.67 ab, xy	5.67 abc, xy	6.33 abcde, x	8.33 abcde, w	8.67 bcd, w	0.938	7.972
Fusarium fujikuroi SC	3	1.00 de, x	1.33 cd, wx	1.67 de, wx	2.67 ef, wx	4.00 de, wx	5.33 de, w	4.065	85.696
Fusarium incarnatum-equiseti SC	1	2.33 bcde, y	2.33 bcd, y	3.00 cde, y	4.33 bcdef, xy	6.67 bcde, wx	7.67 bcd, w	2.481	31.772
Fusarium oxysporum SC	1	3.00 abcde, z	3.67 abcd, yz	5.67 abc, y	8.67 a, x	10.67 abc, x	20.00 a, w	2.219	14.484
Fusarium oxysporum SC	2	3.33 abcd, x	4.33 abcd, x	5.33 abcd, wx	7.33 abc, wx	9.67 abcd, wx	13.33 abc, w	8.217	63.953
Fusarium solani SC	1	4.33 abc, x	4.33 abcd, x	5.00 abcde, wx	5.33 abcdef, wx	6.33 cde, wx	7.67 bcd, w	3.138	32.070
Fusarium solani SC	2	3.00 abcde, w	4.00 abcd, w	6.33 abc, w	7.67 abc, w	9.33 abcde, w	10.00 bcd, w	7.442	62.231
Lasiodiplodia pseudotheobromae	1	5.33 ab, x	6.33 a, wx	8.00 a, wx	8.67 a, wx	12.67 ab, w	13.00 bc, w	6.748	42.147
Neofusicoccum parvum	1	4.67 abc, x	4.67 abc, x	7.33 ab, wx	8.33 a, wx	8.67 abcde, wx	12.33 bc, w	5.337	39.130
Pestalotiopsis maculans	1	0.00 e, x	1.67 cd, wx	3.00 cde, wx	3.00 def, wx	7.00 abcde, w	7.00 bcde, w	6.345	98.774
LSD $^{(a-f)}$		3.330	3.609	3.827	3.857	6.205	6.899		
CV (%)		63.494	55.980	45.225	37.623	46.197	44.217		

[a] Average of the three individual seedlings' disease rating scores

[b] Certain fungal species were used in duplicate, indicated by Isolate 1 or 2.

*Statistical analysis performed using One way ANOVA and Fisher's Least significance difference (LSD) post hoc test; CV % = coefficient of variation. Letters a-f represemt statistical differences in the columns, while letters w-z represent statistical differences in the rows.

Table 4.9. The average disease rating scores (maximum 20) for pathogenicity Trial 2, a repeat of Trial 1 inoculated seven days after Trial 1.

Treatment	**Isolate**	**Week**					**LSD $^{(w-z)}$**	**CV (%)**
		1	**2**	**3**	**4**	**5**		
Negative control		1.00 d, x	1.00 d, x	1.00 e, x	2.17 d, xw	3.17 c, w	2.020	66.633
Alternaria alternata	1[b]	3.33 abcd, y	5.33 bcd, xy	6.33 bcde, xwy	8.00 bc, xw	8.67 ab, w	3.288	28.538
Alternaria alternata	2	5.67 abc, x	5.67 abc, x	6.67 abcde, x	8.67 abc, xw	14.00 a, w	6.475	43.759
Bipolaris sp.	1	7.33 a, w	10.00 a, w	11.00 a, w	12.33 a, w	14.67 a, w	9.348	46.429
Bipolaris zeae	1	5.00 abcd, w	7.33 ab, w	7.67 abcd, w	8.33 abc, w	10.67 ab, w	5.886	41.477
Botryosphaeria dothidea	1	3.33 abcd, x	3.33 bcd, x	3.67 ed, x	5.67 cd, xw	11.33 ab, w	6.693	67.295
Colletotrichum fructicola	1	2.67 bcd, y	2.67 cd, y	4.33 cde, xy	8.00 bc, xw	8.33 ab, w	3.930	41.543
Colletotrichum fructicola	2	6.33 ab, y	7.00 abc, xy	8.00 abcd, xy	8.67 abc, xw	10.33 ab, w	2.301	15.681
Diaporthe rhusicola	1	1.67 cd, x	7.00 abc, w	8.33 abc, w	9.00 abc, w	10.67 ab, w	5.081	38.084
Didymella sp.	1	3.33 abcd, x	6.00 abc, xw	6.33 bcde, xw	7.33 bcd, xw	10.00 ab, w	6.196	51.604
Fusarium fujikuroi SC	1	4.33 abcd, w	7.67 ab, w	9.33 ab, w	10.33 ab, w	11.33 ab, w	7.262	46.415
Fusarium fujikuroi SC	2	5.33 abcd, x	6.33 abc, xw	6.67 abcde, xw	8.33 abc, xw	12.67 ab, w	6.742	47.108
Fusarium fujikuroi SC	3	3.67 abcd, w	3.67 bcd, w	4.67 cde, w	5.67 cd, w	6.67 bc, w	5.498	62.099
Fusarium incarnatum-equiseti SC	1	3.33 abcd, x	5.33 bcd, x	6.00 bcde, x	8.00 bc, xw	12.33 ab, w	6.034	47.380
Fusarium oxysporum SC	1	5.33 abcd, x	6.33 abc, xw	6.33 bcde, xw	7.33 bcd, w	8.00 abc, w	1.879	15.492
Fusarium oxysporum SC	2	2.67 bcd, y	4.67 bcd, xy	6.67 abcde, xwy	8.00 bc, xw	9.33 ab, w	4.175	36.621
Fusarium solani SC	1	5.33 abcd, w	7.00 abc, w	7.67 abcd, w	8.33 abc, w	9.33 ab, w	6.526	47.615
Fusarium solani SC	2	2.33 bcd, y	5.00 bcd, xy	5.67 bcde, xwy	8.67 abc, xw	10.33 ab, w	4.767	40.944
Lasiodiplodia pseudotheobromae	1	4.00 abcd, x	7.33 ab, x	8.67 abc, xw	9.00 abc, xw	13.33 ab, w	5.867	38.089
Neofusicoccum parvum	1	5.00 abcd, x	6.33 abc, x	6.33 bcde, x	8.33 abc, xw	13.33 ab, w	6.052	42.288
Pestalotiopsis maculans	1	2.67 bcd, x	5.00 bcd, xw	8.00 abcd, w	9.00 abc, w	9.00 ab, w	4.767	38.917
LSD $^{(a-e)}$		4.443	4.623	4.355	4.157	7.086		
CV (%)		67.675	49.103	39.453	31.104	41.937		

[a] Average of the three individual seedlings' disease rating scores

[b] Certain fungal species were used in duplicate, indicated by Isolate 1 or 2.

*Statistical analysis performed using One way ANOVA and Fisher's Least significance difference (LSD) post hoc test; CV % = coefficient of variation. Letters a-e represemt statistical differences in the columns, while letters w-z represent statistical differences in the rows.

Table 4.10. The overall average disease rating scores per fungal species, recorded over a five-week pathogenicity trial period.

Treatment	Week[a]					LSD	CV (%)
	1	2	3	4	5		
Negative control	0.75	0.92	0.92	1.83	2.75	1.09	63.94
Alternaria alternata	3.92	4.67	5.58	6.92	9.00	3.14	42.17
***Bipolaris* sp.**	5.00	6.58	7.33	8.50	9.92	3.90	43.95
Botryosphaeria dothidea	3.17	4.00	5.50	7.00	12.17	4.89	64.62
Colletotrichum fructicola	3.25	3.83	5.33	7.00	9.08	2.77	40.81
Diaporthe rhusicola	1.83	5.83	6.67	7.83	9.83	3.09	40.59
Didymella sp.	4.50	5.83	6.67	7.33	9.33	2.84	35.44
Fusarium fujikuroi SC	4.21	5.46	6.42	7.46	8.96	2.10	29.03
Fusarium incarnatum-equiseti SC	2.83	3.83	4.50	6.17	9.50	3.60	56.46
Fusarium oxysporum SC	3.58	4.75	6.00	7.83	9.42	1.91	25.45
Fusarium solani SC	3.75	5.08	6.17	7.50	8.83	2.70	36.28
Lasiodiplodia pseudotheobromae	4.67	6.83	8.33	8.83	13.00	3.43	34.64
Neofusicoccum parvum	4.83	5.50	6.83	8.33	11.00	3.09	35.56
Pestalotiopsis maculans	1.33	3.33	5.50	6.00	8.00	4.01	69.75
LSD	2.31	2.68	2.65	2.69	4.53		
CV (%)	59.04	49.34	39.55	33.25	42.04		

*The top five fungal species with the highest overall average disease rating scores have been highlighted in bold.

**The overall average disease rating scores were determined for Trials 1 and 2, and duplicate fungal treatments per fungal species.

[a] The results have been shown for the first five weeks based on the lack of data from Trial 2, week 6 due to the premature termination of the trial.

The combined average ratings per fungal species (Table 4.10), including both Trials 1 and 2 and each respective duplicate isolate, ranged from 2.75 (negative control) to 13.00 (*L. pseudotheobromae*) after the five-week trial period.

To analyse the amount of time taken for each fungal isolate to develop symptoms on their respective seedlings, a more calculative approach was taken. The respective mean and median of the Week 1 disease ratings were calculated and averaged for Trials 1, 2 and the combined results. The averaged mean and median score assumed the role of the minimum disease rating (MDR) threshold.

The mean, median and MDR values for Trial 1 were 3.17, 3.50, and 3.33, respectively; for Trial 2 were 3.98, 3.67, and 3.83, respectively; and for the combined results were 3.40, 3.67, and 3.53, respectively. The individual ratings of each fungal isolate for each week were subsequently compared to the MDR of Week 1, and ratings from each week that were below the MDR threshold were considered below the threshold and the symptoms considered 'underdeveloped'. Both *A. alternata*, *Bipolaris* sp., *F. fujikuroi* SC isolates 1 and 2, *F. solani* SC isolate 1, *Neofusicoccum parvum* (Pennycook & Samuels)

Crous, Slippers & A.J.L. Phillips, and *Lasiodiplodia pseudotheobromae* treatments were consistently above the MDR in Week 1 of Trials 1 and 2, and the combined results. The negative control, *Botryosphaeria dothidea* (Moug.) Ces. & De Not., *Colletotrichum fructicola* Prihastuti, L. Cai & K.D. Hyde isolate 1, *D. rhusicola*, *F. incarnatum-equiseti* SC, and *Pestalotiopsis maculans* (Corda) Nag Raj treatments were consistently below the MDR in in Week 1 of Trials 1 and 2, and the combined results.

In Trial 1 (Table 4.8), most fungal treatments passed the MDR threshold (3.33) before the end of Week 1, with the exceptions of: the *A. alternata* isolate 2, *B. dothidea*, *D. rhusicola*, *F. oxysporum* SC isolate 1, and *F. solani* SC isolate 2 treatments passed the threshold only after Week 1; the *C. fructicola* isolate 2 and *F. incarnatum-equiseti* SC treatments after Week 3; and the *F. fujikuroi* SC isolate 3 and *P. maculans* treatments after Week 4. Similarly, most of the fungal treatments in Trial 2 (Table 4.9) passed the MDR threshold (3.83) before the end of Week 1, with the exceptions of: the *A. alternata* isolate 1, *D. rhusicola*, *Didymella* sp. Sacc., *F. incarnatum-equiseti* SC, *F. oxysporum* SC isolate 2, *F. solani* SC isolate 2, and *P. maculans* treatments passed the threshold only after Week 1; the *C. fructicola* isolate 1 and *F. fujikuroi* SC isolate 3 treatments after Week 2; and the *B. dothidea* treatment after Week 3. Overall (Table 4.10), the *B. dothidea*, *C. fructicola*, *D. rhusicola*, and *F. incarnatum-equiseti* SC treatments only passed the MDR threshold (3.53) after Week 1, while the *P. maculans* treatment only passed the MDR threshold after Week 2. The *A. alternata*, *Bipolaris* sp., *Didymella* sp., *F. fujikuroi* SC, *F. oxysporum* SC, *F. solani* SC, *L. pseudotheobromae*, and *N. parvum* treatments surpassed the MDR threshold before the end of Week 1.

The negative control treatment was consistently the lowest rating throughout the different weeks for both Trials 1 and 2, and the combined results. The *Fusarium fujikuroi* SC isolate 3 treatment consistently had low ratings in both Trials 1 and 2, while the *P. maculans* treatment consistently had low ratings in Trial 1 and the combined results. In contrast, the *L. pseudotheobromae* treatment consistently had high ratings throughout the weeks for both Trials 1 and 2, and the combined results. Similarly, the *N. parvum* treatment consistently had high ratings in Trial 1 and the combined results, while the *Bipolaris* sp. treatment had high ratings in Trial 2 and the combined results. Almost consistently throughout the six-week Trial 1 time period, the *A. alternata* isolate 1 and 2, *C. fructicola* isolate 1 and *P. maculans* treatments had ratings significantly similar to the negative control. In contrast, the *L. pseudotheobromae* and *N. parvum* treatments both had ratings significantly different to the negative control. The *F. oxysporum* SC isolate 1 and 2, *F. fujikuroi* SC isolate 3, and *P. maculans* treatments almost consistently had significantly similar ratings to the negative control over the five-week Trial 2 period, while the *Bipolaris* sp. treatment had consistently significantly higher rating to the controls over the five-week period.

By the end of the six-week Trial 1 period (Table 4.8), only the *F. oxysporum* SC isolate 1 and *B. dothidea* treatments were significantly similar to *F. oxysporum* SC isolate 2 treatment. All of the fungal treatments were significantly similar to each other with the exception of the *F. oxysporum* SC isolate 1, *F. fujikuroi* SC isolate 3, *A. alternata* isolate 2 and the negative control treatments, while the *A. alternata* isolate 1, *F. fujikuroi* SC isolate 3, and *P. maculans* treatments were all significantly similar to the negative control. When comparing the individual progression of symptom development over the six-week period, all of the fungal treatments indicated a significantly different Week 6 rating to their respective Week 1 rating, with the exception of the *A. alternata* isolate 1, *C. fructicola* isolate 1, *F. fujikuroi* SC isolate 1, and *F. solani* SC isolate 2 treatments.

After the five-week Trial 2 period (Table 4.9), all of the fungal treatments were significantly similar to the *A. alternata* isolate 2 and *Bipolaris* sp. treatments, with the exception of the *F. fujikuroi* SC isolate 3 and negative control treatments. The *F. oxysporum* SC isolate 1 treatment was significantly similar to all of the fungal and negative control treatments, while the *F. fujikuroi* SC isolate 3 treatment was similar to the negative control and all fungal treatments except for the *A. alternata* isolate 2 and *Bipolaris* sp. treatments. When comparing the individual progression of symptom development over the five-week period, all of the fungal treatments indicated a significantly different Week 5 rating to their respective Week 1 rating, with the exception of the *Bipolaris* sp., *B. zeae*, *F. fujikuroi* SC isolates 1 and 3, and *F. solani* SC isolate 1 treatments.

According to the ratings in Week 5 the overall average disease rating scores (Table 4.10), the top five highest rating species were *L. pseudotheobromae* (13.00); *B. dothidea* (12.17); *N. parvum* (11.00); *Bipolaris* sp. (9.92); and *Diaporthe rhusicola* (9.83). The *L. pseudotheobromae* treatment remained the highest disease rated fungal treatment each week over the five-week trial period, while the *B. dothidea* and *N. parvum* treatments indicated the largest increase in disease rating over the five-week period. These three fungal species appeared to have the most severe impact on the marama bean seedlings in terms of the disease symptoms observed and the subsequent disease rating scores they received. The *A. alternata, Bipolaris* sp., *C. fructicola, D. rhusicola, Didymella* sp., *F. incarnatum-equiseti* SC, *F. fujikuroi* SC, *F. oxysporum* SC, *F. solani* SC and *P. maculans* treatments appeared to have a slower, less severe impact on the marama bean seedlings regarding disease symptoms and subsequent rating scores, with their overall week five rating scores ranging between 8.00 and 9.92. However, the *B. dothidea*, *D. rhusicola*, *L. pseudotheobromae,* and *P. maculans* treatments have the greatest predicted exponential growth over a potential longer trial period (Figure 4.4).

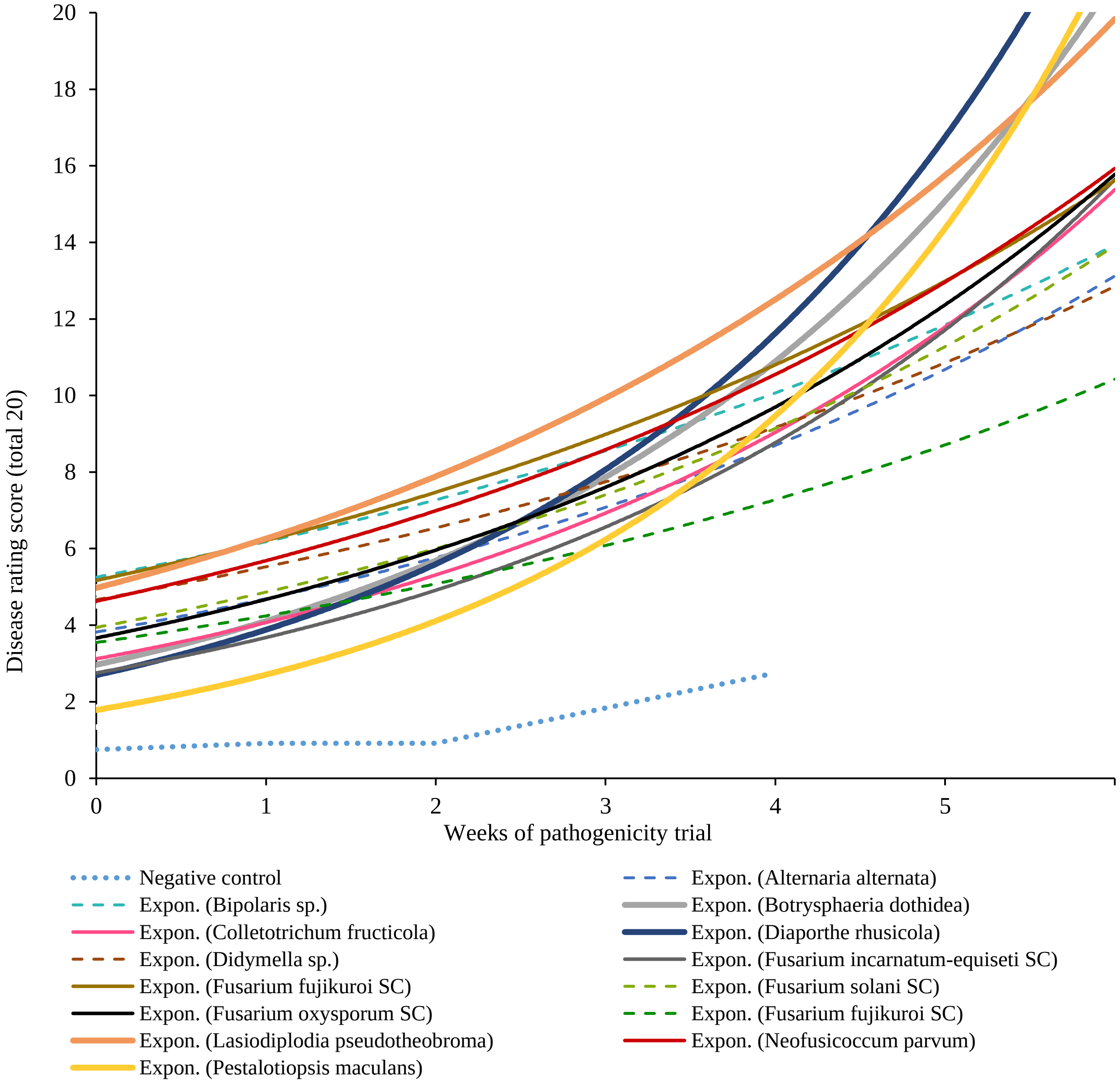

Figure 4.4. An exponential forecast predicting how the different fungal species and their respective disease symptoms and ratings would progress over time after the five-week trial period. Fungal species with higher rates of exponential growth are highlighted with bolder lines.

After termination of the pathogenicity trial, the seedlings were harvested and processed for wet and dry weight measurements of the above (leaves and stems) and below (roots and tubers) ground material. The individual and average seedling above and below ground wet and dry weight measurements per fungal treatment were recorded (Appendix 6.9, Tables 6.8 to 6.12). The average above and below ground wet and dry weight measurements for both Trials 1 and 2, and for duplicate fungal treatments were calculated (Appendix 6.9, Tables 6.8 to 6.12). Table 4.11 indicates the average above and below ground dry weight measurements (g) per fungal species treatment for Trial 1, as well as the difference in weight compared to the control. Table 4.12 presents the respective data for Trial 2.

When considering the Trial 1 above ground weight measurement differences compared to the control, the *F. oxysporum* SC isolate 1 treatment presented the largest negative difference in wet weight (-0.50 g), followed by the *L. pseudotheobromae* treatment (-0.45 g). The *F. solani* SC isolate 1 treatment indicated the largest negative difference in dry weight (-0.10 g). The *L. pseudotheobromae* treatment presented the largest negative below ground wet weight measurement (-8.68 g), followed by the *B. dothidea* treatment (-7.68 g), while the *B. dothidea* treatment presented the largest negative difference in below ground dry weight measurements (-1.49 g). The *D. rhusicola* treatment indicate the largest positive difference in above ground wet and dry weight measurements when compared to the control (0.61 g and 0.42 g, respectively), while *A. alternata* isolate 2 presented the largest positive difference in below ground wet and dry weight measurements (12.29 g and 3.05 g, respectively).

In Trial 2, the *N. parvum* treatment presented the greatest negative difference compared to the control for the wet and dry, above and below ground weight measurements (-1.11g; -0.38g; -19.57g; -5.91g respectively). The treatments presenting the greatest positive difference in weight measurements compared to the control include *A. alternata* isolate 1 (wet above ground - 0.19g), *C. fructicola* isolate 1 (dry above ground - 0.13g), *F. oxysporum* SC isolate 2 (wet below ground - 7.09g), and the control treatment (dry below ground - 0.00g).

For both Trials 1 and 2, the *F. solani* SC isolate 1 and *L. pseudotheobromae* treatments consistenly presented wet and dry weight measurements less than the control treatment. The *A. alternata* isolate 2, *Bipolaris* sp., *B. zeae*, *C. fructicola* isolate 2, *D. rhusicola*, *F. fujikuroi* SC isolate 1, and *N. parvum* treatments presented above and below ground wet and dry weight measurements greater than the control in Trial 1, but less than the control in Trial 2. In contrast, the *A. alternata* isolate 1 and *F. solani* SC isolate 2 treatments generally presented above and below ground wet and dry weight measurements less than the control in Trial 1, but greater than the control in Trial 2. Treatments in Trial 1 that presented above and below, dry and wet weight measurements less than control included *F. incarnatum-equisetii* SC, *F. solani* SC isolate 2, while the respective Trial 2 weight measurements were mixed greater than and less than the control. Treatments in Trial 2 that presented above and below, dry and wet weight measurements less than control included *B. dothidea*, *Didymella* sp., *F. fujikuroi* SC isolate 3, *F. oxysporum* SC isolate 1, and *P. maculans*. The respective Trial 1 weight measurements were mixed greater than and less than the control.

Table 4.11. The average dry and wet weight measurements (g) above ground (leaves and stems) and below ground (roots and tubers) material harvested from the pathogenicity Trial 1 per fungal species inoculated, as well as a comparison of the respective dry and wet weight measurements to the respective control.

	Above ground material				**Below ground material**			
	Wet mass (g)	**c/t Control[a]**	**Dry mass (g)**	**c/t Control**	**Wet mass (g)**	**c/t Control**	**Dry mass (g)**	**c/t Control**
Control	1,02 abcde	0,00	0,43 bc	0,00	35,35 ab	0,00	7,94 a	0,00
*Alternaria alternata*_1	0,83 bcde	-0,19	0,55 abc	0,12	31,65 ab	-3,70	7,97 a	0,03
*Alternaria alternata*_2	1,62 a	0,60	0,71 ab	0,28	47,64 a	**12,29**	10,99 a	**3,05**
Bipolaris sp._1	1,49 abc	0,47	0,63 abc	0,20	38,18 ab	2,83	9,61 a	1,67
*Bipolaris zeae*_1	1,34 cde	0,32	0,62 bc	0,19	43,31 b	7,96	8,99 a	1,05
*Botryosphaeria dothidea*_1	0,74 ab	-0,28	0,48 abc	0,05	27,67 ab	-7,68	6,45 a	**-1,49**
*Colletotrichum fructicola*_1	0,94 abcde	-0,08	0,65 abc	0,22	32,38 ab	-2,97	7,20 a	-0,74
*Colletotrichum fructicola*_2	1,16 abcde	0,14	0,64 abc	0,21	39,20 ab	3,85	9,21 a	1,27
*Diaporthe rhusicola*_1	1,63 a	**0,61**	0,85 a	**0,42**	37,63 ab	2,28	8,52 a	0,58
Didymella sp._1	0,78 bcde	-0,24	0,45 bc	0,02	36,16 ab	0,81	7,57 a	-0,37
Fusarium fujikuroi SC_1	1,05 abcde	0,03	0,64 abc	0,21	38,55 ab	3,20	8,70 a	0,76
Fusarium fujikuroi SC_2	0,93 abcde	-0,09	0,60 abc	0,17	34,81 ab	-0,54	9,03 a	1,09
Fusarium fujikuroi SC_3	0,88 bcde	-0,14	0,53 abc	0,10	35,31 ab	-0,04	7,68 a	-0,26
Fusarium incarnatum-equiseti SC_1	0,65 cde	-0,37	0,38 c	-0,05	31,77 ab	-3,58	6,78 a	-1,16
Fusarium oxysporum SC_1	0,52 e	**-0,50**	0,39 bc	-0,04	38,49 ab	3,14	6,87 a	-1,07
Fusarium oxysporum SC_2	0,77 cde	-0,25	0,60 abc	0,17	37,91 ab	2,56	10,39 a	2,45
Fusarium solani SC_1	0,61 de	-0,41	0,33 c	**-0,10**	33,95 ab	-1,40	7,57 a	-0,37
Fusarium solani SC_2	0,60 de	-0,42	0,39 bc	-0,04	29,80 b	-5,55	6,63 a	-1,31
*Lasiodiplodia pseudotheobroma*_1	0,57 e	-0,45	0,39 bc	-0,04	26,67 b	**-8,68**	6,96 a	-0,98
*Neofusicoccum parvum*_1	1,30 abcd	0,28	0,71 ab	0,28	38,23 ab	2,88	9,09 a	1,15
*Pestalotiopsis maculans*_1	0,95 abcde	-0,07	0,45 bc	0,02	34,34 ab	-1,01	7,70 a	-0,24
LSD	**0.72**		**0.32**		**17.32**		**4.79**	
CV (%)	**44.95**		**36.00**		**29.46**		**35.53**	

*The average dry and wet weight measurements for both above and below ground material were calculated using the three individual seedling measurements for Trial 1.

**Statistical analysis performed using One-way ANOVA and Fisher's Least significance difference (LSD) post hoc test; CV % = coefficient of variation

[a] The mass compared to the control (c/t Control) for both above and below ground material has been colour coded: mass smaller than the control (0.00 g) in red; mass greater than control (0.00 g) in green. The highest and lowest values for the above and below ground mass compared to the controls are highlighted in bold.

Table 4.12. The average dry and wet weight measurements (g) above ground (leaves and stems) and below ground (roots and tubers) material harvested from the pathogenicity Trial 2 per fungal species inoculated, as well as a comparison of the respective dry and wet weight measurements to the respective control.

	Above ground mass (g)				Below ground mass (g)			
Treatment	**Wet mass**	**c/t Control**[a]	**Dry mass**	**c/t Control**	**Wet mass**	**c/t Control**	**Dry mass**	**c/t Control**
Control	1,69 ab	0,00	0,61 abc	0,00	42,40 ab	0,00	10,49 a	**0,00**
*Alternaria alternata*_1	1,88 a	**0,19**	0,70 ab	0,09	43,87 ab	1,47	8,86 abcd	-1,63
*Alternaria alternata*_2	1,02 ab	-0,67	0,46 abcd	-0,15	38,08 abc	-4,32	7,91 abcde	-2,58
Bipolaris sp._1	1,09 ab	-0,60	0,43 abcd	-0,18	29,30 bc	-13,10	6,41 bcde	-4,08
*Bipolaris zeae*_1	0,89 ab	-0,80	0,50 abcd	-0,11	30,19 abc	-12,21	6,74 bcde	-3,75
*Botryosphaeria dothidea*_1	1,02 ab	-0,67	0,48 abcd	-0,13	36,38 bc	-6,02	6,81 cde	-3,68
*Colletotrichum fructicola*_1	1,82 a	0,13	0,74 a	**0,13**	44,26 ab	1,86	10,27 ab	-0,22
*Colletotrichum fructicola*_2	1,50 ab	-0,19	0,60 abc	-0,01	37,49 abc	-4,91	7,83 abcde	-2,66
*Diaporthe rhusicola*_1	1,02 ab	-0,67	0,58 abc	-0,03	41,24 ab	-1,16	8,21 abcd	-2,28
Didymella sp._1	0,89 ab	-0,80	0,48 abcd	-0,13	29,59 bc	-12,81	7,34 abcde	-3,15
Fusarium fujikuroi SC_1	0,76 ab	-0,93	0,40 bcd	-0,21	35,22 abc	-7,18	6,87 abcde	-3,62
Fusarium fujikuroi SC_2	0,99 ab	-0,70	0,56 abcd	-0,05	42,91 ab	0,51	9,85 abc	-0,64
Fusarium fujikuroi SC_3	0,61 b	-1,08	0,36 cd	-0,25	29,74 bc	-12,66	6,71 bcde	-3,78
Fusarium incarnatum-equiseti SC_1	1,82 a	0,13	0,70 ab	0,09	42,37 ab	-0,03	9,31 abcd	-1,18
Fusarium oxysporum SC_1	1,08 ab	-0,61	0,44 abcd	-0,17	36,64 abc	-5,76	7,58 abcde	-2,91
Fusarium oxysporum SC_2	0,83 ab	-0,86	0,53 abcd	-0,08	49,49 a	**7,09**	8,96 abcd	-1,53
Fusarium solani SC_1	1,33 ab	-0,36	0,61 abc	0,00	37,76 abc	-4,64	9,34 abcd	-1,15
Fusarium solani SC_2	0,94 ab	-0,75	0,52 abcd	-0,09	42,55 ab	0,15	10,45 a	-0,04
*Lasiodiplodia pseudotheobroma*_1	1,36 ab	-0,33	0,52 abcd	-0,09	32,92 abc	-9,48	7,54 abcde	-2,95
*Neofusicoccum parvum*_1	0,58 b	**-1,11**	0,23 d	**-0,38**	22,83 c	**-19,57**	4,58 e	**-5,91**
*Pestalotiopsis maculans*_1	0,90 ab	-0,79	0,39 bcd	-0,22	30,21 bc	-12,19	5,81 de	-4,68
LSD	**1,14**		**0,33**		**16,67**		**3,62**	
CV (%)	**60,47**		**38,19**		**27,39**		**27,52**	

*The average dry and wet weight measurements for both above and below ground material were calculated using the three individual seedling measurements for Trial 2.

**Statistical analysis performed using One-way ANOVA and Fisher's Least significance difference (LSD) post hoc test; CV % = coefficient of variation

[a] The mass compared to the control (c/t Control) for both above and below ground material has been colour coded: mass smaller than the control (0.00 g) in red; mass greater than control (0.00 g) in green. The highest and lowest values for the above and below ground mass compared to the controls are highlighted in bold.

The *D. rhusicola* and *C. fructicola* treatments presented the largest positive increase in above ground dry weight when compared to the control, indicating an infection that did not greatly affect the development of the above ground material. Regarding the above ground dry weight (g) measurements, only the *D. rhusicola* treatment was significantly higher than the negative control treatment in Trial 1, while the *N. parvum* treatment was the only treatment in Trial 2 that was significantly different to the negative control treatment.

4.2.3.2 Koch's postulates

A single leaf was obtained from each seedling per fungal treatment of the pathogenicity Trials 1 and 2, followed by subsequent culturing on 50% PDA. From the plated out leaf samples, fungal pure cultures were obtained on 100% PDA. Confirmation of the isolates was obtained through culture morphology visually and using microscopy. The following fungal species were confirmed to be the same as the fungal isolates that were used to inoculate the respective marama bean seedlings in both pathogenicity Trials 1 and 2: *A. alternata*; *F. fujikuroi* species complex (SC); *F. oxysporum* SC; *F. incarnatum-equiseti* SC; *N. parvum*; *Bipolaris* sp.; *Didymella* sp.; *B. dothidea*; *P. maculans*; *D. rhusicola*; and *L. pseudotheobromae*.

Fusarium solani SC and *C. fructicola* were the only fungal species that could not successfully be re-isolated from diseased material to confirm that it was the same as the fungal isolates that were used to inoculate the respective marama bean seedlings in both pathogenicity trials.

4.2.3.3 *In vivo* multi-mycotoxin analysis

Multi-mycotoxin analysis of the fungal infected seeds removed from pots before the pathogenicity trial started, confirmed the presence of 0.021 mg/kg of FB_1 in one of the four samples analysed, as well as 0.061 mg/kg of ZEA and 0.066 mg/kg of FB_1 in another of the four samples analysed, all within the limit of detection. No other mycotoxins were detected in the seed samples.

Multi-mycotoxin analysis of the dried and ground seedlings from each of the identified potentially mycotoxigenic fungal species used to inoculate the respective trials confirmed the presence (within the limit of detection) of 0.014 mg/kg ZEA in the *A. alternata* isolate 1 treatment; 0.016 mg/kg ZEA in the *F. fujikuroi* SC isolate 2 treatment; and 0.024 mg/kg ZEA in the *F. oxysporum* SC isolate 1 treatment. No mycotoxins were detected in the *A. alternata* isolate 2, *F. fujikuroi* SC isolates 1 and 3, *F. oxysporum* SC isolate 2, *F. solani* SC isolates 1 and 2, or *F. incarnatum-equiseti* SC treatment seedlings.

4.3 Discussion

Categorization of all of the molecular-identified fungal species into one of the four groups [red, yellow, white, and black] allowed for focus to be placed on species that have a higher potential to be pathogenic to marama beans. The red potential pathogen group constituted 25% of the fungal species previously isolated and identified, and consisted of fungal species known to be pathogenic to crops or plants and known to cause diseases of varying severity (Velasquez et al., 2015). The yellow weak or postharvest group constituted 8% of the fungal species. Weak pathogens, also referred to as opportunistic pathogens, are pathogens that require optimal conditions for infection and disease development, are likely not highly virulent, or that may infect as a secondary infection after another pathogen has caused primary infection (Abdullah et al., 2017; Charkowski & Hurst, 2013). The white group (42% of the fungal species) was characterized by fungi only of interest under certain conditions. These conditions may include extreme weather conditions such as drought or flooding, postharvest conditions, storage conditions, among others. The remaining 25% were categorized in the black group, labelled as saprophytes. These included fungal species known to have a saprophytic nature whereby they survive on dead and decaying organic matter (Velasquez et al., 2015).

A total of 49 fungal species were selected for the DLA. Several of these species had multiple isolates, separated based on distinct morphological culture differences, resulting in 69 isolates being selected for analysis in the DLA trials. For the DLA, focus was placed on the red and yellow group fungi due to their nature as known pathogens on other crops, while representative isolates of the white group were selected for each species identified.

Based on the various disease symptoms, as well as various factors such as time and trial size constraints, prevalence of the pathogen on other crops, general severity of the diseases caused by the pathogen, and pre- or postharvest disease conditions, the 58 successful isolates were narrowed down to 20 fungal isolates to be used in the pathogenicity trial. Many of the isolates in the yellow and white groups are common postharvest pathogens – the ability of the fungus to cause disease symptoms on the already harvested (detached) leaves may not serve as a good indicator of the ability of these fungi to cause disease on growing seedlings. As a result, the isolates within the white group were excluded from further analysis, while the isolates in the red and yellow groups were prioritized. Three isolates within the yellow group and seventeen isolates within the red group were selected for further analysis based on the respective symptoms each produced, as well as their ability to cause diseases on other crops – *A. alternata* isolates 1 and 2; *Bipolaris* sp.; *Bipolaris zeae*; *B. dothidea*; *C. fructicola* isolates 1 and 2; *D. rhusicola*; *Didymella* sp. isolate 3; *F. fujikuroi* SC isolates 1, 2 and 3; *F. incarnatum-*

equiseti SC; *F. oxysporum* SC isolates 2 and 3; *F. solani* SC isolates 1 and 2; *L. pseudotheobromae*; *N. parvum*; and *P. maculans*.

Prior to conducting the pathogenicity trial, several factors regarding the propagation and growth of the marama beans required investigation. These factors included determining the preferred location for the pathogenicity trial, the scarification method and pot size, the optimal soil components and respective soil drainage rate to create the ideal soil combination for marama bean propagation.

There are conflicting reports regarding seed preparation of the marama bean seeds. Seed scarification provides a hole through the seed coat through which water can move, and is necessary for seeds that have hard seed coats, such as marama bean seeds (Travlos et al., 2007a). Although the hard seed coats are beneficial to plants such as legumes for the purposes of prolonging the seed's lifespan to enable the plant to germinate over different generations (Travlos et al., 2007a), the hardness of the seed coats also presents a problem for reliable germination in farm-like and commercialised conditions, which would need to be overcome before the marama bean can be commercialised successfully (Travlos et al., 2007a). Several reports state that marama bean seed scarification is very effective along with low temperature seed exposure, however soaking the seeds in water should be avoided as the water will promote seed rot, while boiling water will inactivate the seeds (Chingwaru, et al., 2015; Department of Agriculture, Forestry and Fisheries, 2014; National Research Council, 2006; Omotayo & Aremu, 2021). In contrast, Travlos et al. (2007c, 2007b) reported effective germination of marama bean seeds was achieved through soaking the seeds in water for 20 hours, using dry heating methods such as an oven preheated between 100 and 150°C for 5 min, or alternatively soaking the seeds in sulfuric acid for 20 min followed by soaking in water. Batanani (2020) reported a higher marama bean germination rate in scarified seeds compared to non-scarified seeds, using a scarification method of a drill and mini circular saw attachment in order to create an aperture in the hard seed coat to allow imbibition. Odhiambo (2020) investigated the effect of various climatic conditions on the germination of *Tylosema fassoglense* (sprawling Bauhinia) seeds in Kenya. Several germination conditions were investigated, including the role of water imbibition, manual scarification, and temperature on marama bean seed germination. Water imbibition rates were determined for scarified (using a nail clipper to chip off part of the seed coat) and non-scarified seeds, and calculated by weighing the seeds at several intervals over a 52-hour period. The scarified seeds indicated a higher water imbibition rate than non-scarified seeds. Manual scarification was reported based on the final germination percentage achieved by the scarified and non-scarified seeds, using the previously watered imbibed seeds. Both the scarified seeds and the non-scarified seeds germinated; however, the scarified seeds indicated a higher germination rate over the six-day period compared to the non-scarified seeds. Regarding the seed scarification method used for this pathogenicity trial, there appeared to be a slight significant difference in germination rate between the secateur and wood file scarified seeds in favour of the secateur method, with a seed emergence rate of 27% when using the wood file compared to the 54% emergence rate when using secateurs. The scarification by secateur method was selected largely due to the higher emergence rates observed, as well as the large number of seeds required for the pathogenicity trial – the secateurs were more efficient in damaging the hard seed coat to create a hole for the water to enter, without damaging the seed's cotyledons within and inhibiting germination.

Tylosema esculentum naturally grows in arid climatic conditions with high temperatures and dry, drought-like conditions with low annual rainfall levels (Coetzer et al., 2011; Mitchell et al., 2005). While investigating the effect of several climatic conditions for marama bean seed germination, the effect of temperature on seed germination was determined through germinating seeds at different temperature intervals ranging from 10 to 40°C, over a 60-day period (Odhiambo, 2020). The results indicated that 10°C and 40°C had the lowest germination rates, temperatures between 15 and 35°C indicated similar germination rates, however it was concluded that 30 to 35°C was the optimal temperature range for seed germination (Odhiambo, 2020). Various legume grain crops such as soybeans (*Glycine max* (L.) Merr.), cowpeas (*Vigna unguiculata* (L.) Walp.), chickpeas (*Cicer arietinum* L.), *Vicia sativa* L., *Onobrychis viciifolia* (Scop.) and lentils (*Lens nigricans* (M. Bieb.)), have been reported to have similar temperature ranges – a base temperature between 0 and 10°C, optimal temperature ranges between 20 and 35°C, and ceiling temperature ranges between 32 and 45°C (Covell et al., 1986; Hu et al., 2015; Odhiambo, 2020; Raveneau et al., 2011; Tribouillois et al., 2016). Legumes and legume crops are typically water sensitive to both high levels of watering and drought conditions - too little water results in a decreased yield and drought stress, while too much water can result in decreased yield, water stress, rotting, and eventual death (Chibarabada et al., 2019; Ghosh & Panja, 2016; Modi & Mabhaudhi, 2017). However, several legumes, such as cowpeas, chickpeas, pigeon peas (*Cajanus cajan* (L.) Millsp.), and Bambara groundnut (*Vigna subterranean* (L.) Verdc.), have been identified as potential legume crops for arid and semi-arid areas where conventional legume crops may struggle, due to their natural adaptation to survive in water-scarce or drought-like conditions (Chibarabada et al., 2019; Ghosh & Panja, 2016; Modi & Mabhaudhi, 2017).

Four locations with varying climatic conditions were investigated for use during the pathogenicity trial, with the aim of determining the ideal trial location with the optimal climatic conditions to aid in marama bean seed germination and seedling growth. The various climatic conditions of each location were observed for the duration of each trial. Trial 1 in the ARC Roodeplaat campus greenhouse was conducted at a controlled temperature of 32°C, and a high humidity level was observed over the trial period. The high temperature and humidity contributed to the high soil moisture levels that were maintained throughout the duration of the trial, regardless of when watering occurred. The trial was scheduled to be watered once a week; however, the pots were placed on a bench in the greenhouse with drip trays which contributed to the high soil moisture levels. Due to space limitations within the greenhouse, the humidity and the constant soil moisture levels maintained, the location was excluded from the pathogenicity trial. Trial 2 was conducted in the UP Cycad and indigenous nursery, Innovation Africa @UP on Hillcrest campus, in an enclosed area covered by 60% black shade cloth, which received four to six hours of sunlight daily. The temperature ranges included a min temperature range of 12 to 26°C, and max temperature range of 19 to 36°C. However, shaded conditions occurred for longer periods of time due to the nursery being surrounded by more brush and trees than the shade tunnel of Trial 3. The shade tunnel in Trial 3 was covered by 30% white shade cloth and located in an open field setting where the trial received full day sun. The min and max temperature ranges for Trial 3 were the same as Trial 2. While temperature, rainfall, and air movement could not be controlled in either of the locations, the rainfall patterns were carefully observed for the purpose of manual watering to prevent overwatering. Soil moisture readings recorded randomly throughout the three-week trial period generally presented lower moisture readings from the pots in Trial 3 (the shade tunnel) compared to the readings of those in Trial 2 (the Cycad nursery). The full day sun and lower grade shade cloth netting could contribute to higher temperatures within the shade tunnel, resulting in the soil drying out faster and maintaining lower soil moisture readings between watering. The large amount of tunnel space available and the open setting allowing for better air movement within the tunnel reduced the potential of cross contamination between fungal treatments in the pathogenicity trial. Trials 1, 2 and 3 were equally adequate in terms of location, each with different pros and cons, while Trial 4 was determined to be unsuitable for the pathogenicity trial due to the specific location and space available. When considering the climatic conditions offered by the shade tunnel compared to the other locations, the shade tunnel offered the most optimal conditions for the growth of the marama beans, and was therefore selected for the location of the pathogenicity trial.

Previous studies have indicated that the marama bean focuses the initial growth after germination on establishing the tuber and root system during the first vegetative growth period, before remaining dormant during the winter season when the plants die-back prior to the next vegetative growth period (Coetzer et al., 2011; Cole et al., 2014; DAFF, 2014). The development of a secure tuber and root system to survive the winter die-back period is essential for the survival of the marama bean, and is preferable when conducting a field or pot trial over an extended period of time, particularly a trial investigating the ability of the plant to survive the intentional introduction of known pathogens. The deep penetrating tap root allows the plant to access water deep below the surface in the root zone, sustaining the plant during drought or winter conditions (Cullis et al., 2018). Therefore, an adequate growing environment with sufficient space is necessary for marama bean development. Three pot sizes were used to determine the ideal pot size for growing the marama beans seedlings – 12.5 cm, 15 cm, and 20 cm. When comparing the seedling emergence for the different pot sizes at the conclusion of Trials 2 to 4, the 15 cm pots had the highest seed emergence rate of 60%, followed by the 20 cm pots with an emergence rate 50%, and finally the 12.5 cm pots with an emergence rate of 40%. However, the success of the specific pot size with regards to the seedling emergence rate could be attributed to the respective soil combination rather than the pot size, thus the seed emergence rate was an inconclusive method to determine ideal pot size for growing marama bean seedlings. When the seedlings were removed at random from the different pot sizes and soil combinations from trials 2 to 4, it was noted that the seedlings in the smaller pots had larger above ground development than below ground development compared to that of the 20 cm pot seedlings. The long tap roots curling around the base of the 20 cm pots offered an indication that the pot sizes were possibly still small for the marama beans, but more optimal than the 12,5 and 15 cm pots in which the seedlings developed shorter tap roots. The longer tap roots also indicate that the seedlings were able to grow in the pots as they would in the natural environment, without the pots hindering typical marama bean seedling growth. These observations were confirmed during the different collection trips executed to collect tuber and leaf material for Chapter 3. At the different collection sites, the plants and typically the seedlings with more extensive above ground development tended to have smaller tubers and root systems, compared to that of the plants with less extensively developed above ground material. The tubers in all cases developed in a long cylindrical manner varying in thickness, with long tap roots that extended deep into the ground, making the removal of the entire tuber and root near impossible. Based on these observations, as well as the seed emergence rates and the larger amount of space available within the pot for plant development, the 20 cm pots were selected for the pathogenicity trial. However, as mentioned previously, the success of the either scarification method, trial location or pot size cannot be directly attributed to specific method, location or size used, as the soil combination itself may have had a greater impact on seed emergence (Table 4.5).

The type of soil the plants were found in at the different locations from which plant material was collected for analysis also seemed to play a role in the tuber development – the plants in more densely compacted clay type soils tended to have thinner tubers that were closer to ground level, while plants in looser loam type soils tended to have thicker tubers that were buried deeper beneath ground level. The effect of the soil type on tuber development could be seen also in the soil trials – the soil trials containing clay topsoil that became denser and more compact with watering seemed to develop tubers that were smaller in diameter compared to those in the loose river sand and/or perlite trials that maintained the less compact structure with watering, enabling larger tubers with larger diameters to develop. However, the soil type did not seem to affect the tap root length in the wild populations that were visited during the collection trips. Regardless of the soil type, all of the plants appeared to have long tap roots that extended deep into the ground, with the only difference being the thickness of the root which was likely determined by the soil type. In light of this observation, it could be concluded that the larger issue regarding pot size is the depth of the pot rather than the diameter – shallower pots could inhibit the growth of the tap root, causing the seedling to stunt the tap root and tuber growth, developing a short and round tuber rather than a long cylindrical tuber. *Tylosema esculentum* found in the Kalahari typically grows in sandy soils which are considered low in quality due to the lack of organic matter and nutrients (Chimwamurombe, 2010; Coetzer et al., 2011; Mitchell et al., 2005; National Research Council, 2006). Thomas (2004) reported on the soil types in which marama beans were found in Botswana and Namibia, with "Brown fine aeolian sand" with or without "limestone/dolomite concretions" being prevalent in both countries. Ten *T. esculentum* seeds were planted in alluvial filled flower beds (sand, clay, gravel, silt and organic material deposited by rivers (Hosch, 2009)) during an experiment in Texas, USA in 1981 to propagate the legume with 100% germination rate in the USA (Powell, 1987). Takundwa (2014) propagated *T. esculentum* seeds in potting soil with a 33% germination rate. Travlos & Karamanos (2006) investigated the effect of different soil textures on marama bean vegetative growth using sand, clay, clay loam, and sandy clay loam soil types. Various leaf, stem and tuber measurements were used to determine which soil types would best suit marama bean growth, and concluded that clay and clay loam soils are unsuitable for vegetative growth, while sandy soils with good drainage are more ideal (Travlos & Karamanos, 2006). Based on the information available regarding the propagation of marama beans, various soil combinations were used to conduct the different soil trials in order to determine the ideal soil combination to grow the seedlings for the pathogenicity trial.

The soil combinations used in Trial 1 consisted of filtered sand, compost, loam, vermiculite and Hygromix in different ratios. Hygromix is commonly used in plant propagation due to its high water holding capacity, providing sufficient water to the plants (Mathowa et al., 2016). However, a high water holding capacity is not ideal for a plant such as the marama bean that thrives in drought-like conditions. Although filtered sand and vermiculite are all commonly used in plant propagation trials due to their high drainage abilities (Costa et al., 2015), the low emergence rate of the soil combinations containing filtered sand and/or vermiculite could be attributed to the high humidity in the greenhouse, waterlogging due to the presence of the drip trays, or the presence of high water retaining components such as loam soil and Hygromix. Trials 2, 3 and 4 consisted of river sand, perlite, compost, clay topsoil, loam topsoil in different combinations and ratios. The soil trials with a portion of clay or loam topsoil tended to have the lowest seed emergence rate, likely due to the high-water retention and soil moisture rates. Loam soil is composed of clay, silt and sand in combination, and tends to retain enough soil moisture for the plant to use, while still being able to drain effectively to prevent waterlogging (Boughton, 2022). In comparison, clay soil is composed of 25% clay, which reduces the drainage ability. Clay soil is slower to warm than loam soil and other finer grain soils, further reducing the drainage ability and increasing the amount of water retained after watering (Boughton, 2022). Clay soil also tends to compact and harden after watering, which may have an influence on the growth of the plant roots. While removing seedlings from Trials 1 to 4 to observe the below ground material development, many of the seeds within the clay or loam topsoil combinations that did not emerge had rotted before germinating, which may have been a result of the high soil moisture levels in those soil combinations. The results of Trial 5 confirmed the unsuitable nature of the clay and loam topsoil soil combinations, as all of the soil combinations with either clay or loam measured between seven and ten on the soil moisture meter one week after watering. If this type of soil was used for the pathogenicity trial, the watering schedule would have had to be amended to less than once a week. However, with the openness of the shade tunnel and rainfall as an uncontrollable variable, any clay or loam soil would likely have been waterlogged for the duration of the pathogenicity trial.

The soil combinations including river sand or perlite in Trials 2 to 5 proved to be the most effective combinations in terms of seedling emergence and soil drainage, alongside the perlite soil combinations (Table 4.5 and Figure 4.2). The river sand used for this study consisted of large particles, allowing for large spaces between the sand particles and subsequently a faster drainage rate, despite the presence of fine sand powder present due to the unwashed nature of the river sand. Nutrients cannot be retained by sand particles, largely due to the particle size and acting as a filter when watered (Larum, 2021). Perlite refers to volcanic rock with amorphous shape that is typically formed when obsidian is hydrated and rapidly heated, allowing the perlite particles to expand between four and 20 times their original size, creating the commonly known expended perlite used for agriculture and horticulture (Maxim et al., 2014). Due to the large particle sizes of the perlite, as well as the large pores created between the perlite particles, it is known for the high drainage capability, light weight, and soil aeration ability (Haasbroek, 2018). However, due to the structure and composition of perlite particles, nutrients cannot be retained by the perlite particles (Haasbroek, 2018). In general, due to the inability of river sand and perlite to retain nutrients, neither are commonly used in traditional crop propagation, but they have become invaluable soil components for the propagation of succulents and other drought-tolerant plants. The soil combinations of river sand only (Mix 3), river sand: perlite (Mix 7), and river sand: perlite: compost (Mix 9 and 10) were identified as the soil combinations of interest due to the presence of river sand and/or perlite for the drainage ability, as well as compost for the nutrient composition provided. After the seven-day trial, Mix 3 and 7 both had a final soil moisture reading of one, Mix 9 had a final reading of five, while Mix 10 had a final reading of two. Although soil Mix 3 and 7 presented the lower soil moisture readings after the seven-day trial, soil Mix 10 was selected for the pathogenicity trial due to the high ratio of river sand and perlite to compost, awarding the soil combinations a good drainage rate while providing nutrients through the compost portion.

Although the results of the various soil trials did not offer a direct or clear protocol for marama bean propagation (specifically regarding the scarification method, ideal pot size, ideal growing location, soil type), the results did offer enough information to form a guideline regarding which seed preparation and trial conditions would be more suitable for the pathogenicity trial for this study. Based on previous research regarding marama bean germination rates, nearly double the required number of marama seeds were used for germination in the pathogenicity trial, in order to ensure that a minimum of 126 seedlings were obtained. From the total 240 secateur scarified seeds that were planted in the selected 20 cm pots containing the river sand: perlite: compost (8:8:1) soil combination in the shade tunnel on Hillcrest campus, only 156 seeds emerged – indicating a 65% emergence rate. The low germination rate highlights the necessity of the soil and drainage trials performed to identify the most optimal growth conditions. Had any or all of the selected conditions been sub-optimal for marama bean germination or growth, the germination rate observed may have been much lower. The low germination rate also identifies a great challenge for commercialisation and farming of the marama bean with regards to the specific optimal growth and climatic conditions required by the marama bean for optimal yield production. More extensive agronomy studies are required in order to formulate effective production guidelines for future marama bean farming practices.

The 156 seedlings were grown and monitored over a three-month period prior to thinning out and transplanting the seedlings to ensure a single seedling per pot – due to the randomized planting of seeds, some pots two seedlings per pot, some pots a single seedling was found, and in other pots there were no seedlings. As a result, pots with two seedlings were randomly thinned to one seedling per pot, with many of the seedlings being transplanted into the empty pots to ensure the 126 individually planted seedlings requirement was met, while the additional seedlings were transplanted into the remaining six pots that were available. During the relocation of the seedlings, the remaining 85 ungerminated seeds were removed from their respective pots, and noted to have extensive fungal infection. These seeds were selected for multi-mycotoxin analysis to detect the presence of mycotoxins on or within the seeds, which would indicate the presence of mycotoxin producing fungal species, as well as adequate climatic conditions which are required for mycotoxin production (Ciegler, 1978; Fernández-Cruz et al., 2010). Multi-mycotoxin analysis of the dried and ground infected seed samples indicated the presence of ZEA and FB_1 in low but detectable quantities. The presence of mycotoxin producing fungal species and mycotoxins may have contributed to the low germination rate of the seedlings planted for the pathogenicity trial, highlighting another challenge for the commercialisation and farming of the marama bean (Ismaiel & Papenbrock, 2015). Due to the surface sterilization of each of the soil components within the soil combination selected, it is unlikely that the fungal infection would have occurred from the soil. The fungi may have been present on the seeds at the time of planting, or alternatively introduced to the seeds from the external environment, such as from the air or from the water used to imbibe the seeds. It could be theorized that the rate of infection by the fungal species had outcompeted the rate of seed germination in the case of the ungerminated seeds, whereas the rate of germination of the emerged seedlings may have outcompeted the fungal infection rate thereby allowing successful seedling emergence.

In order to determine the amount of time in weeks for each seedling in each fungal treatment to develop disease symptoms, focus is typically placed on the specific week when the treated seedlings develop symptoms and show an increase in disease rating. However, due to the wide range of symptoms presented by the various fungal species and the disease rating score system that was established, a calculative approach was taken to determine the amount of time for symptom development per fungal treatment. For Trials 1 and 2 and the combined results, the mean and median rating scores for Week 1 were calculated and average to obtain a minimum disease rating (MDR) threshold serving as an indicator for the amount of time taken for seedlings to develop symptoms in each fungal treatment or where a specific fungal treatment started to develop considerable symptom severity. After one week of the pathogenicity trial, the *A. alternata*, *Bipolaris* sp., *Didymella* sp., *F. fujikuroi* SC, *F. oxysporum* SC, *F. solani* SC, *L. pseudotheobromae*, and *N. parvum* treatments typically rated above the MDR for the respective trials. In contrast, the *B. dothidea*, *C. fructicola*, *D. rusicola*, *F. incarnatum-equiseti* SC, and *P. maculans* treatments typically rated below the MDR for the respective trials, indicating that considerable symptom severity only developed from Week 2 or onwards depending on the fungal treatment. Over the five- and six-week trial periods, the *L. pseudotheobromae*, *N. parvum* and *Bipolaris* sp. treatments tended to have significantly higher disease severity ratings compared to the other fungal treatments and the negative control, while the *P. maculans* and *F. fujikuroi* SC isolate 3 treatments tended to have significantly lower ratings. However, the combined Trial 1 and 2 results indicated that the *B. dothidea*, *D. rhusicola*, and *P. maculans* treatments, along with the *N. parvum* and *L. pseudotheobromae* treatments, experienced the greatest increase in disease severity over the five-week period, as well as the greatest potential exponential increase in disease rating after the five-week period. As such, the *D. rhusicola*, *Bipolaris* sp., *N. parvum*, *B. dothidea* and *L. pseudotheobromae* treatments had the highest disease ratings after the five-week period.

The most prevalent disease symptoms recorded were black leaf spots, leaf lesions, stem lesions and leaf discolouration, followed by vein lesions and leaf wilting with a lower prevalence. Seedlings with no symptoms or complete wilting of the above ground parts were the least prevalent observation groups. Black leaf spots are common symptoms among many pathogens, including *L. pseudotheobromae* and *N. parvum*, where infection by *L. pseudotheobromae* has been reported to cause leaf spots on the legume *Cynometra malaccensis* Meeuwen (Gomdola et al., 2020) and branch black spots, necrosis and withering on *Ormosia pinnata* (Lour.) Merr. (Li et al., 2020). *Lasiodiplodia pseudotheobroma* has also been reported to cause fruit rot on longan (*Dimocarpus longan*) during postharvest storage, shoot dieback and canker on apples in China, canker disease on Sacha inchi (*Plukenetia volubilis* L.), *Acacia confusa* Merr., *Paulownia fortunei* (Seem.) Hemsl., *Mangifera sylvatica* Roxb., *Albizia falcataria* (L.) Fosberg, and *Eucalyptus* spp., and lesions and necrosis on baobab trees (*Adansonia digitata* s.l.) (Pipattanapuckdee et al., 2019; Wang & Song, 2021; Xue et al., 2019; Zhao et al., 2010). Leaf spots are also common symptoms produced by *N. parvum* infection, as reported on *Ginkgo biloba* (Mirhosseini et al., 2014). Along with leaf spots, *N. parvum* is responsible for various other symptoms and diseases, including trunk diseases and canker formation on grapevines, and pod rot of *Theobroma cacao* L. (Massonnet et al., 2017; Puig et al., 2021). However, *N. parvum* has also been reported as an endophyte on *Dendrobium harveyanum* and *D. moschatum* (Ma et al., 2021). Leaf spots are also a common symptom developed due to infection by different *Bipolaris* species, among other symptoms such as leaf blight, root rot and foot rot, especially prevalent in field and grass crops such as maize (*Zea mays* L.), rice (*Oryza sativa* L.) and limpograss (*Hemarthria altissima* (Poir.) Stapf & C.E. Hubb.), a grass crop native to southern Africa (Manamgoda et al., 2014; Xue et al., 2016). Though dieback of plants and seedlings is a common symptom of many fungal pathogens, *B. dothidea* is a known causal agent of dieback of the legume *Cassia tora* Linn. (Huixiang et al., 2008). *Botryosphaeria dothidea* has also been reported to cause branch dieback and necrotic rot of olive fruit, necrosis on grapevine, canker formation on pistachio trees, canker disease of almonds, and canker and blight of walnut trees (Z. Ma et al., 2001; Moral et al., 2019; Phillips, 2002). Branch dieback, wood canker, shoot and fruit blight and necrotic leaf lesions are some examples of symptoms produced by infection by *D. rhusicola* and other *Diaporthe* species, as well as resulting from coinfection by *D. rhusicola* and *Neofusicoccum* species (Agustí-Brisach et al., 2019; Chen et al., 2014; Dissanayake et al., 2017; López-Moral et al., 2020; Luna et al., 2022).

Several of the aforementioned disease symptoms were also recorded on the negative control replicate seedlings. A possible explanation for the slow increase in disease symptoms and rating scores of the negative control seedlings over the five-week period could be natural occurrence of fungal species on or in the seeds at the time of planting the pathogenicity trial, which was observed with the ungerminated seeds covered in extensive fungal growth that were removed from the pots during the seedling transplanting stage. Due to the surface sterilization of each of the soil components within the soil combination selected, it is unlikely that fungal infection would have occurred from the soil. However, fungal presence is natural in any circumstance, particularly in fields. As such, fungal associations from the surrounding areas may have caused the symptoms observed on the negative control seedlings, as well as all of the other seedlings. The consistency of the symptoms between different seedlings per fungal trial indicate that the symptoms likely originated from the same source, in this case the fungal species intentionally inoculated on each seedling.

In order to confirm that the respective fungal species and isolates used to inoculate the seedlings in each treatment could be re-isolated after the pathogenicity trial, and was likely the cause of the disease symptoms observed on the respective seedlings, Koch’s postulates was employed (Segre, 2013). Segre (2013) reiterated that the four criteria of Koch’s postulates that need to be met are: “(1) The microorganism must be found in diseased but not healthy individuals; (2) The microorganism must be cultured from the diseased individual; (3) Inoculation of a healthy individual with the cultured microorganism must recapitulate the disease; and finally (4) The microorganism must be re-isolated from the inoculated, diseased individual and matched to the original microorganism”.

In line with the criteria necessary for Koch’s postulates, the fungal species used during the pathogenicity trial were found in and cultured from marama bean plant material; the fungal species were successfully used to inoculate healthy marama bean seedlings; and all of fungal species except the *C. fructicola* and *F. solani* SC isolates were successfully re-isolated and identified from the respective marama bean trials following the conclusion and harvesting of the pathogenicity trial. The confirmation of the presence of the fungal species in each respective fungal trial indicates that there is a strong possibility that the fungal species was the cause of the symptoms observed on the seedlings, therefore it can be deduced that the fungal species may be pathogenic to the marama bean. Regarding the *C. fructicola* and *F. solani* SC isolates that were not re-isolated from the seedlings, it is possible that the inoculated fungus was overgrown by saprophytes that were naturally associated with the seedling at the same time, or that the species did not successfully infect the leaves. Various factors may contribute to one species overgrowing another, such as the respective growth rates of each species on the plants and on the media used for isolation (Rand, 2004). Endophytic or saprophytic species may overgrow the inoculated species on the plant due to optimal conditions for the saprophyte or endophyte compared to the inoculated pathogen. This would result in the inoculated species causing disease on the plant, but difficulty in re-isolating the inoculated species. Similarly, on the media used for isolation, the inoculated species may have been isolated successfully, but outcompeted for nutrients by other isolated saprophytic or endophytic species with a higher growth rate. During the isolation stage, multiple additional fungal species were observed on the agar plates, the most notable being *Epicoccum*, *Alternaria* and *Cladosporium* species. These fungi are endophytic fungi that often survive in the plant's leaves without causing disease (Bamisile et al., 2018; Saikkonen et al., 1998; Zhang et al., 2006). *Epicoccum*, *Alternaria* and *Cladosporium* species were prevalent in all of the leaf samples collected from the various wild marama bean populations in Chapter 3, which coincides with the observations made from the seedling leaf isolates from the pathogenicity trial. Several *Epicoccum*, *Alternaria* and *Cladosporium* species have been reported to produce antifungal metabolites that could be used against plant pathogens (Bian et al., 2021; Fatima et al., 2016; Fávaro et al., 2012; Qader et al., 2021; Wang et al., 2013). Bian et al. (2021) reported that *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. mycelial growth was suppressed by *Epicoccum dendrobii* Q. Chen, Crous & L. Cai, while the metabolites produced by *E. dendrobii* decreased the biomass and inhibited *C. gloeosporioides* appressorium formation, conidial germination, and lesion development on apple fruit, Chinese fir needles (*Cunninghamia lanceolata* (Lamb.) Hook.), and poplar (*Populus* L.) leaves. *Epicoccum nigrum* Link metabolites were found to be effective in inhibiting growth of *Colletotrichum falcatum* Went, *Fusarium verticillioides* (Sacc.) Nirenberg, *Xanthomonas albilineans* (Ashby) Dowson and *Ceratocystis paradoxa* (De Seynes) C. Moreau (Fávaro et al., 2012). Qader et al. (2021) and Fatima et al. (2016) reported on the various metabolites produced by *E. nigrum* and *A. alternata* which may have potential as antifungal biocontrol mechanisms, while Wang et al. (2013) reported on various metabolites produced by *Cladosporium cladosporioides* that effectively inhibited the growth of several *Colletotrichum* and *Phomopsis* Sacc. & Roum. species. The presence and potential metabolite production by the various *Alternaria*, *Epicoccum* and *Cladosporium* species observed on the pathogenicity trial seedling leaves may have contributed to the inability to re-isolate the *F. solani* SC and *C. fructicola* species.

In line with the investigation into potentially pathogenic fungal species present on the marama bean, the presence of potentially mycotoxigenic fungal species was also determined. Multi-mycotoxin analysis of the dried and ground seedlings inoculated with potentially mycotoxigenic fungal species confirmed the presence of ZEA in the *A. alternata* isolate 1, *F. fujikuroi* SC isolate 2, and *F. oxysporum* SC isolate 1 treatments. The concentrations of mycotoxins detected were fairly low compared to the tolerable daily intake (TDI) levels of ZEA and FB_1 of 0.25 µg/kg body weight and 2.00 µg/kg body weight, respectively (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011; Joint FAO/WHO Expert Committee on Food Additives, 2017). Zearalenone is commonly produced by various *Fusarium* species and produces symptoms such as necrosis, chlorosis, root and shoot growth inhibition, and mortality (Ismaiel & Papenbrock, 2015). Fumonisins are commonly produced by various *Fusarium* species, as well as *A. alternata*, and produce symptoms such as necrosis, chlorosis, wilting, inhibition of seed emergence and root and shoot growth (Abbas & Boyette, 1992; Ismaiel & Papenbrock, 2015) in a wide variety of plants, including cowpeas and various legumes (Ismaiel & Papenbrock, 2015; Kritzinger et al., 2006). The presence of mycotoxins on the seeds and in the seedling samples is significant as it indicates the presence of mycotoxigenic fungal species, and adequate climatic conditions which are required for mycotoxin production (Ciegler, 1978; Fernández-Cruz et al., 2010). Salas et al. (2007) investigated the pathogenicity and mycotoxigenicity of various *Fusarium* species on barley *in vivo* and *in vitro*, and detected the production of various *Fusarium* mycotoxins in the plant material that had been inoculated with the *Fusarium* species *in vitro* and *in vivo*. Similarly, Harris et al. (2007) and Jestoi et al. (2008) were able to detect the production of various *Fusarium* mycotoxins in plant material from maize (Harris et al., 2007), and barley and wheat (Jestoi et al., 2008) that were inoculated with different *Fusarium* species *in vitro* and *in vivo*. Siciliano et al. (2015) investigated the potential of various *Alternaria* species isolated from cabbage, rocket and cauliflower, to produce mycotoxins *in vivo* and *in vitro* on the plant material inoculated with the *Alternaria* species. Most of the *Alternaria* isolates were able to produce at least one mycotoxin *in vitro*, with higher levels of tenuazonic acid generally being produced. However, it was observed that the ability of the fungal species to produce the mycotoxins *in vivo*, particularly tenuazonic acid, was lost (Siciliano et al., 2015). *In vivo* assays, as well as *in vitro* assays, that are used to determine the potential of fungal species to produce mycotoxins on plant material, can be used as a guidance tool and to aid in predicting the potential implications of mycotoxins on a large scale, such as under farming conditions.

It can be concluded that various fungal species have the potential to be both pathogenic and mycotoxigenic to marama beans under farming conditions, with the potential to cause diseases of varying severity, which could present a hurdle in the process of commercialising the legume as a crop. However, this does not diminish the potential of the marama bean to be commercialised. In the current agricultural world, there is not a single crop without a pathogen or disease. The key is understanding the plant and pathogen individually as well as the plant-pathogen interaction, and discovering how to protect, prevent, or manage the various pathogens and diseases associated with the plant. Future studies of the potential of the fungal species isolated from the marama bean to be pathogenic to the marama bean are recommended, in particular a larger study with more marama bean plants at various stages of development, as well as an extended trial period over the summer months or over several growing seasons. Investigating the mycotoxigenic potential of several fungal species can also be recommended, as well as investigating the conditions necessary for mycotoxin production *in vitro* and *in vivo*.

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Chapter 5 - General discussion, conclusions, and recommendations

Image 5. Marama bean seeds in their pod, collected from the wild population in Laudium, Centurion, South Africa. Photo by T. Armfield

5.1 General discussion, conclusion and recommendations

The marama bean is currently a locally important wild-harvested plant widely used by local communities in southern Africa and Namibia that has the potential to become an economically important crop (Cole et al., 2014). The marama bean has the potential to address malnutrition and hunger issues within many arid countries where many conventional and agricultural crops often cannot provide an adequate yield due to the climatic conditions and underdeveloped agricultural infrastructure in many arid countries (Cullis et al., 2018; Jackson, 2017; National Academy of Sciences, 1979). The potential of the marama bean to provide relief in these areas can be attributed to the high protein, oil and nutrient content, the potential health and medicinal benefits that the plants offer, as well as their ability to survive and thrive in arid areas where it is acclimatised to the environmental conditions (Cole et al., 2014; Jackson, 2017; National Academy of Sciences, 1979; Smýkal et al., 2018). These aspects could contribute to the potential value of the plant as a commercial crop in areas lacking crops and food sources with suitable nutrient content.

The environmental conditions required for growth, a general lack of information regarding the production requirements, as well as a lack of information regarding symbiotic associations, such as insect pests, and fungal and bacterial pathogens, are current constraints on marama bean production and commercialisation (Cullis et al., 2018; National Academy of Sciences, 1979; National Research Council, 2006). To date, there are no records of insect pests on the marama bean. Previous reports of bacterial associations indicate potential plant growth promoting (PGP) bacteria in the soil (Kandjimi et al., 2015), as well as seed-associated bacterial endophytes (Chimwamurombe et al., 2016). Reports on studies of fungal associations with *T. esculentum* performed in Namibia indicate the presence of several fungal species isolated from the pods and seeds, the leaves and as leaf endophytes (Chimwamurombe, 2016; Martin et al., 2012; Takundwa et al., 2015; Uzabakiriho et al., 2013). Desite the reports of fungal associations with the marama bean in Namibia, there have also been no reports of pathogens or diseases on the marama bean related to the fungal species previously isolated and identified. Furthermore, there have been no reports of either fungal species, pathogens or diseases on South African marama beans. In addition to the presence of various fungal species and their ability to cause diseases, comes the threat of fungal mycotoxin production. There are currently no reports of mycotoxin presence on marama beans. However, if the seeds are contaminated with mycotoxins, it raises health concerns for those communities that utilize the seeds for food and medicinal purposes.

The identification of fungal species and the presence or absence of mycotoxins on the seeds will form a crucial part of determining whether the marama is a suitable crop for commercial production and utilization, considering the potential of the plant. Therefore, the aims of this study were to investigate the mycoflora and mycotoxins associated with the marama bean in South Africa, and to investigate the potential pathogenicity of selected fungal species isolated from the plant.

An unexpectedly large diversity of 116 fungal species were molecularly identified and classified into 27 families. A large number of the fungal species identified included species that are known to produce various mycotoxins. Despite the extensive fungal presence on the plants, during the investigation of mycotoxins naturally occurring on the plant, it was found that there were no mycotoxins detected on any of the plant material analysed. The absence of mycotoxins naturally present on the marama bean plant material is likely the result of sub-optimal environmental conditions necessary for mycotoxin production. Mycotoxigenic fungi require a range of optimal environmental conditions such as temperature, moisture content and aeration to produce mycotoxins (Ciegler, 1978; Fernández-Cruz et al., 2010). The conditions in which the marama bean naturally grow may not be considered as optimal for mycotoxin production – marama beans tend to grow in areas with high temperatures and low rainfall, often remaining dry throughout most of the growing season (Ciegler, 1978; Mitchell et al., 2005).

Emphasis was placed on the *Aspergillus*, *Penicillium* and *Fusarium* genera due to the large number of mycotoxin producing species found within the three genera. Between the three genera, a total of 40 different species were identified. Of the 40 species identified, eighteen of the species were analysed for *in vitro* mycotoxin production. *Aspergillus sojae* Sakaguchi & K. Yamada ex Murak. and *A. sydowii* (Bainier & Sartory) Thom & Church produced high concentrations of AFB_1 (0.687 ppm) and all three fumonisins (0.1354 ppm FB_1, 0.1867 ppm FB_2 and 0.1363 ppm FB_3), respectively. *Fusarium chlamydosporum* Wollenw. & Reinking and *Penicillium onobense* C. Ramírez & A.T. Martínez produced AFB_1 (0.6131 ppm and 0.8504 ppm, respectively), while *Fusarium scirpi* Lambotte & Fautrey produced 11.5876 ppm FB_2. Fumonisin is expected to be produced by most *Fusarium* species as well as *Alternaria alternata* (Fr.) Keissler (Ismaiel & Papenbrock, 2015), however, it is uncommon to be produced by *Aspergillus* or *Penicillium* species. The ability of the various isolated species to produce mycotoxins *in vitro* indicates a potential for production *in vivo* under the favourable conditions. This may present a potential obstacle for plant production at a commercial level, however, the tendency of the marama bean to grow in such difficult conditions may prove to be the solution – maintaining the natural environmental conditions of the plant may reduce the possibility of mycotoxin production *in vivo*. At the conclusion of the pathogenicity trial, the seedling material harvested was analysed for mycotoxin production. Very low concentrations of zearalenone were detected in the seedling material inoculated with *A. alternata*, *F. fujikuroi* (Nirenberg) species complex (SC), and *F. oxysporum* Schltdl. SC. The presence of the mycotoxin, even in low concentrations, reinforces the problematic potential of several fungal species to produce mycotoxins *in vivo*. However, the specific environmental conditions in which mycotoxins would be produced *in vivo* in a farm-like or commercialised setting would need to be investigated before definite conclusions can be drawn.

While thinning out the seedlings for the pathogenicity, 85 ungerminated seeds were removed from the trial, whereupon it was observed that the seeds were infected by various fungal species, as noted by the various blue and green spores, as well as mycelial growth visible on the seed coats. These seeds were further processed for multi-mycotoxin analysis to determine whether the fungal infection had caused mycotoxin production on the seeds. The presence of zearalenone and fumonisin B_1 was detected in the seed material that was analysed, indicating that the fungal species present on the seeds at the time of harvesting had more favourable conditions for some time in order for the production of mycotoxins. The presence of mycotoxins associated with both the seedlings and ungerminated seeds highlights the need for further and more comprehensive mycotoxin production analysis.

Considering the 65% emergence rate, it could be assumed that the growth conditions such as the soil combination and location’s climatic conditions were suitable for marama bean propagation when compared to previous research (Takundwa, 2014; Travlos & Karamanos, 2006). Individual seed germination abilities could affect the germination rate, as well as several environmental conditions. However, the low germination rate could indicate a larger issue. The fungal infected seeds retrieved from the pathogenicity trial may form part of that issue, and also posed two new questions – were the fungal species already present in or on the seeds before planting, and could the fungal presence have caused an infection of the seeds, thus preventing germination of the seeds during the trial? Marama bean germination is already difficult on small scale due to the hard seed coat present, the added risk of infection due to fungal species present in or on the seeds highlights a greater challenge for marama bean commercialisation and subsequent farming.

While the large diversity of fungal species isolated from the marama bean indicated a low potential for mycotoxin production, it did indicate a higher potential for pathogenicity. Several of the fungal species previously isolated from the marama bean proved to be pathogenic to the marama bean seedlings that were propagated. Through the completion of the pathogenicity trial and Koch’s postulates, *Alternaria alternata*; *Fusarium fujikuroi* SC; *F. oxysporum* SC; *F. incarnatum-equiseti* SC; *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips; *Bipolaris* sp. Shoemaker; *Didymella* sp. Sacc.; *Botryosphaeria dothidea* (Moug.) Ces. & De Not.; *Pestalotiopsis maculans* (Corda) Nag Raj; *Diaporthe rhusicola* Crous; and *Lasiodiplodia pseudotheobromae* A.J.L. Phillips, A. Alves & Crous were confirmed to be pathogenic to the marama bean seedlings. Though the pathogenicity trial was prematurely concluded due to unfavourable growing conditions, the ability of these fungal species to cause disease symptoms of varying severity on the plants may be used as an indication of potential pathogens to be aware of in commercial cultivation. The potential of the eleven species to be pathogenic to the marama bean may be a concern, however, more extensive pathogenicity and field trials would need to be performed before a definite conclusion can be obtained regarding the number of species that are pathogenic to marama beans and severity of the diseases can be ascertained. It should also be noted that only 14 of the 161 species identified were analysed for pathogenicity potential. With the exception of the saprophytic fungal species, investigation into many of the remaining 120 isolated species should be considered, particularly the postharvest species that would affect the harvested marama bean yield. Along with more extensive research into the pathogenicity and mycotoxigenicity of the various fungal isolates, a more comprehensive identification of the fungal species should be considered, using a multigene molecular identification approach that will allow for a more accurate identification of the fungal species. Field trials with conditions similar to the conditions found in small holder farming scenarios may also be worth consideration, to determine the potential of the marama bean as a crop and to progress the marama bean towards commercialisation, as well as to investigate the mycoflora and mycotoxins associated with the marama bean during these field trials.

In conclusion, the identification of fungal species present on the marama bean seeds, leaves and tubers has provided valuable information regarding the potential plant diseases and possible mycotoxins that could be encountered on the seeds and tubers of the plant, a vital component of many rural community's staple diets. The determination of mycotoxins on the plant material, and the ability of the potentially mycotoxigenic fungi to produce mycotoxins *in vivo*, provides information regarding the potential hazards associated with consuming the marama bean seeds and tubers. This information can aid in the advancement of the marama bean from indigenous wild plant to a commercially valuable food crop produced in a variety of areas and communities for various benefits and uses, as well as allow the potential uses of the marama bean to be broadened to enable commercial production and utilization. The investigation of the symptoms and diseases caused by the isolated fungal species offers valuable information regarding the influence that the potential fungal pathogens could have on marama bean cultivation on a larger scale, providing insight into whether the pathogens and associated diseases could be a constraint on marama bean production that would impact the value of the marama bean as a food and commercial crop.

Continuing research on marama bean, and understanding it's cultivation and potential constraints can aid in the commercialisation of the crop, contributing to the goal of the Ten-Year Innovation Plan developed by the Department of Science and Technology (National Research Foundation - Republic of South Africa et al., 2015), stating that South Africa must become a biotechnology world leader through the use of expanding knowledge and indigenous plants with great potential, such as the marama bean. The United Nations (UN) Member States have all adopted the 2030 Agenda for Sustainable Development, an initiative including a list of 17 sustainable development goals (SDGs) aimed at achieving peace and prosperity for people as well as the planet for the present and the future generations. Research into the marama bean's potential as a food crop aligns with several of the UN SDGs, including addressing poverty and hunger in areas where the marama bean is a known but uncultivated food resource; promoting good health and well-being of the people of the community due to the high nutrient profile and medicinal properties of the marama bean seeds and tubers; promoting sustainable and responsible production and consumption of the plant without harming the environment with unsustainable production means; as well as providing education within the communities regarding sustainable farming and the benefits of researching locally grown wild plants for potential food crops (United Nations, 2015).

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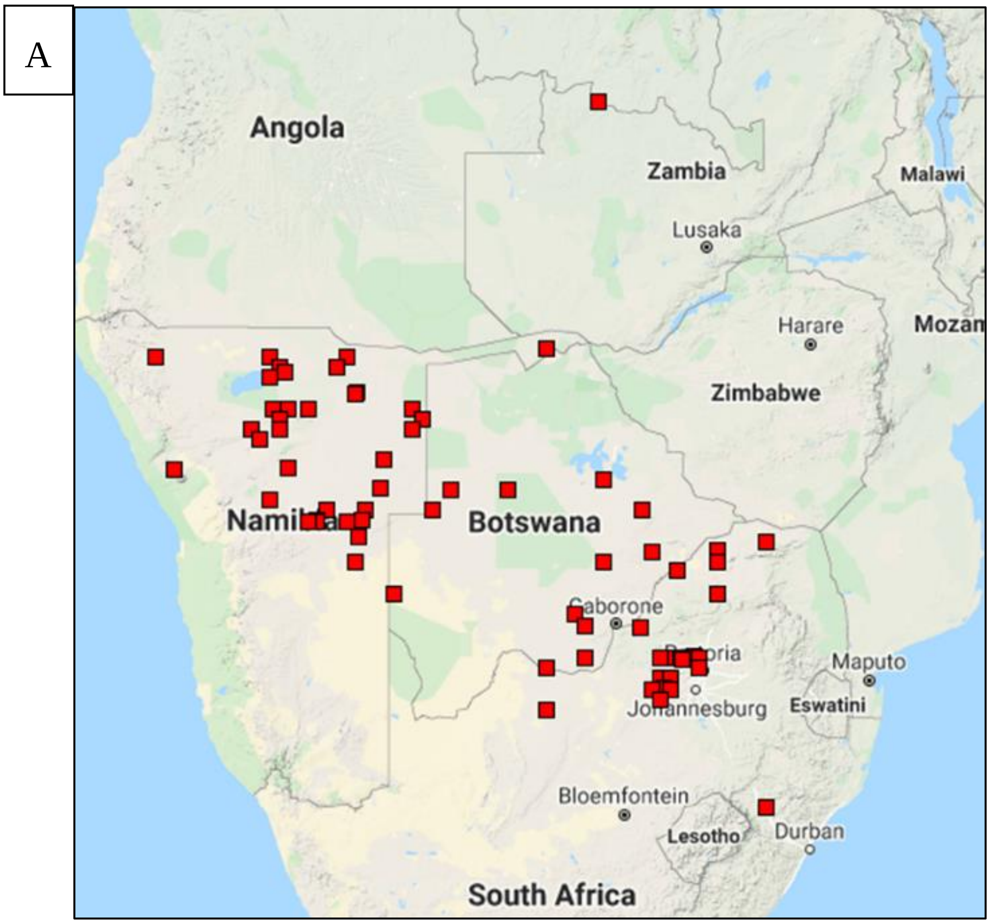
Chapter 6 - Appendices

Appendix 6.1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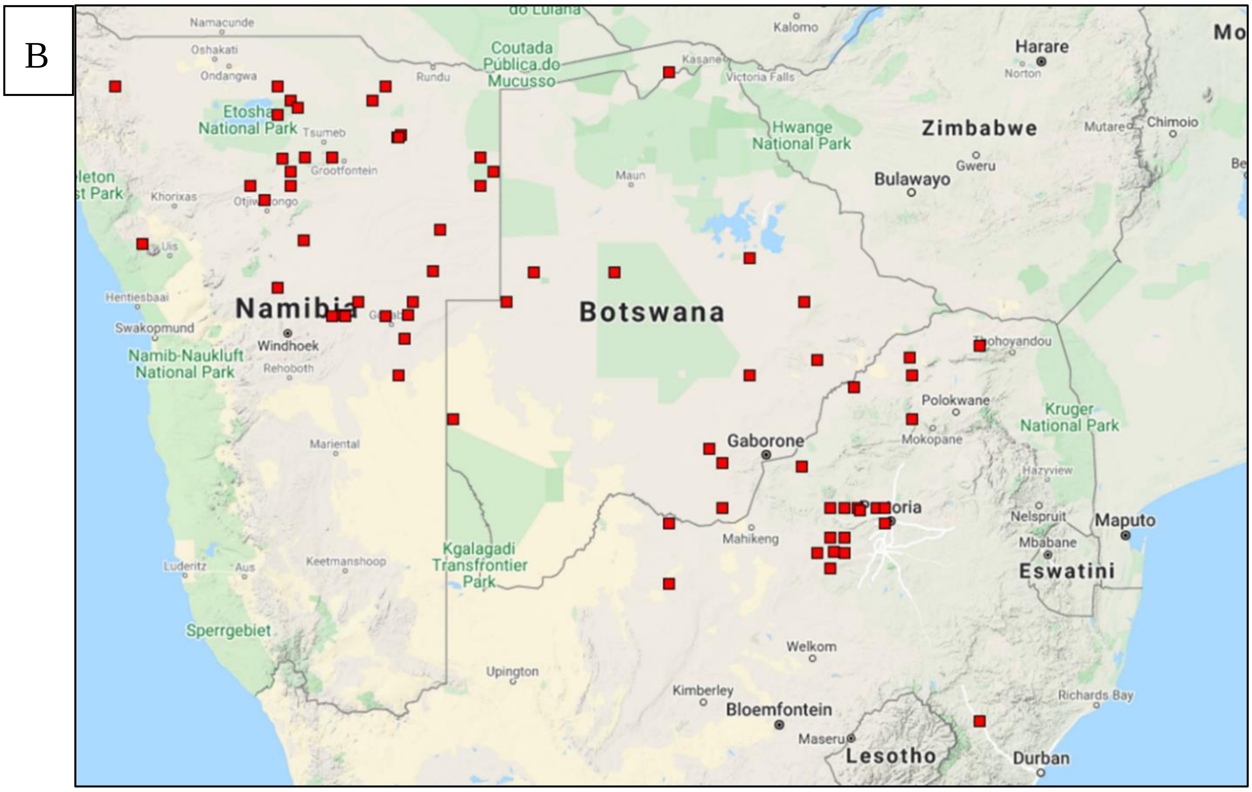
Eswatini
Bloemfontein
Lesotho
Durban
South Africa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Tsumeb
Hwange National Park
Zimbabwe
Mutare
Chimoio
Grootfontein
Gweru
Bulawayo
Maun
Khorixas
Otjiwarongo
Uis
Hentiesbaai
Swakopmund
Namibia
Windhoek
Botswana
Namib-Naukluft National Park
Rehoboth
Thohoyandou
Polokwane
Kruger National Park
Mokopane
Gaborone
Mariental
Hazyview
Nelspruit
Maputo
Mbabane
Mahikeng
Pretoria
Eswatini
Luderitz
Aus
Keetmanshoop
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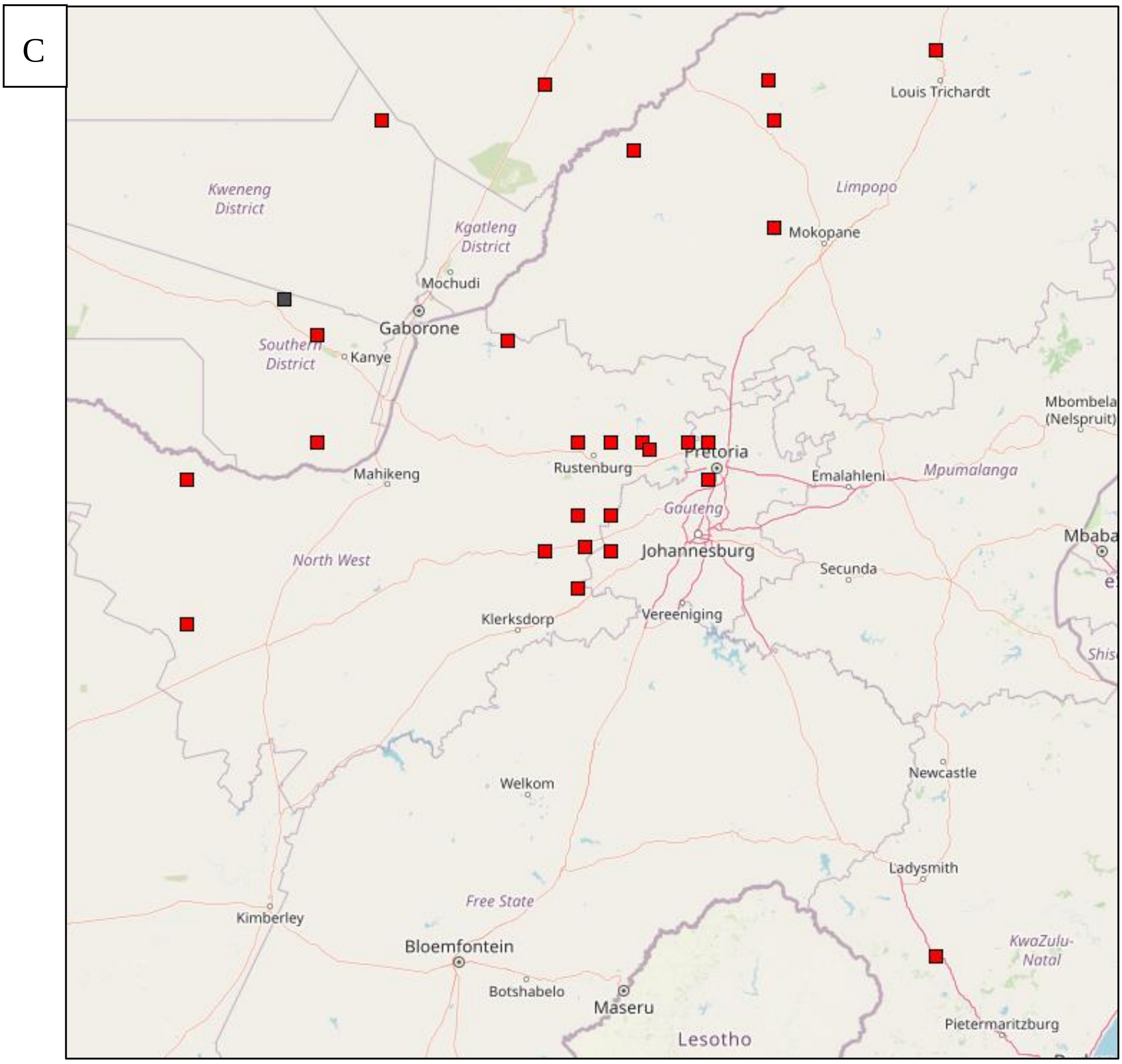

Figure 6.1. The distribution maps of *Tylosema esculentum* obtained from the Plants of Southern Africa (POSA) database, indicating the distribution of *T. esculentum* throughout Africa [A]; throughout Namibia, Botswana and South Africa [B]; and within South Africa, notably in the North West, Limpopo and Gauteng provinces [C]. The red squares indicate specimens which have not yet been checked for accuracy when uploading to the database, therefore some outlier data points may not be accurately recorded.

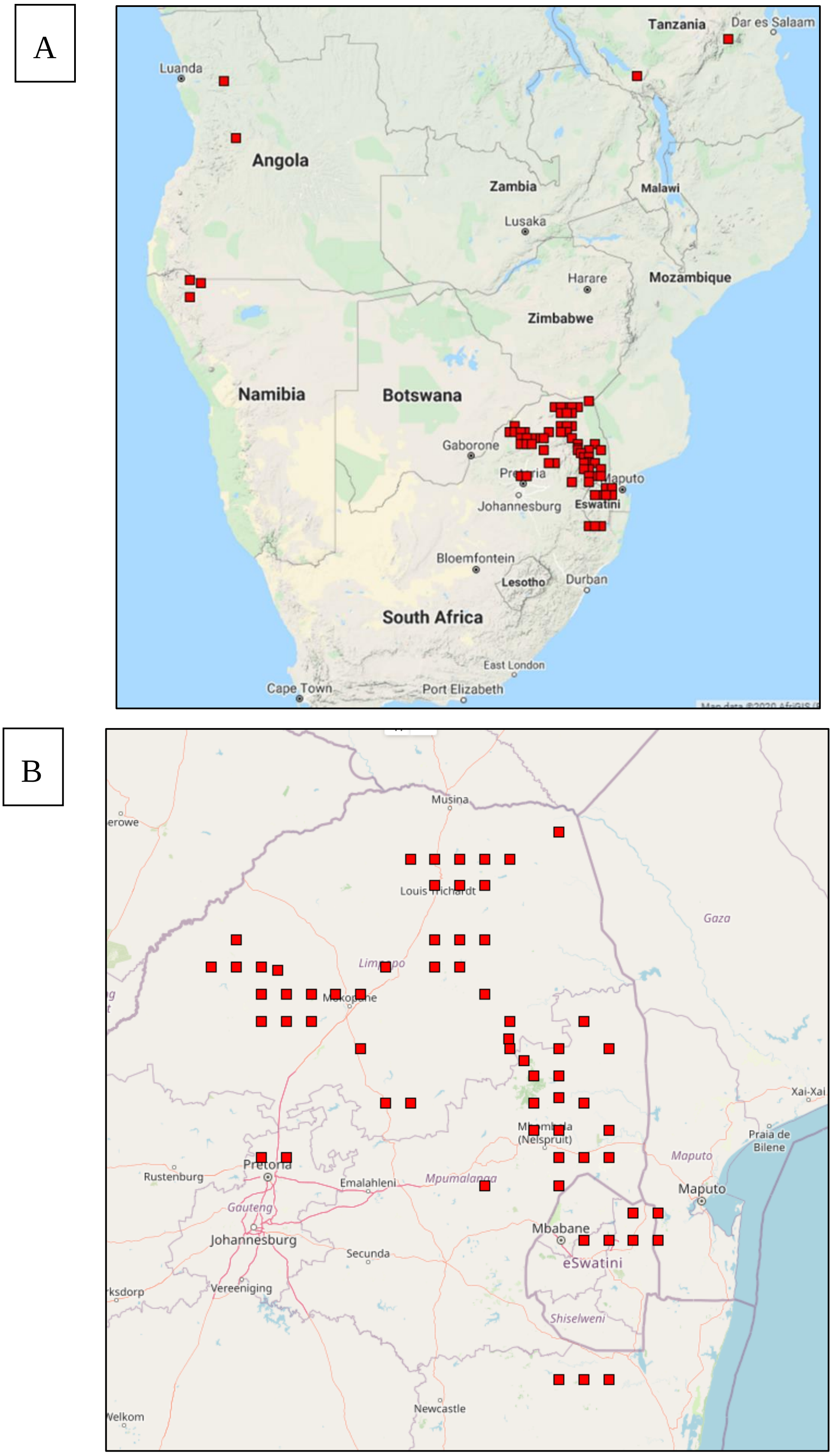

Figure 6.2. The distribution maps of *Tylosema fassoglense* obtained from the Plants of Southern Africa (POSA) database, indicating the distribution of *T. esculentum* throughout Africa [A]; and within South Africa, notably in the Mpumalanga, Limpopo and Gauteng provinces [B]. The red squares indicate specimens which have not yet been checked for accuracy when uploading to the database, therefore some outlier data points may not be accurately recorded.

Appendix 6.2

Table 6.1. A summary of the mycotoxins associated with various fungal isolates, including those previously isolated from the marama bean (*Tylosema esculentum*). The mycotoxin name, main fungal producers, common plant hosts where the mycotoxin or fungi have been isolated, toxicity to humans and animals, and phytotoxicity are indicated.

Mycotoxin	**Main fungal producers**	**Common plant host**	**Toxicity**	**Phytotoxicity**
Alternuene (ALT)	*Alternaria alternata**	Barley, wheat, oats, rice, tomatoes, sweet pepper and berries (Ostry, 2008)	Estrogenic activity (Martins et al., 2020).	Leaf necrosis (Siciliano et al., 2015).
Alternariol (AOH)	*Alternaria alternata** *Alternaria brassicae* (Berk.) Sacc. *Alternaria capsici-anui* Savul. & Sandu *Alternaria citri* Ell. & Pierce *Alternaria cucumerina* (Ell. & Ev.) Elliot *Alternaria dauci (*Kuhn) Groves & Skolko *Alternaria kikuchiana* Tanaka *Alternaria solani** *Alternaria tenuissima** *Alternaria tomato* (Cooke) Jones	Cereals and oilseeds (Sweeney & Dobson, 1998). Tomatoes, carrots, oilseeds, lentils, cereals, legumes, sunflowers and nuts (Escrivá et al., 2017). Barley, wheat, maize, rice, oats, millet, sorghum, soya beans, tomatoes, sweet pepper, apple, berries and nuts (Ostry, 2008)	Affect DNA by causing cell transformation and mutagenicity (Pinto et al., 2017). Food poisoning and fungal keratitis in humans (Troncoso-Rojas & Tiznado-Hernández, 2014). Estrogenic activity (Martins et al., 2020).	Leaf necrosis (Siciliano et al., 2015).
Alternariol monomethyl ether (AME),	*Alternaria solani** *Alternaria alternata** *Alternaria brassicae* *Alternaria capsici-anui*	(Pollock et al., 1982). Cereals (Sweeney & Dobson, 1998). Tomatoes, carrots, oilseeds, lentils, cereals, legumes,	Affect DNA by causing cell transformation and mutagenicity (Pinto et al., 2017).	Leaf necrosis (Siciliano et al., 2015).

	Alternaria citri *Alternaria cucumerina* *Alternaria dauci* *Alternaria kikuchiana* *Alternaria tenuissima** *Alternaria tomato* *Alternaria longipes* (Ell. & Ev.) *Alternaria porii* (Ell.) Cif.	sunflowers and nuts (Escrivá et al., 2017). Barley, wheat, maize, rice, oats, barley, millet, sorghum, soya beans, tomatoes, sweet pepper, apple, berries and nuts (Ostry, 2008)	Food poisoning and fungal keratitis in humans (Troncoso-Rojas & Tiznado-Hernández, 2014). Estrogenic activity (Martins et al., 2020).	
Altertoxins I, II and III (ATX)	*Alternaria tenuissima** *Alternaria alternata** *Alternaria cassiae* Jurair & A. Khan *Alternaria mali* Roberts *Alternaria radicina* Meier, Drechsler & Eddy	*Tribulus terrestris* (Bashyal et al., 2014). Wheat, rice, oats, barley, millet, sorghum, berries and nuts (Ostry, 2008)	Cause more accurate and toxic DNA mutations Estrogenic activity (Martins et al., 2020).	Leaf necrosis (Siciliano et al., 2015).
Chlamydosporol	*Fusarium chlamydosporum**	Rice (Savard et al., 1990). Chickpea, maize, sorghum, wheat, barley, rye, oat, onion, mulberry and *Trifolium pratense* (Visconti et al., 1994)	Appetite and weight loss, cytotoxic effects on cells (Abbas et al., 1992)	No information available.
Cyclopiazonic acid (CPA)	*Penicillium commune** *Penicillium aurantiogriseum* *Penicillium cyclopium* *Penicillium patulum* *Alternaria tenuissima** *Aspergillus flavus*	Acorn, barley, maize, Kodo millet (*Paspalum scrobiculatum*), Navy beans, pecan, peanuts, turnips, walnuts, wheat (Burdock & Flamm, 2000)	Necrosis of liver, gastrointestinal tissue, skeletal muscle and kidney tissues (Gqaleni et al., 1996).	Specific inhibitor of the Ca^{2+}-dependent ATPase and induces calcium leakage in cells, preventing calcium replenishment in cells. Cyclopiazonic acid also desensitizes photoreceptors, causing a decrease in photosynthesis (Dorlöchter et al., 1999).

	Aspergillus oryzae *Penicillium camemberti* *Penicillium urticae*			
Deoxynivalenol (DON) commonly known as vomitoxin	*Fusarium incarnatum** *Fusarium graminearum* Schwabe *Fusarium culmorum*	Wheat (Vesonder & Hesseltine, 1980). Maize (Marasas et al., 1977). Cereals (Sweeney & Dobson, 1998).	Toxic to swine and other domestic animals (Pestka, 2007). Reduced feeding, weight loss and eventually vomiting in animals (Bennett and Klich, 2003). Inhibition of DNA and protein synthesis resulting in decreased levels of cell proliferation (Minervini et al., 2004).	Effects include necrosis, chlorosis and mortality (Ismaiel & Papenbrock, 2015). Inhibits germination and root development, growth retardation, seedling regeneration inhibition (Ismaiel & Papenbrock, 2015).
Diacetoxyscirpenol (DAS) clinically known as anguidine	*Fusarium equiseti** *Fusarium chlamydosporum** *Fusarium graminearum* *Fusarium sambicinum* *Fusarium poae*	Barley, wheat and sorghum (Ahmed & Hasan, 1999). Field beans (Packa, 1991).	In pigs the effects of DAS included radiation-like poisoning in lymphoid and gastrointestinal epithelium tissues and endothelial necrosis (Coppock et al., 1985). Implicated in alimentary toxic aleukia (ALA) in man, which causes diarrhoea, skin and mucosa necrosis, and bone marrow degeneration (Weaver et al., 1978). Symptoms in other domestic animals include diarrhoea and hemorrhaging (Weaver et al., 1978).	Effects include necrosis, chlorosis and mortality (Ismaiel & Papenbrock, 2015). Reduced seedling viability, inhibition of root and shoot growth, and chromosome abnormalities (Ahmed & Hasan, 1999; Ismaiel & Papenbrock, 2015; Packa, 1991).
Fumonisin [specifically, Fumonisin B_1]	*Fusarium moniliforme* Sheldon *Fusarium proliferatum* (Matsushima) Nirenburg *Fusarium nygamai* Burgess and Trimboli	Maize (Doehlert et al., 1994). Sorghum and cereals (Sweeney & Dobson, 1998).	Affects horses, donkeys and mules with equine leukoencephalomalacia (Bennett and Klich, 2003), hydrothorax and pulmonary oedema	Necrosis and wilting, inhibition of seedling emergence, inhibition of root and shoot growth and biomass accumulation (Ismaiel & Papenbrock, 2015).

	*Fusarium incarnatum** *Alternaria alternata f.* sp. *lycopersici* *Fusarium verticilloides (Sacc.) Nirenberg*	Tomatoes, soybean, and other legumes (Ismaiel & Papenbrock, 2015). Cowpea (Kritzinger et al., 2006). Jimsonweed (Abbas & Boyette, 1992)	in pigs, and liver cancer in rats (Ismaiel & Papenbrock, 2015). Fumonisin B1 as a potential cause of oesophageal cancer in humans (Marasas, 1995).	Necrosis and chlorosis, as well as reduced height and biomass accumulation and death (Abbas & Boyette, 1992). Inhibition of radicle elongation (Doehlert et al., 1994).
Mycophenolic acid	*Penicillium brevicompactum** *Penicillium roqueforti* *Penicillium paxilli* *Penicillium olivicolor* *Penicillium canescens [Penicillium raciborskii]* *Penicillium viridicatum*	Ginger tissue extracts (Overy and Frisvad, 2005).	Mycophenolic acid has been shown to inhibit nucleic acid synthesis (Franklin & Cook, 1969). Generally considered as not toxic or barely toxic to humans (Vinokurova et al., 2005).	Considered to be phytotoxic, but useful as an antiviral, fungicidal and antibacterial agent (Vinokurova et al., 2005).
Nivalenol (NIV)	*Fusarium equiseti** *Fusarium chlamydosporum** *Fusarium graminearum* *Fusarium sporotrichoides* *Fusarium crookwellense* *Fusarium nivale*	Cereals (Sweeney & Dobson, 1998). Leguminous plants (Goswami et al., 2008).	Causes gastrointestinal distress in humans (Minervini et al., 2004). Inhibition of DNA and protein synthesis resulting in decreased levels of cell proliferation (Minervini et al., 2004).	General effects include necrosis, chlorosis and mortality (Ismaiel & Papenbrock, 2015). Reduced shoot and root growth (Ismaiel & Papenbrock, 2015).
Ochratoxin A (OTA)	*Aspergillus ochraceus* *Aspergillus carbonarius* *Aspergillus niger* *Penicillium verrucosum*	Maize, grape, peanut (Kumar et al., 2017). Cereals, coffee beans, cocoa beans, vine fruits, wine grapes, beer and peanuts (Astoreca et al., 2007)	Carcinogenic and nephrotoxic (Astoreca et al., 2007)	

Penitrem A	*Penicillium commune** *Penicillium palitans* *Penicillium crustosum* *Penicillium cyclopium* *Penicillium puberulum*	Cottonseed, wheat, maize, barley, oats, pecan (Wagener et al., 1980). Maize, apples and grains (Ciegler, 1978).	Neurotoxin and tremorgen (Wagener et al., 1980).	Inhibition of seed germination, seedling growth, radicle and coleoptile elongation (Rao et al., 2014).
Roquefortine	*Penicillium commune** *Penicillium olsonii** *Penicillium roqueforti*	Cottonseed (Wagener et al., 1980).	Paralytic neurotoxin (Wagener et al., 1980).	Inhibition of seed germination, seedling growth, radicle and coleoptile elongation (Rao et al., 2014).
Tenuazonic acid (TeA)	*Alternaria alternata** *Alternaria tenuissima** *Phoma sorghina** *Alternaria solani** *Magnaporthe grisea* *Alternaria kikuchiana* *Alternaria mali* *Alternaria oryzae* Hara *Alternaria lonipes* *Alternaria japonica* Yoshii	Cottonseed (Davis et al., 1977). Tomatoes (Visconti et al., 1987). *Datura innoxia* Mill. (Janardhanan & Husain, 1984). Rice (Ismaiel & Papenbrock, 2015). Cereals and oilseeds (Sweeney & Dobson, 1998).	TeA is very toxic to several domesticated animal species, often causing disorders such as haemorrhages, as well as inhibition of protein synthesis (Pinto et al., 2017; Martin et al., 2012). Cause of the Onyalai human hematologic disorder disease (Ismaiel & Papenbrock, 2015).	Non-specific phytotoxin on monocotyledonous and dicotyledonous plants. General effects included necrotic spotting and inhibition of germinating seed growth (Ismaiel & Papenbrock, 2015).
Zearalenone (ZEA) also known as F-2 toxin	*Giberella zeae [Fusarium graminearum]* (Schw.) Petch *Fusarium equiseti** *Fusarium culmorum* *Fusarium cerealis* *Fusarium crookwellense*	Cereals (Sweeney & Dobson, 1998). Leguminous plants (Goswami et al., 2008). Maize, oats, barley, wheat, millet, sorghum rice (Ismaiel & Papenbrock, 2015).	Toxic to swine, causing hyperestrogenism (Mirocha & Christensen, 1974). Suspected of causing infertility in dairy cows (Marasas et al., 1977).	Effects include necrosis, chlorosis and mortality (Ismaiel & Papenbrock, 2015). Inhibition of root and shoot growth and biomass accumulation (Ismaiel & Papenbrock, 2015).

	Fusarium semitectum
	Fusarium roseum

*Fungal species previously isolated from the marama bean

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Appendix 6.3

Protocol 1: Zymo Quick-DNA Fungal/Bacterial Miniprep Kit manufacturers instructions:

- For optimal performance, add beta-mercaptoethanol (user supplied) to the Genomic Lysis Buffer to a final dilution of 0.5%(v/v) ie. 500 µL per 100 mL

1. Add 50 – 100 mg (wet weight) fungal or bacterial cells that have been resuspended in up to 200 µL of water or isotonic buffer (eg. PBS) or up to 200 mg of tissue to a ZR BashingBead Lysis Tube (0.1 mm and 0.5 mm).
2. Add 750 µL Lysis Solution to the tube.
3. Secure in a bead beater fitted with a 2 mL tube holder assembly and process at maximum speed for ~5 min.
    a. Required processing time will vary depending on device and application and therefore should be evaluated on a case by case basis.
    b. For example, processing times may be as little as 3 minutes when using high-speed cell disruptors (eg. The portable TerraLyzer Sample Processor, FastPrep -24, or similar) or as long as 20 minutes when using lower speeds (eg. Disruptor Genie, or standard benchtop vortexes). See manufacturer’s literature for operating information.
4. Centrifuge the ZR BashingBead Lysis Tube in a microcentrifuge at 10 000 x g for 1 min.
5. Transfer up to 400 µL supernatant to a Zymo-Spin IV Spin Filter (Orange top) in a Collection Tube and centrifuge at 7000 x g for 1 min.
    a. Snap off the base of the Zymo-Spin IV Spin Filter prior to use.
6. Add 1,200 µL of Genomic Lysis Buffer to the filtrate in the Collection Tube from step 5.
7. Transfer 800 µL of the mixture from Step 6 to a Zymo-Spin IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 min.
8. Discard the flow through from the Collection Tube and repeat Step 7.
9. Add 200 µL DNA Pre-Wash Buffer to the Zymo-Spin IIC Column in a NEW Collection Tube and centrifuge at 10,000 x g for 1 min.
10. Add 500 µL g-DNA Wash Buffer to the Zymo-Spin IIC Column and centrifuge at 10,000 x g for 1 min.
11. Transfer the Zymo-Spin IIC Column to a clean 1.5 mL microcentrifuge tube and add 100 µL (35 µL minimum) DNA Elution Buffer directly to the matrix column. Centrifuge at 10,000 x g for 30 secs to elute the DNA.

Protocol 2: Polymerase Chain Reactions (PCR):

Taq Polymerase	6.25 µL
Forward primer	0.4 µL
Reverse primer	0.4 µL
Sterile distilled water	4.45 µL
Total reaction	11.5 µL

- Distribute 11,5 µL of Mastermix into each pcr tube and add 1 µL DNA of interest into respective tubes.

Cycling conditions:

ITS1F/4 and Bt2a/2b:

Cycles	**Step**	**Temperature (°C)**	**Time**
1	Initial denaturation	94	1 min
35	Denaturing	94	30 s
	Annealing	55	30 s
	Elongation	72	1 min
1	Final elongation	72	8 min
	Rest period	4	Infinity

EF1/2:

Cycles	**Step**	**Temperature (°C)**	**Time**
1	Initial denaturation	95	3 min
35	Denaturing	95	30 s
	Annealing	51	30 s
	Elongation	72	1 min
1	Final elongation	72	7 min
	Rest period	4	Infinity

Protocol 3: PCR cleanup:

ExoSAP	3 µL	37°C	15 min
PCR product (DNA)	7 µL	80°C	20 min

Protocol 4: Cycle sequencing reactions:

BigDye reagent	0.5 µL
Primer – Forward or Reverse	1 µL
Sequencing buffer	2.1 µL
ddH2O	6.4 µL

- Distribute 10 µL of Mastermix into each pcr tube and add 2 µL clean PCR product into respective tubes.

Cycling conditions:

ITS1F/4 and Bt2a/2b:

Cycles	**Step**	**Temperature (°C)**	**Time**
1	Initial denaturation	94	1 min
25	Denaturing	94	30 s
	Annealing	55	30 s
	Elongation	72	1 min
	Rest period	4	Infinity

EF1/2:

Cycles	**Step**	**Temperature (°C)**	**Time**
1	Initial denaturation	95	3 min
25	Denaturing	95	30 s
	Annealing	51	30 s
	Elongation	72	1 min
	Rest period	4	Infinity

Protocol 5: Sodium acetate / ethanol precipitation:

1. In each 0,5mL sequencing tube:

12 uL	Sequencing PCR product
2 uL	NaOAc
16 uL	100% EtOH (add last)

2. Centrifuge at 13 200 / 14 000 rpm for 30 min
3. Discard supernatant
4. Add 250 uL of ice cold 70% EtOH (immediately add EtOH after removing supernatant)
5. Centrifuge at 13 200 rpm for 5 min
6. Discard supernatant and repeat wash step [4 – 5]
7. Dry samples at 90°C for 3 min with lids open OR dry overnight at room temp with lids open

Appendix 6.4

Table 6.2. The fungal isolates of the *Aspergillus*, *Penicillium* and *Fusarium* genera isolated from marama bean plant material and molecularly identified using the ITS region and either the *BenA* or *TEFα* gene regions as a secondary gene region.

Isolate	**Species identity inferred by study**	**Location**	**Tissue type**	**ITS region**			***BenA* region**		
				Species identity	**% Similarity**	**Accession number**	**Species identity**	**% Similarity**	**Accession number**
A1	*Aspergillus sojae*	Sample 5	Seed	*Aspergillus krugeri*	100.00	MK450654	*Aspergillus sojae*	100.00	HQ285598
A2	*Aspergillus sojae*	Sample 3	Seed	*Aspergillus transmontanensis*	100.00	MK450657	*Aspergillus sojae*	100.00	HQ285598
A3	*Aspergillus japonicus*	Sample 5	Seed	*Aspergillus niger*	100.00	MT541880	*Aspergillus japonicus*	100.00	OL711724
A4	*Aspergillus japonicus*	Sample 5	Seed	*Aspergillus niger*	100.00	MT541880	*Aspergillus japonicus*	100.00	OL711724
A5	*Aspergillus sydowii*	Sample 1	Seed	*Aspergillus sydowii*	100.00	MT582755	*Aspergillus sydowii*	100.00	MG832163
A6	*Aspergillus udagawae*	Sample 4	Seed	*Aspergillus udagawae*	99.80	KY808744	*Aspergillus udagawae*	99.44	KY808584
A7	*Aspergillus sojae*	Sample 2	Seed	*Aspergillus transmontanensis*	99.83	MK450657	*Aspergillus sojae*	100.00	HQ285598
A8	*Aspergillus welwitchiae*	Sample 4	Seed	*Aspergillus niger*	100.00	MT628904	*Aspergillus welwitschiae*	100.00	MK451014
A9	*Aspergillus welwitchiae*	Sample 5	Seed	*Aspergillus welwitschiae*	100.00	MH374611	*Aspergillus awamori*	100.00	MG832179
A10	*Aspergillus awamori*	Sample 4	Seed	*Aspergillus niger*	100.00	MT582749	*Aspergillus awamori*	100.00	MG832179
A11	*Aspergillus welwitchiae*	Sample 4	Seed	*Aspergillus welwitschiae*	100.00	ON231673	*Aspergillus awamori*	100.00	MG832179
A12	*Aspergillus pseudodeflectus*	Sample 7	Tuber	*Aspergillus ustus*	100.00	MH865327	*Aspergillus niger*	100.00	HQ632688
A13	*Aspergillus welwitchiae*	Sample 7	Tuber	*Aspergillus niger*	100.00	KF589312	*Aspergillus piperis*	100.00	MN882790
A14	*Aspergillus luchuensis*	Sample 7	Tuber	*Aspergillus piperis*	99.81	MT588789	*Aspergillus nidulans*	100.00	AB524358
A15	*Aspergillus nidulans* var. *dentatus*	Sample 7	Tuber	*Aspergillus nidulans*	100.00	MH864362	*Aspergillus nidulans*	100.00	AB524358
A16	*Aspergillus quadralineatus*	Sample 7	Tuber	*Aspergillus quadrilineatus*	100.00	MK734102	*Aspergillus quadrilineatus*	100.00	OL625674
A17	*Aspergillus nidulans*	Sample 7	Tuber	*Aspergillus nidulans*	100.00	MH864362	*Aspergillus nidulans*	99.74	MG991346
A18	*Aspergillus nidulans*	Sample 7	Tuber	*Aspergillus nidulans*	100.00	MH864362	*Aspergillus nidulans*	100.00	MG991346
A19	*Aspergillus alabamensis*	Sample 3	Tuber	*Aspergillus alabamensis*	100.00	KP987071	*Aspergillus alabamensis*	98.71	JQ286488
A20	*Aspergillus magaliesburgensis*	Sample 3	Tuber	*Aspergillus magaliesburgensis*	100.00	MK450649	*Aspergillus magaliesburgensis*	97.87	MK451116

A21	*Aspergillus ochraceus*	Sample 3	Tuber	*Aspergillus ostianus*	100.00	MT446137	*Aspergillus ochraceus*	100.00	MG832175
A22	*Aspergillus udagawae*	Sample 3	Tuber	*Aspergillus udagawae*	100.00	KY808744	*Aspergillus udagawae*	97.12*	MK451259
A23	*Aspergillus udagawae*	Sample 3	Tuber	*Aspergillus udagawae*	100.00	KY808744	*Aspergillus udagawae*	95.71*	MK451259
A24	*Aspergillus nidulans* var. *dentatus*	Sample 7	Leaves	*Aspergillus nidulans*	100.00	MT316339	*Aspergillus nidulans*	99.74	MG991346
P1	*Penicillium chrysogenum*	Sample 2	Seed	*Penicillium chrysogenum*	100.00	MH865988	*Penicillium chrysogenum*	100.00	JX996933
P2	*Penicillium rubens*	Sample 6	Seed	*Penicillium chrysogenum*	99.81	KU847860	*Penicillium rubens*	100.00*	JX996931
P3	*Penicillium momoi*	Sample 6	Seed	*Penicillium rubefaciens*	100.00	LT558907	*Penicillium rubefaciens*	98.22	KJ834487
P4	*Penicillium caperatum*	Sample 5	Seed	*Penicillium caperatum*	100.00	MK450677	*Penicillium caperatum*	100.00	MK451278
P5	*Penicillium* cf. *onobense*	Sample 3	Seed	*Penicillium brasilianum*	99.59	MH865784	*Penicillium onobense*	97.52	MK451076
P6	*Penicillium* cf. *onobense*	Sample 3	Seed	*Penicillium brasilianum*	99.60	MH865784	*Penicillium onobense*	97.95	MN380965
P7	*Penicillium brevicompactum*	Sample 4	Seed	*Penicillium brevicompactum*	100.00	MN636238	*Penicillium brevicompactum*	100.00	MK451071
P8	*Penicillium brevicompactum*	Sample 6	Seed	*Penicillium brevicompactum*	100.00	MH857204	*Penicillium brevicompactum*	100.00	KT779540
P9	*Penicillium citrinum*	Sample 6	Seed	*Penicillium citrinum*	100.00	MT582768	*Penicillium citrinum*	100.00	GU944550
P10	*Penicillium brevicompactum*	Sample 6	Seed	*Penicillium brevicompactum*	100.00	MK036520	*Penicillium brevicompactum*	100.00	AY674435
P11	*Penicillium brevicompactum*	Sample 6	Seed	*Penicillium brevicompactum*	100.00	MN636238	*Penicillium brevicompactum*	100.00	MK451071
P12	*Penicillium* cf. *onobense*	Sample 3	Seed	*Penicillium brasilianum*	99.62	MH865784	*Penicillium onobense*	97.52	MK451076
P13	*Penicillium brevicompactum*	Sample 5	Seed	*Penicillium brevicompactum*	99.79	MK036520	*Penicillium brevicompactum*	99.79	EU128586
P14	*Penicillium rubens*	Sample 3	Seed	*Penicillium rubens*	100.00	MN944726	*Penicillium rubens*	100.00	JX996931
P15	*Penicillium raperi*	Sample 7	Tuber	*Penicillium raperi*	99.83	MK450712	*Penicillium raperi*	99.19	MK451243
P16	*Penicillium annulatum*	Sample 7	Tuber	*Penicillium rolfsii*	99.77	KY263599	*Penicillium annulatum* (T_)	99.79	JX091514
P17	*Penicillium cluniae*	Sample 7	Tuber	*Penicillium* sp. *'cluniae'*	100.00	MK450684	*Penicillium* sp. *'cluniae'*	100.00	MK451006
P18	*Penicillium ortum*	Sample 3	Tuber	*Penicillium ortum*	100.00	MK450708	*Penicillium ortum*	100.00	MK451132
P19	*Penicillium abidjanum*	Sample 3	Tuber	*Penicillium longicatenatum*	99.50	MK450722	*Penicillium longicatenatum*	92.23*	MK450967
P20	*Penicillium caperatum*	Sample 3	Tuber	*Penicillium caperatum*	100.00	MK450677	*Penicillium caperatum*	100.00	MK451245
P21	*Penicillium* cf. *striatisporum*	Sample 6	Tuber	*Penicillium striatisporum*	99.49	MH859205	*Penicillium striatisporum*	98.10	MN969401

P22	*Penicillium raperi*	Sample 6	Leaves	*Penicillium raperi* (T_)	99.83	MK450712	*Penicillium raperi* (T_)	99.15	GU981622
Isolate	**Species identity inferred by study**	**Location**	**Tissue type**	**ITS region**			**TEFα region**		
				Species identity	**% Similarity**	**Accession number**	**Species identity**	**% Similarity**	**Accession number**
F1	FFSC[a]	Sample 3	Seed	*Fusarium proliferatum*	100.00	MT466521	*Fusarium echinatum*	99.23	MW834273
F2	FCSC[b]	Sample 3	Seed	*Fusarium chlamydosporum*	100.00	MG250446	*Fusarium* sp., *chlamydosporum*	100.00	MH582448
F3	FCSC	Sample 3	Seed	*Fusarium chlamydosporum*	100.00	MG250446	*Fusarium* sp., *chlamydosporum*	100.00	MH582448
F4	*Fusarium lacertarum*	Sample 6	Seed	*Fusarium equiseti*	100.00	MH857318	*Fusarium lacertarum*	99.66	GQ505593
F5	FFSC	Sample 3	Seed	*Fusarium proliferatum*	100.00	MT560212	*Fusarium echinatum*	99.05	MW834273
F6	*Fusarium solani*	Sample 3	Seed	*Fusarium solani*	100.00	MT560378	*Fusarium solani*	99.67	DQ246851
F7	*Fusarium chlamydosporum*	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	98.76	MH854820	*Fusarium* sp., *chlamydosporum*	100.00	MH582447
F8	*Fusarium chlamydosporum*	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	99.62	MH854820	*Fusarium* sp., *chlamydosporum*	100.00	MH582447
F9	*Fusarium scirpi*	Sample 3	Seed	*Fusarium equiseti*	100.00	MT560337	*Fusarium scirpi*	100.00	GQ505592
F10	FOSC[c]	Sample 3	Seed	*Fusarium oxysporum*	100.00	MH864510	*Fusarium oxysporum*	99.62	FJ985418
F11	FCSC	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	100.00	MH859028	*Fusarium* sp., *chlamydosporum*	99.17	MH582448
F12	FFSC	Sample 3	Seed	*Fusarium proliferatum*	100.00	MT466521	*Fusarium echinatum*	99.06	MW834273
F13	FSAMSC[d]	Sample 3	Seed	*Fusarium brachygibbosum*	100.00	JF776653	*Fusarium* sp., *sambucinum*	93.12*	MH582307
F14	FIESC[e]	Sample 3	Seed	*Fusarium flagelliforme*	100.00	MZ890500	*Fusarium* sp., *incarnatum-equiseti*	99.81	MH582433
F15	FFSC	Sample 3	Seed	*Fusarium proliferatum*	100.00	MT466521	*Fusarium echinatum*	98.53	MW834273
F16	FFSC	Sample 3	Seed	*Fusarium proliferatum*	100.00	MT466521	*Fusarium echinatum*	99.13	MW834273
F17	FCSC	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	100.00	MH859028	*Fusarium* sp., *chlamydosporum*	99.17	MH582448
F18	FCSC	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	100.00	MH859028	*Fusarium* sp., *chlamydosporum*	99.08	MH582448
F19	FIESC	Sample 3	Seed	*Fusarium equiseti*	100.00	MT560337	*Fusarium* sp., *incarnatum-equiseti*	99.46	MH582433

F20	FIESC	Sample 3	Seed	*Fusarium equiseti*	100.00	MT560337	*Fusarium* sp., *incarnatum-equiseti*	99.82	MH582434
F21	FCSC	Sample 3	Seed	*Fusarium chlamydosporum*	100.00	MG250446	*Fusarium* sp., *chlamydosporum*	100.00	MH582448
F22	FIESC	Sample 3	Seed	*Fusarium equiseti*	100.00	MT558601	*Fusarium lacertarum*	99.44	GQ505593
F23	FCSC	Sample 3	Seed	*Fusarium chlamydosporum*	100.00	MG250446	*Fusarium* sp., *chlamydosporum*	99.08	MH582448
F24	FIESC	Sample 6	Seed	*Fusarium equiseti*	100.00	MT560337	*Fusarium* sp., *incarnatum-equiseti*	99.44	MH582433
F25	FOSC	Sample 3	Seed	*Fusarium oxysporum*	100.00	MZ890536	*Fusarium oxysporum*	99.64	FJ985418
F26	FCSC	Sample 3	Seed	*Fusarium chlamydosporum* var. *chlamydosporum*	100.00	MH859028	*Fusarium* sp., *chlamydosporum*	99.08	MH582448
F27	FSAMSC	Sample 3	Seed	*Fusarium brachygibbosum*	99.82	MG575493	*Fusarium brachygibbosum*	95.04	GQ505418
F28	FIESC	Sample 6	Seed	*Fusarium equiseti*	99.82	MT560375	*Fusarium lacertarum*	99.35	GQ505593
F29	FIESC	Sample 6	Seed	*Fusarium equiseti*	100.00	MT560323	*Fusarium lacertarum*	99.33	GQ505593
F30	*Fusarium scirpi*	Sample 3	Seed	*Fusarium equiseti*	100.00	MT560323	*Fusarium scirpi*	100.00	GQ505592
F31	FIESC	Sample 6	Seed	*Fusarium equiseti*	100.00	MT560337	*Fusarium* sp., *incarnatum-equiseti*	99.64	MH582434
F32	FSAMSC	Sample 8	Seed	*Fusarium brachygibbosum*	99.82	ON181983	*Fusarium* sp., *sambucinum*	93.43	MH582307
F33	*Fusarium oxysporum*	Sample 7	Tuber	*Fusarium oxysporum*	100.00	MT560381	*Fusarium oxysporum*	100.00	MH582354
F34	FOSC	Sample 7	Tuber	*Fusarium oxysporum*	100.00	KT211520	*Fusarium oxysporum* f. sp. *dianthi*	100.00	FJ985348
F35	FSSC^{f}	Sample 7	Tuber	*Fusarium falciforme*	100.00*	MT251175	*Fusarium falciforme*	99.84	DQ246906
F36	FSSC	Sample 7	Tuber	*Fusarium falciforme*	100.00	MT251175	*Fusarium falciforme*	100.00	DQ246959
F37	*Fusarium oxysporum*	Sample 7	Tuber	*Fusarium oxysporum*	99.82	MH864510	*Fusarium oxysporum*	99.46	MH582351
F38	*Fusarium solani*	Sample 7	Tuber	*Fusarium solani*	99.83	KT313633	*Fusarium solani*	99.84	DQ247118
F39	*Fusarium solani*	Sample 7	Tuber	*Fusarium solani*	100.00	KT313633	*Fusarium solani*	99.69	DQ246851
F40	*Fusarium solani*	Sample 7	Tuber	*Fusarium solani*	99.79	MZ890569	*Fusarium solani*	100.00	DQ246982
F41	*Fusarium solani*	Sample 7	Tuber	*Fusarium solani*	100.00	GU170639	*Fusarium solani*	99.69	DQ246851
F42	FOSC	Sample 7	Tuber	*Fusarium oxysporum*	100.00	MZ890536	*Fusarium oxysporum* f. sp. *lini*	100.00	FJ985344
F43	*Fusarium solani*	Sample 7	Tuber	*Fusarium solani*	100.00	KT313633	*Fusarium solani*	99.84	DQ246982

F44	FSSC	Sample 7	Tuber	*Fusarium falciforme*	100.00	MT251175	*Fusarium falciforme*	99.81	DQ247088
F45	FOSC	Sample 7	Tuber	*Fusarium oxysporum*	100.00	MH864510	*Fusarium oxysporum* f. sp. *lini*	100.00	FJ985344
F46	FSSC	Sample 7	Tuber	*Fusarium solani*	100.00	EF488413	*Fusarium metavorans*	99.69	DQ790484
F47	*Fusarium citri*	Sample 6	Leaves	*Fusarium citri*	99.64	P372	*Fusarium citri*	98.16	JF740715
F48	FIESC	Sample 5	Leaves	*Fusarium lateritium*	99.65	LC171689	*Fusarium* sp., *tricinctum*	94.07	MH582380

*Molecular identity has a low probability score (E value)

**Results obtained using NCBI BLAST search, Genbank and MycoBank databases.

[a] *Fusarium fujikuroi* species complex (FFSC)

[b] *Fusarium chlamydosporum* species complex (FCSC)

[c] *Fusarium oxysporum* species complex (FOSC)

[d] *Fusarium sambucinum* species complex (FSAMSC)

[e] *Fusarium incarnatum-equiseti* species complex (FIESC)

[f] *Fusarium solani* species complex (FSSC)

Appendix 6.5

Table 6.3. The complete list of reference strains used to obtain the *Aspergillus*, *Fusarium* and *Penicillium* phylogenetic trees, including the ITS, *BenA* and *TEFα* gene accession numbers.

Phylogenetics name	**Section**	**Host**	**Country**	**Accession numbers**	
				ITS	***BenA***
*Aspergillus ochraceus*_CECT:2969	*Circumdata*			FM956457	FM995528
*Aspergillus ochraceus*_T_CBS108.08	*Circumdata*	Unknown	Unknown	EF661419	EF661322
*Aspergillus ostianus*_T_CBS103.07	*Circumdati*			EF661421	EF661324
*Aspergillus alliaceus*_NRRL4181	*Flavi*			EF661556	AY160978
*Aspergillus alliaceus*_T_CBS536.65	*Flavi*	Dead blister beetle	USA	EF661551	EF661465
*Aspergillus magaliesburgensis*_T_CMV007A3	*Flavi*	Antlion	South Africa	MK450649	MK451116
*Aspergillus oryzae*_KCCM60241	*Flavi*	Rice wine	Korea	HQ285609	HQ285590
*Aspergillus oryzae*_T_CBS102.07	*Flavi*	Unknown	Unknown	EF661560	EF661483
*Aspergillus sojae*_CBS134.52	*Flavi*			MH856961	-----
*Aspergillus sojae*_KCCM60354	*Flavi*	Rice wine	Korea	HQ285620	HQ285598
*Aspergillus sojae*_T_CBS100928	*Flavi*	Koji of soy sauce	Japan	KJ175434	EF203168
*Aspergillus aureolus*_CBS137453	*Fumigati*			KY808743	KY808583
*Aspergillus aureolus*_NRRL20643	*Fumigati*			EF669945	EU014109
*Aspergillus aureolus*_NRRL2391	*Fumigati*			EF669952	EF669810
*Aspergillus aureolus*_T_CBS105.55	*Fumigati*	Soil	Ghana	EF669950	EF669808
*Aspergillus udagaweae*_CBS142231	*Fumigati*			KY808752	KY808590
*Aspergillus udagaweae*_CBSDTO308-H6	*Fumigati*			KY808746	KY808586
*Aspergillus udagaweae*_T_CBS114217	*Fumigati*	Soil	Brazil	AB250781	AF132226
*Aspergillus udagaweae*_CMV010I9	*Fumigati*	Mealy bug on citrus	South Africa	-	MK451179
*Aspergillus latus*_CBS236.65	*Nidulantes*		South Africa	MH858552	-----
*Aspergillus latus*_DTO047-H2	*Nidulantes*			-----	KU866810
*Aspergillus latus*_IFM54207	*Nidulantes*			AB248971	AB248313
*Aspergillus latus*_T_CBS492.65	*Nidulantes*			KF465768	AB248334

Aspergillus nidulans var. *dentatus*_CBS114.63	*Nidulantes*			-----	AY573552
Aspergillus nidulans var. *dentatus*_IFM42001	*Nidulantes*			AB248995	AB248337
Aspergillus nidulans var. *dentatus*_T_IFM42028	*Nidulantes*			AB248965	AB248307
*Aspergillus nidulans*_CBS240.90	*Nidulantes*	Unknown	Netherlands	KU866629	KU866868
*Aspergillus nidulans*_CMXY2059	*Nidulantes*			MG991576	MG991346
*Aspergillus nidulans*_DTO322-H9	*Nidulantes*	Moldy bamboo	China	KU866638	KU866873
*Aspergillus nidulans*_NRRL32573	*Nidulantes*			EF652458	EF652282
*Aspergillus nidulans*_T_CBS589.65	*Nidulantes*	Unknown	Belgium	EF652427	EF652251
*Aspergillus quadrilineatus*_CBS113684	*Nidulantes*		India	KU866607	KU866850
*Aspergillus quadrilineatus*_T_CBS591.65	*Nidulantes*	Soil	USA	EF652433	EF652257
*Aspergillus striatus*_T_CBS283.67	*Nidulantes*			EF652470	EF652294
*Aspergillus sydowii*_CMXY18899	*Nidulantes*			MG991624	MG991339
*Aspergillus sydowii*_NRRL250	*Nidulantes*			AY373868	LC589352
*Aspergillus sydowii*_NRRL4768	*Nidulantes*			EF652473	JN853935
*Aspergillus sydowii*_T_CBS593.65	*Nidulantes*	Unknown	Unknown	EF652450	EF652274
*Aspergillus aculeatus*_ATHUM5028	*Nigri*			EU982028	EU982087
*Aspergillus aculeatus*_CMV007C9	*Nigri*	Soil	South Africa	MK450627	MK451118
*Aspergillus aculeatus*_T_CBS172.66	*Nigri*			FJ629320	FJ629271
*Aspergillus aculeatus*_T_NRRL5094	*Nigri*			AAU65309	LC589337
*Aspergillus awamori*_MUT<ITA>:2099	*Nigri*	*Holoturia poli*	Tunisia	-----	MG832179
*Aspergillus awamori*_NBRC4115	*Nigri*			LC573563	LC573621
*Aspergillus awamori*_NRRL4760	*Nigri*			KF288141	KF288131
*Aspergillus awamori*_NRRL4948	*Nigri*			KF288139	KF288129
*Aspergillus awamori*_T_CBS557.65	*Nigri*	Grapes	Italy	AM087614	AY820001
Aspergillus foetidus var. *acidus*_CBS121.28	*Nigri*			FJ491683	FJ491690
Aspergillus foetidus var. *acidus*_NBRC4122	*Nigri*			AB573936	AB574107
Aspergillus foetidus var. *acidus*_NRRL4750	*Nigri*			KF288145	KF288135
Aspergillus foetidus var. *acidus*_NRRL4784	*Nigri*			KF288143	KF288133
Aspergillus foetidus var. *acidus*_T_CBS564.65	*Nigri*	Unknown substratum	Japan	FJ629329	FJ629280
*Aspergillus japonicus*_NRRL4839	*Nigri*			EF661219	EF661081

*Aspergillus japonicus*_T_CBS114.51	*Nigri*	Saito	Unknown	AJ279985	HE577804
*Aspergillus luchuensis*_5Si	*Nigri*	Complementary food	Nigeria	-----	MH063939
*Aspergillus luchuensis*_NBRC4308	*Nigri*			LC573595	LC573652
*Aspergillus luchuensis*_NBRC6086	*Nigri*			LC573599	LC573655
*Aspergillus luchuensis*_T_CBS205.80	*Nigri*	Unknown	Unknown	JX500081	JX500062
*Aspergillus niger*_CBS113.50	*Nigri*			FJ629339	FJ629290
*Aspergillus niger*_DTO391-A1	*Nigri*	Cocoa	*Nigeria*	MN788114	MN787912
*Aspergillus niger*_NBRC4043	*Nigri*			LC573610	LC573666
*Aspergillus niger*_T_CBS554.65	*Nigri*	Tannin-gallic acid fermentation	USA	EF661186	EF661089
Aspergillus sp._CMXY5154	*Nigri*			MG991584	MG991402
*Aspergillus welwitschiae*_CMV005D2	*Nigri*	Soil	South Africa	MK450667	MK451040
*Aspergillus welwitschiae*_CMV005H5	*Nigri*	*Allium sativum*	South Africa	MK450668	MK451061
*Aspergillus welwitschiae*_DTO:180-A9	*Nigri*	Indoor hourse dust	South Africa	KJ775591	KP329865
*Aspergillus welwitschiae*_T_CBS139.54	*Nigri*	*Welwitschia mirabilis*	Namibia	FJ629340	FJ629291
*Aspergillus alabamensis*_CMV003A6	*Terrei*	Hominy chop animal feed	South Africa	-	MK450947
*Aspergillus alabamensis*_NRRL29810	*Terrei*			EF669589	EF669522
*Aspergillus alabamensis*_T_CBS125693	*Terrei*			KP987071	KP987049
*Aspergillus alabamensis*_UAB20	*Terrei*	Wound	USA	FJ531193	FJ491731
Aspergillus cf *alabamensis*_CBS142981	*Terrei*			LT798912	LT798945
*Aspergillus pseudodeflectus*_CBS596.65	*Usti*			EF591742	EF591732
*Aspergillus pseudodeflectus*_CMV005I2	*Usti*	*Colophospermum mopane*	South Africa	MK450643	MK451065
*Aspergillus pseudodeflectus*_NRRL278	*Usti*			EF652456	EF652280
*Aspergillus pseudodeflectus*_T_CBS756.74	*Usti*	Desert soil	Egypt	EF652507	EF652331
*Penicillium annulatum*_CBS135123	*Aspergilloides_Rolfsiorum*			JX091423	JX091516
*Penicillium annulatum*_CBS135124	*Aspergilloides_Rolfsiorum*			JX091424	JX091517
*Penicillium annulatum*_CBS135125	*Aspergilloides_Rolfsiorum*			JX091425	JX091515
*Penicillium annulatum*_T_CBS135126	*Aspergilloides_Rolfsiorum*			JX091426	JX091514
Penicillium novae *zeelandiae*_T_CBS137.41*	*Atroveneta*		New Zealand	MH856089	MN969390
*Penicillium brevicompactum*_CMV012C1	*Brevicompacta_Brevicompacta*	Salami	South Africa	-----	MK451242

*Penicillium brevicompactum*_CV0144	*Brevicompacta_Brevicompacta*			JX091532	JX091532
*Penicillium brevicompactum*_DTO236C3	*Brevicompacta_Brevicompacta*			KJ775097	KJ775097
*Penicillium brevicompactum*_MUT5193	*Brevicompacta_Brevicompacta*	*Padina pavonica*	Italy	-----	KT779540
*Penicillium brevicompactum*_NRRL28139	*Brevicompacta_Brevicompacta*			DQ645795	DQ645795
*Penicillium brevicompactum*_T_CBS257.29	*Brevicompacta_Brevicompacta*	Unknown	Belgium	AY484912	AY674437
*Penicillium canescens*_T_CBS300.48*	*Canescentia*	Soil	England	AF033493	JX140946
*Penicillium chrysogenum*_CBS131527	*Chrysogena_Chrysogena*			JX997059	JX996921
*Penicillium chrysogenum*_CBS132209	*Chrysogena_Chrysogena*			JX996988	JF909943
*Penicillium chrysogenum*_CBS776.95	*Chrysogena_Chrysogena*	Lechuguilla cave	USA	JX997114	JX996933
*Penicillium chrysogenum*_T_CBS306.48	*Chrysogena_Chrysogena*	Unknown	USA	AF033465	JF909955
*Penicillium rubens*_CBS401.92	*Chrysogena_Chrysogena*	Gypsum, building materials	Netherlands	JX996931	JX996931
*Penicillium rubens*_CV0361	*Chrysogena_Chrysogena*			JX091412	JX091547
*Penicillium rubens*_CV0378	*Chrysogena_Chrysogena*			JX091413	JX091548
*Penicillium rubens*_T_CBS129667	*Chrysogena_Chrysogena*	Unknown	Unknown	JX997057	JF909949
*Penicillium tardochrysogenum*_T_CBS132200	*Chrysogena_Chrysogena*			JX996898	JX996898
*Penicillium citrinum*_CBS122397	*Citrina_Citrina*			GU944565	GU944548
*Penicillium citrinum*_CV0480	*Citrina_Citrina*			JX140860	JX141012
*Penicillium citrinum*_CV0506	*Citrina_Citrina*			JX140861	JX141013
*Penicillium citrinum*_T_CBS139.45	*Citrina_Citrina*	Unknown	Unknown	AF033422	GU944545
*Penicillium corylophilum*_T_CBS312.48	*Exilicaulis_Corylophila*	Unknown	Unknown	AF033450	JX141042
*Penicillium momoi*_CN002D5	*Exilicaulis_Corylophila*			GBxxxxxx	GBxxxxxx
*Penicillium momoi*_CN002D6	*Exilicaulis_Corylophila*			GBxxxxxx	GBxxxxxx
*Penicillium momoi*_DTO179F3	*Exilicaulis_Corylophila*			KJ775661	KJ775154
*Penicillium momoi*_T_CBS139157	*Exilicaulis_Corylophila*			JX140895	JX141073
*Penicillium rubefaciens*_T_CBS145.83	*Exilicaulis_Corylophila*	Sandy soil under pine tree	Spain	KC411677	KJ834487
*Penicillium erubescens*_T_CBS318.67	*Exilicaulis_Erubescentia*	Nursery soil	South Africa	AF033464	HQ646566
*Penicillium striatisporum*_T_CBS705.68	*Exilicaulis_Erubescentia*	*Acacia karroo*, leaf litter	South Africa	AF038938	MN969401
*Penicillium abidjanum*_T_CBS246.67	*Lanata-Divaricata_Dalearum*	Savannah soil	Ivory Coast	GU981650	GU981650
Penicillium sp. nov *abidjanum*_PPRI10607	*Lanata-Divaricata_Dalearum*			MK450722	MK451261
*Penicillium brefaldianum*_T_CBS235.81	*Lanata-Divaricata_Janthinella*	Unknown	Unknown	AF033435	GU981623

*Penicillium caperatum*_PPRI25802	*Lanata-Divaricata_Janthinella*	Soil	South Africa	MK450678	MK451245
*Penicillium caperatum*_PPRI25895	*Lanata-Divaricata_Janthinella*	Wood in mine	South Africa	MK951937	MK451278
*Penicillium caperatum*_PPRI6261	*Lanata-Divaricata_Janthinella*			MK450677	MK450916
*Penicillium caperatum*_T_CBS443.75	*Lanata-Divaricata_Janthinella*	Soil	Papau New Guinea	KC411761	GU981660
*Penicillium cluniae*_PPRI25773	*Lanata-Divaricata_Janthinella*			MK450683	MK451006
*Penicillium cluniae*_PPRI25774	*Lanata-Divaricata_Janthinella*			MK450684	MK451041
*Penicillium cluniae*_T_CBS326.89	*Lanata-Divaricata_Janthinella*			KF296406	MN969376
*Penicillium janthinellum*_T_CBS340.48	*Lanata-Divaricata_Janthinella*	Soil	Nicaragua	GU981585	GU981625
*Penicillium ortum*_CBS135667	*Lanata-Divaricata_Janthinella*			JX091430	JX091519
*Penicillium ortum*_CBS135668	*Lanata-Divaricata_Janthinella*			JX091429	JX091518
*Penicillium ortum*_T_CBS135669	*Lanata-Divaricata_Janthinella*	Soil	South Africa	JX091427	JX091520
*Penicillium raperi*_PPRI25889	*Lanata-Divaricata_Janthinella*	Soil	South Africa	MK450715	MK451243
*Penicillium raperi*_PPRI9606	*Lanata-Divaricata_Janthinella*			MK450712	MK451105
*Penicillium raperi*_PPRI9611	*Lanata-Divaricata_Janthinella*			MK450713	MK451106
*Penicillium raperi*_T_CBS281.58	*Lanata-Divaricata_Janthinella*	Soil	United Kingdom	AF033433	GU981622
*Penicillium reticulisporum*_T_CBS122.68	*Lanata-Divaricata_Janthinella*	Soil	Japan	AF033437	MN969394
*Penicillium ochrochloron*_T_CBS357.48	*Lanata-Divaricata_Rolfsiorum*	Copper sulfate solution	USA	GU981604	GU981672
*Penicillium brasilianum*_T_CBS253.55	*Lanata-Divaricata_Simplicissima*	Bat	Brazil	GU981577	GU981629
Penicillium cf *onobense*_PPRI4067	*Lanata-Divaricata_Simplicissima*	Soil	South Africa	MK450706	MK451076
*Penicillium cremeogriseum*_T_CBS223.66	*Lanata-Divaricata_Simplicissima*	Forest soil	Ukraine	GU981624	GU981624
*Penicillium daleae*_T_CBS211.28	*Lanata-Divaricata_Simplicissima*	Soil	Poland	GU981649	GU981649
*Penicillium onobense*_CCDCA11372	*Lanata-Divaricata_Simplicissima*	Soil	Brazil	-----	MN380965
*Penicillium onobense*_CN014H3	*Lanata-Divaricata_Simplicissima*		-----	MT957436	MT957436
*Penicillium onobense*_T_CBS174.81	*Lanata-Divaricata_Simplicissima*	Soil	Spain	GU981575	GU981627
*Penicillium ortum*_CMV007H9	*Lanata-Divaricata_Simplicissima*	*Aspalathus linearis*	South Africa	MK451132	MK451132
*Penicillium simplicissimum*_T_CBS372.48	*Lanata-Divaricata_Simplicissima*	Flannel bag	South Africa	GU981588	GU981632

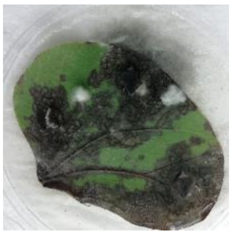
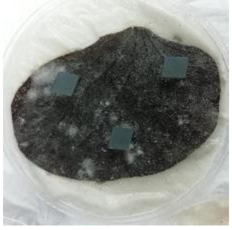
Phylogenetics name	**Section**	**Host**	**Country**	**ITS**	***TEFα***
*Fusarium illudens*_NRRL22090*	*Dimerum*	*Beilschmiedia tawa*	New Zealand	AF178393	AF178326
*Fusarium plagianthi*_NRRL22632*	*Dimerum*	*Hoheria glabrata*	New Zealand	AF178417	AF178354
Fusarium oxysporum f. sp. *dianthi*_NRRL36356	*Elegans*	Unknown	Argentina	MH856497	FJ985348

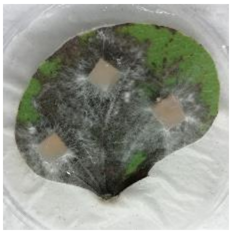
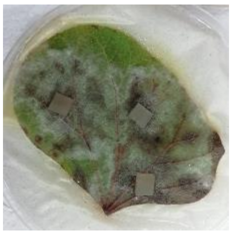
Fusarium oxysporum f. sp. *lini*_NRRL36286	*Elegans*	*Linum usitatissium*	Unknown	MH855399	FJ985344
Fusarium oxysporum f.sp. *hoodiae*_CBS132475	*Elegans*	Root of *Hoodia gordonii*	South Africa	KR071660	KR071771
*Fusarium oxysporum*_CBS130301	*Elegans*	Human	USA	MH865885	MH485017
*Fusarium oxysporum*_CBS18132	*Elegans*	Potato	USA	MH855270	MH484958
*Fusarium oxysporum*_MRC2325	*Elegans*	Carrot	USA	-	MH582351
*Fusarium oxysporum*_NRRL38885	*Elegans*	Unknown	USA	-	FJ985418
*Fusarium oxysporum*_T_CBS144134	*Elegans*	Potato	Germany	-	MH485044
*Fusarium brachygibbosum*_CBS131252	*Fusarium* (formerly *Discolor*)	Wheat	Iran	JX162372	JX118981
*Fusarium brachygibbosum*_NRRL34033	*Fusarium* (formerly *Discolor*)	Human foot cellulites	USA	GQ505450	GQ505418
Fusarium sambucinum SC_MRC2568	*Fusarium* (formerly *Discolor*)			-	MH582308
*Fusarium sambucinum*_MRC2486	*Fusarium* (formerly *Discolor*)	Peanuts	USA	-	MH582307
*Fusarium citri*_NRRL25084	*Gibbosum*	*Adelphocoris*	Austria	JF740883	JF740715
*Fusarium equiseti*_NRRL20697	*Gibbosum*	Beet	Chile	GQ505683	GQ505594
*Fusarium equiseti*_NRRL36466	*Gibbosum*	Potato peel	Denmark	GQ505742	GQ505653
*Fusarium equiseti*_T_NRRL26419	*Gibbosum*	Soil	Germany	GQ505688	GQ505599
*Fusarium flagelliforme*_NRRL26921	*Gibbosum*	Wheat	Germany	GQ505689	GQ505600
*Fusarium flagelliforme*_NRRL31011	*Gibbosum*	*Thuja* sp.	Germany	GQ505695	GQ505606
*Fusarium incarnatum*_NRRL32866	*Gibbosum*	Human	USA	GQ505704	GQ505615
*Fusarium incarnatum*_T_CBS13273	*Gibbosum*	*Trichosanthes dioica*	Malawi	-	MN170476
Fusarium incarnatum-equiseti SC_MRC2435	*Gibbosum*	Unknown	Unknown	MH582458	MH582434
Fusarium incarnatum-equiseti SC_MRC2558	*Gibbosum*	Soy bean	Japan	MH582455	MH582431
Fusarium incarnatum-equiseti SC_MRC2559	*Gibbosum*	Soy bean	Japan	MH582457	MH582433
Fusarium incarnatum-equiseti SC_NRRL34036	*Gibbosum*	Human ethmoid sinus	USA	GQ505451	MH582380
Fusarium incarnatum-equiseti SC_PPRI12549	*Gibbosum*	Soil	South Africa	-	KY586234
*Fusarium lacertarum*_MRC2609	*Gibbosum*			MH582459	MH582435
*Fusarium lacertarum*_NRRL20423	*Gibbosum*	Lizard skin	India	GQ505682	GQ505593
*Fusarium lacertarum*_NRRL36123	*Gibbosum*			GQ505732	GQ505643
*Fusarium lacertarum*_NRRL52753	*Gibbosum*			JF740923	JF740828
*Fusarium scirpi*_NRRL13402	*Gibbosum*	Pine soil	Australia	GQ505681	GQ505592
*Fusarium fujikuroi*_CBS26254	*Liseola*	Oryza sativa	India	KR071670	KR071744

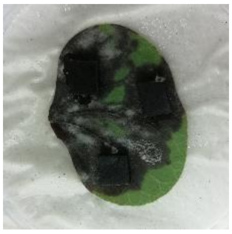
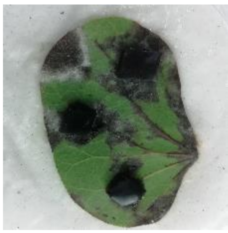
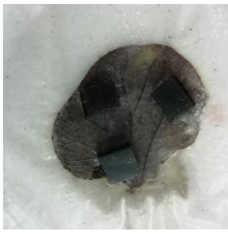
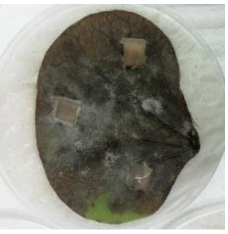
*Fusarium fujikuroi*_NRRL43470	*Liseola*	Unknown	Illinois, USA	DQ790538	DQ790494
*Fusarium fujikuroi*_T_CBS22176	*Liseola*	Oryza sativa	China Taiwan	NR111889	AB725605
*Fusarium lactis*_NRRL31630	*Liseola*	*Capsicum annuum*	South Africa	-	FR870289
*Fusarium nygamai*_CBS120995	*Liseola*	*Sorghum* sp. root	Australia	KR071694	KR071723
*Fusarium nygamai*_CBS57294	*Liseola*	*Cajanus indicus*	Netherlands	X94174	MW402141
*Fusarium nygamai*_T_CBS749.97	*Liseola*	*Sorghum bicolor* root	Australia	MH862671	MW402151
*Fusarium proliferatum*_T_CBS48096	*Liseola*	Soil	Papua New Guinea	-	MN534059
*Fusarium falciforme*_CBS135512	*Martiella* and *Ventricosum*	Human	Mexico	KM401895	KM401894
*Fusarium falciforme*_CBS138971	*Martiella* and *Ventricosum*	Unknown	India	KT716201	KT716212
*Fusarium falciforme*_NRRL28565	*Martiella* and *Ventricosum*	Human wound	USA	DQ094379	DQ246906
*Fusarium falciforme*_NRRL32331	*Martiella* and *Ventricosum*	Human leg	USA	DQ094428	DQ246959
*Fusarium falciforme*_NRRL32778	*Martiella* and *Ventricosum*	Horse eye	USA	DQ094549	DQ247088
*Fusarium falciforme*_T_CBS47567	*Martiella* and *Ventricosum*	Human	Puerto Rico	MH859035	LT906669
*Fusarium falciforme*_VG296	*Martiella* and *Ventricosum*	Citrus	South Africa	MW173062	MW248761
*Fusarium solani*_CBS127118	*Martiella* and *Ventricosum*	Soil	USA Iowa	MH864425	LR583637
*Fusarium solani*_CBS18129	*Martiella* and *Ventricosum*	Potato	Germany	MH855035	HE647958
*Fusarium solani*_NRRL22783	*Martiella* and *Ventricosum*	Seal skin	USA	DQ094335	DQ246851
*Fusarium solani*_NRRL25388	*Martiella* and *Ventricosum*	Human eye	India	MH582401	MH582421
*Fusarium solani*_NRRL32484	*Martiella* and *Ventricosum*	Human cancer	USA	DQ094449	DQ246982
*Fusarium solani*_NRRL32810	*Martiella* and *Ventricosum*	Human eye	USA	DQ094577	DQ247118
*Fusarium solani*_T_CBS140079	*Martiella* and *Ventricosum*	Potato	Slovenia	KT313633	KT313611
Fusarium chlamydosporum var. *chlamydosporum*_CBS22061	*Sporotrichiella*	Soil	South Africa	MH858030	MN120770
Fusarium chlamydosporum var. *chlamydosporum*_CBS44567	*Sporotrichiella*	Wheat	Australia	MH859028	MN120752
Fusarium chlamydosporum var. *chlamydosporum*_T_CBS14525	*Sporotrichiella*	Banana	Hondoras	NR172283	MN120754
*Fusarium chlamydosporum*_MRC117	*Sporotrichiella*	Bean hay	South Africa	MH582471	MH582447
*Fusarium chlamydosporum*_MRC35	*Sporotrichiella*	Millet	South Africa	MH582472	MH582448
*Fusarium chlamydosporum*_NRRL43630	*Sporotrichiella*			GQ505458	GQ505426

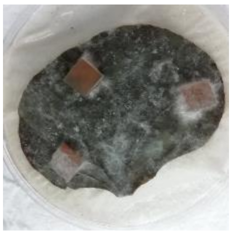
Appendix 6.6

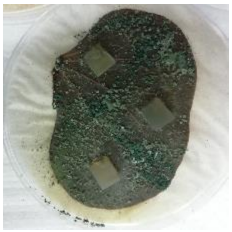
Table 6.4. Results of the detached leaf assays (DLA) performed, indicating disease symptom development after 14 days. Each fungal sample consisted of three replicates. Certain fungal species have replicates due to varying culture morphology.

Potential pathogens (red group)			
*Alternaria alternata** isolate 1		*Alternaria alternata** isolate 2	
Alternaria alternata isolate 3		*Alternaria alternata* isolate 4	
Bipolaris sp.*		*Bipolaris zeae**	
*Colletotrichum fructicola** isolate 1		*Colletotrichum fructicola** isolate 2	
Colletotrichum fructicola isolate 3		*Curvularia mebaldsii*	
*Diaporthe rhusicola**		*Fusarium chlamydosporum*	
Fusarium fujikuroi SC isolate 1*		*Fusarium fujikuroi* SC isolate 2*	

Fusarium fujikuroi SC isolate 3*		*Fusarium incarnatum equiseti* SC*	
Fusarium lacertarum isolate 1		*Fusarium lacertarum* isolate 2	
Fusarium oxysporum		*Fusarium oxysporum* SC isolate 1	
Fusarium oxysporum SC isolate 2*		*Fusarium oxysporum* SC isolate 3*	
Fusarium sambucinum SC isolate 1		*Fusarium sambucinum* SC isolate 2	
Fusarium solani isolate 1		*Fusarium solani* isolate 2	
Fusarium solani SC isolate 1*		*Fusarium solani* SC isolate 2*	
*Neofusicoccum parvum**		*Neopestalotiopsis clavispora*	

Pestalotiopsis maculans		*Pestalotiopsis maculans**	
Pestalotiopsis sp.		*Neopestalotiopsis sporophytica*	
Weak or postharvest pathogens (yellow group)			
Actinomucor elegans		*Botryosphaeria dothidea**	
Cunninghamella echinulate		*Didymella* sp. isolate 1*	
Didymella sp. isolate 2		*Didymella* sp. isolate 3	
*Lasiodiplodia pseudotheobroma**		*Phoma* sp. isolate 1	
Phoma sp. isolate 2		Uncultured *Phoma*	
Only in certain conditions (white group)			

Aspergillus awamori		*Aspergillus japonicus*	
Aspergillus luchuensis		*Aspergillus nidulans var. dentatus*	
*Aspergillus sojae***		*Cladosporium delicatulum*	
Cladosporium halotolerans		*Cladosporium pseudocladosporioides*	
Epicoccum nigrum		*Epicoccum sorghinum*	
Penicillium annulatum		*Penicillium brevicompactum*	
Penicillium caperatum		*Penicillium cluniae*	
Penicillium momoi		*Penicillium onobense*	

Penicillium raperi		*Penicillium rubens* isolate 1**	
Penicillium rubens isolate 2		*Stagonosporosis cucurbitacearum*	
Talaromyces macrosporus		*Talaromyces pinophilus*	
Trichoderma asperellum		*Trichoderma atroviride*	
Trichoderma harzianum			
Control groups			
X		Y	
Z			

*The twenty fungal samples selected for the pathogenicity trial.
**The two isolates selected for the pilot detached leaf assays

Appendix 6.7

Table 6.5. A visual representation of the number of plates (total three) indicating disease symptoms during the detached leaf assay (DLA) trial conducted over a two week period, whereby results were recorded daily except incubation days 1-3. These results represent all of the 69 fungal isolates selected for analysis during the DLA.

		Day										
	Fungal sample molecular identity	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Red group	*Alternaria alternata* isolate 1**	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Alternaria alternata isolate 2**	🟩	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Alternaria alternata isolate 3	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Alternaria alternata isolate 4	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Bipolaris sp.**	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	*Bipolaris zeae***	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Colletotrichum fructicola isolate 1**	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥
	Colletotrichum fructicola isolate 2**	🟩	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Colletotrichum fructicola isolate 3	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Curvularia mebaldsii	▨	▨	🟩	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟥
	*Diaporthe rhusicola***	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium chlamydosporum SC	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC isolate 1**	▨	▨	🟩	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC isolate 2**	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium fujikuroi SC isolate 3**	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium incarnatum-equiseti SC**	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium lacertarum isolate 1	▨	▨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
	Fusarium lacertarum isolate 2	▨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Fusarium oxysporum	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium oxysporum SC isolate 1	▨	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Fusarium oxysporum SC isolate 2**	🟩	🟩	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium oxysporum SC isolate 3**	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium sambucinum SC isolate 1	🟩	🟩	🟩	🟩	🟩	🟨	🟨	🟥	🟥	🟥	🟥
	Fusarium sambucinum SC isolate 2	▨	🟩	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
	Fusarium solani isolate 1	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium solani isolate 2	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥
	Fusarium solani SC isolate 1**	▨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Fusarium solani SC isolate 2**	▨	▨	▨	▨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	*Neofusicoccum parvum***	▨	▨	🟩	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Neopestalotiopsis clavispora	▨	▨	🟩	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟨
	Neopestalotiopsis saprophytica	🟩	🟩	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥
	Pestalotiopsis maculans isolate 1	🟩	🟩	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥
	Pestalotiopsis maculans isolate 2**	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Pestalotiopsis sp.	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
Yellow group	*Actinomucor elegans*	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	*Botryosphaeria dothidea***	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Cunninghamella echinulata	🟩	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Didymella sp. isolate 1	🟩	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Didymella sp. isolate 2	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Didymella sp. isolate 3	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	*Lasiodiplodia pseudotheobroma***	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Phoma sp. isolate 1	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Phoma sp. isolate 2	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Uncultured Phoma	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
White group	*Aspergillus awamori*	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Aspergillus japonicus	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Aspergillus luchuensis	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Aspergillus nidulans var. *dentatus*	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Aspergillus sojae[a]	🟩	🟩	🟩	🟩	🟨	🟨	🟨	🟥	🟥	🟥	🟥

	Cladosporium delicatulum	🟩	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Cladosporium halotolerans	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Cladosporium pseudocladosporioides	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Epicoccum nigrum	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Epicoccum sorghinum	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium annulatum	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Penicillium brevicompactum	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium caperatum	⬜	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium cluniae	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium momoi	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩
	Penicillium onobense	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium raperi	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Penicillium rubens[a] isolate 1	🟩	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟥	🟥	🟥
	Penicillium rubens isolate 2	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Stagonosporosis cucurbitacearum	🟩	🟩	🟩	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥
	Talaromyces macrosporus	🟩	🟩	🟨	🟨	🟨	🟨	🟥	🟥	🟥	🟥	🟥
	Talaromyces pinophilus	🟩	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨
Black	*Trichoderma asperellum*	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥
	Trichoderma atroviride	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟥
	Trichoderma harzianum	🟩	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨	🟨

*Colours were assigned according to the number of plates (total three) that indicated disease symptoms. No plates = grey; One plate = green; Two plates = yellow; Three plates = red.

[a] Fungal isolate selected as the pilot trial isolates.

**Isolates selected for further analysis during pathogenicity trial.

Appendix 6.8

Table 6.6. The individual seedling and average disease rating scores per fungal trial in Trial 1 recorded weekly over the pathogenicity trial duration.

Fungus inoculated	**Isolate**	**Week**					
		1	**2**	**3**	**4**	**5**	**6**
Control A1		0	0	0	1.5	2.5	3
Control A2		1.5	2	2	2	2.5	3.5
Control A3		0	0.5	0.5	1	2	3
Control A average		**0.50**	**0.83**	**0.83**	**1.50**	**2.33**	**3.17**
Alternaria alternata	**1**	2	2	2	2	4	5
Alternaria alternata	**1**	2	2	3	3	5	6
Alternaria alternata	**1**	7	7	7	7	8	9
***Alternaria alternata* average**	**1**	**3.67**	**4.00**	**5.33**	**7.00**	**7.67**	**9.00**
Alternaria alternata	**2**	1	2	4	4	4	4
Alternaria alternata	**2**	6	6	6	7	8	11
Alternaria alternata	**2**	2	4	6	10	11	12
***Alternaria alternata* average**	**2**	**3.00**	**5.00**	**6.00**	**8.50**	**9.50**	**11.50**
Bipolaris sp.	**1**	3	4	6	8	9	9
Bipolaris sp.	**1**	4	4	4	5	5	6
Bipolaris sp.	**1**	5	5	6	7	7	8
***Bipolaris* sp. average**	**1**	**4.00**	**4.33**	**5.33**	**6.67**	**7.00**	**7.67**
Bipolaris zeae	**1**	4	6	7	8	8	8
Bipolaris zeae	**1**	4	4	5	7	9	9
Bipolaris zeae	**1**	3	4	4	5	5	7
***Bipolaris zeae* average**	**1**	**3.67**	**4.67**	**5.33**	**6.67**	**7.33**	**8.00**
Botryosphaeria dothidea	**1**	9	9	11	12	13	13
Botryosphaeria dothidea	**1**	0	0	4	4	6	8
Botryosphaeria dothidea	**1**	0	5	7	9	20	20
***Botryosphaeria dothidea* average**	**1**	**3.00**	**4.67**	**7.33**	**8.33**	**13.00**	**13.67**
Colletotrichum fructicola	**1**	0	0	0	0	2	2
Colletotrichum fructicola	**1**	0	3	6	7	20	20
Colletotrichum fructicola	**1**	0	0	2	5	5	5
***Colletotrichum fructicola* average**	**1**	**0.00**	**1.00**	**2.67**	**4.00**	**9.00**	**9.00**
Colletotrichum fructicola	**2**	5	5	6	7	9	12
Colletotrichum fructicola	**2**	2	4	8	9	11	11
Colletotrichum fructicola	**2**	5	5	5	6	6	6
***Colletotrichum fructicola* average**	**2**	**4.00**	**4.67**	**6.33**	**7.33**	**8.67**	**9.67**
Diaporthe rhusicola	**1**	1	5	5	6	10	11
Diaporthe rhusicola	**1**	1	1	2	6	8	8
Diaporthe rhusicola	**1**	4	8	8	8	9	9
***Diaporthe rhusicola* average**	**1**	**2.00**	**4.67**	**5.00**	**6.67**	**9.00**	**9.33**
Didymella sp.	**1**	4	4	7	7	8	11
Didymella sp.	**1**	6	6	6	7	8	9
Didymella sp.	**1**	7	7	8	8	10	12
***Didymella* sp. average**	**1**	**5.67**	**5.67**	**7.00**	**7.33**	**8.67**	**10.67**

Fusarium incarnatum-equiseti SC	**1**	1	1	2	4	6	7
Fusarium incarnatum-equiseti SC	**1**	4	4	5	6	8	8
Fusarium incarnatum-equiseti SC	**1**	2	2	2	3	6	8
***Fusarium incarnatum-equiseti* SC average**	**1**	**2.33**	**2.33**	**3.00**	**4.33**	**6.67**	**7.67**
Fusarium fujikuroi SC	**3**	6	6	7	7	8	10
Fusarium fujikuroi SC	**3**	4	6	7	8	8	9
Fusarium fujikuroi SC	**3**	5	5	7	9	10	10
***Fusarium fujikuroi* SC average**	**3**	**5.00**	**5.67**	**7.00**	**8.00**	**8.67**	**9.67**
Fusarium solani SC	**1**	6	6	6	6	6	7
Fusarium solani SC	**1**	3	3	3	3	5	6
Fusarium solani SC	**1**	4	4	6	7	8	10
***Fusarium solani* SC average**	**1**	**4.33**	**4.33**	**5.00**	**5.33**	**6.33**	**7.67**
Fusarium solani SC	**2**	0	0	0	2	6	7
Fusarium solani SC	**2**	5	8	9	9	9	9
Fusarium solani SC	**2**	4	4	10	12	13	14
***Fusarium solani* SC average**	**2**	**3.00**	**4.00**	**6.33**	**7.67**	**9.33**	**10.00**
Fusarium oxysporum SC	**1**	4	4	5	6	9	20
Fusarium oxysporum SC	**1**	2	4	6	10	11	20
Fusarium oxysporum SC	**1**	3	3	6	10	12	20
***Fusarium oxysporum* SC average**	**1**	**3.00**	**3.67**	**5.67**	**8.67**	**10.67**	**20.00**
Fusarium oxysporum SC	**2**	7	7	8	9	12	20
Fusarium oxysporum SC	**2**	0	0	0	3	5	6
Fusarium oxysporum SC	**2**	3	6	8	10	12	14
***Fusarium oxysporum* SC average**	**2**	**3.33**	**4.33**	**5.33**	**7.33**	**9.67**	**13.33**
Fusarium fujikuroi SC	**1**	5	6	6	6	8	8
Fusarium fujikuroi SC	**1**	5	5	5	7	9	9
Fusarium fujikuroi SC	**1**	5	6	6	6	8	9
***Fusarium fujikuroi* SC average**	**1**	**5.00**	**5.67**	**5.67**	**6.33**	**8.33**	**8.67**
Fusarium fujikuroi SC	**2**	1	1	1	2	2	3
Fusarium fujikuroi SC	**2**	0	1	2	4	8	10
Fusarium fujikuroi SC	**2**	2	2	2	2	2	3
***Fusarium fujikuroi* SC average**	**2**	**1.00**	**1.33**	**1.67**	**2.67**	**4.00**	**5.33**
Lasiodiplodia pseudotheobroma	**1**	8	8	8	9	9	9
Lasiodiplodia pseudotheobroma	**1**	4	6	7	8	9	10
Lasiodiplodia pseudotheobroma	**1**	4	5	9	9	20	20
***Lasiodiplodia pseudotheobroma* average**	**1**	**5.33**	**6.33**	**8.00**	**8.67**	**12.67**	**13.00**
Neofusicoccum parvum	**1**	5	5	9	10	10	20
Neofusicoccum parvum	**1**	5	5	5	7	7	8
Neofusicoccum parvum	**1**	4	4	8	8	9	9
***Neofusicoccum parvum* average**	**1**	**4.67**	**4.67**	**7.33**	**8.33**	**8.67**	**12.33**
Pestalotiopsis maculans	**1**	0	0	2	2	2	2
Pestalotiopsis maculans	**1**	0	5	5	5	13	13
Pestalotiopsis maculans	**1**	0	0	2	2	6	6
***Pestalotiopsis maculans* average**	**1**	**0.00**	**1.67**	**3.00**	**3.00**	**7.00**	**7.00**

*SC is Species Complex

Table 6.7. The individual seedling and average disease rating scores per fungal trial in Trial 2 recorded weekly over the pathogenicity trial duration

Fungal treatment	**Isolate**	**Week**					
		1	**2**	**3**	**4**	**5**	**6**
Control A1		0	0	0	1.5	2	2
Control A2		2	2	2	3	5	4
Control A3		1	1	1	2	2.5	0
Control A average		**1.00**	**1.00**	**1.00**	**2.17**	**3.17**	**2.00**
Alternaria alternata	1	6	7	8	8	9	-
Alternaria alternata	1	2	5	6	10	10	-
Alternaria alternata	1	2	4	5	6	7	-
***Alternaria alternata* average**	**1**	**3.33**	**5.33**	**6.33**	**8.00**	**8.67**	**-**
Alternaria alternata	2	4	4	5	8	20	-
Alternaria alternata	2	9	9	11	11	12	-
Alternaria alternata	2	4	4	4	7	10	-
***Alternaria alternata* average**	**2**	**5.67**	**5.67**	**6.67**	**8.67**	**14.00**	**-**
Bipolaris sp.	1	4	5	5	6	7	-
Bipolaris sp.	1	8	12	14	15	20	-
Bipolaris sp.	1	10	13	14	16	17	-
***Bipolaris* sp. average**	**1**	**7.33**	**10.00**	**11.00**	**12.33**	**14.67**	**-**
Bipolaris zeae	1	5	5	5	6	8	-
Bipolaris zeae	1	4	6	7	7	8	-
Bipolaris zeae	1	6	11	11	12	16	-
***Bipolaris zeae* average**	**1**	**5.00**	**7.33**	**7.67**	**8.33**	**10.67**	**-**
Botryosphaeria dothidea	1	2	2	2	4	7	-
Botryosphaeria dothidea	1	3	3	4	5	7	-
Botryosphaeria dothidea	1	5	5	5	8	20	-
***Botryosphaeria dothidea* average**	**1**	**3.33**	**3.33**	**3.67**	**5.67**	**11.33**	**-**
Colletotrichum fructicola	1	0	0	3	7	8	12
Colletotrichum fructicola	1	5	5	7	10	10	15
Colletotrichum fructicola	1	3	3	3	7	7	9
***Colletotrichum fructicola* average**	**1**	**2.67**	**2.67**	**4.33**	**8.00**	**8.33**	**12.00**
Colletotrichum fructicola	2	7	8	9	10	11	-
Colletotrichum fructicola	2	7	7	9	9	10	-
Colletotrichum fructicola	2	5	6	6	7	10	-
***Colletotrichum fructicola* average**	**2**	**6.33**	**7.00**	**8.00**	**8.67**	**10.33**	**-**
Diaporthe rhusicola	1	2	2	5	6	9	-
Diaporthe rhusicola	1	0	9	10	10	11	-
Diaporthe rhusicola	1	3	10	10	11	12	-
***Diaporthe rhusicola* average**	**1**	**1.67**	**7.00**	**8.33**	**9.00**	**10.67**	**-**
Didymella sp.	1	6	10	10	11	14	-
Didymella sp.	1	4	5	6	6	9	-
Didymella sp.	1	0	3	3	5	7	-
***Didymella* sp. average**	**1**	**3.33**	**6.00**	**6.33**	**7.33**	**10.00**	**-**
Fusarium incarnatum-equiseti SC	1	3	4	6	8	10	-
Fusarium incarnatum-equiseti SC	1	6	6	6	7	7	-
Fusarium incarnatum-equiseti SC	1	1	6	6	9	20	-
***Fusarium incarnatum-equiseti* SC average**	**1**	**3.33**	**5.33**	**6.00**	**8.00**	**12.33**	**-**
Fusarium fujikuroi SC	3	2	3	6	9	10	-
Fusarium fujikuroi SC	3	9	13	14	14	15	-
Fusarium fujikuroi SC	3	2	7	8	8	9	-
***Fusarium fujikuroi* SC average**	**3**	**4.33**	**7.67**	**9.33**	**10.33**	**11.33**	**-**

Fusarium solani SC	1	4	8	8	10	11	-
Fusarium solani SC	1	9	10	11	11	12	-
Fusarium solani SC	1	3	3	4	4	5	-
Fusarium solani **SC average**	**1**	**5.33**	**7.00**	**7.67**	**8.33**	**9.33**	**-**
Fusarium solani SC	2	7	7	7	9	10	-
Fusarium solani SC	2	0	5	5	11	13	-
Fusarium solani SC	2	0	3	5	6	8	-
Fusarium solani **SC average**	**2**	**2.33**	**5.00**	**5.67**	**8.67**	**10.33**	**-**
Fusarium oxysporum SC	1	5	7	7	8	8	-
Fusarium oxysporum SC	1	6	7	7	8	9	-
Fusarium oxysporum SC	1	5	5	5	6	7	-
Fusarium oxysporum **SC average**	**1**	**5.33**	**6.33**	**6.33**	**7.33**	**8.00**	**-**
Fusarium oxysporum SC	2	5	6	10	10	11	-
Fusarium oxysporum SC	2	0	4	4	5	8	-
Fusarium oxysporum SC	2	3	4	6	9	9	-
Fusarium oxysporum **SC average**	**2**	**2.67**	**4.67**	**6.67**	**8.00**	**9.33**	**-**
Fusarium fujikuroi SC	1	5	6	7	8	20	-
Fusarium fujikuroi SC	1	9	9	9	10	10	-
Fusarium fujikuroi SC	1	2	4	4	7	8	-
Fusarium fujikuroi **SC average**	**1**	**5.33**	**6.33**	**6.67**	**8.33**	**12.67**	**-**
Fusarium fujikuroi SC	2	7	7	8	8	10	-
Fusarium fujikuroi SC	2	1	1	3	3	3	-
Fusarium fujikuroi SC	2	3	3	3	6	7	-
Fusarium fujikuroi **SC average**	**2**	**3.67**	**3.67**	**4.67**	**5.67**	**6.67**	**-**
Lasiodiplodia pseudotheobroma	1	6	6	7	8	9	-
Lasiodiplodia pseudotheobroma	1	0	9	10	10	11	-
Lasiodiplodia pseudotheobroma	1	6	7	9	9	20	-
Lasiodiplodia pseudotheobroma **average**	**1**	**4.00**	**7.33**	**8.67**	**9.00**	**13.33**	**-**
Neofusicoccum parvum	1	4	5	5	7	7	-
Neofusicoccum parvum	1	3	6	6	9	20	-
Neofusicoccum parvum	1	8	8	8	9	13	-
Neofusicoccum parvum **average**	**1**	**5.00**	**6.33**	**6.33**	**8.33**	**13.33**	**-**
Pestalotiopsis maculans	1	2	5	8	11	11	13
Pestalotiopsis maculans	1	0	1	7	7	7	8
Pestalotiopsis maculans	1	6	9	9	9	9	12
Pestalotiopsis maculans **average**	**1**	**2.67**	**5.00**	**8.00**	**9.00**	**9.00**	**11.00**

*SC is Species Complex

Appendix 6.9

Table 6.8. The individual seedling and average above ground [stems and leaves] weight measurements per fungal treatment in the pathogenicity Trial 1.

		Wet mass (g)				**Dry mass (g)**			
Treatment	**Isolate**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**
Negative control	A	0.99	1.07	1.01	1.02	0.49	0.49	0.31	0.43
Alternaria alternata	1	0.66	0.71	1.12	0.83	0.50	0.40	0.75	0.55
Alternaria alternata	2	2.42	1.10	1.34	1.62	0.73	0.70	0.70	0.71
Bipolaris sp.	1	2.14	1.89	0.44	1.49	0.83	0.72	0.33	0.63
Bipolaris zeae	2	1.64	1.73	0.65	1.34	0.91	0.56	0.40	0.62
Botryosphaeria dothidea	1	0.35	1.09	0.79	0.74	0.23	0.65	0.55	0.48
Colletotrichum fructicola	1	0.95	0.85	1.02	0.94	0.57	0.60	0.78	0.65
Colletotrichum fructicola	2	0.36	0.78	2.33	1.16	0.24	0.59	1.09	0.64
Diaporthe rhusicola	1	1.47	1.22	2.21	1.63	0.93	0.91	0.70	0.85
Didymella sp.	1	1.02	0.47	0.84	0.78	0.58	0.29	0.48	0.45
Fusarium fujikuroi SC	1	0.67	1.34	1.15	1.05	0.55	0.59	0.77	0.64
Fusarium fujikuroi SC	2	1.08	0.66	1.04	0.93	0.63	0.57	0.60	0.60
Fusarium fujikuroi SC	3	1.36	0.75	0.53	0.88	0.75	0.41	0.44	0.53
Fusarium incarnatum-equiseti SC	1	0.67	0.46	0.83	0.65	0.49	0.16	0.48	0.38
Fusarium oxysporum SC	1	0.51	0.66	0.38	0.52	0.40	0.42	0.36	0.39
Fusarium oxysporum SC	2	0.38	0.87	1.05	0.77	0.32	0.64	0.83	0.60
Fusarium solani SC	1	0.65	0.44	0.75	0.61	0.34	0.21	0.44	0.33
Fusarium solani SC	2	0.53	0.56	0.70	0.60	0.38	0.31	0.49	0.39
Lasiodiplodia pseudotheobroma	1	0.70	0.61	0.40	0.57	0.35	0.53	0.29	0.39
Neofusicoccum parvum	1	1.21	1.21	1.47	1.30	0.63	0.45	1.05	0.71
Pestalotiopsis maculans	1	0.76	1.22	0.88	0.95	0.32	0.73	0.31	0.45

*SC is Species Complex

Table 6.9. The individual seedling and average above ground [stems and leaves] weight measurements per fungal treatment in the pathogenicity Trial 2.

		Wet mass				**Dry mass**			
Treatment	**Isolate**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**
Negative control	B	2.20	2.31	0.56	1.69	0.79	0.83	0.22	0.61
Alternaria alternata	1	3.06	0.64	1.93	1.88	1.09	0.40	0.61	0.70
Alternaria alternata	2	0.66	1.30	1.09	1.02	0.34	0.48	0.56	0.46
Bipolaris sp.	1	1.84	0.68	0.76	1.09	0.72	0.32	0.25	0.43
Bipolaris zeae	2	1.12	0.78	0.76	0.89	0.39	0.56	0.54	0.50
Botryosphaeria dothidea	1	1.30	0.59	1.17	1.02	0.38	0.34	0.72	0.48
Colletotrichum fructicola	1	2.81	1.74	0.91	1.82	1.01	0.61	0.59	0.74
Colletotrichum fructicola	2	1.38	2.35	0.76	1.50	0.64	0.70	0.46	0.60
Diaporthe rhusicola	1	0.81	0.83	1.42	1.02	0.50	0.58	0.67	0.58
Didymella sp.	1	0.97	0.58	1.13	0.89	0.64	0.34	0.47	0.48
Fusarium fujikuroi SC	1	0.43	0.49	1.35	0.76	0.22	0.51	0.46	0.40
Fusarium fujikuroi SC	2	0.84	1.28	0.86	0.99	0.42	0.79	0.46	0.56
Fusarium fujikuroi SC	3	0.79	0.13	0.90	0.61	0.46	0.05	0.57	0.36
Fusarium incarnatum-equiseti SC	1	3.21	1.76	0.49	1.82	0.89	0.70	0.51	0.70
Fusarium oxysporum SC	1	2.08	0.59	0.57	1.08	0.65	0.35	0.31	0.44
Fusarium oxysporum SC	2	0.77	0.90	0.81	0.83	0.53	0.60	0.46	0.53
Fusarium solani SC	1	1.40	1.01	1.58	1.33	0.61	0.74	0.49	0.61
Fusarium solani SC	2	0.81	1.08	0.93	0.94	0.36	0.75	0.45	0.52
Lasiodiplodia pseudotheobroma	1	2.80	0.60	0.68	1.36	0.79	0.29	0.49	0.52
Neofusicoccum parvum	1	0.98	0.49	0.26	0.58	0.26	0.26	0.18	0.23
Pestalotiopsis maculans	1	0,50	1,46	0,75	0,90	0,32	0,48	0,38	0,39

*SC is Species Complex

Table 6.10. The individual seedling and average below ground [tubers and roots] weight measurements per fungal treatment in the pathogenicity Trial 1.

		Wet mass				**Dry mass**			
Treatment	**Isolate**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**
Negative control	A	40.21	37.47	28.37	35.35	9.60	8.33	5.88	7.94
Alternaria alternata	1	49.41	17.70	27.83	31.65	9.75	5.01	9.16	7.97
Alternaria alternata	2	49.08	42.31	51.52	47.64	9.99	10.57	12.40	10.99
Bipolaris sp.	1	48.60	41.25	24.70	38.18	11.36	10.82	6.66	9.61
Bipolaris zeae	2	51.69	42.89	35.35	43.31	9.93	8.97	8.06	8.99
Botryosphaeria dothidea	1	22.65	26.54	33.82	27.67	4.96	7.27	7.12	6.45
Colletotrichum fructicola	1	31.70	15.23	50.22	32.38	6.35	2.80	12.44	7.20
Colletotrichum fructicola	2	25.48	39.91	52.20	39.20	4.51	8.84	14.29	9.21
Diaporthe rhusicola	1	38.09	43.05	31.76	37.63	9.46	8.08	8.02	8.52
Didymella sp.	1	31.34	31.46	45.67	36.16	7.18	5.84	9.68	7.57
Fusarium fujikuroi SC	1	42.50	28.82	44.33	38.55	8.37	6.77	10.96	8.70
Fusarium fujikuroi SC	2	34.04	29.90	40.50	34.81	9.73	6.70	10.66	9.03
Fusarium fujikuroi SC	3	50.56	35.35	20.01	35.31	11.07	6.45	5.52	7.68
Fusarium incarnatum-equiseti SC	1	37.03	28.52	29.77	31.77	7.67	5.75	6.92	6.78
Fusarium oxysporum SC	1	52.38	35.97	27.13	38.49	9.55	6.05	5.00	6.87
Fusarium oxysporum SC	2	27.40	33.30	53.02	37.91	5.22	7.79	18.16	10.39
Fusarium solani SC	1	37.80	39.47	24.59	33.95	9.04	7.02	6.65	7.57
Fusarium solani SC	2	30.82	31.00	27.57	29.80	7.51	6.43	5.96	6.63
Lasiodiplodia pseudotheobroma	1	15.27	35.64	29.09	26.67	4.98	10.28	5.63	6.96
Neofusicoccum parvum	1	36.40	25.94	52.35	38.23	7.55	6.06	13.66	9.09
Pestalotiopsis maculans	1	26.53	46.09	30.41	34.34	6.99	10.49	5.62	7.70

*SC is Species Complex

Table 6.11. The individual seedling and average below ground [tubers and roots] weight measurements per fungal treatment in the pathogenicity Trial 2.

		Wet mass				Dry mass			
Treatment		**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**	**Seedling 1**	**Seedling 2**	**Seedling 3**	**Average**
Negative control	B	41.91	44.375	40.925	42.4	11.665	9.98	9.825	10.49
Alternaria alternata	1	65.66	27.73	38.23	43.87	11.63	5.62	9.33	8.86
Alternaria alternata	2	37.93	34.86	41.44	38.08	7.23	7.59	8.91	7.91
Bipolaris sp.	1	40.57	26.34	20.99	29.3	10.47	5.51	3.26	6.41
Bipolaris zeae	2	26.49	34.3	29.78	30.19	5.56	8.06	6.61	6.74
Botryosphaeria dothidea	1	31.27	30.96	46.91	36.38	7.13	5.39	7.9	6.81
Colletotrichum fructicola	1	60.6	37.7	34.49	44.26	13.47	7.04	10.31	10.27
Colletotrichum fructicola	2	46.02	36.63	29.82	37.49	6.68	9.91	6.9	7.83
Diaporthe rhusicola	1	37.3	37.28	49.13	41.24	7.67	7.9	9.07	8.21
Didymella sp.	1	35.94	27.51	25.31	29.59	9.71	5.56	6.75	7.34
Fusarium fujikuroi SC	1	32.4	40.44	32.83	35.22	5.47	7.46	7.68	6.87
Fusarium fujikuroi SC	2	52.6	44.3	31.83	42.91	11.58	10.59	7.37	9.85
Fusarium fujikuroi SC	3	40.01	19.06	30.15	29.74	8.84	4.22	7.07	6.71
Fusarium incarnatum-equiseti SC	1	39.7	51.37	36.05	42.37	10.14	10.62	7.16	9.31
Fusarium oxysporum SC	1	53.04	34.91	21.97	36.64	11.39	5.76	5.58	7.58
Fusarium oxysporum SC	2	64.88	34.6	48.98	49.49	9.88	9.15	7.84	8.96
Fusarium solani SC	1	41.4	40.04	31.83	37.76	9.56	9	9.46	9.34
Fusarium solani SC	2	32.86	56.62	38.16	42.55	7.51	15.39	8.44	10.45
Lasiodiplodia pseudotheobroma	1	33.12	24.6	41.04	32.92	9.61	5.37	7.64	7.54
Neofusicoccum parvum	1	21.57	33.49	13.44	22.83	4.57	6.2	2.97	4.58
Pestalotiopsis maculans	1	37.2	32.91	20.53	30.21	4.93	7.45	5.05	5.81

*SC is Species Complex

Table 6.12. The dry and wet weight measurements (g) for the below ground (roots and tubers) and above ground (leaves and stems) parts of marama bean seedlings harvested from of the pathogenicity Trials 1 and 2 and their averages per fungal species.

Fungus inoculated	**Below ground (tubers and root)**				**Above ground (stems and leaves)**			
	Wet mass (g)		**Dry mass (g)**		**Wet mass (g)**		**Dry mass (g)**	
	Trial 1	**Trial 2**	**Trial 1**	**Trial 2**	**Trial 1**	**Trial 2**	**Trial 1**	**Trial 2**
Negative control	35.35	42.40	7.94	10.49	1.02	1.69	0.43	0.61
Alternaria alternata	39.64	40.98	9.48	8.39	1.23	1.45	0.63	0.58
Bipolaris zeae	40.75	29.75	9.30	6.58	1.42	0.99	0.63	0.46
Botryosphaeria dothidea	27.67	36.38	6.45	6.81	0.74	1.02	0.48	0.48
Colletotrichum fructicola	35.79	40.88	8.21	9.05	1.05	1.66	0.65	0.67
Diaporthe rhusicola	37.63	41.24	8.52	8.21	1.63	1.02	0.85	0.58
Didymella sp.	36.16	29.59	7.57	7.34	0.78	0.89	0.45	0.48
Fusarium fujikuroi SC	36.81	35.77	8.53	7.57	0.98	0.78	0.60	0.43
Fusarium incarnatum-equiseti SC	31.77	42.37	6.78	9.31	0.65	1.82	0.38	0.70
Fusarium oxysporum SC	38.20	43.06	8.63	8.27	0.64	0.95	0.50	0.48
Fusarium solani SC	31.88	40.15	7.10	9.89	0.61	1.14	0.36	0.57
Lasiodiplodia pseudotheobroma	26.67	32.92	6.96	7.54	0.57	1.36	0.39	0.52
Neofusicoccum parvum	38.23	22.83	9.09	4.58	1.30	0.58	0.71	0.23
Pestalotiopsis maculans	34.34	30.21	7.70	5.81	0.95	0.90	0.45	0.39

* The average mass values were calculated for both Trial 1 and 2 per fungal isolate. Where certain fungal species had duplicate fungal treatments, the average masses of all seedlings per fungal isolate were combined