CYANOGEN AND MYCOTOXIN REDUCTION FOR CASSAVA (*MANIHOT ESCULENTA* CRANTZ) CULTIVATED SOIL

BY

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"The future belongs to those who believe in the beauty of their dreams"

- Eleanor Roosevelt

"The future belongs to those who challenge the present"

-Eleanor Roosevelt

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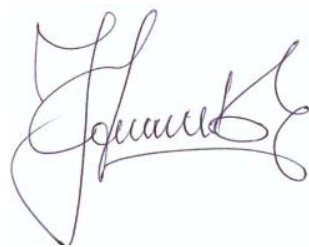Cape Peninsula University of Technology (CPUT)

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DECLARATION

I, **Elie Fereche Itoba Tombo,** hereby declare that the contents of this thesis represent my own unaided work and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily that of the Cape Peninsula University of Technology and its sponsors.

All intellectual concepts, theories and methodologies used in this thesis and published in various scientific journals and/or peer-reviewed conference proceedings were derived solely from the candidate and first author of the published manuscripts. Where appropriate, intellectual property of others was acknowledged by using appropriate references. The contribution of co-authors for conference and published manuscripts was in a training capacity (Mhlangabezi Golela, Anda Waxa, in-service training), research assistance (Margaret Kena, John Baptist Mudumbi, Joseph Andrew Bell, Lukhanyo Mekuto, Enoch Akinpelu) and supervisory capacity (Prof. S.K.O. Ntwampe and Dr. A.N Paulse) to meet the requirements for the doctoral degree award.

Signature: **Date:** 30/10/2017

ABSTRACT

The management of agricultural soil and its sustainable use, namely productivity, is paramount to the agricultural industry worldwide. Large-scale agricultural product producers and scientists emphasise using environmentally benign methods to increase agricultural production such as taking a green chemistry approach to agricultural activities and/or using cultivation techniques for the bio-augmentation of agricultural soil. Some of these agricultural products, such as cassava (*Manihot esculenta*), produce cyanogens which promote the infestation of a cyanogen-resistant microbial species known to produce mycotoxins during decomposition. Although cyanogens and mycotoxins are important components in the functioning of the earth system and agricultural soil, their cumulative effects can result in reduced soil productivity, hence degradation. Furthermore, the presence of mycotoxins in the environment and agricultural produce is hazardous to the environment, including the biotic communities in soil and humans. Therefore, an environmentally benign (green chemistry approach) method for the reduction of cyanogens and mycotoxins was proposed for this research study.

The method investigated had to be applicable *in-situ* for the biodegradation of cyanogens and mycotoxins. Their reduction from decomposing cassava in cultivated soil, which can be used on a small and large scale, would mitigate deleterious effects of a less reported, unknown mycotoxins producer (fungal species), *Cunninghamella bertholletiae* (KT275316)*,* found to be a free cyanide- (CN^-) resistant isolate*.* The *C. bertholletiae* was isolated from decomposing cassava tubers and silt, subsequent to culturing on potato dextrose agar (PDA) and in an equivalent volume of nutrient broth (NB) containing KCN (4mg/40mL) at 30 °C for 120 hrs. The isolate demonstrated an ability to biodegrade CN^- into NH_3 and NO_3. NH_3 and NO_3 are nitrogenous by-products produced when young cassava plants are cultivated in a controlled environment, with 80% of the initial CN^- concentration being efficiently degraded to NH_3/NO_3 at a conversion rate of 77.5% and 72.5% (fungus from silt and cassava), respectively, within 120 hrs.

From this research, it was observed that Sub-Saharan Africa is the largest contributor to the CN^- load into the environment; from cassava cultivation as per FAO data. The quantity of CN^- released was estimated at 0.025×10^{-3} to 6.71 ppq, with further increases of 60.5% being projected to be released into the environment by 2024. As such, it was hypothetically assumed that numerous species in cassava-cultivated soil become CN^- resistant as they are exposed to CN^- from decomposing cassava, becoming pathogenic thus antigonistic towards other biota in cassava-cultivated soil.

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Consequently, the pathogenicity of the isolate was investigated against organisms (n = 12) from cassava-cultivated soil. The isolate demonstrated inhibitory pathogenic activity against some soil bacterial communities such as *Oligella ureolytica, Acinetobacter* sp., *Pseudomonas luteola* and *Sphingomonas paucimobilis*. The isolate also demonstrated minor antagonistic effects against *Myroides* sp., *Stenotrophomonas maltophilia*, *Candida lipolytica*, *Cryptococcus albidus* and *Rhodotorula* sp.. Further research to identify extracellular metabolites produced by this organism, using a fermentation method was also carried out using a liquid state fermentation technique. 30 mL Erlenmeyer flasks containing 25 mL of NB/KCN (source of CN⁻) at 37 °C for 168 hrs, with a volume of (5 mL), extracts from the fermentation being filtered, centrifuged, mixed with chloroform for a liquid-liquid extraction procedure subsequent to a nitrogen-facilitated blow-down technique and reconstitution with 100% analytical grade methanol, for LC/MS-TOF 6230 analysis. The analysis revealed that the isolate was able to produce the mycotoxins/secondary metabolites, Fumonisin B1 (FB1) and Deoxynivalenol (DON).

Though the isolate (KT275316) demonstrated the ability to biodegrade cyanide as well as produce mycotoxin, an environmentally benign strategy (green chemistry method) with a potential to biodegrade $CN^-/NH_3/NO_3/NO_2$ for the biodegradation of mycotoxins was evaluated, including the identification of biodegradation by-products post-biodegradation treatment. Thus, plant extracts from *Nepenthes mirabilis* were found to contain enzymes such as carboxylesterase, β-glucosidase, β-glucoronidase and phosphatidyl inositol phospholipase C (identified using both quantitative and qualitative methods). The plant extracts were used with treated samples from the fermentation and were subjected to biodegradation. Thus, resulting in biodegradation by-products such as Heptadecanone Octadecanamide, Octadecenal for FB1 and Tolmetin for DON, respectively. For future research, it is therefore recommended that plant extracts with similar properties to those observed for *N. mirabilis* extracts (juice) be sought for application of the proposed method.

Keywords: Agricultural soil; cassava; carboxylesterase; *Cunninghamella bertholletiae*; cyanogens; LC/MS-ToF; mycotoxins; *Nepenthes mirabilis.*

LIST OF OUTPUTS

The following outputs are contributions by the candidate to scientific development and knowledge during the doctoral candidacy (2013 to 2017):

Peer reviewed publications/DHET-accredited conference proceedings

1. **Itoba-Tombo, E.F.,** Ntwampe, S.K.O., Bell, J.J.A., Mudumbi, J.B.N & Golela, T.M. **2017.** A decade's (2014–2024) perspective on cassava's (Manihot esculenta Crantz) contribution to the global hydrogen cyanide load in the environment. *International Journal of Environmental Studies*, 74(1): 28-41.

2. **Itoba-Tombo, E.F.,** Ntwampe, S.K.O. & Mudumbi, J.B.N. **2017.** Leaching of cyanogens and mycotoxins from cultivated cassava into agricultural soil: effects on groundwater quality*.* In *Aflatoxin-Control, Analysis, Detection and Health Risks.* Abdulra'uf, L.B. (Ed.). InTechOpen, ISBN: 978-953-51-3458-9; http://dx.doi.org/10.5772/intechopen.68715.

3. **Itoba-Tombo, E.F.,** Ntwampe, S.K.O., Waxa, A., Paulse, A. & Akinpelu, E. A. **2016.** Screening of fungal (*Cunnighamella bertholletiae*) pathogenic activity on microbial communities in cassava- (*Manihot esculenta* crantz) cultivated soil. International Conference on Advances in Science, Engineering, Technology & Natural Resources (ICASETNR-16). Nov. 24 - 25, 2016. Parys (South Africa). ISBN: 978-93-84468-79-8.

4. **Itoba-Tombo, E.F.**, Waxa, A. & Ntwampe, S.K.O. **2015.** Isolation of an endophytic cyanide-resistant fungus, *Cunninghamella bertholletiae,* from *Manihot esculenta* and cassava-cultivated soil for environmental engineering applications. 7th International Conference on Latest Trends in Engineering and Technology (ICLTET'2015). Nov. 26-27, 2015. Irene, Pretoria (South Africa). http://dx.doi.org/10.15242/IIE.E1115047.

International/Local conference(s): Oral presentation/Posters

5. **Itoba-Tombo, E.F.,** Ntwampe, S.K.O. & Mudumbi, J.B.N. **2014.** Cyanogen-loading effects on terrestrial ecosystem and groundwater quality in agricultural soil. Abstract No. P1. 034 (conference proceeding p.123). Abstract and poster presentation at the First Global Soil Biodiversity Conference. 2-5 December 2014. Dijon, France. https://dspace.uevora.pt/rdpc/bitstream/10174/12805/Book%20of%20Abstracts.

6. **Itoba-Tombo, E.F.,** Ntwampe, S.K.O. & Mudumbi, J.B.N. **2013.** *In-situ* cyanogen and hydrogen cyanide reduction for *Manihot esculenta* cultivation. Oral presentation from conference proceedings at the Cape Peninsula University of Technology (CPUT). Post-Graduate Conference on 5 November 2013.

ACKNOWLEGDEMENTS

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DEDICATION

My parents

Tombo Francois and Apendi Marthe

My wife and children

Itoba Tombo Andisiwe Portia (nee Nani Andisiwe Portia)

Itoba Tombo Princia Babalwa

Itoba Tombo Frans Presly

BIOGRAPHICAL SKETCH

Elie Fereche Itoba Tombo was born in Makoua (Region de la cuvette) in the Republic of Congo-Brazzaville. He attended primary school in Liranga (Likouala Region), secondary school in Makoua, high school at the Lycee Agricole Amilcar Cabraal, where he completed his *Baccalaureat* in Agricultural Sciences in 1990, and tertiary studies at the University Marien Ngouabi of Brazzaville. He obtained a Diploma of General Studies (DEUG) in Geography in 1994, a Bachelor Degree in Physical Geography in 1996 and C2 Certificate for a Master's degree in Climatology in 1997 from the University Marien Ngouabi of Congo - Brazzaville-. Later, he obtained an Honours Degree of Natural Sciences (Earth and Environmental Sciences) in 2005 from the University of the Western Cape (UWC), Cape Town, South Africa, and a Master of Technology (M. Tech) degree in Environmental Management in 2010 from the Cape Peninsula University of Technology, Cape Town, South Africa. He enrolled for his doctoral studies in Environmental Health in the Department of Environmental and Occupational Studies in 2013. He worked as a part-time lecturer and has currently been working as a lecturer of Environmental Geology, Ecology and Environmental Management at the Cape Peninsula University of Technology since 2007.

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CLARIFICATION OF BASIC TERMS AND CONCEPTS

Terms/Concepts and Descriptions

Biological reduction - The removal or reduction of harmful substances into more elementary compounds using a natural process through the action of microorganisms, namely microbial activity (USEPA, 2010).

Cyanogen - Water soluble chemical compounds which have a pseudo-halogen molecule consisting of a CN group (Lary, 2004).

Cytochrome P450 gene - A gene found in *Manihot esculanta* (cassava) cultivars which has the function of producing CYP79D1 and CYP79D2 enzymes. CYP79D1 and CYP79D2 enzymes catalyse the conversion of *L*-valine and *L*-isoleucine to the corresponding oximes. This is the first step in linamarin and lotaustralin biosynthesis in cassava (Andersen *et al.*, 2000), which are precursors of hydrogen cyanide (HCN).

Green chemistry – An environmentally benign approach which focuses on the design of products and/or processes that use natural products or processes, thus lessening the environmental burden associated with hazardous substances.

In-situ - refers to a specific location, which can be classified as an original place or position.

Manihot esculenta - is also known as cassava. It is a tropical perennial plant from the Euphorbiaceae family whose vegetative propagation is done through the cutting of the stem. The leaves and tubers are consumed because of their nutritional content as sources of protein and vitamins.

Sub-Saharan Africa - The area within the continent of Africa consisting of all countries that are fully or partially located south of the Sahara desert, excluding Sudan.

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GLOSSARY

Abbreviations/Symbols	Definition/Description
ATSDR	Agency for Toxic Substances and Disease Registry
COD	Chemical oxygen demand
DCM	Dichloromethane
DOM	Dissolved organic matter
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
GMO	Genetically modified organism
HCN	Hydrogen cyanide
HPLC	High-performance liquid chromatography
k	Kinetic rate constant (hr^{-1}/day^{-1})
KCN	Potassium cyanide
NA	Nutrient agar
NPK	Nitrogen, Phosphorous, and Potassium
NOB	Nitrite-oxidizing bacteria
N_xO_y	Nitrogenous gasses ($_{x,\ y}$ number of atoms)
OM	Organic matter
PAH's	Polycyclic aromatic hydrocarbons
PAR	Photosynthetically active reaction
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PoPs	Persistent organic pollutants
NaCN	Sodium cyanide

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rDNA	Ribosomal deoxyribonucleic acid
rpm	Revolutions per minute
SPE	Solid phase extraction
TAN	Tropical Ataxic Neuropathy
TN	Total nitrogen
TOC	Total organic carbon
TOM	Total organic matter
UN	United Nations
UNEP	United Nations Environment Programme
UNCTAD	United Nations Conference on Trade and Development
μ	Specific growth rate (h^{-1})
WHO	World Health Organisation

CONTENTS OF THE THESIS

This PhD research was done at the Department of Environmental and Occupational Studies while laboratory analyses were conducted at the following laboratories: Bioresource Engineering Research Group (*BioERG*) laboratory, Department of Biotechnology; the Microbiology laboratory, Department of Environmental and Occupational Studies; and the AgriFood Technology Station, Department of Food and Technology, all at the Cape Town and Bellville campuses of the Cape Peninsula University of Technology, South Africa.

The thesis has the following chapters:

Chapter 1: It covers the introductory part to the research with a background of the research, problem statement, including hypothesis, research questions, aims with objectives, delineation and significance of the research.

Chapter 2: Focuses on the broad literature review on the effects of cyanogens and cyanide load on the environment, their presence in terrestrial ecosystems and thus the possibility of contaminating groundwater, compromising its quality and that of agricultural soil. It also places an emphasis on biological treatment for cyanogenic compounds as well as production of toxins and/or mycotoxins as secondary metabolites of bacteria, including fungal species such as fumonisins, ochratoxins, deoxynivalenol (DON) and aflatoxins, to name few, from decomposing cultivated agricultural produce.

Chapter 3: Presents an overview of all materials and methods used for experiments and analyses which allowed for the generation of the research data. The subsequent chapters, Chapters 4, 5, 6 and 7, highlight and discuss results obtained from these methods.

Chapter 4 Deals with the spatial and temporal modelling of cassava production globally through mapping, using ArcGIS as well as cassava's estimated contribution to the global hydrogen cyanide load into the environment as well as linearised projections. Moreover, the production of various gases from healthy cassava plants is also discussed.

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Chapter 5:   Covers the isolation of unreported/unique cyanide-resistant organism(s), having a cyanide-resistant threshold of >200 mg/L, which is the maximum threshold for most organisms (Hodek *et al.* 2002), namely *Cunninghamella bertholletiae*, isolated from decomposing cassava (*Manihot esculenta*) as a unique isolate which has never been reported to be resistant to high free cyanide concentrations. Overall, the focus was on isolates which were found to facilitate cassava decomposition with the potential of producing toxins and/or mycotoxins, while having an ability to biodegrade CN^- and its by-products.

Chapter 6:   Reports on isolate(s) isolated in Chapter 5, focusing on unreported/unique isolate(s) having a high free cyanide concentration tolerance and their pathogenic ability/activity on other microorganisms found in cassava- (*Manihot esculenta*) cultivated soil. This was to assess the antagonistic effect of the cyanide-resistant isolate(s), extracellular bio-products and its ability to modify and/or change the micro biota of the soil, thus contributing to its modification.

Chapter 7:   Focuses on the detection of secondary metabolites/mycotoxins produced by the free cyanide-tolerant isolate, namely *Cunninghamella bertholletiae*. Furthermore, a remediation strategy for the *in-situ* application in agricultural soil to mitigate against cyanogen and mycotoxin loading and effects is proposed.

Chapter 8:   This chapter focuses on the overall summary, conclusions and recommendations for future research.

Chapter 9:   Lists the bibliography used, including references which were not cited in-text for the thesis.

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CHAPTER 1

INTRODUCTION

CHAPTER 1

1.   INTRODUCTION

1.1 Introduction

Manihot esculenta, commonly known as cassava, is a perennial plant which is consumed by the population of sub-Saharan Africa countries, with its tubers and leaves being found to have nutritional potential as a dietary supplement for protein and minerals, including vitamins (Soto-Blanco & Górniak, 2010; Mburu *et al.*, 2012). The produce from this plant is toxic due to its free cyanide (CN^-) content and its precursors, such as Linamarin, Lotaustralin, and other cyanogenic glycosides such as 2-((6-O-(β-D-apiofuranosyl)-β-D-glucopyranosyl)oxy)-2-methylbutanenitrile), which are enzymatically transformed into CN^- (Prawat *et al.*, 1995; Andersen *et al.*, 2000).

In the past few years, researchers have developed several methods for the reduction of CN^- in cassava, using genetically modified cultivars (Koehorst-Van Putten *et al.*, 2012), suppressing the functionality of cytochrome P450 genes (producers of enzymes CYP79D1 and CYP79D2) and thus inhibiting the biological conversion of Linamarin and Valine to CN^- (Prawat *et al.*; 1995; Andersen *et al.*, 2000). However, owing to the decomposition of cassava tubers due to harsh environmental conditions, some of the cyanogens leach into the soil in which they are cultivated. Therefore, there is a need to explore a user-friendly method for *in-situ* cyanogen and CN^- conversion during cassava cultivation, particularly when some of the tubers decompose prior to harvesting (McMahon *et al.*, 1995, Padmaja & Steinkraus, 1995) which will require minimal maintenance for application even in remote rural areas where cassava is cultivated and consumed in large quantities. The reduction of both CN^- and its precursors for large *in-situ* application will thus involve a suitable biocatalyst or biological extracts, either introduced to the soil or using an *ex-situ* system whereby the biocatalyst extracts or products are produced for physical application in the soil for cyanogen/cyanide load reduction. Furthermore, there is limited information on CN^-, its precursors decomposition by-products (N_xO_y) load into the environment globally which is attributed to cassava cultivation, including gaseous constituents produced by healthy cassava plants.

Any agricultural produce intended for human consumption, should meet a set of criteria for edible agricultural products, such as being free of contaminants and harmful microbial species, free of infections and having a suitable nutritional content (Henson & Caswell, 1999; Wagacha &

Muthomi, 2008). The introduction of unsuitable species, even in the form of extracts produced by a microorganism which is not an indigenous species to the area, can result in arable land degradation and the disruption of local terrestrial ecosystems. Therefore, a compatibility study must be done to assess the suitability of a selected biocatalyst and/or extracts for *in-situ* cyanogen and mycotoxin/toxin reduction for cassava cultivation.

Similarly, various types of biocatalysts under varying conditions can produce mycotoxins/toxins. Thus, the presence of mycotoxins/toxins, either in produce, soil or in extracts/products used to reduce cyanogen content, can render the cassava tubers inedible (Hocking, 1997; Wagacha & Muthomi, 2008; Afsah-Hejri *et al.*, 2013; Ediage *et al.*, 2014). This can result in the incompatibility of such extracts with soil micro-biota, which can exacerbate land degradation/deterioration, loss of soil fertility and excessive reed growth.

The most common microbial species present in agricultural soil are: *Pseudomonas* sp*., Nocardia* sp*., Achromobacter* sp*., Flavobacterium* sp*., Bdellovibrio* sp*., Nitrosomonas* sp*., Nitrobacter* sp*., Aspergillus* sp*., Penicillium* sp*.* and *Cunninghamella* sp*.* (Jenkinson, 1981; Rillig & Mummey, 2006; Vineela *et al.*, 2008). Previous studies demonstrated that fungi such as *Aspergillus* sp. and *Fusarium* sp. produce mycotoxins/toxins which spoil harvested agricultural products (such as tubers), thus rendering them inedible (Munkvold & Desjardins, 1997; Kuti & Konoru, 2006: Manjula *et al.*, 2009: Afsah-Hejri *et al.*, 2013: Adetunji *et al.*, 2014: Matumba *et al.*, 2014). See Appendix 2. These mycotoxins include Ochratoxin A, Fumonisin B1*,* Pyranonigrin A, Tensidol B, Funalenone, Naphtho-y-pyrones and Malformins (Ottesen *et al.*, 2010; Perrone *et al.*, 2011)*.*

Due to the fact that fungi such as *Aspergillus* sp. and *Penicillium* sp. are well known mycotoxin producers (Andersen *et al.*, 2000; Stoilova *et al.*, 2006; Perrone *et al.*, 2011; Umsza-Guez *et al.*, 2011), the current study was aimed at unknown or less-reported species with the potential of producing mycotoxins/toxins which can withstand CN$^-$ released by cassava tubers while facilitating the decompositions of the tubers *in-situ*, that is in agricultural soil. Thus, amongst these species, *Cunninghamella* sp. was the isolate found to meet this criterion after an extended experimentation period which could also withstand varying environmental conditions in which cassava was cultivated, thus becoming persistent in agricultural soil.

Cunninghamella sp*.* is commonly found in soil (Asha & Vidyavathi, 2009; Budziszewska *et al.*, 2010; Lima *et al.*, 2016) and has been found to even grow on some waste such as ageing

sawdust (Sing *et al.,* 2014). Furthermore, various strains of *Cunninghamella* sp. were found to grow on agricultural produce such as maize, peanuts and kola nuts (Adebajo *et al.,* 1994; Hocking, 1997; O'Donnell *et al.,* 2001). However, fungal species such as *Cunninghamella* sp. in general and *Cunninghamella bertholletiae* in particular, were also demonstrated to be pathogenic to humans, causing ailments ranging from sinus, pneumonia, skin infections and with the potential of infecting internal organs such as vocal cords, lungs, small intestines, heart, kidneys, liver and the pancreas (Schell *et al.,* 1982; Ortin *et al.,* 2004; Righi *et al.,* 2008).

It is not clear if *Cunninghamella* sp. produces such mycotoxins/ toxins and whether it has a high tolerance for CN^- produced during cassava decomposition. It was therefore proposed that an evaluation be conducted to assess the potential of the CN^- resistant and/or degrading isolate (*C. bertholletiae*) to produce mycotoxins/toxins. Furthermore, the compatibility and/or pathogenicity of the isolate of other soil micro-biota in which cassava is propagated must be evaluated. This could culminate in the determination of whether the tuber infected with such species was edible and to develop appropriate mitigation strategies to counter the effects of such isolates in soil. The successful implementation of the proposed *in-situ* and/or *ex-situ* biological method would enable communities to use the method, not only in rural areas, but also in small and large-scale plantations. An added benefit would be the conversion of cyanogen/CN^- to ammonium nitrogen and other nitrogenous compounds that could support nitrogen dynamics in the soil, as cassava growth requires a soil rich in nutrients-NPK (Polthanee & Kotchasatit, 1999).

Cassava cultivation can happen in approximately six months to two year (maximum) cycles although it can be as short as six months (Howeler, 2002). Thus, after harvesting, there is often nutrient depletion in the soil. Therefore, the fallow field is uncultivated for at least six months to one year in order to allow the soil to recuperate. This creates a viable remediation and recovery strategy using a green chemistry approach so that the cyclical propagation strategy for cassava currently being used can be modified to reduce recovery time, enhance produce output and reduce the need for inorganic fertilizers (as used in some rural communities). In other words, to develop a strategy for soil bio-augmentation, the conversion of both volatilised CN^- and toxin/mycotoxin from decomposing cassava for improved agricultural soil quality.

1.2 Hypothesis and research questions

1.2.1 Hypothesis

Unique and/or common soil microorganism(s) which have not been reported to: 1) facilitate cassava decomposition, 2) be resistant to CN^-, and 3) produce toxins/mycotoxins, can be isolated from cassava and/or cassava-cultivated soil. Furthermore, an environmentally benign method (using a green chemistry approach) can be proposed to mitigate CN^- and toxin/mycotoxin load (leaching) into agricultural soil from decomposing cassava, thus lessening the possibility of groundwater contamination.

1.2.2 Research questions

From the challenges highlighted, the following research questions were of considerable interest:

- Is there any concentration levels of CN^- and its gaseous by-products released from cultivated healthy cassava plants? Subsequently,

- What is cassava's contribution to the global CN^- load in the atmosphere and its possible impact on the environment?

- Are there any unique microorganisms and/or less-reported organisms which facilitate cassava decomposition and are resistant to CN^- with the potential of producing toxins/mycotoxins?

- Are the identified microorganisms pathogenic against other microorganisms in cassava-cultivated soil?

- What are the metabolites (i.e. toxins/mycotoxins) produced by the identified isolates which can render the cassava tubers inedible and thus accumulate in cassava-cultivated soil?

- What will be a suitable, benign environmental method, that is non-invasive and based on the green chemistry approach, which can be proposed for large-scale cyanogen and toxin/mycotoxin reduction to improve the health of arable agricultural soil?

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1.3 Research aims and objectives

This research was separated into numerous phases with each phase having its own objectives: Phase 1: (Aim 1): assessment of 1) the concentration of gases (HCN, NH$_3$, NO$_2$) released from healthy cultivated cassava plants; 2) the contribution of cassava cultivation to the global free cyanide (CN$^-$) load in the atmosphere/environment; Phase 2: (Aim 2): isolation, identification and characterisation of unique and/or common soil microorganism(s) which have not been reported to facilitate cassava decomposition, which are both resistant to CN$^-$ and have the potential of producing toxins/mycotoxins; Phase 3: (Aim 3): assessment of the pathogenicity of the isolate(s) identified in Phase 2: (Aim 2) against the microbial communities in cassava-cultivated soil; Phase 4: (Aim 4): identification of the isolate secondary metabolites/mycotoxins and assessment of a suitable mitigation strategy for mycotoxins/CN$^-$ and its reduction of by-products.

Phase 1: Aim 1: To assess the concentration of gases (HCN, NH$_3$, and NO$_2$) released from healthy cassava plants as well as the determination of cassava's contribution to the global CN$^-$ load into the atmosphere. In order to achieve this aim, the research devolved into the following objectives:

Objective 1: Quantification of the concentration of gases (HCN, NH$_3$, NO$_2$) released from cultivated cassava plants in a controlled environment.

Objective 2: Determination of the contribution of cassava to global CN$^-$ load in the atmosphere, its possible long-term impact in the environment using projections-based Food And Agriculture Organization (FAO) data, that is, linearised models and Geographic Information System (GIS) to map such distribution continentally (focusing on cassava-producing countries).

Phase 2: Aim 2: Isolation, identification, and characterisation of unique and/or common soil microorganism(s) which have not been reported to facilitate cassava decomposition, which are both resistant to CN$^-$ and have the potential of producing toxins/mycotoxins. To achieve this aim, this part of the study focused on the following objectives:

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Objective 1: Isolation, identification and characterisation of organisms from decomposing cassava and cassava-cultivated soil which are free cyanide tolerant/resistant. These organisms had to withstand a free cyanide-tolerance threshold >200 mg/L. As such, a concentration was found to be the maximum that most organisms could tolerate.

Objective 2: Assess the ability of the isolate to biodegrade free cyanide and its by-products; that is, nitrogenous by-products associated with free cyanide biodegradation and which can leach into agricultural soil, thus contaminate groundwater sources.

Phase 3: Aim 3: Screening of the isolate (identified in Phase 2: Aim 2) for pathogenic activity against microbial communities in cassava-cultivated soil. To achieve this aim, this part of the study focused on the following objectives:

Objective 1: Isolation, identification and characterisation of a few culturable and common (n = 12) soil organisms from cassava-cultivated soil.

Objective 2: Screening the isolate (identified in Phase 2: Aim 2) for pathogenic activity against a few (n = 12, from objective 1 above) soil microorganisms (bacteria and fungi) from cassava-cultivated soil.

Phase 4: Aim 4: Identification of secondary metabolites/mycotoxins/toxins produced by the cyanide-resistant isolate and identify (propose) a mitigation strategy for the reduction of these mycotoxins; that is, a method/strategy which can be applied *in-situ*. To achieve this aim, this part of the study focused on the following objectives:

Objective 1: Identification of secondary metabolites/mycotoxins produced by the cyanide-degrading isolate(s) using suitable analytical instruments, which can 1) render the cassava tubers inedible, and 2) contaminate arable agricultural land, including groundwater sources.

Objective 2: Assessing a proposed method of mitigation (biological/environmentally benign method) which can be applied *in-situ* for the reduction of these secondary metabolites/mycotoxins/toxins in cassava-cultivated soil. This method must also have the potential to facilitate CN^- reduction, its biodegradation by-products.

1.4 Significance of the study

This research study contributed to the following:

The monitoring/quantification of cyanogen/mycotoxin loading into the environment, soil largely used for cassava cultivation and the proposal of a biological method for the reduction of such cyanogen/mycotoxin loading. The proposed method was suitable for adaptation and for use even in rural areas that cultivate cassava as a primary and, perhaps, a main nutritional source.

1.5 Delineation of the study

In this study, the following aspects were not studied:

- Economic evaluation and feasibility of the proposed mycotoxin/CN$^-$ mitigation method on a large scale, or *in-situ* trial studies. Such evaluation/studies can be undertaken as an additional project as highlighted in the recommendations of this thesis;

- Microbial growth kinetics of all isolates identified, characterised and used in this study. Microbial growth was assessed by both microscopic and visual inspection during experimental culturing, using appropriate media;

- Assessment of the availability of a proposed extracts method as this can be a research study on its own. See recommendations section (Chapter 8).

CHAPTER 2

LITERATURE REVIEW

CHAPTER 2

2.   LITERATURE REVIEW

2.1   Introduction

Cyanogens have been widely demonstrated to be an important component in various living organisms within the earth's system (Conn, 1981; Figueira *et al.*, 1996; Jones, 1998). These compounds have been reported to have an influential role in the lives of several organisms on earth (Conn, 1981; Figueira *et al.*, 1996) and are characterised by the presence of a carbon and nitrogen functional group held together by a triple bond (C≡N). The simplest form, which is predominant in the environment, is hydrogen cyanide (HCN), with nitriles (Cumbana *et al.*, 2007, Montagnac *et al.*, 2009, Ubalua, 2010) and cyanogenic glycosides (CGs) being other forms of these compounds (Conn, 1981; Cressey *et al.*, 2013; Hidayat *et al.*, 2016).

Generally, free cyanide originates from both anthropogenic and natural processes (Dash *et al.*, 2006; Cumbana *et al.*, 2007). The anthropogenic sources of cyanide range from effluents discharged from various industrial wastewater treatment plants, agricultural runoff, mining activities and electroplating industries (Siller & Winter, 1998; White *et al.*, 2000; Akcil & Mudder, 2003; Ebbs, 2004; Magnusson *et al.*, 2012; Dash *et al.*, 2014), including the application of some cyanides containing insecticides, which culminate in environmental contamination (McQueen, 2015).

Free cyanide and cyanogenic glycosides (CGs) have also been generated in plants and agricultural produce such *as Manihot esculenta* (cassava), with the waste generated through processing such produce contributing to the cyanide load into the environment. During cassava harvesting and processing, plant-borne hydrolases result in the conversion of CGs into by-products which are released into the environment, although sometimes this is due to produce rot, a consequence of microbial contamination of the produce and wastewater generated from processing such produce (Siller & Winter, 1998; Ubalua, 2010; Okechi *et al.*, 2012).

As a result of produce-facilitated microbial decay, due to the availability of pathogenic organisms in soil where the produce grows, mycotoxins are also produced. Mycotoxins are fungal secondary metabolites that also have a negative impact on the wellbeing of humans and animals (Alassane-Kpembi *et al.*, 2013; Klarić *et al.*, 2013).

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Mycotoxins co-occur with other bacterial toxins in spoiled agricultural produce such as cassava. Previous studies on mycotoxins/toxins revealed that these compounds are also hazardous to animals and humans (Adetunji *et al.*, 2014; Delgado *et al.*, 2014; Ediage *et al.*, 2014). Generally, it has been reported that CGs as well as mycotoxins/toxins occur naturally in flora and organisms (fungi/bacteria) as a result of biosynthesis, with their prevalence being quantifiable in many agricultural products such as cassava, apples, spinach, apricots, cherries, peaches, plums, quinces, almonds, sorghum, lima beans, corn, yams, chickpeas and cashews (Kuti & Konoru, 2006; Cressey *et al.*, 2013; Ediage *et al.*, 2014). See Appendix 2.

Although some microorganisms and plants synthesise these compounds for their survival when exposed to harsh environmental conditions, their cumulative production can contribute to ecological disturbances. Furthermore, it was also found that various arthropods and invertebrates produced cyanogens as a defence mechanism and for controlling mating behaviour (Gleadow & Woodrow, 2002; Ismaiel & Papenbrock, 2015), although on a minute scale. Research by Jones (1998) indicated that plants, including microorganisms, are known to be major producers of these compounds owing to their physiology. Thus, the presence and loading of these cyanogens and mycotoxins/toxins into terrestrial ecosystems is largely overlooked, although they have some negative effects on the physico-chemical and biological properties of soil, particularly arable land as well as the environment in general (Jones, 1998; Okechi *et al.*, 2012).

Previous studies have stated that cyanogen and mycotoxin loading in agricultural soil can have a serious impact, disturbing the local terrestrial ecosystem functionality (Rillig & Mummey, 2006; Okechi *et al.*, 2012). Current evidence suggests that most studies on agricultural produce such as cassava, known for its high cyanogen content, have predominantly focused on the production of the crop for nutritional and industrial purposes, with its effects on soil (including the surrounding environment) being overlooked (Ubalua, 2010; Okechi *et al.*, 2012). Accordingly, minimal research has been completed on cyanogen and mycotoxin loading, including their behaviour and movement in soil that can culminate in groundwater contamination. Generally, a large quantity of agricultural produce, such as cassava tubers, perishes prior to harvesting for a variety of reasons. Although free cyanide and mycotoxin toxicity is widely reported, their level of toxicity is also influenced by cumulative exposure and the continuation of their release from produce into the environment. Cyanogen and mycotoxin loads and their movement in soil, including their potential to contaminate underground water which is used in impoverished communities where cassava is cultivated mostly as a source of protein and starch, are largely

under-reported (Brakhage, 1998; Brakhage *et al.*, 1999; Brakhage & Schroeckh, 2011; Brakhage, 2013).

The highlights of an extended literature review are condensed and reported here:

☐ There are similarities in the movement of cyanogens and mycotoxins, including their degradation by-products in soils due to mass transfer processes influenced by the moisture content in the soil;

☐ Cyanogen and mycotoxins can hypothetically distort the soil's characteristics with seepage into groundwater systems which is of paramount concern, negatively impacting on terrestrial and aquatic life and water quality;

☐ This seepage will culminate in prolonged, cumulative human and animal exposure.

The sustained exposure within a terrestrial ecosystem that serves as a habitat for a multitude of living organisms, albeit at varying concentrations for both cyanogens and mycotoxins from diverse sources, is detrimental. Methods can be developed to reduce their voracity with mitigation strategies implementable in communities with low literacy levels to quantify and reduce these compounds concentrations in soil as well as in groundwater sources (Barclay *et al.*, 1998; Baxter & Cummings, 2006).

2.2 Cyanogen and mycotoxin reduction

Several methods for cyanogen reduction have been proposed and include physical, chemical and biological methods (Figueira *et al.*, 1996; Akcil *et al.*, 2003; Akcil & Mudder, 2003; Ebbs, 2004; Cumbana *et al.*, 2007; Dash *et al.*, 2014). However, it has been reported that some of these methods require high input costs and sophisticated knowledge and/or training to implement successful removal strategies (Figueira *et al.*, 1995; Gurbuz *et al.*, 2004; Hossain *et al.*, 2005). Meanwhile, scientists have embarked on research to intensify reduction methods for these toxicants in the environment by using techniques which are deemed environmentally benign. Such novel ways of reducing both cyanogen and mycotoxin levels in the environment, including agricultural produce destined for consumption, are generally considered cost effective

when compared with long-term outcomes of the lack of implementation of control measures (White *et al.*, 2000; Siritunga & Sayre, 2003; Vanhoutte *et al.*, 2016).

2.2.1 Biological reduction of cyanogens

The biological reduction of CGs as a source of cyanide, as well as mycotoxins, has gained popularity and has been a huge research focus area (Siller & Winter, 1998; White *et al.*, 2000; Siritunga & Sayre, 2003; Hossain *et al.*, 2005; Vanhoutte *et al.*, 2016). As such, genetically modified cassava cultivars with a suppressed cytochrome P450 gene functionality (producers of enzymes *CYP79D1* and *CYP79D2*), may inhibit the infiltration of linamarin as it can be converted into free cyanide from valine (Andersen *et al.*, 2000).

Furthermore, other biological treatments for free cyanide involve the application of microorganisms known to be toxin producers. These include bacteria such as *Pseudomonas* sp*., Nocardia* sp., *Achromobacter* sp., *Flavobacterium* sp., *Bdellovibrio* sp., as well as nitrifiers (bacteria) such as *Nitrosomonas* sp., *Nitrobacter* sp*., Sphingomonas* sp*., Exophiala* sp*., Curtobacterium* sp*., Eubacterium* sp*.* and fungi such as *Aspergillus* sp. and *Penicillium* sp*.* (Figueira *et al.*, 1995; Figueira *et al.*, 1996; Siller & Winter, 1998; White *et al.*, 2000; Akcil *et al.*, 2003; Ebbs, 2004; Hossain *et al.*, 2005; Dash *et al.*, 2006; Vanhoutte *et al.*, 2016). See Appendix 2. Amongst these microorganisms, *Aspergillus* sp. and *Penicillium* sp*.* are the most prevalent species able to grow successfully in stringent weather conditions, with some including *Cunninghamella* sp. being abundant in soil (Schell *et al.*, 1982; Zheng & Chen, 2001), and have the ability to grow on a variety of agricultural produce, namely maize, peanuts and tubers (Adebajo *et al.*, 1994; Hocking, 1997; Sing *et al.*, 2014) (Appendix 2).

In soil consisting of fungal biocatalysts of different origins, scientific evidence seems to indicate that agricultural produce appears to be susceptible to spoilage due to substrate availability which results in the proliferation of spoilage causing microorganisms (Adebajo *et al.*, 1994; Wagacha & Muthomi, 2008; Bhat *et al.*, 2010; Adetunji *et al.*, 2014; Matumba *et al.*, 2014; Ediage *et al.*, 2014). It has also been reported that fruit or produce has trace elements such as Ca, Na, K, Zn, low relative molecular weight hydrocarbons, including proteins and moisture, providing conditions which facilitate microbial growth and thus proliferation of spoilage organisms (Hocking, 1997; Afsah-Hejri *et al.*, 2013; Ediage *et al.*, 2014). Owing to this, some microorganisms produce hydrolases, reducing primary compounds in produce to by-products, furthering physico-

chemical changes in the environment in which they are growing (Knudsen, 2006; Rillig & Mummey, 2006). These conditions seem to be ideal conditions in which cyanide-reduction organisms proliferate, conditions which are nutrient-rich as a result of nutrient availability from decaying produce.

Some of the cyanogens are reduced to by-products such as bicarbonate and ammonia. The ammonia, formed during the process, is further utilised by the microorganisms as a source of nitrogen, supporting increased microbial growth (Henrikson *et al.*, 2009; Heinl *et al.*, 2011; Umsza-Guez *et al.*, 2011). In the agricultural industry, the reduction of both cyanogens and related compounds is complex as *in-situ* quantification of such processes is reported on minimally. The development of processes and strategies which are environmentally benign, those of biological origin, is gaining popularity due to their simplicity and beneficial advantages as they are considered less harmful and can be beneficial for economic management for improving the profitability of the manufacture of commercial agro-produce (Barclay *et al.*, 1998; Gurbuz *et al.*, 2004; Dash *et al.*, 2014; Sing *et al.*, 2014). Owing to the exposure to cyanogen, including by-products from cyanogen conversion/transformation, some species become tolerant, thus evolving biologically.

For example, Sing *et al. (*2014) successfully isolated a fungus, namely, *Cunninghamella* sp*.* UMAS SD12 from sawdust, with an ability to biodegrade 51.7% Pentachlorophenol (PCP) within 15 days in a controlled static environment. However, more research needs to be conducted to assess direct evolvement of the microbial ecosystem as other microorganisms that constitute a community for the betterment of soil, can reduce the viability of such soils and/or resulting in some organisms producing extracellular secondary metabolites such as mycotoxins.

2.2.2 Biological reduction of mycotoxins

There are numerous mycotoxins which are known to contaminate agricultural produce such as cassava. Amongst the mycotoxins, Fumonisin B1 and Deoxynivalenol (DON) are common (see Appendix 2). The biodegradation of Fumonisin B1 and DON can be achieved through direct conversion using detoxification processes with different pathways (Vanhoutte *et al.*, 2016).

For example, Fumonisin biodegradation was observed through the elimination of the tricarballylate side chains and amino groups. The enzymatic hydrolysis of such mycotoxins might involve carboxylesterases and aminotransferases from bacteria such as *Sphingomonas* and *Sphingopysis* sp. normally found in soil, which have the ability to detoxify recalcitrant, persistent organic pollutants (PoPs) such as Polycyclic aromatic hydrocarbons (PAH's) (Duvick *et al.*, 1998; Duvick *et al.*, 2003; Heinl *et al.*, 2011; Amodu *et al.*, 2013; Vanhoutte *et al.*, 2016). Other researchers have reported degradation or detoxification of Fumonisin B1 including the by-products of toxins by oxidative deaminase from *Exophiala* sp., a common soil organism (Duvick *et al.*, 1998; Blackwell *et al.*, 1999; Duvick *et al.*, 2003; Vanhoutte *et al.*, 2016). *Bacillus* sp., including non-saccharomyces yeast commonly found in soil, was also suggested to destabilise functional groups in the structure of mycotoxins and thus reduce their amino acid to functional groups albeit at elevated pH (Camilo *et al.*, 2000).

In most instances, the biodegradation process of most mycotoxins involves a consortium of organisms which utilise a variety of degradation pathways (Blackwell *et al.*, 1999; Duvick *et al.*, 2003). Overall, the initial biodegradation stage starts at extracellular level by deamination or facilitation by esterase with the last biodegradation step involving microbial/enzymatic decoupling of the aliphatic chain within the mycotoxin molecule (Vanhoutte *et al.*, 2016). For example, the first biodegradation steps of DON, using *Curtobacterium* and *Eubacterium* sp., were found to be initiated by the de-epoxidation step, which is subsequently followed by oxidation (Guan *et al.*, 2009; Vanhoutte *et al.*, 2016).

2.3   Toxicity of cyanide as a cyanogen and mycotoxins from cassava

2.3.1   Toxicity of cassava

Worldwide, cassava is utilised as a primary foodstuff for many communities of Africa, Asia and South America (Padmaja & Steinkraus, 1995; Fasuyi, 2005; Montagnac *et al.*, 2009; Mburu *et al.*, 2012; Hidayat *et al.*, 2016). Cassava's toxicity is due to cyanogens such as linamarin, lotaustralin and 2-((6-O-(b-D-apiofuranosyl)-b-D-glucopyranosyl)oxy)-2-methylbutanenitrile) that are biologically transformed into hydrogen cyanide (Prawat *et al.*, 1995; Andersen *et al.*, 2000). As a result of enzymatic hydrolysis, linamarin is transformed in plant tissue into acetone cyanohydrin (Montagnac *et al.*, 2009). At an increased temperature of >30°C and a soil pH of 5, respectively, conditions associated with arid regions which are suitable for microbial proliferation

and thus the contamination of agricultural produce and spoilage, acetone cyanohydrin is released, resulting in its decomposition into acetone and hydrogen cyanide (Andersen *et al.*, 2000; Montagnac *et al.*, 2009) (see Figure 2.1). Several studies have been done on the impact of the cultivar on humans as a result of direct ingestion (Kamalu, 1995; Ernesto *et al.*, 2002; Siritunga & Sayre, 2003; Fasuyi, 2005; Soto-Blanco & Górniak, 2010) as cyanide concentration in the tuber is estimated to be as high as 450 mg/kg (Hidayat *et al.*, 2016). An illustration of the enzymatic hydrolysis of linamarin to hydrogen cyanide is illustrated in Figure 2-1 below.
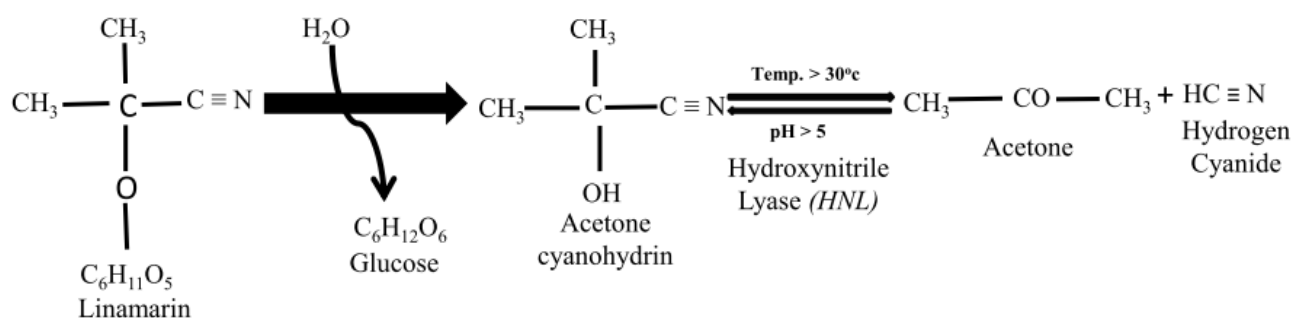
Figure 2-1: Enzymatic hydrolysis of linamarin to hydrogen cyanide

Thus, its prolonged consumption may be toxic (Hidayat *et al.,* 2000; Hidayat *et al.,* 2016). However, there is minimal information on hydrogen cyanide loading into arable land in which cassava is cultivated. Furthermore, free metal ions which are also exposed to hydrogen cyanide in such a soil, can form metallic cyanide complexes under suitable conditions, prolonging the prevalence of cyanide-based compounds in the soil further, which might leach into groundwater.

2.3.2 Impact of cyanogens on biochemical and physical properties of agricultural soil

Although the conditions and diversity of habitats contribute to and thus influence the biochemical and physical properties of arable soil (Knudsen, 2006; Rillig & Mummey, 2006), a high cyanogen load in such a soil can have a negative impact on the soil's microbial population, with sustained exposure and an increased concentration of cyanogens hindering the microbial activity and thus the functionality of soil microorganisms leading to the deformation of the

biochemical, including the physical properties of the soil. A high hydrogen cyanide concentration load in such a soil was found to contribute to an increase in the total organic carbon (TOC) and chemical oxygen demand (Dzombak *et al.*, 2005), reducing the ability of *Nitrobacter* sp. to sustain nitration processes (Kim *et al.*, 2008; Kim *et al.*, 2011). Therefore, an increase in the hydrogen cyanide loading could lead to an imbalance between *Nitrospira* and *Nitrobacter* sp., resulting in a higher count of species with a hydrogen cyanide-resistant ability. The change in the microbial population balance could lead to a stunted growth and/or variations in the growth of a cultivar. This can easily culminate in the dominance of the species which can be a spoilage organism with free cyanide-resistant characteristics contributing to spoilage patterns/microbial contamination of the produce in question.

For mitigation strategies during the post-harvesting period, preparation of soil for re-cultivation could lead to inadequate organic matter (OM), a variation in total nitrogen (TN) content and availability, which can interfere with soil biochemical and physical properties (Meeussen *et al.*, 1994; Dzombak *et al.*, 2005).

Research on the physicochemical characteristics of cassava-cultivated soil has shown a correlation between continuous cassava cultivation and a decline in the soil's physico-chemical properties (Howeler, 1998). Therefore, continuous cultivation of cassava, which normally happens in impoverished communities, could result in a decrease in soil quality, bulk density, organic carbon (OC), OM, trace elements, moisture, infiltration rates, including holding capacity and aggregate stability. Howeler (2002) reported further that the average nutrient removal rate per ton of cassava tubers harvested is equivalent to N = 2.53 (38%), P = 0.37 (49%), K = 2.75 (56%), Ca = 0.44 (16%) and Mg = 0.26 (30%). Thus, cyanogen loading was reported to have an indirect impact on the C:N ratio, which resulted in a pH increase with depth, while it was found that OC, TN, and OM distortions had been entrenched.

Similarly, Matsumura and Kojima (2003) examined the relationship between cyanogen concentration, pH and soil moisture, determining that, with an increase in cyanogen levels, soil pH increases with moisture content availability, further supporting the retention of cyanogens with such a pH.

The concentration of cyanogenic compounds was shown to vary from soil to groundwater and from one site to another (Meeussen *et al.*, 1994; White & Markwiese, 1994; Kjeldsen, 1999;

Ubalua, 2010), which suggested that the discrepancies in distribution could be due to the mobility of the contaminants (Meeussen *et al.*, 1994).

2.3.3 Production of mycotoxins

Terrestrial ecosystems are populated by a diversity of microorganisms that contribute to and maintain the ecological as well as the biological balance. These organisms contribute to the characteristics of the soil which directly influences soil productivity and crop yield in the agricultural sector (Brookes, 2001; Chen & Hu, 2004; Knudsen, 2006; Rillig & Mummey, 2006; Vineela *et al.*, 2008). However, some have been shown to exhibit pathogenicity toward mature produce. For example, during the growth and up to the harvest stage of cassava tubers*,* several pathogenic organisms with mycotoxin production potential can dominate several other species of bacteria and fungi on the tuber and in cassava-cultivated soils (Sing *et al.*, 2014). Some of these organisms are even resistant to the free cyanide in cassava, and with their inherent characteristics, such as their predisposition for survival, they produce mycotoxins such as Ochratoxin A, Aflatoxins, Fumonisin B*,* Pyranonigrin A, Tensidol B, Funalenone, Naphtho-y-pyrones, Deoxynivalenol *(DON)* and Malformins (Perrone *et al.*, 2006; Stoilova *et al.*, 2006; Perrone *et al.*, 2011; Varga *et al.*, 2011). (See Appendix 2)*.* Research revealed that exposure to mycotoxins pre/post-harvest and their presence in soil can render the cassava tubers inedible (Shifrin *et al.*, 1996; Stoilova *et al.*, 2006), leading to cumulative and increased levels due to sustained use of pre-recovery land for cultivation to produce an essential food source, a method which will affect the soil's ecology.

2.3.4 Mycotoxins effects on soil ecology

Soil ecology is influenced by biochemical processes, biotic relations and physical conditions which are paramount to its health (Brookes, 2001; Chen & Hu, 2004; Knudsen, 2006; Rillig & Mummey, 2006; Vineela *et al.*, 2008). The biochemical characteristics of the soil used for cultivation is related to its microbial diversity as well as its pollutant content (Knudsen, 2006), with the soil's microbial community playing a transformative role with regard to the soil nutrient availability, health and fertility, all of which enhance the soil's quality, including its productivity (Vineela *et al.*, 2008). Furthermore, the microbial ecology of any soil facilitates the flow of nutrients through immobilisation processes, which result in the soil's bio-augmentation (Wani & Lee, 1995;

Rillig & Mummey, 2006), contributing to the soil structure that assists in the formation of nutrient-rich aggregates.

According to Knudsen (2006), soil aggregates are created due to microbial activity, albeit at a microscopic level, linking banding soil particles, while the extracellular polysaccharides from bacterial cells play a role in holding soil aggregates together (Vineela *et al.*, 2008). With subsequent structuring and compaction, parameters influence on the quality of the soil's texture: porosity, aeration, moisture permeability and circulation, including organic matter content (Schjønning *et al.*, 1999; Rillig & Mummey, 2006). Soil grain cohesion, porosity, permeability and organic matter content are vital for soil quality and fertility, particularly for soil demarcated for sustaining the production of agricultural produce. All these parameters are indispensable for the sustainable use of arable soil for food production and productivity for crop yield (Knudsen, 2006; Vineela et al., 2008). Additionally, soil health can also be affected by surface, subsurface and ground water supply, including groundwater quality, which has been contaminated with mycotoxin, assisting the mobility of these toxins to soil free of contaminants.

Soil moisture content is also vital for soil functionality as it serves as a water reservoir for the terrestrial ecosystem, playing a major role in the water cycle between surface and subsurface water, thus affecting the quality of groundwater when contaminated (Vervier *et al.*, 1992; Fetter & Fetter, 1999). High mycotoxin loading into the soil may thus impact its functionality. Thus the interaction of pollutants with microorganisms, invertebrates, vertebrates and cultivated crops, which eventually leads to the reduction of groundwater quality, may lead to the increase of sustained leaching or periodic contamination of the water, to which humans can easily be exposed. The distribution of pollutants in a terrestrial hydrological cycle might have long-term disastrous consequences for surface, subsurface and groundwater supply (Fetter & Fetter, 1999) to impoverished communities.

Previous studies on mycotoxin mobility in soil revealed that the movement of these contaminants is influenced by processes such as deposition, decomposition, distribution and accumulation (Ubalua, 2010), while the concentration of the compounds increases with soil depth. Soil with a high moisture content creates conditions that lead to the increase of the contaminants' ability to be transferred, based on processes such as infiltration, percolation and leaching into groundwater (Meeussen *et al.*, 1994; Fetter & Fetter, 1999). The land detoxification bioprocesses and strategies may involve extended periods during which the land is unusable.

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Furthermore, several studies on the effects of cassava effluents on soil, including microbiota, found that a high mycotoxin concentration in soil is harmful to some soil microorganisms. Some of these mycotoxins are produced because of inhabitative competition. This means that organisms will produce these mycotoxins to limit the proliferation of others, particularly under nutrient-depleted conditions.

Previous studies revealed that mycotoxins from cassava are mobile in soil and destroy the resident soil's organisms (Baumgarte & Tebbe, 2005; Rui *et al.*, 2005). Additionally, Okechi *et al.* (2012) have shown that the effects of cassava effluents on soil microbial populations culminate in a discrepancy between bacterial and fungal populations at different pH levels and soil depths. The study indicated varying bacterial populations from the upper layers of the soil which increased in comparison to those recorded in the lower (deeper) soil layers, with high concentrations of the mycotoxins and their producers being observed in the lower soil layers, a process furthered by leaching. Similar total fungal population counts revealed a similar phenomenon at surface, subsurface and deeper soil layers.

2.3.5 Cyanogen and mycotoxin movement behaviour in soil

The behaviour of cyanogen and mycotoxins in soil, groundwater and the environment is largely controlled by numerous biochemical reactions and processes. There are similarities and differences between the processes involved in the behaviour of each contaminant, which is controlled by the conditions the contaminants undergo when in the soil and groundwater. These processes are primarily influenced by numerous biochemical processes and by the structure, properties and behaviour of the compounds in the environment. According to Kjeldsen (1999), the behaviour of cyanogen and mycotoxins, from soil into groundwater, is largely influenced by processes such as degradation, transformation and complexation (see Figure 2.2).

Furthermore, exposure to humans and wildlife, including environmental contamination, is also directly associated with pathways such as volatilisation, dermal contact and ingestion of degradation by-products from the transformation of the primary toxicant/pollutant due to its transportation pathways, facilitated by leaching mechanisms into groundwater (Shifrin *et al.*, 1996; Kjeldsen, 1999). Thus, when not monitored, the environmental prevalence and exposure of these contaminants can be harmful to human health/wildlife, thus environmental health.

For example, the concentration of leached iron-cyanide complexes in groundwater in a cyanide-contaminated environment ranged between 2 to 12 mg/L in lower soil levels (Meeussen *et al.*, 1994; Shifrin *et al.*, 1996). The prevalence of such complexes can be influenced by the reactivity of free metal ions and their interaction with free hydrogen cyanide from cassava. These compounds may be transformed (through decomposition) to free cyanide at a later stage; although, most are stable with a long half-life, thus may enter an aquifer through processes such as infiltration and leaching.

The movement of cassava cyanogen and mycotoxin movement in agricultural soil is illustrated in Figure 2-2.
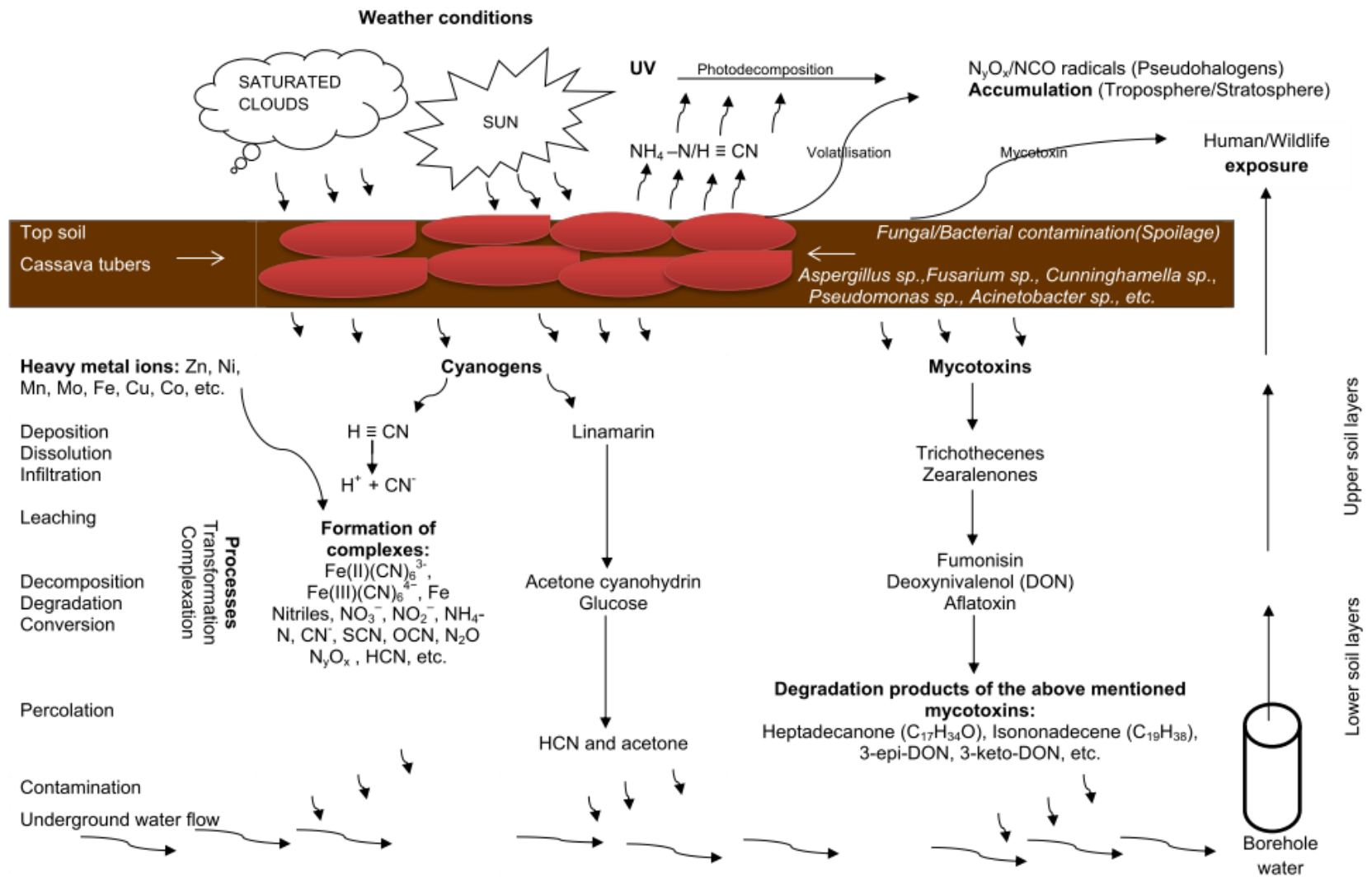
Figure 2-2: Cassava cyanogen and mycotoxin movement in agricultural soil. Key: NO_3^- (nitrate), NO_2^- (nitrite), Fe $(CN)_6^{3-}$ (ferrocyanide), $Fe(III)(CN)_6^{4-}$ (ferricyanide), SCN (thiocyanide), NH_4-N (ammonium nitrogen), CN^- (cyanide ion), - OCN (cyanate), N_2O (nitrous oxide), N_yO_x (nitrogen oxides)

It is also important to point out that the behaviour of contaminant movement in terrestrial and/or aquatic ecosystems and the environment in general, is also influenced by parameters such as periodic wash-off, moisture saturation and time. Based on the stability of each individual contaminants, including their by-products, the mobility can also be spontaneously influenced by their rate of conversion, thus degradation, and even become volatilised under suitable conditions (Kjeldsen, 1999; Zidenga *et al.*, 2012), depending on their vapour pressure. Time or length of exposure is a very important aspect, particularly where human exposure is assessed, which is generally neglected or unclear in many recent studies. Similarly, contamination gradients must be established because of groundwater flow, including level variations as well as the influence of the immediate surroundings by the water-body, which might contribute to acute exposure levels. Furthermore, from the produce itself, volatilised compounds can undergo photo-decomposition due to UV effects contributing to pseudohalogen accumulation in the Tropo- and Strato-sphere (see Figure 2.2).

2.4   Cyanogen and mycotoxin effects on humans/animals

As previously stated, cassava can be toxic when consumed in large quantities due to its cyanogen content (Prawat *et al.*, 1995). A prolonged consumption of cassava in different forms can be harmful for humans in particular, owing to inadequacies in post-harvest treatment techniques (Ernesto *et al.*, 2002; Fasuyi, 2005; Montagnac *et al.*, 2009). For instance, studies on cassava cyanide effects in humans revealed that a permanent consumption of low-level concentrations of cyanide from poorly processed cassava, could result in goiters and Tropical Ataxic Neuropathy (TAN) (Shifrin *et al.*, 1996; Siritunga & Sayre, 2003), while a high consumption of the produce could result in neurological disorders, such as *Konzo* (Kamalu, 1995; Soto-Blanco *et al.*, 2002). Most post-harvest cyanogen removal techniques focus on free cyanide-removal techniques, without accounting for transformed varieties, including cyanide complexes and thiocyanates, which are not reduced by common treatment technologies because of their characteristics.

A team of researchers conducting studies on the thiocyanate concentration in urine samples of pupils who consumed cassava in Mozambique, revealed that a mean concentration of urinary thiocyanate in schoolchildren ranged from 225 to 384 mol/L, while a mean total of cyanogen concentrations in processed cassava flour varied with the seasons and years from 26 to 186 mg/L (Ernesto *et al.*, 2002). Similarly, a study by Shifrin *et al.* (1996) revealed that

mycotoxins could easily be absorbed through dermal contact, ingestion and inhalation, highlighting that some mycotoxins are hazardous and are proposed to be carcinogens facilitating mutation in human's cells, an effect that can be postulated to suggest their facilitation of cancer formation in humans.

In animals, on the other hand, an increased consumption of tuber debris and/or waste of produce processing, could lead to neuronal disturbances, weight loss and dysfunctional thyroid glands (Kamalu, 1995; Soto-Blanco *et al.*, 2002; Soto-Blanco & Górniak, 2010). Observations reported by Wade *et al.* (2002) on cassava waste in fish, such as the Nile Tilapia *(Oreochromis Niloticus)*, revealed that some cyanogen caused oedema, gill lamella's telangiectasia, gill enlargement, formation of vacuoles and liver cell deterioration. Similar health outcomes for humans and animals were observed in acute mycotoxin exposure, including ingestion. These outcomes were, inter alia, weight loss, bleeding of internal organs, respiratory diseases (Asthma, Pneumonia), diarrhea, liver and kidney cancer, and skin irritation (Bankole & Mabekoje, 2004; Bhat *et al.*, 2010; Afsah-Hejri *et al.*, 2013). Therefore, a large-scale propagation of agricultural produce with cyanogens, which are susceptible to spoilage by mycotoxin producers, requires continuous monitoring to ascertain its quality. Such produce should be free of both cyanogens and mycotoxins, primarily if it is destined for human and animal consumption and/or exposure. In this case, required strategies for the reduction of exposure must be implemented.

2.5 Summary

Cassava, in general, and cassava tubers, in particular, are indispensable for daily self-nourishment of several poor communities worldwide owing to their nutritional value. However, when exposed to environmental processes, as well as bacterial and fungal attacks that can occur prior to harvesting, the produce is susceptible to releasing cyanogen and mycotoxin compounds that are hazardous to humans, animals and the environment. These contaminants, and the mobility of their by-products into the terrestrial ecosystem, are similar and are facilitated by environmental processes such as transformation, complexation, percolation and volatilisation as they can travel from surface and subsurface to the underground water level, which can result in exposure to both animals and humans. The presence of these compounds in arable land can lead to their accumulation, which can negatively affect soil properties, groundwater quality and the environment, thus contributing to a decline in the production of useful produce such as cassava tubers. Monitoring, particularly in communities that use such an arable soil on a continuous basis,

can limit the intoxification of humans and animals, by effectively implementing suitable reduction strategies, thus minimising environmental pollution. Therefore, continuous monitoring, quality assurance and novel *in-situ* biological reduction methods (for treatment of the contaminants) are paramount to ensuring a healthier agricultural soil, clean surface and groundwater quality.

Limitations of current research studies include (but are not limited to):

Minimal and/or limited research on:

➢ Common soil organisms and/or unique pathogenic microorganisms which:

☐ facilitate cassava (*Manihot esculanta* Crantz) decomposition and which are also resistant to cyanogens such as free cyanide from decomposing cassava,

☐ have not been previously reported to produce toxins and/or mycotoxins which can contaminate agricultural produce commonly cultivated in some arid regions as a primary food source in countries such as those in sub-Saharan Africa, and

☐ produce other extracellular bio-products that have a negative impact on other resident organisms (soil biota).

Furthermore, previous studies have also indicated the need to:

➢ Develop suitable agricultural soil remediation strategies (methods) by using a green chemistry approach in order to improve soil productivity, in particular to:

☐ reduce the impact of consistent toxin and/or mycotoxin including cyanogen loading into agricultural soil from decomposing produce such as cassava.

CHAPTER 3

RESEARCH DESIGN, MATERIALS AND METHODS

CHAPTER 3

3. RESEARCH DESIGN, MATERIALS AND METHODS

3.1 Overview of research design and methodology

The overview of the research design and methodology used is sequentially listed below to address the following:

- Quantification of the concentration of gases (HCN, NH_3, NO_2) released from young cassava plants as well as determination of the cassava contribution to the global cyanide load into the atmosphere. See Chapter 4;

- Isolation, identification and characterisation of a previously unreported cyanide tolerant strain, which facilitates cassava decay with a potential of producing mycotoxins. See Chapter 5;

- Screening of the isolates (isolated in Chapter 5) and pathogenic activity on the microbial communities in cassava-cultivated soil. See Chapter 6;

- Identification of secondary metabolites/mycotoxins produced by the cyanide-resistant isolate and identification of mitigation means of reduction of these mycotoxins including CN^- and its degradation by-products. See Chapter 7.

3.1.1 Quality of Chemical reagents

Chemical reagents used were all certified analytical grade reagents and/or test kits, such as PowerBiofilm DNA isolation kit (MOBIO Laboratories Inc., CA- USA). Primers for ITS1, ITS2 and ITS4 (see Appendix 1), as well as PCR reagents (Inqaba Biotec, S.A.) and a master mix (Lasec Pty Ltd, S.A.). The absolute chloroform and methanol used were purchased from an international supplier through the local distribution office (Sigma-Aldrich Corporation, S.A.).

3.1.2 Mapping of cassava production and estimated HCN into the environment from cassava production

Geographic Information System software (ArcGIS, ESRI Pty Ltd, Italy) and Quantum GIS (QGIS, Open Source Geospatial Foundation) were used to map cassava production, including global distribution. The geospatial analysis used in GIS-mapping enables the characterization of maps giving information on a specific commodity using historical data for analysis (Gregory & Healey, 2007). The analysis focused on the highest cassava producers, namely Africa, Asia and South America. Thus, cassava-growing countries were shaded using a key marker, whereby a single key marker was equivalent to an estimated 72×10^{-5} ppq (Africa), 36×10^{-5} ppq (Asia) and 41×10^{-5} ppq (South America). The countries that do not have records of producing cassava were left blank (Figures 4.2,3,4). Overall, most cassava-producing countries are inland, with the highest cassava producers being within the inter-tropical convergence zone (ITCZ), where it is relatively hot and rainy throughout the year (Chiang *et al.*, 2000; Garibaldi, 2012).

3.1.3 Reliability of Food and Agriculture Organisation (FAO) data

The Food and Agriculture Organisation (FAO) data raise concerns. Firstly, the coverage is limited to FAO member countries, with many developing countries having remotely accessible areas due to poor infrastructure. This affects the recording of cassava production. Secondly, the collection and recording of data in many developing countries are not adequate; data collection is often done manually prior to being characterised at the nearest data-logging site. In the case of missing data, statistical methods can be used to correct errors (Karp *et al.*, 2007; Zuur *et al.*, 2010). Statisticians often determine (by estimation) the national, regional, continental and world aggregates to compile secondary derived statistics by using supply and actual usage accounts from the database to ascertain the consistency of particular data sets (Karp *et al.*, 2007).

Thus, statistical methods such as average yield or average production, estimation of mean values, and so forth, had to be used to correct errors.

3.1.4 Model development and HCN projection load into the environment

Global cassava production data were obtained from the Food and Agriculture Organisation's statistical database. Approximately a decade (for example, 2002 to 2013) was considered for the projection (2014 to 2024) of corresponding HCN load (ppq) into the environment using a linear projection (Eq. 3.1) with correlation coefficients (R^2) being Africa – 0.93, Asia – 0.84 and South America – 0.92:

$$P_x = P_b + kt \tag{3.1}$$

whereby $\quad k = \left(P_b\text{-}P_{ref}\right)/\left(t_b\text{-}t_{ref}\right)$ \hfill 3.2

where P_x (ppq) is the projected HCN load at time t (yr), k (ppq/yr). The projected incremental rate of HCN load, P_b (ppq) is the estimated HCN load projection where t_b, P_{ref} (ppq) is the start of the projection, t_b is the estimated time of the projection and t_{ref} is the reference (initial) year.

3.1.5 Statistical analysis of FAO data

A statistical analysis of FAO data (2002 to 2013) (FAOSTAT, 2014) and the estimated projection trend up to 2024 was done using MS Excel® v2010. For modelling the HCN load into the environment, 250 HCN per mass (kg) was used as per FAO data (see Table 3.1). For the data presentation, 1 quadrillion was represented as HCN concentration divided by 10^{15}. The mean absolute deviation (MAD) between the model and the actual data set was calculated (Eq. 3.3).

$$\text{MAD} = \frac{\sum |\text{errors}|}{n} \tag{3.3}$$

where Σ |*errors*| is the sum of the absolute values of errors between the projection and actual HCN environmental loading values (ppq), while n is the number of periods (yr) under consideration.

Table 3-1: HCN content in cassava

Type: Non GMO	Non-bitter	Bitter	Very bitter	References
Tubers & Leaves	<100 mg HCN/kg FW*	100–450 mg HCN/kg FW*	>450 mg HCN/kg FW*	(Sundaresan *et al.*, 1987; (Nambisan & Sundaresan, 1994)
		10–500 mg HCN/kg DW*		(Siritunga & Sayre, 2003)
		6–250 mg HCN /kg FW*		(Piero *et al.*, 2015)
		420 mg HCN/kg FW*		(Sundaresan *et al.*, 1987)
		200–1,300 mg HCN/kg DW*		(Montagnac *et al.*, 2009)
Type: GMO		**Non-bitter**		(Siritunga & Sayre, 2004)
Tubers & Leaves		10 < 50 mg HCN/kg FW*		(Nambisan, 2011)
		6 < 50 mg HCN/kg FW*		(Piero *et al.*, 2015)

HCN estimated at an average of 250 mg HCN per kg dry weight cassava tubers (DWCT) in mature plants

*DW: Dry weight, *FW: Fresh weight

3.1.6 Cassava plant health studies

The overall plant health of cassava under greenhouse conditions, characterised by PAR and chlorophyll content measurements, was assessed. These two processes are important for plant growth assessment under normal and controlled environments to assess and quantify developmental functions such as leaf lifespan, nitrogen content, hydraulic conduction, chlorophyll fluorescence and reflectance (Bornman & Teramura, 1993). Although chlorophyll and PAR fluorescence wavelength range is between 400 and 700 $\mu mol/m^2 s$ (Pavlovic *et al.*, 2015), a healthy plant in a controlled environment has a relative photosynthesis efficiency range of between 380 and 780 $\mu mol/m^2 s$ (Pavlovic *et al.*, 2015), while its chlorophyll content can range from between 15 and 200 $\mu mol/m^2$ (Gitelson & Merzlyak, 1997; Merzlyak *et al.*, 1997; Barker & Pilbeam, 2015). Plant leaves contain pigments (chlorophyll) that react with photochemical energy (photons) during the photosynthesis process. During the process, CO_2, water molecules and photons are used to produce the plant's energy sources (for example, glucose) while the hydrogen atom is removed and replaced by oxygen which is released into the air (Barker & Pilbeam, 2015).

The physical characteristics of the cassava plants used for analysis were: Plant 1: 63.5 cm long; the two branches were 53 cm, 44 cm and 37 cm long; the length of the leaves on each branch was approximately 31 cm. Another plant, Plant 2, was 1.05 cm long with minimal sprouting of branches. The leaves were 43 cm, 38 cm and 30 cm long.

Prior to cultivation in black bags, cassava cuttings of 15cm were placed into rectangular plastic trays for 6-10 days, depending on the plants' growth, then transferred into 4 kg black plastics bags with soil and placed on top of a warm bed to allow for growth for 2-3 months. The plants were water sprinkled for 2-3 min periodically, with the temperature in the greenhouse ranging from 19-25 °C. At five months, plants were transferred into 10 kg bags for rooting development and further growth. See Figure 3-1.

(a)　　　　　　　　　　　(b)

(c)　　　　　　　　　　　(d)
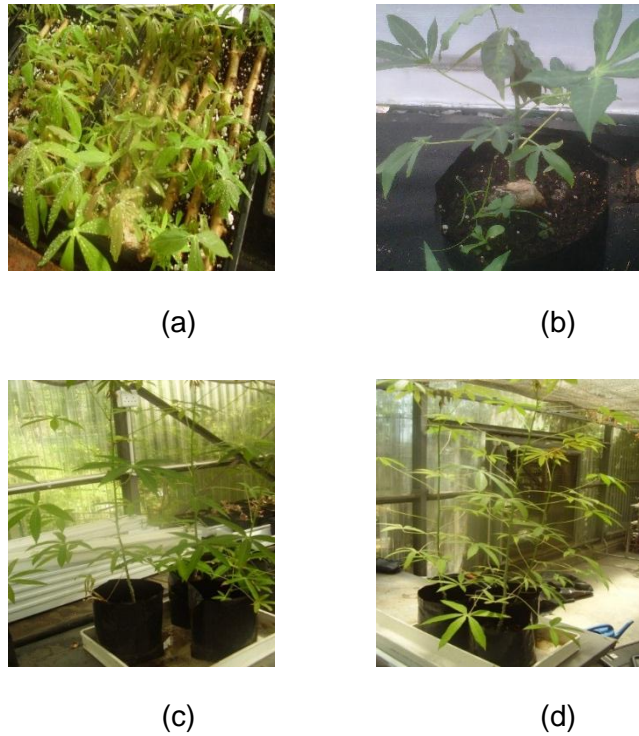
Figure 3-1: Cassava plant growth stages. (a) cassava cuttings in plastic trays; (b) cassava plants in small (4kg) plastic bags; (c)/(d) cassava plants in small (10kg) plastic bags

3.1.7 Determination of plant's chlorophyll content and photosynthetically active radiation (PAR)

A Chlorophyll Content Meter (CCM, Model-200 plus, ADC BioScientific Ltd, England) and a Photosynthetically Active Radiation (PAR) meter (AccuPAR PAR/LAI Ceptometer, Model LP-80, Decagon Devices Inc., USA) were used to assess the chlorophyll content and PAR from cassava leaves according to the manufacturers' instructions. During measurements, three readings from three different leaves of each plant were taken. Each leaf reading was averaged and the readings were repeated thrice. Finally, a standard deviation for each reading was calculated (Eq. 3.4):

$$Standard\ Deviation = \frac{\sum |(x - \bar{x})|}{n} \qquad\qquad 3.4$$

where x – measure value (μmol/m^{2}s), $\bar{x}$ – average of the reading (μmol/m^{2}s), n – number of times the measurements were taken.

The PAR actual signal was obtained using the following (Eq. 3.5):

$$S_{act} = S_{obs} + S_{dev} \qquad\qquad 3.5$$

where S_{act} - is *the* Actual signal, S_{obs} - Observed signal and S_{dev} - Signal deviation.

3.1.8 Determination of gases (HCN, NH$_3$, NO$_2$) volatilised from cassava plants

A gas analyser device was constructed (see Figure 3-2), using rectangular plastic containers (ADDIS® clear storage) of 56 L each. The containers were sealed using silicon glue. ATI Gas analysers (Wire Gas Transmitters, Models: B12-22-1-0200-1, range: 0–200 mg HCN/L; B12-15-1-2000-1, range: 0–500 mg NH$_3$/L; B12-26-1-0200-1, range: 0–200 mg NO$_2$/L) were placed on the inside of the constructed device at about 80 cm height, and an air pump (Resun® AC-9906) was placed at the bottom of the gas analyser device. A paperless recorder – Fiji Model PHF61B11-E1OYV-F (N & Z Instrumentation & Control Pty Ltd, South Africa) was used to determine the quantity of HCN, NH$_3$ and NO$_2$ volatilised from the healthy cassava plants. Gas analysis was recorded at 24-hr intervals.

GT: Gas Transmitter
P-1: Air Pump 1
Ps : Power supply
PC: Computer
Fpr: Fiji paperless recorder
C1: Cassava plant

(a)   (b)   (c)

NH$_3$-GT   HCN-GT   NO$_2$-GT

C1

Ps

Signal from instruments
(meters) gas analysers

Air

P-1

Air

Fpr

PC
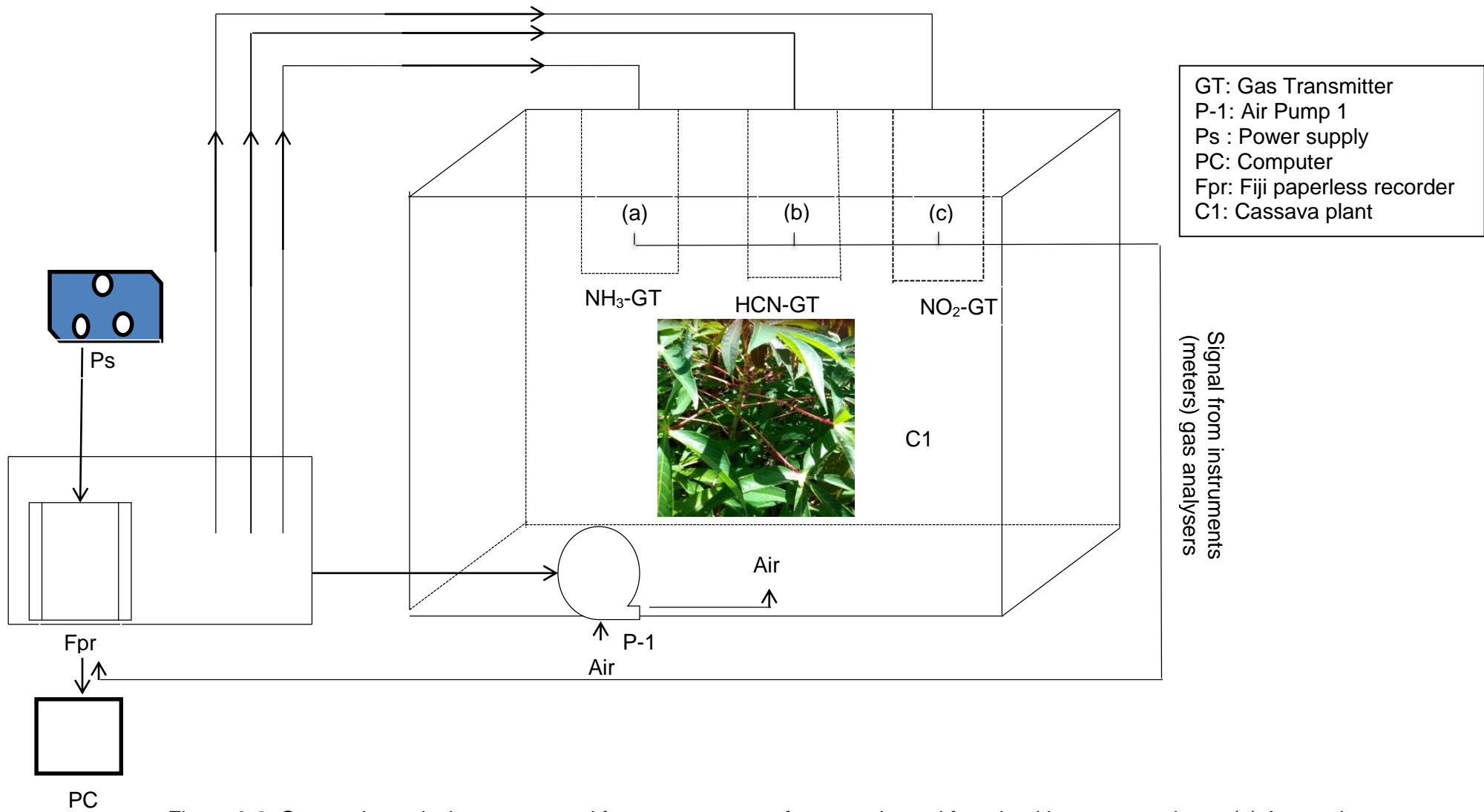
Figure 3-2: Gas analyser device constructed for measurement of gases released from healthy cassava plants. (a) Ammonia, (b) HCN and (c) NO$_2$ meter

3.2 Isolation, characterisation and identification of a cyanide-resistant organism from cassava (Manihot esculenta Crantz) and cassava-cultivated soil

3.2.1 Analytical methods: Microbial isolation, characterisation and identification

3.2.1.1 Cassava sampling and isolation of cyanide-resistant organisms

Cassava tubers were collected from Mbizana local municipality (31.5667° S, 29.4000° E), Eastern Cape Province, South Africa. Tubers were washed in sterile, distilled water, air dried and wrapped in tin foil and refrigerated at 4 °C before use. The organisms harvested from rotting cassava (see Appendix 1), were placed in Potato Dextrose Agar (PDA) while the silt, which was attached to the cassava, was added to PDA plates and stored at an ambient temperature for at least 7 days. The isolates (bacteria and fungi) were sub-cultured several times to obtain pure colonies (cultures). Colonies were then sub-cultured into nutrient broth containing KCN (4mg/40mL) as a source of free cyanide. Samples were placed in an orbital (incubator) shaker (LABWIT- ZWY-240, Shangai Zhicheng Analytical, Shanghai-China) at 30 °C and 130 rpm for 120 hrs. Subsequently, cyanide-resistant isolates were then re-cultured on PDA for 168 hrs at an ambient temperature. The organism isolated with cyanide resistance was found to be a fungus after identification of numerous isolates which had previously been found to be cyanide-resistant and mycotoxin producers. The primary aim was to isolate an organism which met the following criteria: a) previously unreported free cyanide resistance and b) a potential mycotoxin producer, with the potential of being a pathogenic organism to humans.

3.2.1.2 Microscopic characterisation of the isolate

Identification was done on the basis of microbial morphological characteristics. Macroscopic features, such as colour, texture, sporangiophore development and their arrangement, were used during the characterisation process (Bignell, 2010; Machida & Gomi, 2010). Initial observation and identification were done with the addition of lactophenol cotton blue staining reagent onto a glass slide, to which the fungal sample was mobilised and observed microscopically using an Olympus CX21 microscope (model CX21FS1, Olympus Corporation, Tokyo- Japan) at X 100.

3.2.1.3 DNA extraction and sequencing

Fungal DNA extraction was done using the PowerBiofilm DNA isolation kit (MO BIO Laboratories Inc., CA, USA) since the cyanide-resistant organisms were preliminarily found to be fungi. BF1 and BF4 solutions were heated at 55 °C for 510 min prior to use. A mass (0.05-0.20 g) of mycelium was placed into a clean 2 mL Eppendorf tube and centrifuge at 13,000 x g for 1 min. The excess liquid was removed using a micropipette. The mycelium was re-suspended in 350 µL of the BF1 solution and transferred to the PowerBiofilm bead tube. A volume of 100 µL of Solution BF2 was added and vortexed subsequent to incubating the PowerBiofilm bead tube at 65 °C for 5 min using a hot water bath (MERCK hotwater boiler).

Thereafter, the PowerBiofilm bead tube was further vortexed for 10 min followed by centrifugation at 13,000 x g for 1 min at ambient temperature and the supernatant was transferred into a clean 2 mL Eppendorf tube. A volume of 100 µL, of Solution BF3 was added, vortexed and refrigerated at (4 °C) for 5 min, subsequent to centrifugation at 13,000 x g for 1 min at an ambient temperature. The supernatant was transferred into a clean 2 mL Eppendorf tube. Solution BF4 (900 µL) was added followed by vortexing; with 650 µL of the supernatant being transferred into a spin filter tube and centrifuged at 13,000 x g for 1 min. The eluent was discarded. This was repeated until all the supernatant was sufficient to be transferred into another Eppendorf tube (2 mL). A volume, 650 µL, of Solution BF5 was added subsequent to centrifugation at 13,000 x g for 1 min at an ambient temperature. The eluent was discarded and 650 µL of Solution BF6 was added subsequent to centrifuging at 13,000 x g for 1 min at an ambient temperature. The eluent was discarded subsequent to centrifugation again at 13,000 x g for 2 min to remove the residual wash.

To the filter tube, 100 µL of Solution BF7 was added, then centrifuged at 13,000 x g for 1 min, followed by discarding the filter tube. The remaining DNA solutions in the tubes for cassava tuber isolates were labelled A and B, while the isolates from silt obtained from the cassava tubers were labelled C and D. These samples were stored at -20 °C for further use.

3.2.1.4 Spectrophotometric DNA concentration quantification

DNA concentration for isolates from both the decaying cassava and silt were quantified using the Thermo Scientific-Nanodrop 2000/2000C UV-Vis spectrophotometer (Thermo Fisher

Scientific-NanoDrop products Wilmington, Delaware, USA). The instrument quantifies the DNA concentration in ng/μL calculated from the optical density at 260 nm, 230 nm and 280 nm, to determine the purity of the isolated DNA on the basis of ratios (ODs): (260/230 and 260/280) (DeLong *et al.*, 2009; Abadi *et al.*, 2012)

3.2.1.5   PCR reagents preparation, programme and electrophoresis conditions

The PCR reagents were prepared as per the manufacturer's instructions (Inqaba Biotech, S.A.). Nuclease-free water was added to each primer, ITS1, ITS2 and ITS4, in volumes of 350, 360 and 480 μL, respectively, to obtain a final concentration of 100 μM for each. A volume (10 μL) of each primer was transferred into a new 2 mL Eppendorf tube and 90 μL of nuclease-free water was added to make a total volume of 100 μL. A control sample for all the PCR ingredients, like master mix, primers (forward and reverse) and nuclease free water, was added except for the genomic DNA. Polymerase chain reaction was performed at a total volume of 50 μL and the sample volumes were 12.5 μL master mix, 1.5 μL (each) primers (forward and reverse), 2.0 μL genomic DNA and 32.5 μL nuclease-free water. The PCR was performed in a PCR machine (T100™) equipped with a thermal cycler (produced by BIORAD laboratories Inc, USA) (see Appendix 6) using an EconoTaq plus green 2x master mix using the following programme: the instrument cycles started at 95 °C for 5 min, with a 95 °C hold up of two min for DNA's denaturation followed by a thermo cycling and annealing between 72 °C and 54 °C for a 30 sec hold and finally a repeat of 40 cycles, of 72 °C with a 10 min hold at 4°C.

The agarose gel preparation was done as follows: 10 g of agarose powder was poured into an Erlenmeyer flask containing 100 mL of TBE buffer and swirled for mixing, followed by heating in a microwave oven for 1015 min (until the solution became clear/transparent). A volume (10 μL) of SYBR Green dye was added into the warm agarose solution and gently mixed prior to pouring the solution into an Electrophoresis tank to cool down (Liu *et al.*, 2000; Seipp *et al.*, 2010). A Fast DNA ladder (New England BioLabs Inc, USA) of mass value 0.5 μg/Lane with an effective size range of 50 to 10,002bp was used to assess the DNA, using an electrophoresis gel for samples A and B from the decaying cassava tissue and from silt, which are samples C and D.

3.2.1.6　　　DNA sequencing

The universal primers, Internal Transcribed Spacers, (ITS1 and ITS4), were used to amplify the targeted region of the DNA sequences of all samples (A, B, C and D) (White *et al.*, 1990). The ITS target region was amplified using the primers with a target sequence (5' to 3'); ITS1: 'TCCGTAGGTGAACCTGCGG' and ITS4: 'TCCTCCGCTTATTGATATG'. PCR products were gel-extracted, purified and sequenced in the forward and reverse directions on an ABI PRISM™ 3500xl genetic analyser. Sequences were analysed using a CLC Main Workbench v.7, followed by a BLAST search provided by NCBI (htt://www.ncbi.nlm.nih.gov), while a Neighbour Joining (NJ) phylogenetic tree was constructed using ClustalX2.1.

3.2.1.7　　　Fermentation sample preparation and chemical analysis

Sterile Schott bottles with a 250 mL volume containing nutrient broth/KCN (4mg/40mL) (see Appendix 3) and inoculated with (0.2-0.5 mg) fungal mycelia were placed in an orbital (incubator) shaker ((LABWIT- ZWY-240, Shangai Zhicheng Analytical, Shanghai-China)) at 30 °C and 130 rpm for 240 hrs. Syringes were used to collect mycelia from cassava (A, B) and soil (C, D), both samples containing free cyanide subsequent to transferring the samples into 2mL sampling tubes. Samples were then centrifuged at 13500 rpm for 5 min. The supernatant collected was analysed for free cyanide, ammonium-nitrogen and nitrate-nitrogen using Mercks' cyanide (CN^-) (09701), ammonium (NH_4^+-N) (00683) and nitrates (NO_3-N) (14773) test kits, respectively (Mekuto *et al.*, 2013). The concentration levels of free cyanide, ammonium-nitrogen and nitrate-nitrogen were analysed using Mercks' cyanide (CN^-) (09701), ammonium (NH_4^+-N) (00683) and nitrates (NO_3-N) (14773) test kits and Merck Spectroquant Nova 60 (Merck Chemicals Pty Ltd, S.A). According to Lambert *et al.* (1975), the cyanide test kits' functionality is based on the reaction of chloramine-T and pyridine-barbituric acid with cyanide, while the ammonium one is based on Berthelot's reaction (the combination of phenolic compounds, chlorine and ammonium ions) which results in the formation of indophenol dyes (Patton & Crouch, 1977); whereas, the nitrate (NO_3-N) test kit methodology is based on sulphuric acid and a benzoic acid derivative reaction. A reaction of nitrite ions with 4-aminobenzenesulfonic acid and 1-aminonaphtalene leads to a formation of a reddish pink colour observable at 520 nm wavelength (Kolmert *et al.*, 2000; Rider & Mellon, 1946). A Crison Basic 20 pH meter was used to measure the pH.

3.3 Screening of the cyanide-resistant isolate pathogenic activity on the microbial communities in cassava-cultivated soil

3.3.1 Analytical methods: pathogenic activity assessment

3.3.1.1 Soil sample preparation

A small quantity of cassava-cultivated soil, which is soil in which cassava has been cultivated, was poured into a clean 250 mL sterile Schott bottle containing deionised distilled water for dilution (10^3 dilutions). Clean 250 mL sterile Schott bottles (n = 2), containing nutrient agar (NA), were used to dilute cyclohexamine (1.75mL/250mL), while ampicillin (500µL/250mL) was diluted into a potato dextrose agar (PDA) after autoclaving the contents, namely NA and PDA, at 121 °C for 15 min and cooled to an ambient temperature. To the still-warm contents, and prior to solidification, cyclohexamine and ampicillin were added prior to pour plating into sterile petri dishes. The slurry sample (10^3 dilution) was spread onto the petri dishes containing cyclohexamine and ampicillin, using a sterile glass loop for bacterial and fungal growth, respectively. This was followed by incubation of the petri dishes at 37 °C using an incubator (KIMIX-PROLAB instruments, Switzerland) for 72 hrs in order to facilitate soil microbial community growth.

3.3.1.2 Microbial isolation and identification from cassava-cultivated soil

After 72 hrs, microorganisms were sub-cultured numerous times to obtain pure colonies. Pure colonies of bacteria and fungi were then identified and characterised on the basis of their morphological characteristics and structure; that is, their shape and colour (gram positive or gram negative) when observed under a microscope (Olympus CX21 -model CX21FS1, Olympus corp., Tokyo, Japan) at X100 magnification using a staining method. For fungal identification, lactophenol cotton blue staining reagent was used, using a glass slide. Furthermore, biochemical identification for both bacterial and fungal communities was done using a VITEK 2 system v07.01 (BioMérieux Inc., France) at the CPUT AgriFood Technology Stations' laboratory. The VITEK 2 systems have an automated system for accurate microbial phenotype identification using biochemical tests with colorimetric reagent cards (Pincus, 2006) for specific identification of multiple microorganisms such as bacteria, fungi and yeast.

The VITEK 2 preparation procedure was as follows: VITEK 2 GP cards were stored at 2 - 8 °C prior to experiments. All other reagents were stored at an ambient temperature prior to their use. A volume (3.0 mL) of sterile saline solution (0.45-0.5% of NaCl, with pH: 4.5-7.0) was aseptically transferred into a corresponding clean (12x75 mm) polystyrene test tube. All agar plates with individual isolates were marked with the specimen number, followed by incubation of pure colonies at 35-37 °C for 18-24 hrs. A sufficient quantity of 18-24hrs old pure colonies were transferred into individual saline tubes in order to reach a density equivalent to McFarland standard of 0.50-0.63 which was assessed using a VITEK 2 DensiChek™, with test tubes containing individual cultures being inserted into the instrument, within 30 min post preparation. Furthermore, the VITEK GP cards were also placed in the instrument according to the user manual instructions (Pincus, 2006).

3.3.1.3 Screening pathogenic activity of the isolate of interest on cassava soil microbial communities

A mass (0.2-0.5 mg) of fungal mycelia (isolate of interest) was placed at the centre of fresh PDA petri dishes containing a diverse microbial community of cassava soil and labelled accordingly. The plates were incubated at 37 °C in an incubator (KIMIX-PROLAB Instruments, Switzerland) for 168 hrs depending on the microbial growth in order to assess pathogenicity and/or pathogenic activity of the isolates (See Figure 3-3) based on visual clearing zones on the bacterial/fungal community in the petri dishes, using the cyanide-resistant organism as the antagonistic microorganism.
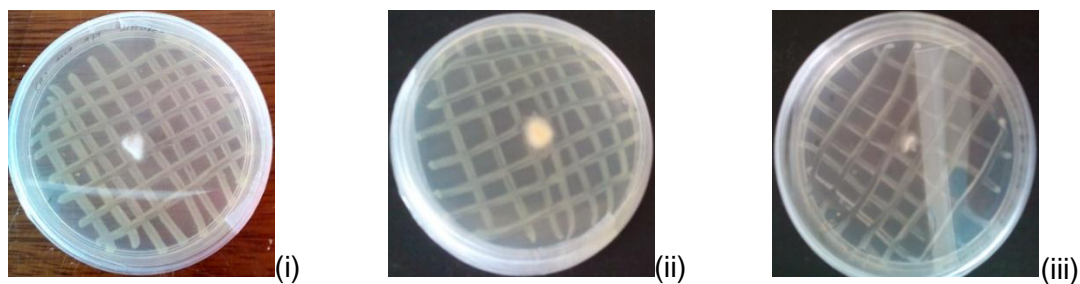
Figure 3-3: An example of the assessment of *C. bertholletiae* pathogenic activity on cassava-cultivated soil microbial (*Oligella ureolytica* after (i) 24hrs,(ii) 48hrs and *Acinetobacter* sp. after (iii) 24hrs.

3.3.1.4   Fungal isolation, identification and characterisation

Cunnighamella bertholletiae was isolated using a culture-based technique from decomposing cassava and cultivated in Potato Dextrose Agar (PDA) petri dishes (Merck, Germany) for 168 hrs at an ambient (25 °C) temperature. Subsequent to sub-culturing, in order to attain pure cultures of *C. bertholletiae,* mycelium were aseptically transferred into a 40 mL Nutrient Broth media (Merck, Germany) in 250 mL airtight multiport shake flasks. To simulate cyanogenic conditions on cassava tubers, free cyanide concentration (as KCN) at 100 mg CN$^-$/L was used while the cultures were incubated for 120 hrs at 30 °C, followed by spread-plating onto PDA plates for a further 168 hrs at an ambient temperature. This was done to further confirm the isolates' resistance and/or tolerance to free cyanide.

A loop full of mycelia from PDA plates were then re-inoculated into Nutrient Broth for 24 hrs for subsequent DNA extraction and sequencing procedures which were conducted as described elsewhere (Section 3.2.1.1). The generated nucleotide sequence was analysed using a CLC main workbench 7 followed by a BLAST (Basic Local Alignment Search Tool) provided by NCBI (National Center for Biotechnology Information), with confirmatory identification classifying the organism *C. bertholletiae,* accession no. KT275316. This method is analogous to that in Section 3.2.1.).

3.3.1.5   Mycotoxins/secondary metabolites production, extraction and identification

3.3.1.5.1   Sample fermentation

C. bertholletiae was grown on PDA for a period of 168 hrs; thereafter, the agar containing mycelia was cut (1x1 cm) from the PDA using a sterile surgical blade. The mycelia was inoculated into 30 mL Erlenmeyer flasks containing 25 mL sterile Nutrient Broth (Merck, Germany) (Ismaiel & Papenbrock, 2015), followed by incubation in an orbital shaker at 37 °C for a period of 168 hrs. The broth (suspension) was filtered using an ethanol (70% v/v) sterile distilled water rinsed No 5 Whatman filter paper, in order to obtain mycelia free extracts (Manjula *et al.*, 2009). The liquid fraction was transferred into centrifuge tubes, followed by centrifugation at 5000 rpm for 10 min using a Megafuge 1.0 (Kendrol Laboratory products, Germany). The supernatant was subsequently transferred into clean 20 mL sampling tubes while the residual pellets were discarded. For the fermentation, both CN- free and CN- containing cultures (KCN 4 mg/40 mL) were used.

3.3.1.5.2 Rapid extraction and analysis of mycotoxins (secondary metabolites) including their biodegradation by-products

Mycotoxins extraction was done using a modified liquid-liquid extraction method (Bankole & Mabekoje, 2004; Lattanzio *et al.*, 2007). A volume (5 mL) of the extracts was transferred into clean tubes containing 5 mL of 100% chloroform (Sigma Aldrich, Germany) and the contents were mixed by inversion (3-5 times) subsequent to centrifugation at 5000 rpm for 10 min using a Megafuge 1.0 (Kendrol Laboratory products, Germany). The extract chloroform phase was transferred into clean polypropylene tubes while the residual Nutrient Broth phase was discarded. The solvent extract phase was steadily evaporated using a blow-down technique to dryness at an ambient temperature for 24 hrs to minimise mycotoxin evaporation using nitrogen (N_2) gas (Afrox, South Africa) (Bily *et al.*, 2004). To the dried tubes, 1 mL of 100% methanol was added (Sigma Aldrich, Germany). A volume (1 mL) of each of the reconstituted extracts was transferred into LC/MS analysis vials using a 1 mL Lyer lock glass syringe, to which a 0.22 µm cellulose syringe filter (Promolab Pty Ltd, South Africa) was subsequently attached for further filtration procedure for each sample, with an analytical grade methanol rinse being used between each sample being processed, using the same syringe. The samples were analysed using a LC/MS-ToF 6230 (Agilent Technologies, Inc. USA) and using mobile phase parameters as listed in Table 3-2, without optimisation (Zide *et al.*, 2017), at an experimental pH of 3.

Table 3-2: LC/MS-ToF elution and mobile phase parameters

Gradient (min)	A (H$_2$O)*	B (MeOH)^Y	Flow (mL/min)
0	85	15	0.4
30	0	100	0.4
33	0	100	0.4
45	85	15	0.4
50	85	15	0.4

*: water contained, 0.1% formic acid, pH 3
^{Y}: Analytical grade methanol

LC separation, was achieved using a 0.4 mL/min flow rate, with an operational run of 50 min and operating with a column (symmetry C8, 3.9 x 150mm column, 5μm), including a C8 guard column, 3.9 x 20mm, 5μm. The column temperature was 30 ^{o}C, while being operated with the periodic reconditioning of the column being at 15 min intervals between samples using a capillary voltage of 3500 v. The mass spectrum scan (m/z) was between 112 to 996 m/z, with the spectra obtained being used for analysis using a mass-hunter software v3.0 (Agilent Technologies, Inc. USA) (see Table 3-3). Furthermore, the migration for identified analytes were recorded, with each compound m/z (M + H$^+$) values, including migration times, being compared to those published elsewhere.

The identification of the mycotoxins and other secondary metabolites from *C. bertholletiae* isolate, including toxin biodegradation by-products, was done using finger printing and/or profile data (Pereira *et al.*, 2006; Draper *et al.*, 2009; Farag *et al.*, 2012), using a mycotoxin/biodegradation by-product database, with the assumption that samples were assumed to lose an electron with the H$^+$ proton being hypothetically the lost ion.

Table 3-3: ToF LC/MS operational parameters summary

Operational parameters	Description
1. LC/MS model and supplier	**HPLC model:** HPLC system and ToF/simple point data system method with full control **LC/MS model:** 6230 **LC/MS supplier:** Agilent Technologies, Inc. USA
2. LC/MS operational conditions and Ionisation mode	**MS Interface Ionisation**: Electrospray ionisation (ESI) **High vacuum pressure:** 1.16×10^{-7} Pa (Torr) **Dry gas temp.:** 350 ^{o}C **Nebulising pressure:** 15 psig **Dry gas flow:** 8.0 L/min **Acquisition mode:** Positive **ESI capillary voltage:** 3500 kv **Mass range:** 112.9855-966.0007 **ToF flow rate:** 3 mL/min **Sample injection volume:** 5μL
3. Column and guard column characteristics, temperature (e.g. operational parameters)	**Column:** symmetry C8, 3.9 x 150mm column, 5μm **Column temperature:** 30°C **Symmetry C8 guard column:** 3.9 x 20mm, 5μm
4. Mobile phase constituents, concentrations, gradients, operational parameters and flow rate	**Mobile phase:** Solvent A: Water, Solvent B: Methanol (mobile phase constituents were analytical grade; Sigma Aldrich, Germany). **(Refer to table 3-2)**

3.3.2   Proposed mitigation strategy implementation

3.3.2.1 *Nepenthes mirabilis* extracts collection: characterisation, preparation and application

Nepenthes mirabilis used during this part of the study was donated by Pan's Carnivores Nursery (Tokai, Cape Town). Pitcher juice (herein referred to as extracts) containing prey was collected from a *N. mirabilis* plant into a 50 mL plastic sampling tube which was sealed to be airtight subsequent to refrigeration at 4 °C prior to preparation and analysis (see Appendix 9).

An assessment of the physicochemical characteristics of the *N. mirabilis* extracts (juice) revealed the following: conductivity: 5.89 S/m, redox potential: 510 mv, specific gravity (SG): 1.02 and a pH of 2.5.

A volume (3 mL) of the *N. mirabilis* extracts (juice) was transferred into sampling tubes (15mL) followed by centrifugation using a Megafuge 1.0 (Kendrol Laboratory products, Germany) at 3000 rpm for 15 min (twice) due to its viscosity with the solid residue formed being discarded. The centrifuged extracts (juice, supernatant) was transferred into clean tubes.

Additionally, as a qualitative method for the analysis, enzymes/biochemical tests, that is, the presence of enzymes in the *N. mirabilis* extracts, the VITEK 2 (GN/GP) preparation procedure was used as follows: VITEK 2 cards were stored at 2 – 8 °C prior to the experiments. All other reagents were stored at ambient temperature prior to their use. A volume (3.0 mL) of sterile saline solution (0.45-0.5% of NaCl, with pH: 4.5- to 7.0) to which the *N. mirabilis* extracts were added, was aseptically transferred into corresponding clean polystyrene test tubes, to attain an optical density equivalent to McFarland standard of 0.50-0.63, which was assessed using a VITEK 2 DensiChek™, with the tubes being inserted into the instrument within 30 min post preparation. Furthermore, the VITEK cards were also placed in the instrument according to the user manual instructions (Pincus, 2006). This method was used as a supplementary method to quantitatively determine enzyme presence in the extracts.

The *N. mirabilis* extract was added to residual fermentation extracts (see Sections 7.3.2.1 for sample preparation and 7.3.2.2 for extraction).

3.3.2.2 Carboxylesterases activity: specificity and quantification

The quantification of carboxylesterases activity was determined by using the method adopted with minor modifications from Ljungquist and Augustinsson (1971), Wheelock *et al.* (2001), Gilham and Lehner (2005) and Schomburg *et al.* (2013). The overall biocatalysis properties of the *N. mirabilis* extract constituents, with a focus on carboxylesterases, are described as indicated in Table 7-4.

The subsequent subsection reports on the development of a spectrophotometric method used for the determination of carboxylesterase activity for the *N. mirabilis* extracts. This method is analogous to the method used by Ljungquist and Augustinsson (1971), Wheelock *et al.* (2001) and Lee *et al.* (2016).

3.3.2.3 Carboxylesterase activity assay

Carboxylesterase assay activity was determined spectrophotometrically at ambient temperature using p-nitrophenyl acetate (PNPA) as the substrate (Wheelock *et al.*, 2001, using a 75% (0.75) dilution while a control sample did contains plant extracts and was undiluted. The activity was measured by determining the rate of biocatalysis of PNPA to p-nitrophenol (PNP) which was spectrophotometrically monitored at 410 nm. The PNPA exhibits minimal absorbance at 410 nm, whereas the PNP absorbs strongly. The extinction coefficient used for PNP was 17000 $M^{-1}.cm^{-1}$ (Ljungquist & Augustinsson, 1971). Activity was then expressed in $U.L^{-1}$, where 1 unit is equivalent to 1 μmol/min; that is, the rate of conversion for PNPA to PNP.

3.3.2.4 Preparation of stock solutions

Sample preparation was done with 300 µL (0.10181 g/L) of PNPA (Sigma Aldrich, Germany) solution (dissolved in acetone) in a form of a substrate with 200 µL of 0.1 M Tris-HCL buffer at a pH of 7.8; 200 μL of sterile distilled water and enzyme solution (extract) of 300 μL for a total volume of 1000 μL. The assay reagents are listed below (Table 3-4).

Table 3-4: Carboxylesterase activity assay reagents

	Blank (μL)	Sample (μL)
0.1M Tris-HCL buffer (pH = 7.8)	200	200
0.6mM PNPA in acetone	300	300
Sterile distilled water	500	200
Enzyme (extract) solution	-	300
Total	**1000 μL**	**1000 μL**

3.3.2.5 Spectrophotometer settings: carboxylesterase activity assay

The JENWAY 6405 UV/Vis spectrophotometer (Agilent Pty, USA) settings used were as follows:

- Kinetics setting, with
- UV/Vis light switched on, for a
- Reading at 410 nm for 2 min at 10 sec. intervals, while the
- Temperature was 25 °C, using an
- Extinction coefficient of 17000 $M^{-1}.cm^{-1}$.

Eq. 3.6 Illustrates the mathematical expression used to quantify the activity of carboxylesterase.

$$\text{activity} \quad (\text{U/L}) = \left[\frac{\frac{dA}{dt} * (\text{dilution} \quad \text{factor})}{\text{extinction} \quad \text{coefficient}} \right] * 60 * 10^{6} \qquad 3.6$$

3.3.2.6 Biodegradation by-products analysis

The residual fermentation extracts, the *N. mirabilis* extracts (300 µL), were added to assess the biodegradation by-products of the compounds identified to have been produced by *C. bertholletiae's* isolate, which is cyanide resistant. This was done using 1.5 mL sealed polypropylene tubes, at ambient temperature, with tubes being left for 168 hrs, such that biodegradation can be ensured.

CHAPTER 4

A DECADE'S (2014–2024) PERSPECTIVE ON CASSAVA'S (*MANIHOT ESCULENTA* CRANTZ) CONTRIBUTION TO THE GLOBAL HYDROGEN CYANIDE LOAD IN THE ENVIRONMENT

RESULTS

CHAPTER 4

4. A DECADE'S (2014–2024) PERSPECTIVE ON CASSAVA'S (*MANIHOT ESCULENTA* CRANTZ) CONTRIBUTION TO THE GLOBAL HYDROGEN CYANIDE LOAD IN THE ENVIRONMENT

4.1 Introduction

Cassava (*M. esculenta*) is a tropical crop used daily in many impoverished communities, particularly on the African, Asian and South American continents (Gleadow & Woodrow, 2002; Gleadow *et al.*, 2009; Mburu *et al.*, 2012), with a billion tons being produced during 2009 to 2013. The continental contribution from Africa (55% for food), Asia (32% for food/biofuel) and South America (13% food/biofuel), suggests sub-Saharan Africa is the largest region of cassava production globally (Koh, 2007; Silayo *et al.*, 2013; Piero *et al.*, 2015). Cassava contains cyanogenic compounds in its leaves, stems and tubers. The glycosides compounds, mainly linamarin, produces hydrogen cyanide (HCN) through its enzymatic hydrolysis (Montagnac *et al.*, 2009; Siritunga & Sayre, 2003) of which the deglycosylation generates acetone cyanodrin which, in turn, decomposes into HCN and acetone largely at a pH greater than 5, as well as at a moderate temperature (30 °C), a temperature which suits HCN volatilisation (Andersen *et al.*, 2000; Siritunga & Sayre, 2003; Montagnac *et al.*, 2009). However, the concentration of cyanogenic compounds differs from tubers to leaves, with leaves having a higher cyanogen concentration during the early growth stages of the cultivar (Montagnac *et al.*, 2009). With HCN estimated at 250 mg HCN per Kg dry weight cassava tubers (DWCT) in mature plants (Howeler, 2002; Howeler *et al.*, 2001; Piero *et al.*, 2015), suggests an astronomical quantity of HCN; that is, 0.25 parts per quadrillion (ppq) was released into the atmosphere between 2009 and 2013 (Liang *et al.*, 2007; Magnusson *et al.*, 2012), a consequence of limited use of genetically modified cultivars (Godfray *et al.*, 2010; Koehorst-van Putten *et al.*, 2012). Previous studies have revealed that genetically modified cultivars produce low cyanogen content (<1%) in comparison with the wild type plants (Siritunga & Sayre, 2004). Thus, even if genetically modified cultivars are produced (cultivated), 0.006-0.01 ppq of HCN will still be produced.

Research on genetic manipulation of (genetically modified) cassava gained popularity in the mid-1990s (Wield *et al.*, 2010; Liu *et al.*, 2011; Mabaya *et al.*, 2015). The project aimed to reduce cyanogen content in cassava, improve its shelf life post-harvest, provide a cleaner

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environment, cultivate fast-growing crops which lead to increased productivity and less use of fertilizers, and to propagate pest-resistant cassava (Koehorst-van Putten *et al.*, 2012; Taylor *et al.*, 2012). However, this initiative experienced challenges and skepticism from a few selected sub-Saharan countries with only Nigeria, Kenya, Ethiopia, Ghana and the Democratic Republic of Congo piloting the genetically modified cassava project (Takeshima, 2010; Wield *et al.*, 2010; Taylor *et al.*, 2012). Environmental activists and non-governmental organisations (NGOs) claimed that Genetically Modified Organisms (GMOs) negatively impacts on communities in a manner that results in: 1) socio-cultural and economic losses of local cassava production and conservation knowledge; 2) high input costs to procure genetically modified cultivars; 3) health and food safety concerns as GMO food and products are prone to allergenicity and toxicity; 4) the invasion of genetically modified genes which can culminate in a gene flow, thus transfer into a local environment, impacting on non-target organisms, resulting in a loss of biodiversity (Siritunga & Sayre, 2003; Taylor *et al.*, 2012). These concerns have resulted in a reluctance to adopt the agricultural use of GMOs which is further hampered by the lack of a regulatory framework and/or strategies associated with the utilisation of GMOs (Takeshima, 2010; Mabaya *et al.*, 2015).

There are several routes/pathways from which cyanides, in general, and hydrogen cyanide, in particular, enter into environmental matrices. The HCN from cassava harvesting and processing, which in most instances is in a form of contaminated wastewater and volatilised gas, can result in bio- and physico-chemical modification of the receiving matrices, such as soil, water and air. In addition, metal cyanides, namely, hexacyanoferrate (III), hexacyanoferrate (II), ferricyanide ($Fe(III)(CN)_6^{4-}$) and other forms of cyanides, such as KCN and NaCN from natural or anthropogenic sources, are converted into CN^- through complexation, decomposition, dissolution, degradation and leaching processes are thus released into the environment. Previous studies on cassava cultivation and cyanogen compounds in the environment revealed a relationship between continuous cassava cultivation and a decrease in soil mineral content and also in other parameters such as potassium, magnesium, organic carbon (OC), organic matter (OM) and bulk density (Howeler , 1998).

Therefore, it is judicious to assess the spatial and temporal distribution of cassava in the environment with the related effects of cyanogen compounds and HCN in particular, because HCN is a pseudo-halogen which is a contributing factor for ozone-layer degradation which could be associated with global warming and consequently climate change (Breton *et al.*, 2013). HCN decomposition leads to the production of CN^- (cyanide) and NCO^- (cyanate), which are additional

sources of nitrogen oxides (N_yO_x), contributors to global warming (Lary, 2005), with nitrous oxide in the troposphere being nearly 300 times higher than that of a century ago (Galloway *et al.*, 2004). Since 2007, Reay *et al.* (2012) have estimated the global atmospheric N_2O concentration 18% higher than in pre-industrial times with its increase being at a rate of about 0.3% per year since 1980 in comparison to the 1.2 petagrams of carbon (Pg C) increase of CO_2 per annum reported in the same period (Van der Werf *et al.*, 2009).

Furthermore, N_yO_x from HCN can contribute to acidification and eutrophication when it and other forms of reactive nitrogen by-products, such as ammonium nitrogen (NH4-N), nitrate (NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), nitrous oxide (N_2O) and nitric oxide (NO), enter water bodies in a form of a liquefied carrier (Reay *et al.*, 2012). Therefore, it is prudent to assess cassava's contribution to HCN release by the largest cassava producers (2002 to 2013) and the projection up to 2024 on the global HCN load in to the environment. Thus, this part of the study has focused only on the global spatial and temporal distribution of cassava and its contribution of HCN release into the environment, with evidence of HCN, NO_2 and NH_3 production from healthy cassava plants being reported.

4.2 Objectives

The objectives for this part of the study were:

- Quantification of the concentration of gases (HCN, NH_3, NO_2) released from cultivated cassava plants in a controlled environment;

- Determination of the contribution of cassava to global CN^- load in the atmosphere, its possible long-term impact in the environment using projections-based FAO data, namely, linearised models and GIS to map such distribution continentally (focusing on cassava-producing countries).

Thus, the HCN released during the period 2002 to 2013 was estimated to be between 0.025×10^{-3} to 6.71 ppq (African), 0.012×10^{-3} to 1.01 ppq (Asian) and 0.007×10^{-3} to 0.920×10^{-3} ppq (South American). Furthermore, a decade's (2014 to 2024) projection of HCN volatilisation displays increases of 60.5% (Africa), 57.7% (Asia) and 50.5% (South America) when compared with the current production. Furthermore, gas released during cassava plants' growth, namely,

HCN, NH$_3$, and NO$_2$, was quantified in healthy plants. These results further indicated the release of pseudo-halogenic gases into the environment, a contributor to climate change.

4.3 Materials and Methods (summary)

Cassava plants were grown in a CPUT greenhouse at the Cape Town Campus while GIS and Quantum GIS software were used for the modelling of the global cassava production and a decade projection of the total HCN release into the environment. The detailed methodology was reported in Chapter 3, Sections 3.1.2 to 3.1.8.

4.4 Results and Discussion

4.4.1 Cassava production and estimated hydrogen cyanide load into the environment

The results of the yearly cassava production revealed variation from one region to another: Africa (55% for daily consumption), Asia (32% for daily consumption/bioenergy) and South America (13% for daily-consumption/bioenergy). See Figure 4-1. This suggests that sub-Saharan Africa is the largest region in cassava production globally (Jansson *et al.*, 2009; Jarvis *et al.*, 2012). The quantity of HCN released per continent from 2002 to 2013 ranged from: 0.025 x 10^{-3} to 6706 x 10^{-3} ppq (African), 0.012 x 10^{-3} to 1010 x 10^{-3} ppq (Asian) and 0.007 x 10^{-3} to 0.920 x 10^{-3} ppq (South American).

Figure 4-1 below illustrates a global hydrogen cyanide load (2002–2013), including a linearised projection (2014 – 2024) with an inset of global cassava production for Africa, Asia and South America.
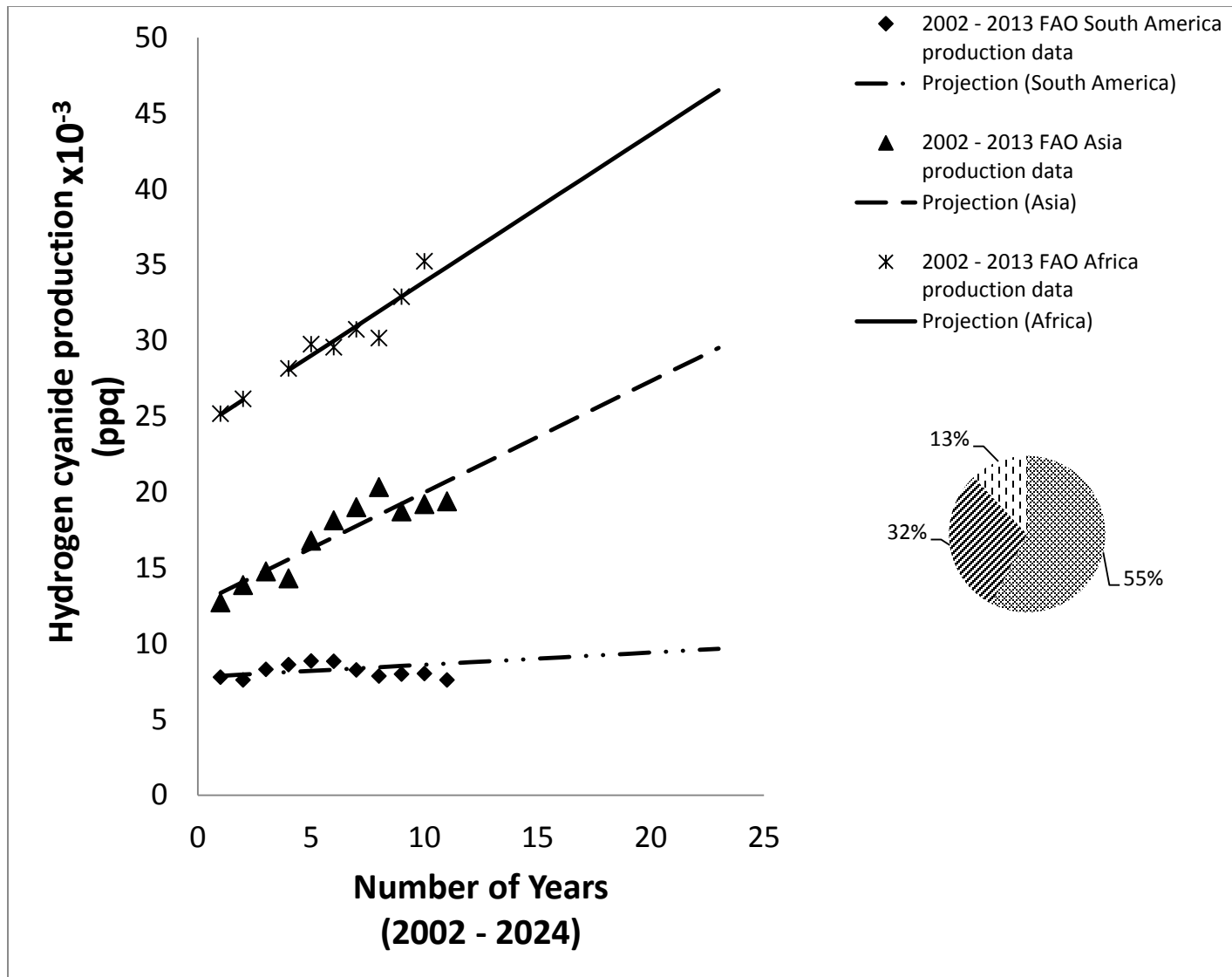
Figure 4-1: Global hydrogen cyanide load (2002–2013), including a linearised projection (2014 – 2024). Inset: Global cassava production for Africa, Asia and South America.

Africa's production of cassava during approximately ten years of study (2002 to 2013) is the highest in the world (Ojeka *et al.*, 2007; Idowu *et al.*, 2015). This may be owing to rapid population growth as well as the population's daily dependence on this cultivar, which constitutes a staple crop. During 2009, the production of cassava for each region was: Africa: 1.21×10^8 tons, Asia: 8.1×10^7 tons and South America: 3.1×10^7 tons, while the estimated production of hydrogen cyanide released during the period under consideration was estimated at: 31.93×10^{-3}, 18.49×10^{-3} and 8.44×10^{-3} ppq, respectively. See Figures 4-2 to 4-4 (Ojeka *et al.*, 2007).

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The mean absolute deviation (MAD) was 10.48, 0.86, 0.05 for African, Asian and South American continents respectively, when the projections were compared with computed data.

Recently, cassava production has increased for several reasons: Africa (55%, self-nourishment only), Asia (32%, for self-nourishment and renewable energy or biofuel), South America (13%, self-nourishment and renewable energy production) (Lamptey *et al.*, 2008, Msangi *et al.*, 2010, Tilman *et al.*, 2009). In most cases the cultivar is used as a staple for the population. Manually processed products such as foufou, gari, manioc, and so on, are sold (Ibitoye, 2011). Increased industrial activity, with high energy demand in Asia and South America, has resulted in production of the cultivar for biofuels (Sundaresan *et al.*, 1987; Tilman *et al.*, 2009; Msangi *et al.*, 2010; Ibitoye, 2011; Nambisan, 2011). Thus, the trend of cassava production globally has increased by 13% to 55% between 2002 and 2013, representing increases of 0.1×10^7 to 0.19×10^8 tons, with a further projected increase to 23.9×10^{-4} and 23.4×10^{-5} by 2024 (see Figure 4-1). The corresponding estimated HCN load in the environment would thus increase by 9.2×10^{-3} to 47.1×10^{-3} ppq HCN from the smallest to the largest continental cassava producers, respectively.

This indicates the overall increase in HCN load in the environment per continent for the next decade (2014 to 2024) to be 60.5 % (Africa), 57.7 % (Asia) and 50.5 % (South America). It has previously been found that there is a direct relationship between increased cassava production and the concentration of cyanide released into the environment (Sundaresan *et al.*, 1987; Ibitoye, 2011). These increases in cassava production and thus cyanide and/or cyanogen loading into the environment, necessitate the proper management of cassava cultivars, including the introduction of GMOs, particularly for Africa, to mitigate the increasing HCN loads which eventually result in residual ammonium nitrogen (NH_4-N), nitrate (NO_3-), nitrite (NO_2-), nitric acid (HNO_3), nitrous oxide (N_2O), nitric oxide (NO) and other sources of nitrogen oxides (N_yO_x). These releases contribute to global warming (Galloway *et al.*, 2004; Lary, 2005).

Changes in global weather patterns resulted in reduced casava production during the warmest years, that is, 2005, 2010 and 2014, with global temperature anomalies of 0.53, 0.67 and 0.70 °C respectively (Shein, 2006; Hansen *et al.*, 2010; Gatti *et al.*, 2014). This does not necessarily correspond with reduced cyanide loading in the environment. This is because higher global temperatures and the warmest years resulted in spoillage of crops and reduced the amount of agricultural produce, including cassava, reaching the market (Zidenga, 2011; Zidenga *et al.*, 2012).

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Global warming and higher temperatures could result in previously colder parts of the world having favourable weather conditions for cassava growth. Thus, in the past, South Africa generally, and the Western Cape province in particular, did not have a record of producing cassava or even growing cassava plants because of cold weather conditions. Recently, cassava plants have been grown in the north-eastern (Allemann & Dugmore, 2004; Allie *et al.*, 2014) and south-western regions of the country which were previously known as cold areas, owing to changes in weather conditions.[1] Thus, favourable growth conditions will lead to an increase in cassava production, resulting in an increase in HCN load release into the environment through volatilisation (Allemann & Dugmore, 2004; Lary, 2005; Allie *et al.*, 2014).

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[1] For example, on Wednesday, 4 March 2015, Cape Town (in the Western Cape) recorded a temperature of 40 °C, the highest in 100 years.

Legend
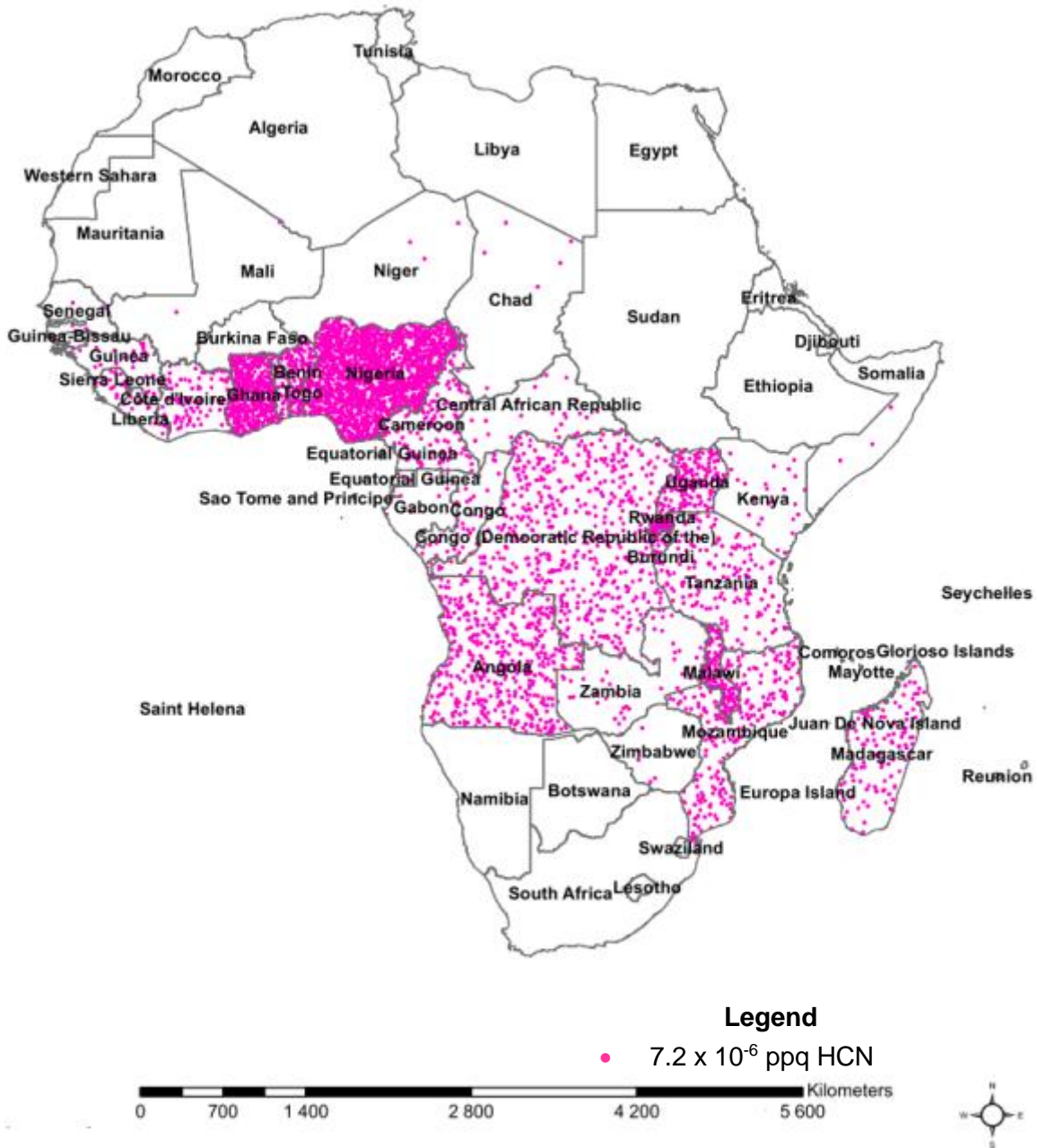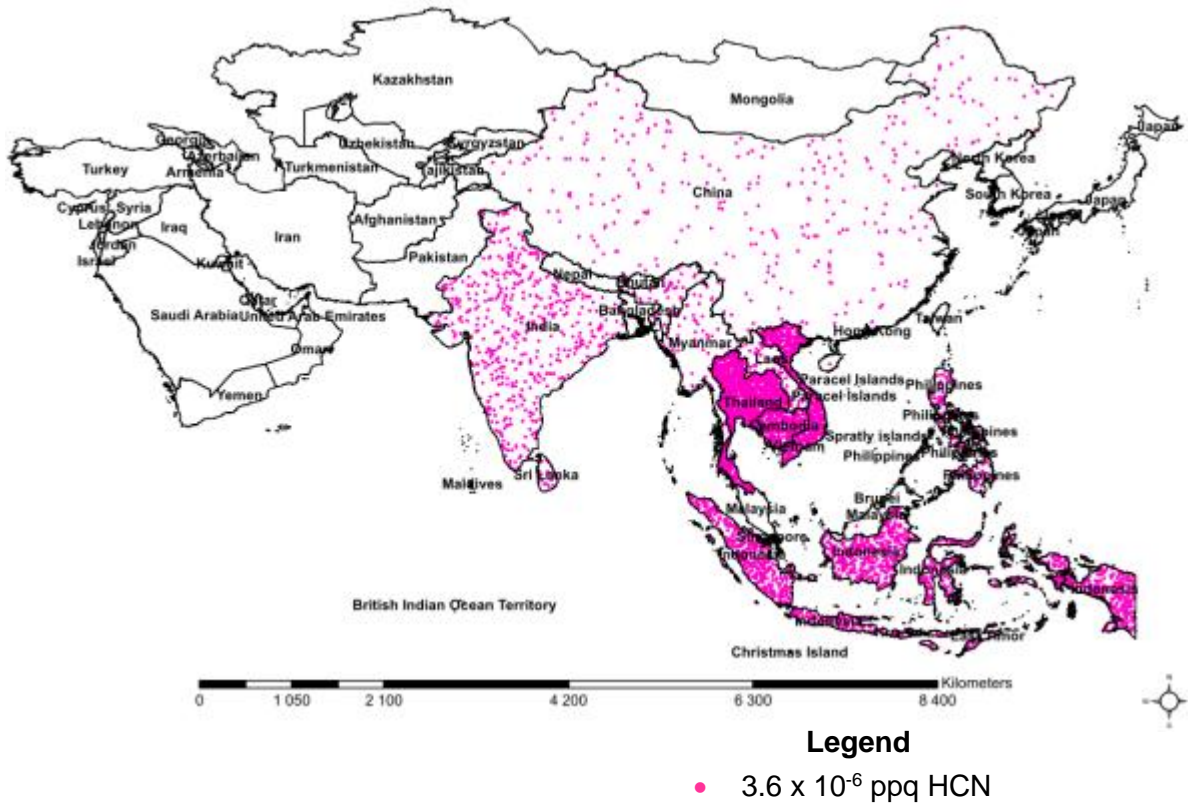
• 7.2×10^{-6} ppq HCN

Figure 4-2: Africa's cassava production and estimated hydrogen cyanide concentration

Figure 4-3: Asia's cassava production and estimated hydrogen cyanide concentration

Legend

- 4.1 x 10^{-6} ppq HCN

Figure 4-4: South America's cassava production and estimated hydrogen cyanide concentration

4.4.2 Assessment of cassava plant gases (HCN, NH$_3$, NO$_2$) volatilised, chlorophyll content and photosynthetically active reaction (PAR)

Plant gases (HCN, NH$_3$, NO$_2$) volatilised from healthy cassava plants (1 and 2) which were 8 to 10 months old with a height range of 55 to 170 cm, showed varying concentrations of gases being produced. The gases released from both plants (1 and 2) measured, using 12 hr intervals, were 4.7 mg/L (HCN), 10.1 mg/L (NH$_3$), 3.6 mg/L (NO$_2$); 40.7 mg/L (HCN); 67.9 mg/L (NH$_3$); and 1.9 mg/L (NO$_2$), respectively. The chlorophyll content of the cassava leaves ranged from 26.55 to 29.5 µmol/m^2 for Plants 1 and 2, respectively. Irradiance PAR on both plants' leaves showed that it ranged from 169.67 to 399.67 µmol/m^{2}s (Table 4-1). The readings indicated that the cassava plants were healthy.

A comparative analysis of the averaged cassava plant gas analysis revealed discrepencies between both plants as well as the two readings of each plant. The initial readings for Plant 1 for HCN and NH$_3$ were lower than the second set of readings, while NO$_2$ values were nearly identical. In contrast, Plant 2 showed higher values for the first readings for all three gases (HCN, NH$_3$, NO$_2$) than for the second readings (Table 4-2). The difference of the values was hypopthesised to be caused by: 1) the transformation of volatilised HCN into NH$_3$ and NO$_2$; 2) a decrease in or insufficient CO$_2$ in the gas analyser device and insufficient solar radiation for photosynthesis; and 3) the length and size of the plants as well as their leaves.

Analysis of the chlorophyll content and PAR for each plant showed minimal differences between the two plants: chlorophyll readings were 28.75 µmol/m^2 with a standard deviation of ± 2.32 (Plant 1 before being in the gas analyser device) and 29.52 µmol/m^2 with a standard deviation of ± 0.69 (after 12 hr in the gas analyser device). Additionally, Plant 2 readings revealed chlorophyll levels of 26.55 and 29.8 µmol/m^2 with a standard deviation of ± 1.45 and ± 1.13 respectively, which are in the range of 15 to 200 µmol/m^2 for healthy plants (Kato & Shimizu, 1987; Cavender-Bares & Bazzaz, 2004; Krizek, 2004). Both plants' chlorophyll content, after 12 hr in the gas analyser device, was nearly identical (Table 4-1).

The PAR value of both plants showed a discrepancy, with Plant 1 having a higher PAR prior and after gas analysis. The discrepency was attributed to the decrease of the cassava plants' photosynthesis activity, which affected radiant power during measurements in the laboratory, as well as to the lack of sufficient solar radiation.

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Table 4-1: Chlorophyll and photosynthetically active radiation (PAR) readings of cassava plants

| | Chlorophyll content ($\mu mol/m^2$) | | | |
| | Plant 1 | | Plant 2 | |
	A	B	A	B
Average	28.75	29.52	26.55	29.8
Stand. Dev.	± 2.32	± 0.69	± 1.45	± 1.13
	PAR ($\mu mol/m^2 s$)			
Average	399.67	260	169.67	88
Stand. Dev.	± 16.8	± 3.3	± 26.8	± 0.7

* A – plant readings prior to being placed into the gas analyser device and B – plant readings taken 12h after volatilised gas analyses

Table 4-2: Average cassava plant gas readings using gas transmitter devices (mg/L)

| Sampling regime/time | Plant 1 | | | Plant 2 | | | Control | | |
	HCN	NH_3	NO_2	HCN	NH_3	NO_2	HCN	NH_3	NO_2
T: (16:00, 19:00, 22:00)	4.7	10.1	3.6	40.7	67.9	1.9	0	0	0
T: (07:00, 10:00, 13:00)	31.5	53.1	3.1	5.3	7.8	1.1	0	0	0

T*: Time intervals for the cassava plants' gas analyses

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4.5 Summary

In recent years, developing countries have increased their cassava (*Manihot esculenta*) production for food security. Cassava contains cyanogen glycosides, mainly as linamarin, which, through bio-catalysis, that is, enzyme hydrolysis, results in hydrogen cyanide (HCN). HCN is released into the environment through numerous ways with subsequent volatilisation. Thus, the HCN released during the period 2002 to 2013 was estimated at between 0.025×10^{-3} to 6.71 ppq (African), 0.012×10^{-3} to 1.01 ppq (Asian) and 0.007×10^{-3} to 0.920×10^{-3} ppq (South American). Furthermore, a decade's (2014 to 2024) projection of HCN volatilisation displays increases of 60.5% (Africa), 57.7% (Asia) and 50.5% (South America) when compared with the current production. Furthermore, gas released during cassava plants' growth, namely HCN, NH_3 and NO_2, was quantified in healthy plants, indicating that varying concentrations of HCN were released even during cultivation. These further indicated the presence of a pseudo-halogenic gas release into the environment, a contributor to climate change.

CHAPTER 5

ISOLATION OF AN ENDOPHYTIC CYANIDE-RESISTANT FUNGUS *CUNNINGHAMELLA BERTHOLLETIAE* FROM *MANIHOT ESCULENTA* CRANTZ AND CASSAVA-CULTIVATED SOIL

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CHAPTER 5

5. ISOLATION OF AN ENDOPHYTIC CYANIDE-RESISTANT FUNGUS CUNNINGHAMELLA BERTHOLLETIAE FROM (MANIHOT ESCULENTA CRANTZ) AND CASSAVA-CULTIVATED SOIL

5.1   Introduction

Manihot esculenta (cassava) is considered a fundamental source of food for many impoverished rural communities in Africa and around world (Soto-Blanco & Górniak, 2010; Piero *et al.*, 2015). In recent years, there is a worldwide interest in cassava as a feedstock for bioenergy and biogas operations (Jansson *et al.*, 2009) in order to meet energy demands both at community and industrial levels in Africa, Asia and South America (Okudoh *et al.*, 2014). However, cassava production and storage for energy production and human consumption have been hampered due to bacterial and fungal attack. These microorganisms (fungi/bacteria) cause food spoilage and such spoilage is characterised by the presence of different types of moulds growing on the tubers, some of which can cause adverse effects on the health of humans (Hocking, 1997; Manjula *et al.*, 2009; Adetunji *et al.*, 2014; Ediage *et al.*, 2014). Human health concerns are related to the consumption of contaminated tubers, inhalation of fungal microspores which often result in cancer of the internal organs, pulmonary diseases, amongst others, even when exposure is by dermal contact (Peraica & Domijan, 2001; Breitenbach *et al.*, 2002; Klich, 2009; Petraitis *et al.*, 2013).

Amongst this multitude of pathogenic fungi, *Cunninghamella* sp., an ambiguous and opportunistic soil fungus, has the ability to speedily and fatally infect humans (Lilleskov *et al.*, 2002; Knudsen, 2006; Righi *et al.*, 2008). Although studies have reported fungal infections on humans through severe tissue invasion (Knutsen *et al.*, 2012; Pashley *et al.*, 2012; Méheust *et al.*, 2014) from crops such as maize and peanuts (Hocking, 1997; Klich, 2009; Ediage *et al.*, 2014; Matumba *et al.*, 2014), few studies have reported the presence of this fungus in cassava tubers. Thus, this part of the study focused on the isolation and characterisation of *C. bertholletiae* from cassava tubers from South Africa, to assess its ability to be resistant to or degrade cyanide. Thus, the fungus can be useful to remediate cyanide-contaminated environments (Lilleskov *et al.*, 2002; Knudsen, 2006; Righi *et al.*, 2008).

Cassava tubers contain cyanide (cyanogenic compounds) which is hydrolysed into hydrogen cyanide that is harmful to human's health when consumed in high doses (Mburu *et al.*,

2012). Thus, microorganisms (fungi/bacteria) exposed to cyanide eventually become cyanide-resistant.

5.2 Objectives

This part of the study focused on the following objectives;

- Isolate, identify and characterise organisms from decomposing cassava and cassava-cultivated soil which are free cyanide-tolerant/resistant. These organisms had to withstand a free cyanide-tolerance threshold >200 mg/L. As such, a concentration was determined to be maximum to that most organisms could tolerate;

- Assess the ability of the isolate to biodegrade free cyanide and its by-products, that is nitrogenous by-products associated with free cyanide biodegradation and which can leach into agricultural soil thus contaminating groundwater sources.

5.3 Materials and Methods (summary)

The microorganism, *C. bertholletiae*, was isolated from cassava and cassava-cultivated soil from cultures grown on PDA and NA as indicated in Chapter 3 (Section 3.2). The microorganisms were grown at an ambient temperature followed by inoculation by nutrient broth containing KCN. The samples were placed into an incubator/shaker at 30 °C and 130 rpm for 120 hrs. The *C. bertholletiae* isolate demonstrated cyanide resistance and biodegradation ability. The fungus growth, isolation, identification, DNA extraction and characterisation procedures are discussed in detail in Chapter 3 (Sections 3.2.1.1 to 3.2.1.7)

5.4 Results and Discussion

5.4.1 *C. bertholletiae* isolation/identification, DNA extraction, phylogenetic tree construction

Cunninghamella sp*.* was grown from a Zambezi species of cassava (*Manihot esculenta*) collected from Mbizana on the 06 August 2014. The species grew well on PDA at an ambient

temperature and were white or grey in colour (at the early growth stage), turning grey after at least 8-10 days of growth and developing woolly mycelia with sporangiophores. A microscopic observation showed elongated mycelia sporangiophores with irregular, sometimes vertical, branches terminated by vesicles with a balloon shape (Álvarez *et al.*, 2011; Guo *et al.*, 2015). The fungus had synchronous sporangioles and was found to grow at a temperature of at least 30 °C.

Samples A, B, C, D sequences revealed that the isolates were identical fungi (*Cunninghamella bertholletiae*) from all samples. The fungi identified from the cassava and silt attached to the cassava showed a 90% similarity to *C. bertholletiae*. Therefore, it was decided that a phylogenetic Neighbour Joining (NJ) tree analysis of the ITS gene regions of one sample (A) was suitably representative of the isolates obtained. Figure 5-1 illustrates extracted DNA bands on agarose gel.
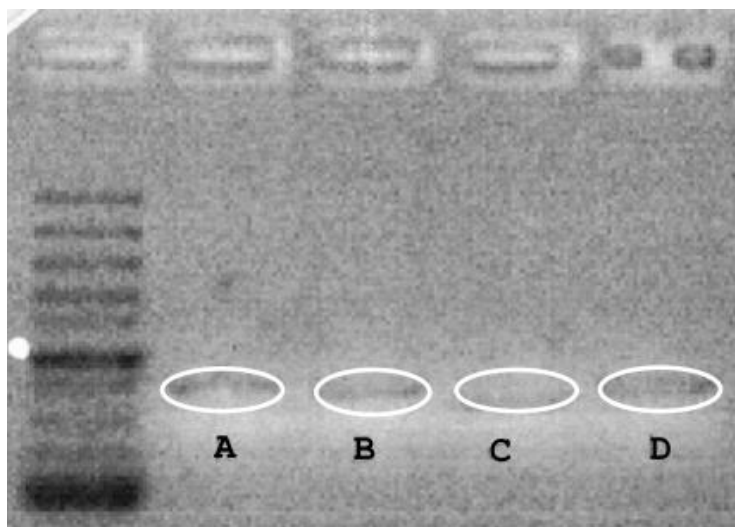
Figure 5-1: Photographic image of an agarose gel indicating the amplification of the ITS target region

5.4.2 Phylogenetic tree analysis

The internally transcribed spacers and DNA sequences of the structural ribosomal of 5.8S rDNA of the isolate were amplified using the universal primers ITS1 and ITS4 in order to amplify the targeted region of 750 bp (White *et al.*, 1990). The amplified sequence was submitted to the GenBank (Bankit) where the accession number KT275316 was allocated (Figure 5-2).

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A phylogenetic tree was generated using LustalX and *Cunninghamella* sp. were clustered according to groups and their clades. The analysis of a phylogenetic tree showed that the isolate was similar to the species of the *C. bertholletiae* strain (CBS190.84), *C. bertholletiae* strain (ATCC42115) *and Polymorpha* sp. strain (CBS779.68). This was followed by a *C. elegans* isolate (CFR-C11)*, C. phaeospora* strain (CBS692.68) *and C. homothallica* strain (IF06736) and then a *C. echinulate* strain (VKAS01)*, C. clavata* strain (CBS 362.95)*, C. bainieri* strain (2A1) *and C. clavata* strain (CBS 100178). The *C. blakesleeana* strain (3C1A) *and M. racemosus* (zygomycetes) strain constituted the out-group species.

Figure 5-2 below illustrate a Phylogenetic (NJ) tree of the isolate (*Cunninghamella bertholletiae*) from cassava cultivated in Eastern Cape based on the analysis of combined ITS gene regions.
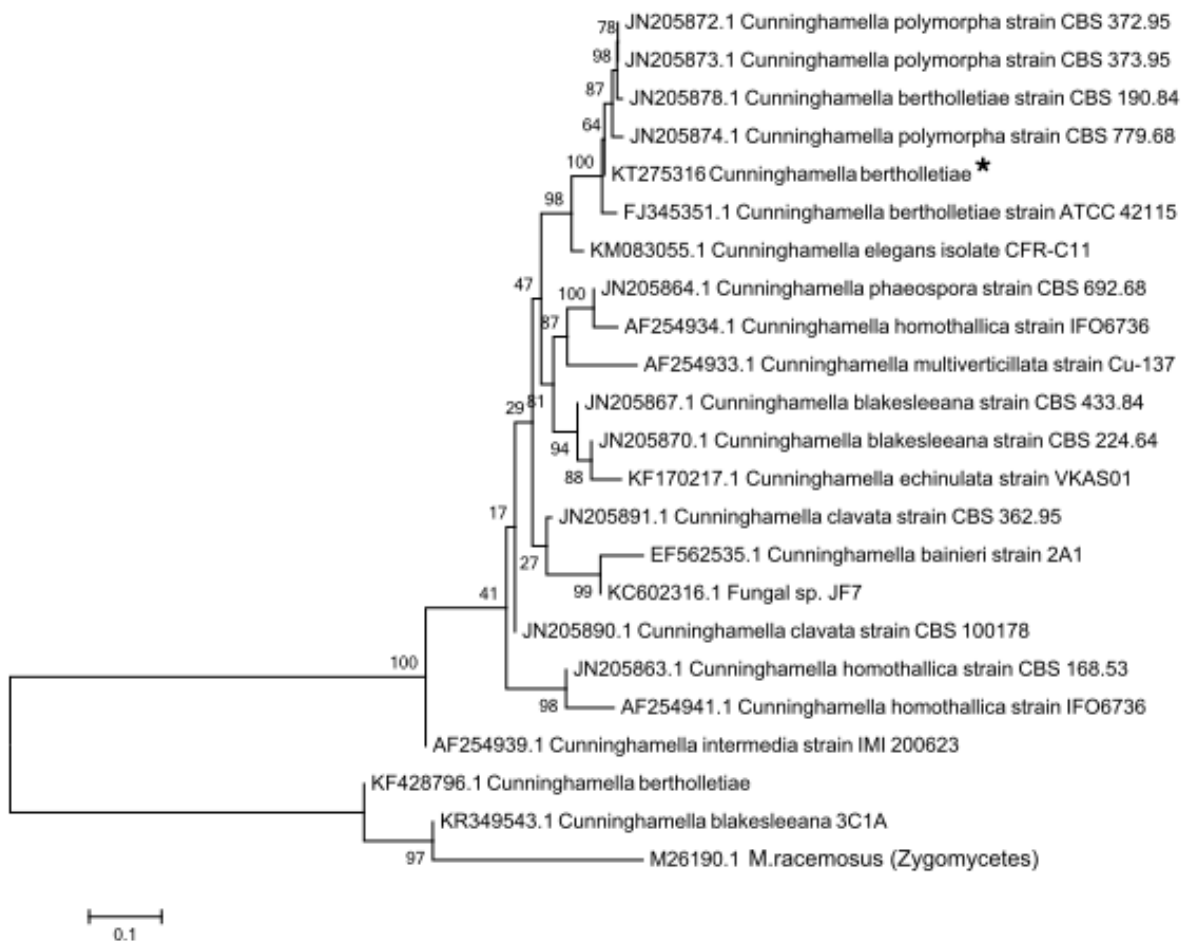
Figure 5-2: Phylogenetic (NJ) tree based on the analysis of combined ITS gene regions (isolate from cassava cultivated in Eastern Cape, South Africa).

5.4.3 Fungal degradation of free cyanide, ammonium-nitrogen and nitrogen-nitrate

Fungus isolated from both cassava and cassava-attached silt was able to biodegrade free cyanide, ammonium-nitrogen and nitrogen-nitrate within 5 days of analysis. Thus, from up to 97.75% of the initial (4 mg/40 mg/L) KCN concentration was biodegraded with nearly 42.5% (isolates from silt) and 37.5% (isolates from cassava) being converted into NH_4^+-N and finally NO_3-N at 77.5% and 72.5% by silt and cassava-isolated fungi, respectively.

Figure 5-4 below illustrates a fungal biodegradation of CN^-, HH_4^+-N and NO_3-N.
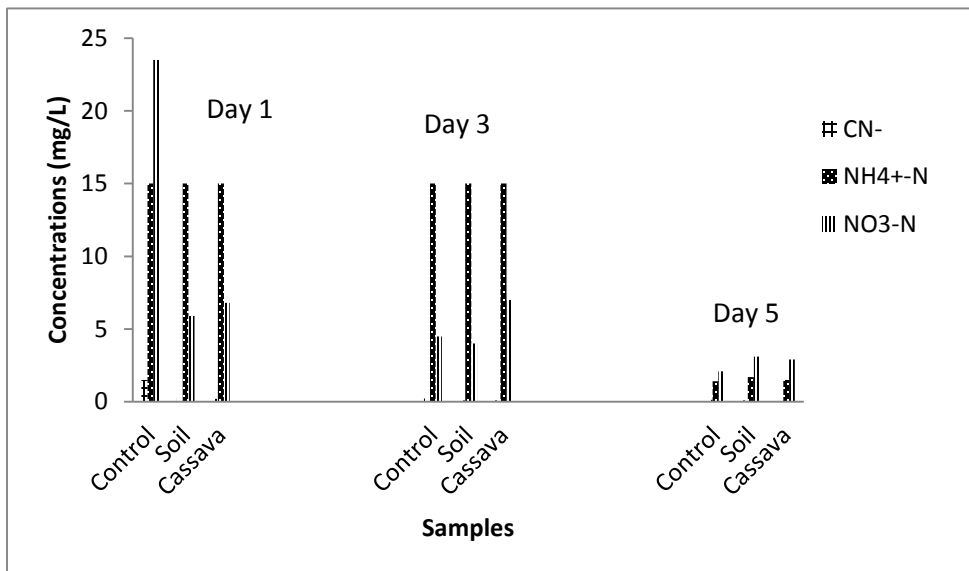
Figure 5-3: Fungal biodegradation of CN^-, HH_4^+-N and NO_3-N

Generally, *Cunninghamella* sp. is often identified through morphological characteristics, microscopic features such as colour, texture and margins, as well as the development of sexual and asexual zygosporic and sporangial levels including processes contributing to their formation and, finally, growth temperature. Additionally, the ITS rDNA sequence analysis of the isolates are also used during the identification and characterisation of the fungal species (Lategan *et al.*, 2012). A study on the amplification of the ITS region and morphological characteristics of *Cunninghamella* sp. revealed twelve species and three varieties (Su *et al.*, 1999; Zheng & Chen,

2001), while amplification of ITS regions of several species found a relationships within *Cunninghamella* sp. based on sequence analysis of rDNA through a consensus tree using neighbour-joining algorithm. Thus, varieties of *C. polymorpha* strains (CBS779.68) and *C. elegans* isolate (CFR-C11) resemble *C. bertholetiae* which demonstrates their closeness to the isolate found on cassava tubers and silt, while *C. blakesleeana* strain 3C1A *and M. racemosus* (Zygomycetes) are different from the *C. bertholletiae* isolate obtained.

Additionally, the *C. bertholletiae* sp. has shown the ability not only to being cyanide resistant, but also degrades the compound successfully. Thus, the fungus can be used for the reduction of free cyanide and cyanogenic compounds produced during the processing of the cultivar in order to mitigate HCN toxicity effects on humans, other organisms and the environment.

5.5 Summary

A fungus, *C. bertholletiae*, was successfully isolated, identified and characterised from cassava and cassava-cultivated soil silt from Mbizana, in the Eastern Cape province of South Africa. The isolate demonstrated the ability to grow at a temperature of at least 30 °C and is able to biodegrade free cyanide and convert it into $NH4^+$-N and NO_3-N and, hypothetically, to N_2 gas. Thus, it can be used for cyanide biodegradation (reduction) in cyanide-polluted soil and the environment.

CHAPTER 6

SCREENING OF FUNGAL *CUNNINGHAMELLA BERTHOLLETIAE* PATHOGENIC ACTIVITY ON MICROBIAL COMMUNITIES IN CASSAVA- (*Manihot esculenta* crantz) CULTIVATED SOIL

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CHAPTER 6

6. SCREENING OF FUNGAL (*CUNNINGHAMELLA BERTHOLLETIAE*) PATHOGENIC ACTIVITY ON MICROBIAL COMMUNITIES IN CASSAVA- (*MANIHOT ESCULENTA* CRANTZ) CULTIVATED SOIL

6.1 Introduction

Cassava (*Manihot esculenta* Crantz) is a crop from a family of Ephorbiaceae which constitutes a main staple food for many communities worldwide. Thus, countries from the African, Asian and Latin American continents massively produce this cultivar for self-consumption and poverty alleviation (Nambisan, 1994; Jarvis *et al.*, 2012) as well as for the industrial production of biofuel and biogas (Tilman *et al.*, 2009; Okudoh *et al.*, 2014). However, the crop contains cyanogenic glycosides compounds which enzymatic hydrolysis transforms into free cyanide during the plant's growth, through damage or from external attack (Siritunga & Sayre, 2004; Montagnac *et al.*, 2009). The CN$^-$ produced from the plant reaches the soil where it is accumulated and impacts negatively on the soil's microbial diversity.

Microorganisms (microbial and fungal communities) have played an important role in the environment and the sustainability of life on earth for millions of years. Bacteria may be aerobic or anaerobic, while some are photosynthetically active and play a role as producers of nutrients for other organisms in the food chain. Fungi are abundant in terrestrial ecosystems and thereby often play the role of decomposers of organic and inorganic material (Knudsen, 2006), which, in turn, are transformed into nutrients for plants' and animals' growth habitat support or its components, increasing the fertility of the soil. These materials or organic matter are thereby transformed into a growth support system for the ecosystem as the decomposed material, now known as humus or compost, contains essential nutrients for growth. Therefore, bacterial and fungal communities within soil influence the good functioning of arable land in general, as well as the terrestrial ecosystem in particular, contributing to suitable conditions for the cultivation of agricultural produce, thus leading to arable land productivity in the process.

The presence of microbial and fungal communities within a particular terrestrial ecosystem, however, can be problematic due to the fact that certain fungi have a more rapid

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growth in a particular environment and consume a larger quantity of substrates than other microorganisms (Scherm *et al.*, 2003; Chanchaichaovivat *et al.*, 2007). Rapid fungal growth can be competitive, inhibiting and pathogenic to some bacterial species (Liu *et al.*, 2013; Mousa & Raizada, 2013; Mehlomakulu *et al.*, 2014). These pathogenic microorganisms often produce metabolic extracellular products such as antimicrobial substances (proteins/toxins), which suppress other microorganisms (like bacterial/yeast) growth. The presence of pathogenic fungi, such as *Cunninghamella* sp., can contribute to an imbalance between the two microbial communities within cassava-cultivated soils.

This part of the research focused on screening fungal (*C. bertholletiae*) pathogenic activity on some microbial communities in cassava-cultivated soil. Soil microbial population was isolated by platting onto PDA growth media followed by biochemical testing, morphological and structural identification in conjunction with blue staining of isolates, in order to assess the pathogenic activity of the *C. bertholletiae* isolated in Chapter 5, with the clearing zones being used as a positive result of the antagonistic effects of the *C. bertholletiae* used.

6.2   Objectives

The objectives of this part of the study were to:

- Isolate, identify and characterise a few culturable common (n = 12) soil organisms in cassava-cultivated soil;

- Screen the isolate (identified in Phase 2: Aim 2) for pathogenic activity against a few (n = 12, from bullet 1 above) soil microorganisms (bacteria and fungi) from cassava-cultivated soil.

6.3   Material and Methods (summary)

Cunninghamella bertholletiae is found in soil and is sometimes endophytic to plants. The fungus was successfully isolated as discussed in Chapter 3 (Sections 3.3.1.1 to 3.3.1.3). Isolates from a cassava-cultivated soil were grown on a nutrient agar containing cyclohexamide (1.75

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mL/250 mL) and PDA with ampicillin (500 µL/250 mL) while the plates were incubated at 37 °C for bacterial and fungal growth.

All procedures for the isolation and assessment of a fungal *C. bertholletiae* pathogenic activity on microcosms of fungi and bacterial communities from cassava-cultivated soil, as highlighted in Section 3.3, with isolates (n = 12) identified using a VITEK 2 system, were conducted on the soil used for this part of the research.

6.4   Results and Discussion

6.4.1 Biochemical identification of microorganisms from cassava-cultivated soil using the VITEK 2 Systems (v07.01)

Identification cards were used for the identification of all bacterial species while yeast-identification cards were used for yeast/fungal identification. Results showed excellent identification with a 95-99% probability for all bacteria and an 86% acceptable probability for yeast/fungi. The biochemical identification (Biochem ID) system revealed that all microorganisms (bacteria and yeast) were Gram-negative as confirmed by identification using microscopic techniques (see Table 6.1), except for *Rhodotorula* sp. and *Cryptococcus albidus* which were confirmed to be Gram-positive.

6.4.2   *C. bertholletiae* pathogenic activity on microbial communities in cultivated soil

Cunninghamella bertholletiae's pathogenic activity against bacteria such as *Oligella ureolytica, Acinetobacter* sp*., Pseudomonas luteola, Sphingomonas paucimobilis, Myroides* sp*., Achromobacter denitrificans, Achromobacter xylosoxidans, Methylobacterium* sp. and *Stenotrophomonas maltophilia*, yeasts such as *Candida lipolytica* and fungi such as *Rhodotorula* sp*.* and *Cryptococcus albidus,* was assessed for a period of 24 to 168 hrs. The results revealed that *C. bertholletiae* was pathogenically active on some bacterial species with a minute antagonistic effect against *Myroides* sp., *S. maltophilia,* as well as *C. lipolytica*. The isolate had a minimal fungicidal effect against *Rhodotorula* sp. and *Cryptococcus albidus*, with growth of these species being insignificantly affected by the co-cultured *C. bertholletiae*.

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C. bertholletiae's pathogenicity against *Pseudomonas luteola* and *Sphingomonas paucimobilis* was imminent and noticeable at 24 hrs after inoculation. At 48 hrs, the fungus showed some growth and antimicrobial activity as bacterial growth decreased. Subsequently, at 120 hrs, fungal growth started to slow down as bacterial growth in the media used decreased to become stagnant, with insignificant growth observed at 168 hrs with bacterial growth being depleted (Figures 6-1). Similarly, *C. bertholletiae*'s growth was observed after 48 hrs with significant antimicrobial activity against *Acinetobacter* sp., which was not noticeable with *Oligella ureolytica.* After 120 hrs, clearing zones were; however, observed for both bacteria, although the *Acinetobacter* sp. was deemed susceptible (or sensitive) to the presence of *C. bertholletiae* than *Oligella ureolytica.* Significantly, co-cultures, after an incubation period of 168 hrs, demonstrated *Acinetobacter* sp. deactivation (Figure 6-1b), while *Oligella ureolytica* could still be observed (Figure 6.1a) in some agar plates.

C. bertholletiae co-culturing also displayed a significant antimicrobial effect on other microbial species that were exposed to the fungus, namely the *Myroides* sp*., Achromobacter denitrificans, Achromobacter xylosoxidans, Methylobacterium* sp., *Stenotrophomonas maltophilia* and *Candida lipolytica* after 24 hrs of inoculation (Figure 6-1e to 6.1i). After 48 hrs and 120 hrs of exposure, respectively, the fungus's pathogenicity became significantly evident on the *Stenotrophomonas maltophilia* and *Candida lipolytica* (Appendix 8, Figure 6-1h and 6-1i) when co-cultured, which was characteristic of the diminished microbial growth patterns of the respective microorganisms which were also observed by their physical destruction (disappearance).

Oligella ureolytica 24hrs   48hrs   120hrs   168hrs   (**a**)
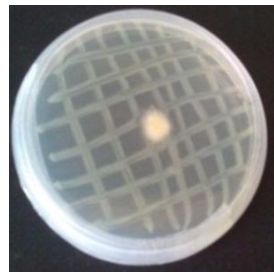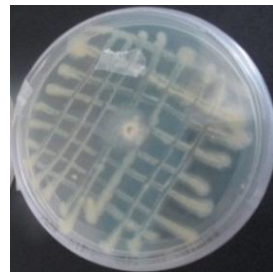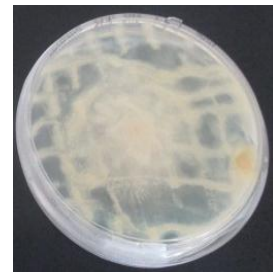
Acinetobacter family 24hrs   48hrs   120hrs   168hrs   (**b**)
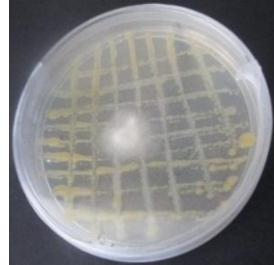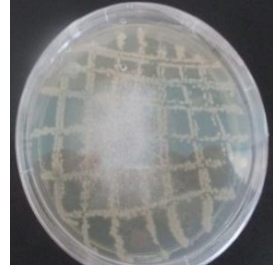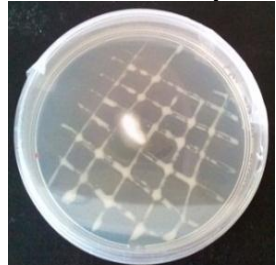
Pseudomonas luteola 24hrs   48hrs   120hrs   168hrs   (**c**)
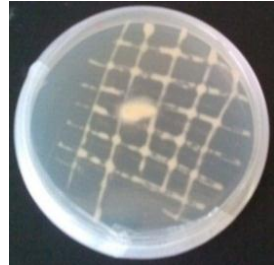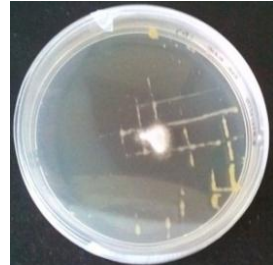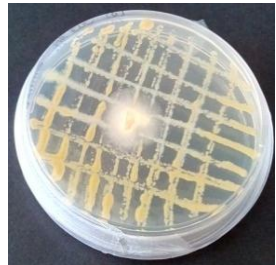
Sphingomonas paucimobilis 24hrs   48hrs   120hrs   168hrs   (**d**)
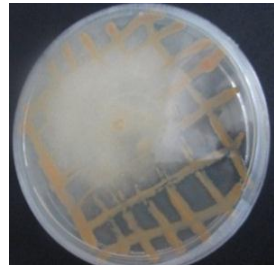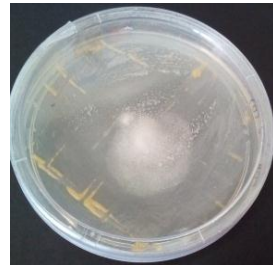
Figure 6-1: *C. bertholletiae* pathogenic activity on bacterial community (from 24-168hrs)
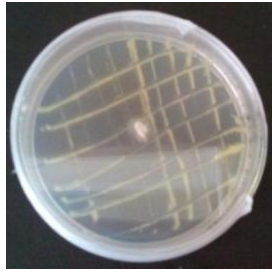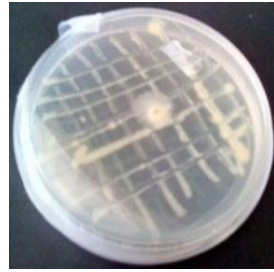
Myroides sp. 24hrs   48hrs   120hrs   (**e**)
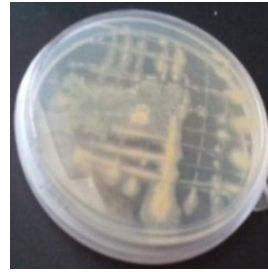
Achromobacter denitrificans/*Achromobacter xylosoxidans* 24hrs   48hrs   120hrs   (**f**)
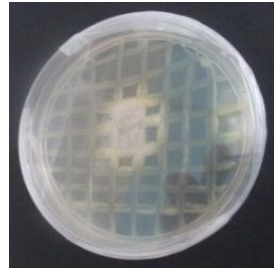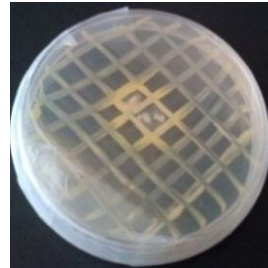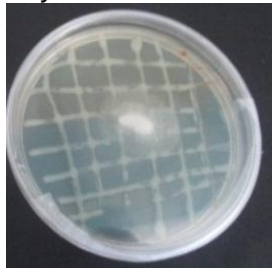
Methylobacterium sp. 24hrs   48hrs (Top view)   Bottom view   (**g**)
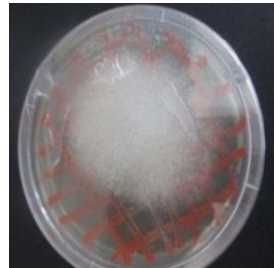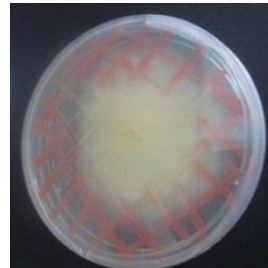
Figure 6-2 *Cont*: *C. bertholletiae* pathogenic activity on bacterial community and yeast (from 24-120hrs)
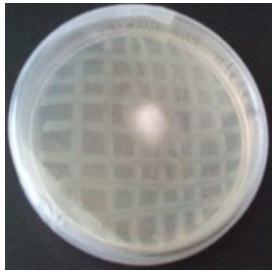
Stenotrophomonas maltophilia
24hrs

48hrs

(**h**)
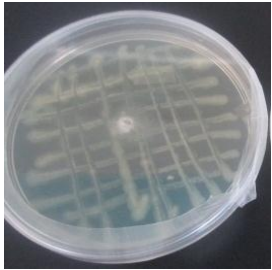
Candida lipolytica 24hrs
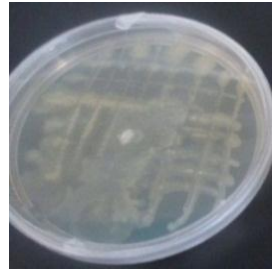
48hrs

(**i**)

Figure 6-3 *Cont: C. bertholletiae* pathogenic activity on bacterial community and yeast (from 24-120hrs)

Achromobacter denitrificans and *Achromobacter xylosoxidans* displayed different characteristics, which resembled or could be regarded as antagonistic/bacterial resistance behaviour. This was concluded as a result of fungal growth ceasing and significant changes in bacterial colour where a bacteria-fungus interphase occurred (Figure 6-1f). However, both *C. bertholletiae* and *Methylobacterium* sp. displayed a similar degree of growth which was not affected by the presence of either species as compared to the other above-mentioned microbial species. Instead, there was some kind of symbiosis (Minerdi *et al.*, 2002; Kobayashi & Crouch, 2009), a mutual and beneficial cohabitation between a fungus (*C. bertholletiae)* and the bacteria which is characterised by mutual growth of both species (Figure 6-1g) under co-culture conditions (Partida-Martinez & Hertweck, 2005; Partida-Martinez *et al.*, 2007; Kobayashi & Crouch, 2009).

However, there were a variation in shapes amongst the microorganisms from rods (*Oligella ureolytica, Candida lipolytica*, cocci (*Acinetobacter family*), rods, chains, some singular, (*Achromobacte* sp.), to rods, small, chains and singular (*Methylobacterium* sp.) and long rods (*Rhodotorula* sp. and *Cryptococcus albidus*).

From these results, *C. bertholletiae* pathogenic activity within agricultural soil can have negative effects on the soil's bacterial community as it suppresses bacterial growth of the *Pseudomonas luteola* and *Achromobacter denitrificans* species (Figure 6-1c and 6-1f). Generally, it is known that the absence of some bacteria in soil can negatively affect soil quality and plant growth as some endophytic microorganisms contribute to the plant's biocontrol/bio-protective mechanism which prevent plant diseases while facilitating nutrient availability and intake rates (De Ingeniis *et al.*, 2009; Kobayashi & Crouch, 2009). Thus, a decrease in microorganism's communities will contribute not only to lowering the soil's biological characteristics (biomass, organic matter formation and content) and physical properties such as soil aggregate formation, aeration, compaction, water circulation abilities (Knudsen, 2006; Rillig & Mummey, 2006; Vineela *et al.*, 2008), but, as well as increasing the plants' vulnerability to diseases and external attack, it will therefore lead to low crop-production and yield.

Previous studies on bacterial/fungal interactions (BFI) have demonstrated that it is important for the environment to have such symbiosis in agriculture and in human health (Kobayashi & Crouch, 2009). Thus, this interaction can be used as a basis of promoting arable soil health, thus its productivity. In agricultural and environmental engineering, BFI are used to improve mushroom production including reducing plant and animal diseases (Moran & Baumann,

2000; Moran & Wernegreen, 2000; Minerdi *et al.*, 2002). Although the fungus can be suited for use as a bio-control agent for plant pathogens in prevention and treatment, it is, however, unsuitable on cassava tubers as it can be pathogenic.

Table 6-1: VITEK- Soil microorganisms identification

	Samples no.	Growth Media	Microscopic Identification	Biochemical Identification	Microscopic Identification	Classification
			NA (Nutrient agar)- Cyclohexamine			
	1	NA+Cyclo	-	-	Rods	*Oligella ureolytica*
	2	NA+Cyclo	-	-	Cocci	*Acinetobacter* sp.
	3	NA+Cyclo	-	-	Short Rods	*Myroides* sp.
	4	NA+Cyclo	-	-	rods, chains, some singular	*Achromobacter denitrificans*
Bacterial community			-	-	rods, chains, some singular	*Achromobacter xylosoxidans*
	5	NA+Cyclo	-	-	Rods, small and singular	*Stenotrophomonas maltophilia*
	6	NA+Cyclo	-	-	Rods	*Sphingomonas paucimobilis*
	7	NA+Cyclo	-	-	rods, small chains and singular	*Methylobacterium* sp.
	8	NA+Cyclo	-	-	rods and spores	*Pseudomonas luteola*
			PDA (Potato dextrose agar)- Ampicillin			
Yeast	9	PDA+Ampi	-	-	Rods	*Candida lipolytica*
		PDA+Amp		+	long rods	*Rhodotorula* sp.
Fungi	10	PDA+Amp		+	long rods	*Cryptococcus albidus*

6.5 Summary

Pure colonies of microorganisms isolated from cassava-cultivated soil were identified using the VITEK 2 biochemical test including morphological and structural characteristics using microscopic techniques for bacteria with lactophenol cotton blue staining method for fungi. Results revealed numerous bacterial species namely, *Oligella ureolytica, Acinetobacter* sp., *Sphingomonas paucimobilis, Myroides* sp., *Achromobacter denitrificans, Achromobacter xylosoxidans, Stenotrophomonas maltophilia, Sphingomonas paucimobilis, Methylobacterium* sp., *Pseudomonas luteola*, yeast *Candida lipolytica* and fungi such as *Cryptococcus albidus, Rhodotorula* sp. Pathogenic activity on bacterial communities revealed that the *C. bertholletiae* isolate which had previously been found to be cyanide-resistant, is pathogenic against some species and thus inhibits and suppresses bacterial growth of *O. ureolytica, Acinetobacter* sp., *P. luteola* and *S. paucimobilis*. Furthermore, it was found to be slightly antagonistic against *Myroides* sp. and *S. maltophilia,* as well as *C. lipolytica*. However, the fungus demonstrated insignificant pathogenicity against *C. albidus* and *Rhodotorula* sp. Overall, the results from this part of the study indicated that *C. bertholletiae* was pathogenic against some bacteria and yeast found in cassava-cultivated soil.

CHAPTER 7

RAPID IDENTIFICATION OF CYANIDE-TOLERANT *CUNNINGHAMELLA BERTHOLLETIAE*'S TOXINS FROM DECOMPOSING CASSAVA: MITIGATION STRATEGY FOR TOXIN REDUCTION USING *NEPENTHES MIRABILIS* EXTRACTS

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CHAPTER 7

7. RAPID IDENTIFICATION OF CYANIDE-TOLERANT *CUNNINGHAMELLA BERTHOLLETIAE'S* TOXINS FROM DECOMPOSING CASSAVA: MITIGATION STATEGY FOR TOXINS REDUCTION USING *NEPENTHES MIRABILIS* EXTRACTS

7.1 Introduction

Post-harvest storage for cassava is often shortened due to produce maceration caused by bacterial and fungal infestation (Hocking, 1997; Zidenga *et al.*, 2012). Fungal species such as *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp. and *Cunninghamella* sp. produce secondary metabolites and toxins that affect the storage longevity and quality of agricultural produce such as cassava (Wagacha & Muthomi, 2008; Ellefson *et al.*, 2012; Zidenga *et al.*, 2012; Matumba *et al.*, 2014;). These secondary metabolites, that is, mycotoxins, which have a negative impact on agricultural products, lead to economic losses due to the contamination of produce, which includes inedibility. Generally, toxins are biosynthetic compounds produced by numerous microorganisms in a natural or in a controlled environment.

These microorganisms include the fungus, *Cunninghamella bertholletiae,* which is pathogenic to humans and animals (Rickerts *et al.*, 2000; Zhang *et al.*, 2002), while its toxins in the environment and on consumable commodities constitute an environmental hazard and a health risk to consumers (Ueno *et al.*, 1997; Brakhage *et al.*, 1999; Hussein & Brasel, 2001; Brakhage, 2013; Selvaraj *et al.*, 2015). Some fungi, including their metabolites, are able to colonise several plant parts as they are endophytes, culminating in infestation of agricultural products, such as tomatoes, maize, potatoes, beans, peanuts, yams, wheat and cassava (Hocking, 1997; Wagacha & Muthomi, 2008; Amadi & Adeniyi, 2009; Adetunji *et al.*, 2014; Delgado *et al.*, 2014; Ediage *et al.*, 2014) and dairy products such as milk, including cheese (Wagacha & Muthomi, 2008; Ellefson *et al.*, 2012; Streit *et al.*, 2013; Warth *et al.*, 2014; Selvaraj *et al.*, 2015). Humans' or animals' consumption of contaminated produce may lead to foodborne toxin-related intoxication (Zhang *et al.*, 2002; Matumba *et al.*, 2014), culminating in the degeneration of human internal organs including their functionality as well as the promotion of diseases such as cancer (Schell *et al.*, 1982; Ueno *et al.*, 1997; Rickerts, 2000; Williams *et al.*, 2004; Pestka & Smolinski, 2005; Van De Walle *et al.*, 2010; Ediage *et al.*, 2014)

There are several varieties of mycotoxins and extracellular metabolites such as Aflatoxins (AFB1, AFB2, AFG1 and AFG2), Fumonisins (FB1, FB2), Deoxynivalenol (DON), and Ochratoxins (A, B and C), amongst others, which are produced by numerous fungal species, some of which are deleterious to plants/agricultural produce, humans and animals (Hocking, 1997; Munkvold & Desjardins, 1997; Williams *et al.*, 2004; Afsah-Hejri *et al.*, 2013; Marroquín-Cardona *et al.*, 2014). However, their production occurs under favourable environmental conditions, such as a high temperature, adequate moisture/humidity including sufficient availability of nutrients for the producers, the nutrient source of which, in most cases, is decaying produce (Kimanya *et al.*, 2008; Van der Fels-Klerx *et al.*, 2012; Srey *et al.*, 2014). These concerns have prompted researchers to find cheap, efficient and cost-effective ways to reduce or manage mycotoxin-producing organisms, including mycotoxin contamination when produced (Brakhage & Schroeckh, 2011; Streit *et al.*, 2013; Selvaraj *et al.*, 2015; Vanhoutte *et al.*, 2016) to limit sequential effects including the contamination of produce.

In previous studies, it was found that *C. bertholletiae*, a common soil organism, which was isolated from decomposing cassava, was both cyanide-resistant with the ability to biodegrade free cyanide while being antagonistic towards other soil organisms (Chapters 5, 6 and 7). Currently, there is minimal literature available on mycotoxins produced by C. bertholletiae. Similarly, there is minimal research on a mitigation strategy which can be classified as environmentally benign for toxin reduction with the potential to also reduce free cyanide and total nitrogen (from the biodegradation of free cyanide), which is implementable (*in-situ*) in such a way that it is able to also minimise antagonistic effects of the cyanide resistant C. bertholletiae on other arable soil microorganisms.

Therefore, the aim of this part of the study was to propose and assess a method for rapid identification of mycotoxins, thus secondary metabolites from *C. bertholletiae* isolated; furthermore, to quantitatively assess a proposed mitigation strategy using plant extracts (a green chemistry approach), from *Nepenthes mirabilis,* known to contain a variety of enzymes (Adlassnig *et al.*, 2011; Lee *et al.*, 2016). Overall, this is for mycotoxin reduction. For such a mycotoxin mitigation strategy to be successful, enzymes associated with deamination or mechanisms biocatalytically facilitated by esterases for the decoupling of aliphatic chains in mycotoxins (Vanhoutte *et al.*, 2016), must be identified using both quantitative and qualitative methods to ensure the usability of the *N. mirabilis* extracts.

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7.2 Objectives

The objectives for this part of the study was to:

- Identify secondary metabolites/mycotoxins produced by the cyanide degrading isolate(s), using suitable analytical instruments which can; 1) render the cassava tubers inedible, and 2) contaminate arable agricultural as well as groundwater sources;

- Assess a proposed mitigation method (biological/environmentally benign method) which can be applied *in-situ* for the reduction of these secondary metabolites/mycotoxins/toxins in cassava-cultivated soil. This method must also have the potential to facilitate CN$^-$ reduction, its biodegradation by-products.

7.3 Material and Methods (Summary)

Secondary metabolites/mycotoxins produced by the cyanide resistant *Cunninghamella bertholletiae* from decomposing cassava were successfully extracted, analysed and identified (Sections 3.3.1.1 to 3.3.1.3). Toxin extraction was done through a fermentation process in which fungal mycelia were cultured in a 30 mL Erlenmeyer flask containing nutrient broth, followed by incubation in an incubator at 37 °C for 168 hrs. To implement the proposed mitigation strategy for mycotoxins reduction in cassava-cultivated soil using *N. mirabilis* extracts (juice) and enzymes, namely carboxylesterase, an activity assay was done while, the control samples did not have plant extracts and were undiluted as discussed in Chapter 3 (Sections 3.3.2.1 to 3.3.2.5).

7.4 Results and Discussion

7.4.1 Results

7.4.1.1 Mycotoxin identification

Mycotoxins produced by the isolated *C. bertholletiae's* were assessed via a fermentation technique in a nutrient broth medium with the liquid-liquid extraction method being done using chloroform, subsequent to a blow-down technique of the extracts and reconstitution in absolute methanol. The compounds listed in Table 7-1 were identified based on their molecular composition (structural features) and mass to charge ratio (m/z), using a LC/MS-ToF. See spectra in Figure 7-1.

Compound identification is important due to observed consequential outcomes of the infested cassava as by-products of mycotic infestation are hazardous both to humans and animals if such agricultural produce is consumed. From the results, both Fumonisin (FB1) and Deoxynivalenol (DON) were identified as the prevalent compounds associated with the fermentation of the cyanide resistant isolate, *C. bertholletiae.* FB1 mycosis outcomes in animals and humans are liver and esophageal cancer (Stockmann-Juvala & Savolainen, 2008), destruction of renal and nerve tissues, profound oxidative stress, heart and pulmonary diseases and degenerative mutation of cells (Al-Fakih, 2014).

FB1 detection on LC/MS ToF was done, based on a method developed by Plattner (1995) and Munkvold and Desjardins (1997), a method in which the compound produced a moderate signal under a positive acquisition mode.

Table 7-1: *C. bertholletiae's* mycotoxins and secondary metabolites were identified using mass to charge ratio LC/MS-ToF

Compounds	Molecular formula	Molar mass (g/mol) [ion form M + H^{+}]	(m/z)
Fumonisin B1[x]	$C_{34}H_{59}NO_{15}$	721.83	722.395 [a]
Deoxynivalenol (DON) [y]	$C_{15}H_{20}O_6$	297	259 [b]

[x]: detected in CN^{-} cultures, [y]: detected in free CN^{-} cultures, [a]:similar to that observed by Sulyok *et al.*, 2015.)
[b]: similar to that observed by Sulyok *et al.*, 2015.

For FB1 counts (level), a 4 x 10^3 count value was observed which was higher than that of 1.9 x 10^3 for DON. Similarly, and according to Plattner (1995) and Malone *et al.* (1998), DON detection is easily achieved through HPLC/LC-MS and UV methods. A LC/MS–ToF method, as described in Section 3.2.3, was used without modification nor optimisation, to identify the biodegradation by-products for each mycotoxin identified.

Table 7-2: Identified biodegradation by-products of mycotoxins/secondary metabolites

Mycotoxins/secondary metabolites	Biodegradation by-products identified	Molar mass (g/mol) [ion form M+H$^+$]	Mass (m/z) to charge ratio
Fumonisin B1	$C_{17}H_{34}O$ Heptadecanone [x]	254.45	256.270 [c]
	$C_{18}H_{37}NO$ Octadecanamide [y]	283.29	285.282 [d]
	$C_{18}H_{36}O$ Octadecenal [z]	266.46	267.268 [e]
Deoxynivalenol (DON)	$C_{15}H_{15}NO_3$ Tolmetin [w]	257.11	258.111 [f]

[x, y, z]: Identified by Vanhouette *et al.* (2016) and Benedetti *et al.* (2006) as a biodegradation by-products of fumonisin B1

[w, c, d, e, f]: Identified by (NIST, 2011)

Two peaks with a retention time of 23.79 min (Peak A, Figure 7-1) and 35.124 min (peak B, Figure 7-1), with a molecular formula of $C_{34}H_{59}NO_{15}$ and $C_{15}H_{20}O_6$, analogous to FB1 and DON, respectively. The peaks, A and B, were directly associated with ion m/z of 722.395 and 259, when the ESI was operated in a positive mode [ion form: M+H$^+$]. From the analysis by. using finger printing, including profile data in a mycotoxin database, the compound was attentively identified as both FB1 (peak A, Figure 7-1) and DON (peak B, Figure 7-1). A combination of the molecular weight, the structure, including m/z ratio, confirmed the identification of the compounds namely, to be FB1 and DON. It is paramount to indicate that FB1 was detected in a culture in which CN$^-$ (as KCN) was supplemented hypothetically, indicating that the FB1 production was perhaps influenced by the strenuous conditions to which the culture was subjected in comparison to DON.

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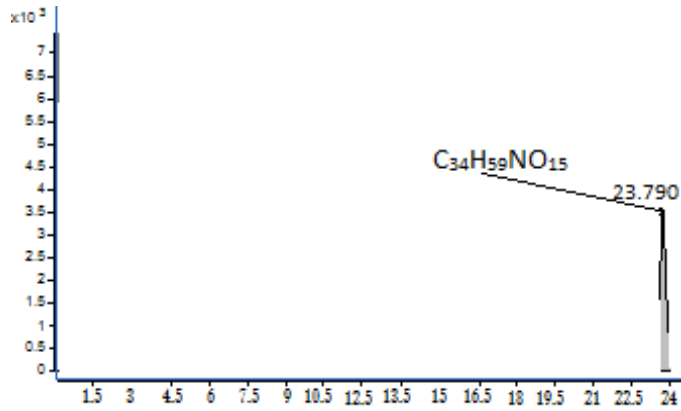
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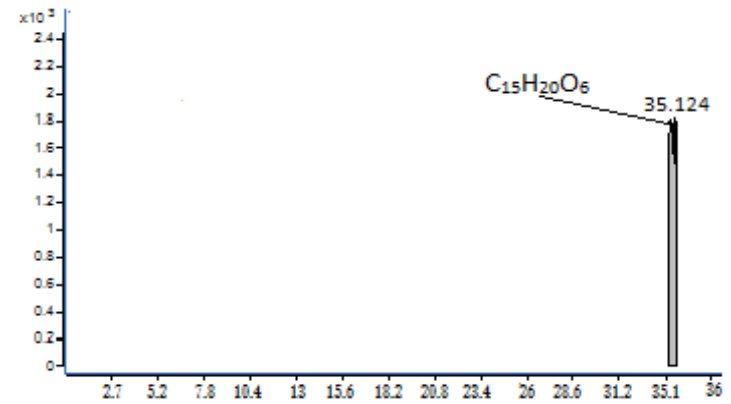
B

Figure 7-1: Molecular features and the extracted ion chromatograms (EICs) for secondary metabolites: (a) Fumonisin B1, (b) Deoxynivalenol (DON)

7.4.1.2 Biodegradation by-products identification

To the reported residual extracts of the cyanide resistant *C. bertholletiae,* in which FB1 and DON were, identified *N. mirabilis* extracts were added. This was for an assessment for which the feasibility of fungal mycotoxins/toxins (FB1 and DON) biodegradation into by-products could be identified using the LC/MS-ToF method, with Heptadecanone, Octadecanamide and Octadecenal being successfully identified from FB1 samples with only Tolmetin being identified in DON samples, respectively (Table 7-3).

Previous studies revealed that a biodegradation of Fumonisin B1 yielded by-products such as Heptadecanone, Octadecanamide and Octadecenal (Benedetti *et al.*, 2006; Vanhouette *et al.*, 2016). An intermediate such as Tolmetin can be a by-product of DON degradation (Figure 7-2). By using a similar identification strategy to that used to identify FB1 and DON, it was clear that *N. mirabilis* had a deleterious effect on both DON and FB1, although it had previously been difficult to biodegrade when compared to other mycotoxins (Benedetti *et al.,* 2006). From the spectra, the by-product counts indicated Octadecenal (1.1×10^2) > Octadecanamide (1×10^2) > Heptadecanone (0.9×10^2) with molecular ion peaks at m/z [M+H$^+$], 267.268, 285.282 and 256.270, respectively.

Furthermore, for DON residual extracts, the by-products observed when subjected to the *N. mirabilis* extracts (juice), were indicative of Tolmetin; that is, with the ESI spectra showing a molecular ion peak at m/z in a positive ion mode which was consistent with the molecular formula ($C_{15}H_{15}NO_3$). See Figure 7-2. Due to the nature of the *in-situ* proposed mitigation strategy, it is prudent to ascertain whether the applied *N. mirabilis* extracts (juice) comprises biocatalytic agents or enzymes known to facilitate the biodegradation of mycotoxins, using both qualitative and quantitative techniques.

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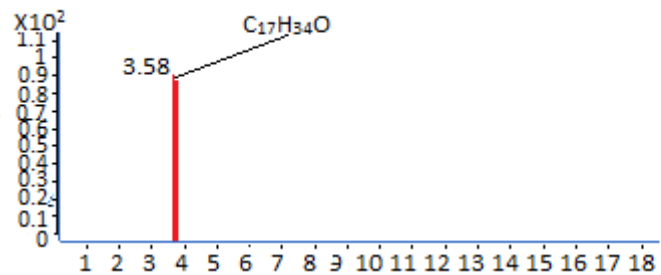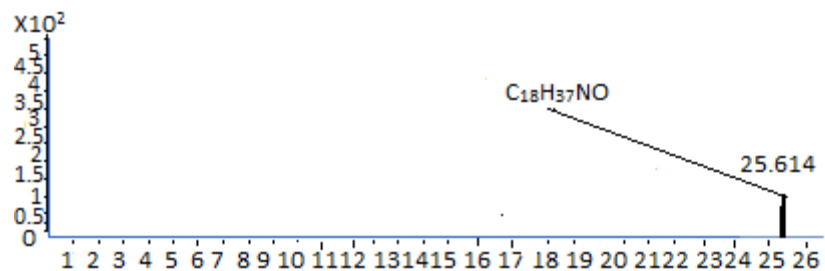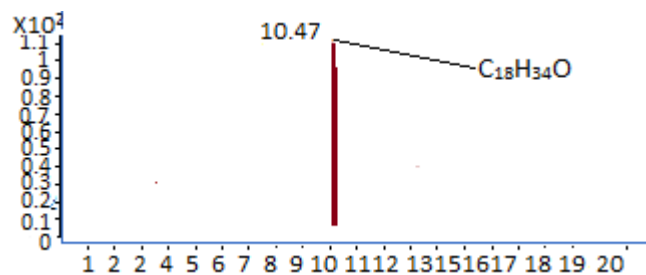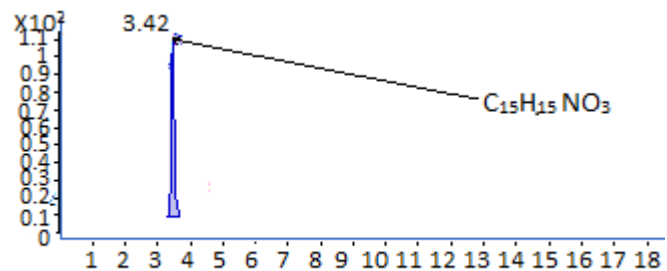
Figure 7-2: Molecular features and the extracted ion chromatograms (EICs) of mycotoxins/toxins' biodegradation by-products: (a) Heptadecanone, (b) Octadecanamide, (c) Octadecenal and (d) Tolmetin

7.4.1.3. Enzyme/biochemical activity assays

The extracts' activity assay had to be successfully done at ambient temperature. Thus, the carboxylesterase activity (quantitative) and other biochemical assays (using the VITEK system, qualitative) were done. For the *N. mirabilis* extracts for carboxylesterase, P-nitrophenyl acetate (PNPA) was used as a substrate at 75% dilution and 410 nm absorbance. For biochemical assays, as highlighted in Table 7-3, numerous enzymes were positively identified, while the calculation of carboxylesterase activity was found to be 7.8 U.L^{-1}.

Table 7-3: Carboxylesterase activity and qualitatively identified enzymes

Enzymes	Activity/outcome	Comment
Carboxylesterase	7.8 (U/L)	see Table 7.4
β-glucosidase,	++*	[a]Known to facilitate hydrolysis of cyanogenic glucosides, like linamarin, taxiphylin, and so on
β-glucuronidase	++*	[b]Known to facilitate hydrolysis of β-glucuronic acid residues
Phosphatidyl inositol phospholipase C	++*	[c]Breaks down L-arginine, a known highly concentrated constituent in cassava, into ammonium-nitrogen and citrulline

*- also identified by Kanokratana *et al.* (2016) and Takeuchi *et al.* (2011) to be prevalent in *N. mirabilis* extracts (juice)

[a]- Nartey (1968) and Yeoh (1989)

[b]- Borgen (2002) and Pereira *et al.* (2005)

[c]- Terra and Ferreira (1994)

Table 7-4: Overall biocatalysis properties associated with carboxylesterases (Schomburg *et al.,* 2013)

Mechanism/Applicability	Functionality/specificity
Hydrolysis	1) Hydrolysis of carboxylic- , esters or thioester functional groups; 2) Facilitates the biocatalysis of reactions associated with enzymes, arylesterase, lysophospholipase, acetylesterase, acylglycerol lipase, acylcarnitine hydrolase, palmitoyl-CoA hydrolase, amidase and aryl acylamidase;
Biodegradation/Environmental applicability	3) Shown to facilitate the biodegradation of Fumonisin and Deoxynivalenol (DON) (this study).

7.5 Discussion

7.5.1 Mycotoxins identified from the cyanide resistant *Cunninghamella* sp.

It is important to observe that toxins such as Fumonisins, DON, Aflatoxin, Ochratoxins, and so on, are produced by several types of microorganism species in environmental and biological matrices, while their extraction and identification methods vary in numerous studies (Adebajo *et al.,* 1994; Plattner, 1995; Dutton, 1996; Hocking, 1997; Peraica & Domijan, 2001; Hodek *et al.,* 2002; Manjula, *et al.* 2009; Bhat *et al.* 2010; Ottesen *et al.,* 2010; Afsah-Hejri *et al.,* 2013; Adetunji *et al.,* 2014; Al-Fakih, 2014; Ismaiel & Papenbrock, 2015). Some previous studies also revealed that mycotoxins are produced from multiple fungal and/or microbial sources in a single matrix or from a single species in numerous matrices. Bily *et al.* (2004) extracted toxins (deoxynivalenol; 15-acetyldeoxynivalenol (15-ADON) produced by *Fusarium graminearum* from several matrices, such as pig serum, insect larvae and maize grain, through the analysis for their identification using different optimised chromatographic conditions, with most methods being based on the use of an LC/MS.

From the sub-Saharan African continent, Ediage *et al.* (2014) reported that numerous

toxin- producing fungi can be found in several common agricultural products consumed by the populace like cassava, maize and peanuts, revealing that fungal species such as *Aspergillus* sp., *Fusarium* sp. and *Penicilum* sp. are common contaminants of such produce, with further classification of the toxins as Aflatoxins B1, B2 G1, Fumonisins B1, B2, B3, DON and Ochratoxins, particularly in cassava chips and cassava flakes sold in local markets.

For effective contaminant monitoring, leading to quantification, Platter (1995) recommended to identify mycotoxins such as DON and fumonisins (FB1, FB2 and FB3) in food products, crops and stored grains, the use of High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS) was advocated. The reason for this is that recently, and due to technological advancements, Rubert *et al.* (2013) indicated and identified mycotoxins such as DON, Fumonisins, Aflatoxins, Ochratoxins, Zearalenone, Beauvericin, Nivalenol and T-2, HT-2, using a Liquid chromatography triple quadrupole mass spectrometer (HPLC-QqQ-MS/MS) device which was deemed suitable. The use of such a device can detect traces of mycotoxins, even in several beers from European countries, which is indicative of global grain/agricultural produce contamination challenges. This is thus not limited to sub-Saharan Africa or other developing countries.

Some scholars, including Amadi and Adeniyi (2009), identified mycotoxins produced by *Aspergillus flavus* in maize (*Zea mays*) and rice (*Oryza sativa*) using Thin Layer Chromatographic (TLC) with Aflatoxin B1 and Fumonisin B1 being the primary analytes identified. As such, and due to the multitude of methods developed and assessed, a method modified by Bily *et al.* (2004) for toxin extraction from a fermentation broth was adopted and was thus used to produce mycotoxins from the cyanide resistant *C. bertholletiae*, with the extracts being used for LC/MS-ToF analysis due to the methods of usability, reproducibility and rapidity, while incurring minimal input/sample-processing costs. By using this method, the analysis reported herein successfully identified primary mycotoxins, FB1 and DON, and other secondary metabolites, 11-Ketotestosterone 3 and Silibinin. The results are available but not discussed and reported in this section.

An analysis of the concentration for each identified compound was indicative of concentration differences from one compound to another (between the mycotoxins as well as the secondary metabolites). Difference in acquisition time is directly related to the compound's structural characteristics, thus mobility through the C8 column used.

From both environmental and public health perspectives, the identified mycotoxins can

significantly contribute towards the deleterious effects in arable land used for cassava production, especially for impoverished communities that cultivate the plant and use its tubers as their staple food, and animals that consume fresh and rotten tubers, including waste from processed cassava. Previous studies have revealed Deoxynivalenol's (DON) health hazards due to cumulative or continuous exposure to humans and animals. Consumption of a high concentration of DON or exposure through contaminated processed and/or non-processed agricultural commodities, can result in diarrhea, vomiting, suppression of the digestive system's cell growth, including reproduction, rectal and anal bleeding, with clinical outcomes indicating the inhibition of cells in the small intestines to absorb proteins or nutrients (Rotter, 1996; Deshaies, 1999; Sergent *et al.,* 2006; Van De Walle *et al.,* 2010; Al-Fakih, 2014). Furthermore, disturbances and inhibition of blood cells were also reported (Pestka & Smolinski, 2005; Van De Walle *et al.,* 2010) which may ultimately lead to death. Some clinical indicators due to continuous consumption, albeit even in small doses from contaminated food sources, include psychological and eating disorders, culminating in weight loss and stunted growth in humans (Van De Walle *et al.,* 2010).

Similarly, FB1 cumulative consumption as a result of contaminated agricultural products such as maize, peanuts and others, can lead to cancer in organs such as the esophagus, liver, including the swelling of the pharynx and the prevalence of sores in gastric organs and rectal bleeding (Chu & Li, 1994; Peraica & Domijan, 2001). Furthermore, dermal exposure to these mycotoxins through contact and/or inhalation was also reported to cause skin irritation/rash, irritation of respiratory organs, with symptoms being chest pain, and periodic headaches (Gelderblom *et al.,* 1992; Chu & Li, 1994; Dutton, 1996; Peraica & Domijan, 2001).

7.5.2 Biodegradation by-products: outcomes of the mitigation strategy

Previous studies have demonstrated that the application of an appropriate mitigation strategy as a means in which the reduction of mycotoxins in general, including for cassava (*M. esculenta* crantz) soil, and its cultivated soil, biological means are preferable due to their environmental benignity. Some have suggested a bio-augmentation strategy, including techniques for contaminated soil and matrices, using bacteria such as *Sphingomonas* sp*., Nocardioides* sp., *Devosia mutans, Spseudomonas* sp. and *Exophiala* sp., to name a few (Duvick *et al.*, 1998; 2003; Benedetti *et al.*, 2006). For this study, a different approach was assessed, using ready-made plant extracts to minimise microbial imbalances which could be profoundly attributed to the introduction of alien species.

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According to Duvick *et al.* (2003), the biodegradation of Fumonisin, using *Exophiala* sp., produces by-products such as 2-oxo-12,16-dimethyl-3,5,10,14,15-icosanepentol hemiketal and N-acetylated aminopentol backbone (N-acetylAP1). However, a study by Benedetti *et al.* (2006) revealed that Fumonisin biodegradation by a combination of *Delftia/Comamonas* sp. yields compounds such as eicosane ($C_{20}H_{42}$), isononadecene ($C_{19}H_{38}$), including heptadecanone ($C_{17}H_{34}O$), octadecenal ($C_{18}H_{34}O$) and octadecanamide ($C_{18}H_{37}NO$), some of which were identified as being by-products of FB1 biodegradation using *N. mirabilis* extracts in this study.

Additionally, previous studies on a biodegradation of DON by bacteria revealed that the mycotoxin was able to be biodegraded into 3-epi-DON, a remedial strategy which can be achieved through the action of bacteria such as *Nocardioides* sp. (Ikunaga *et al.*, 2011; Vanhoutte *et al.*, 2016), 3-keto-DON,15-acetyl DON, 3-acetyl DON and HT-2 toxin using *Devosia mutans* and *Eubacterium* sp. through biocatalytic processes such as de-epoxidation/deacylation (Young *et al.*, 2007; Vanhoutte *et al.*, 2016).

Generally, it was established that bacterial abilities to biodegrade toxins such as Fumonisin can be achieved through enzymatic biocatalysis (Duvick *et al.*, 2003; Heinl *et al.*, 2009; Vanhoutte, 2016). Thus, bacteria such as *Sphingomonas* sp. have been found to have the ability to biodegrade mycotoxins such as Fumonisins by enzymatic action facilitated by carboxylesterases and aminotransferases, that contribute to the detoxification of the toxins by decoupling molecular components of toxins and hydroxyl bonds through the process of deamination, detoxification and degradation of amino/fatty acid chains (Duvick *et al.*, 2003; Hartinger *et al.*, 2011; Heinl *et al.*, 2011; Vanhoutte *et al.*, 2016).

From this study, extracts of *Nepenthes mirabilis* were thus used as a feasible alternative for the biodegradation of fungal mycotoxins/toxins (Fumonisin and DON) with assays (n = 2) confirming the prevalence of carboxylesterases, β-glucosidase, β-glucoronidase and Phosphatidyl inositol phospholipase C, which counts as a larger enzymatic profile than individual microbial species, as highlighted in Table 7-3.

Currently, this mitigation approach has not been studied nor reported in the literature survey undertaken for a period of 5 years (2013 to 2017). It is therefore suggested that plant-based extracts such as those of *Nepenthes mirabilis,* pitcher juice, be used for the biodegradation of mycotoxins/toxins on agricultural products such cassava and in cassava-cultivated soil.

Furthermore, a few skeptics could express concern about the use of the extracts on mycotoxin- contaminated soil because of its low pH (2.5), including availability, which can be addressed by using appropriate buffers and suitable plant extracts with similar enzymatic characteristics. Overall, the application of a low pH extract in agricultural soil should not be a major concern because a soil's pH can be amended by an application of lime. To corroborate this, a study by Bezdicek *et al.* (2003) revealed that the application of lime on agricultural soil with a low pH increases the soil's pH, improving its respiration capacity, while retaining the soil's microbial community profile at an acceptable level of 100 ugCg.

7.6 Summary

This part of the study revealed the production of mycotoxins from a free cyanide-resistant and/or tolerant *Cunninghamella bertholletiae*. Fumonisin B1 and Deoxynivalenol (DON) were successfully identified as extracellular metabolites from this isolate. Human intoxication from these mycotoxins, directly or indirectly, is through dermal contact, inhalation or consumption of contaminated food or agricultural produce which can be hazardous to humans and animals. Therefore, control measures for food and animal feed contamination is needed in order to decrease the levels of these compounds, focusing on future research to establish preventative protocols and/or mitigation strategies that would ensure the eradication of these hazardous compounds, using an environmentally benign approach which was assessed using *Nepenthes mirabilis* extracts (juice). The application of the extracts to a liquid matrix culminated in the biodegradation of mycotoxins, namely Fumonisin B1 and DON, with the subsequent formation of the following biodegradation by-products: Heptadecanone, Octadecanamide, Octadecenal from fumonisin B1 and Tolmetin from DON, which are easier to biodegrade by other microbial communities in agricultural soil.

At this stage, there is a need to find alternative indigenous plants extracts with similar characteristics to that of *N. mirabilis*, because of its scarcity.

The following are scientific advancements emanating from this study:

- The isolation, identification and characterisation of a less reported cyanogen and cyanide tolerant/resistant fungus (*Cunninghamella bertholletiae)* with the ability to degrade free

cyanide and total nitrogen with an ability to produce toxins/mycotoxin that are contaminants in agricultural produce;

- The development of an environmentally benign remediation strategy (method) with a potential for the reduction of toxin/mycotoxin and cyanogen load in agricultural soil.

CHAPTER 8

OVERALL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

CHAPTER 8

8. OVERALL SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

8.1 Overall summary

Cyanogens and mycotoxins are mostly generated by/or found on several agricultural products worldwide. High concentrations of these components in products such as cassava (*M. esculenta)* renders the produce inedible and harmful to humans and environmental health. *M. esculenta* is used as a primary source of food for many communities globally. Cyanogens such as linamarin contained in the produce are released/volatilised during its cultivation, harvesting, processing and cooking. An assessment of the quantity of cyanogens, thus CN⁻ , released during one of these stages (namely, cultivation) elucidated its contributing role to the overall cyanide load into the environment. The presence of Mycotoxins in cassava-cultivated soil caused by cyanide-resistant microorganisms (bacteria/fungi) can contribute towards producing spoilage and environmental contamination. This research aimed to identify cyanide-resistant isolate(s), with an efficacy to decompose cassava and produce mycotoxins and to propose an environmentally benign reduction method for cyanogens (including hydrogen cyanide) and mycotoxin biodegradation in cassava-cultivated soil.

Numerous research studies have suggested several methods for the reduction of cyanogens and CN⁻ in particular, as well as mycotoxins in agricultural products in general, which are deemed unsuitable due to either the use of chemical compounds and/or cost. Thus, the populace that consumes the produce on a daily basis and those living in rural areas cannot use these methods. It is therefore mandatory to propose a low-cost, environmentally benign biotechnological method for the reduction of cyanogens (CN⁻) and mycotoxins for *in-situ* application in cassava-cultivated soil.

To attain the above objectives, cassava was grown (in a controlled environment) at the Cape Peninsula University of Technology's Cape Town Campus. After 10 months of growth, volatilised gases (HCN, NH_3, NO_2) from cassava plants, the chlorophyll content as well as PAR were determined. The gas volatilised was: 14.4 mg/L (NH_3), 5.8 mg/L (HCN) and 5.8 mg/L (NO_2) being minimum values, with maximum values of 147.4 mg/L, 90.3 mg/L and 7.7 mg/L of NH_3, HCN, NO_2 being observed respectively. As an indication of the health of the plants when volatilised, gas measurements were made. A chlorophyll content measured on the cassava plant

leaves ranged from 26.55 to 29.5 µmol/m^2 with PAR being at a maximum of 122.1 to 196.7 µmol/m^{2}s (Chapter 4).

Additionally, using ArcGIS, and Quantum GIS (QGIS), the global HCN from cassava tuber harvesting was mapped, followed by projections (2014 to 2024) for HCN release for the largest cassava producers, namely Africa, Asia and South America, using FAO data. This information indicated that the estimated HCN released from cassava harvesting from 2002 to 2014 ranged from 0.025x10^{-3} to 6.71 ppq (Africa), 0.012 x10^{-3} to 1.01 ppq (Asia) and 0.007x10^{-3} to 0.920x10^{-3} ppq (South America), while the projection of HCN volatilisation for the next 10 years (2014 to 2024), indicating increases estimated at 60.5% (Africa), 57.7% (Asia) and 50.5% (South America).

Furthermore, an assessment of fungal pathogenic activity against cassava-cultivated soil consortia, the identification of mycotoxins from cyanide-resistant isolate, identified as *C. bertholletiae,* was successfully completed. The fungus identification was based on morphology, microscopic structure, nucleotide sequences and phylogenetic analysis. The fungus has the potential to be used as a free cyanide and total nitrogen degrader (Chapter 5).

Fungal pathogenic activity of the *C. bertholletiae* against microorganisms in cassava-cultivated soil, revealed an inhibition of some bacterial growth accompanied by the complete antagonistic effect of some bacterial species, *Oligella ureolytica, Acinetobacter* sp*., Pseudomonas luteola* and *Sphingomonas paucimobilis,* while there was a partial antagonistic effect against *Myroides* sp*., Stenotrophomonas maltophilia* and *Candida lipolytica*. Additionally, the fungus's pathogenic ability on other soil fungi such as *Cryptococcus albidus* and *Rhodotorula* sp*.,* was found to be insignificant (Chapter 6).

By using a fermentation method, the cyanide-resistant organism (identified in Chapter 5) was able to produce mycotoxins such as Fumonisin B1 (FB1) and Deoxynivalenol (DON) and other secondary metabolites, respectively (not a focus of this research under CN$^-$ and CN$^-$-free culture conditions) (Chapter 7). With regard to the mitigation or bioremediation strategies with a potential to also facilitate CN$^-$ and total nitrogen reduction, *Nepenthes mirabilis* extracts were successfully evaluated to specifically biodegrade FB1 and DON with carboxylesterases, β-glucosidase, β-glucoronidase and Phosphatidyl inositol phospholipase C being amongst enzymes quantitatively and qualitatively being identified as some of the constituents in the extracts. Such constituents are known for the detoxification of mycotoxins and other constituents associated with cassava decomposition via hydrolysis of side chains and amino groups, thus causing destabilisation.

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8.2 Overall conclusion

In conclusion, the research undertaken has confirmed the hypotheses and probable outcomes to the research questions asked. Thus, the study established that during cassava cultivation, a significant concentration of CN^- and its gaseous by-products, NO_2 and NH_3, are being released into the atmosphere (through volatilisation), as well as into the environment in general, which can contaminate soil and groundwater sources in particular, through decomposition, complexation, transportation and leaching. This was indicative of free cyanide loading into the atmosphere and subsequently into the environment which can negatively impact on the soil's physical, chemical and biological properties, culminating in direct and deleterious consequences on agricultural productivity of soil used for cassava cultivation.

Furthermore, a less-reported cyanide resistant fungus, identified as *C. bertholletiae,* was successfully isolated, identified and characterised. The novelty of the research was not only on the isolates' tolerance and ability to biodegrade free cyanide and convert it into NH_4^+-N and NO_3-N and eventually into N_2 gas, but also on its ability to produce mycotoxins/toxins (FB1, DON), including other secondary metabolites which were not of interest in this study.

Additionally, a synergistic co-culturing of the fungus, *C. bertholletiae,* and other cassava-cultivated soil microorganisms, demonstrated the isolates' pathogenic activity on numerous bacterial species such as *Oligella ureolytica, Ac*inetobacte*r* sp.*, Pseudomonas luteola, Sphingomonas paucimobilis, Myroides* sp.*, Stenotrophomonas maltophilia* and *Candida lipolytica,* while it showed a mutualistic and beneficial symbiosis with other bacterial species such as *Methylobacterium* sp.

For the bioremediation of soil contaminated by mycotxins/toxins, this study proposed an environmentally benign method in which extracts from a carnivorous plant (*Nepenthes mirabilis*) were shown to be able to successfully biodegrade FB1 and DON, producing biodegradation by-products such as Heptadecanone, Octadecanamide Octadecenal and Tolmetin which were successfully identified. This method can be further developed using plant extracts with similar properties (characteristics) as a biodegradation means of mycotoxin reduction in contaminated agricultural (arable) soil.

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8.3 Recommendations: future studies

Further studies on cyanide movement from decomposing cassava are therefore needed in order to fully comprehend the movement including complexation of cyanides specifically in the environment.

The option to cultivate genetically modified cassava, particularly in regions with high cassava production including decomposition and whereby communities are reluctant to use genetically modified cultivars in order to decrease both environmental and public health outcomes, can be investigated further.

As the *C. bertholletiae* isolate has proven to biodegrade cyanides, it can be used for environmental engineering purposes particularly for the biodegradation of cyanide compounds in matrices such as wastewater from large gold mining operations.

Finally, the economic evaluation and feasibility of the proposed mycotoxin/CN$^-$ mitigation method (proposed) on a large scale, or *in-situ/*trial studies, are needed in order to comprehend the financial implications and to evaluate the extracts of indigenous plants with the potential of producing extracts with similar characteristics to those observed for *Nepenthes mirabilis* to ensure the sustainability of the proposed method, and to consider alternative plant sources.

9. BIBLIOGRAPHY

9.1. Bibliography

Abadi, M. D. M., Ashraf, N., Chamsaz, M. & Shemirani, F. 2012. An overview of liquid phase microextraction approaches combined with UV–Vis spectrophotometry. *Talanta,* 99: 1-12.

Abbas, H. K., Boyette, D. & Hoagland, R. 1995. Phytotoxicity of *Fusarium,* other fungal isolates, and of the phytotoxins fumonisin, fusaric acid, and moniliform to Jimsonweed. *Phytoprotection*, 76(1): 17-25

Abbas, H. K., Cartwright, R. D., Shier, W. T., Abouzied, M. M., Bird, C. B. & Rice, L. G., Ross, P. F., Sciumbato, G. L. & Meredith, F. I. 1998. Natural occurrence of fumonisins in rice with *Fusarium* sheath rot disease. *Plant Disease*, 82(1): 22-25.

Adebajo, L. O., Idowu, A. A. & Adesanya, O. O. 1994. Mycoflora, and mycotoxins production in Nigerian corn and corn-based snacks. *Mycopathologia,* 126(3): 183-192.

Adetunji, M., Atanda, O., Ezekiel, C. N., Sulyok, M., Warth, B., Beltrán, E., Krska, R., Obadina, O., Bakare, A. & Chilaka, C. A. 2014. Fungal and bacterial metabolites of stored maize (*Zea mays*, L.) from five agro-ecological zones of Nigeria. *Mycotoxin Research,* 30(2): 89-102.

Adlassnig, W., Peroutka, M. & Lendl, T. 2011. Traps of carnivorous pitcher plants as a habitat: composition of the fluid, biodiversity and mutualistic activities. *Annals of Botany*, 107(2): 181-194.

Afsah-Hejri, L., Jinap, S., Hajeb, P., Radu, S. & Shakibazadeh, S. 2013. A review on mycotoxins in food and feed: Malaysia case study. *Comprehensive Reviews in Food Science and Food Safety,* 12(6): 629-651.

Akcil, A. 2003. Destruction of cyanide in gold mill effluents: biological versus chemical treatments. *Biotechnology Advances,* 21(6): 501-511.

Akcil, A., Karahan, A. G., Ciftci, H. & Sagdic, O. 2003. Biological treatment of cyanide by natural isolated bacteria (*Pseudomonas* sp.). *Minerals Engineering,* 16(7): 643-649.

Akcil, A. & Mudder, T. 2003. Microbial destruction of cyanide wastes in gold mining: process review. *Biotechnology Letters,* 25(6): 445-450.

Al-Fakih, A. A. 2014. Overview on the fungal metabolites involved in mycopathy. *Open Journal of Medical Microbiology,* 4(1): 38-63.

Alassane-Kpembi, I., Kolf-Clauw, M., Gauthier, T., Abrami, R., Abiola, F. A., Oswald, I. P. & Puel, O. 2013. New insights into mycotoxin mixtures: The toxicity of low doses of type B

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trichothecenes on intestinal epithelial cells is synergistic. *Toxicology and Applied Pharmacology,* 272(1): 191-198.

Allemann, J. & Dugmore, N. 2004. Potential for cassava production in the Bathurst region of the Eastern Cape Province of South Africa. *African Crop Science Journal,* 12(3): 275-281.

Allie, F., Pierce, E. J., Okoniewski, M. J. & Rey, C. 2014. Transcriptional analysis of South African cassava mosaic virus-infected susceptible and tolerant landraces of cassava highlights differences in resistance, basal defense and cell wall associated genes during infection. *BMC genomics,* 15(1): 1-30.

Álvarez, E., Cano, J., Stchigel, A. M., Sutton, D. A., Fothergill, A. W., Salas, V., Rinaldi, M. G. & Guarro, J. 2011. Two new species of Mucor from clinical samples. *Medical Mycology,* 49(1): 62-72.

Amadi, J. E. & Adeniyi, D. O. 2009. Mycotoxin production by fungi isolated from stored grains. *African Journal of Biotechnology,* 8(7): 1219-1221.

Amodu, O. S., Ojumu, T. V. & Ntwampe, S. K. O. 2013. Bioavailability of high molecular weight polycyclic aromatic hydrocarbons using renewable resources. In*:* Petre, M. & Petre, V. (Eds). *Environmental Biotechnology - New Approaches and Prospective Applications.* Rijeka, Croatia: INTECH.

Andersen, M. D., Busk, P. K., Svendsen, I. & Møller, B. L. 2000. Cytochromes P-450 from Cassava (*Manihot esculenta* Crantz) catalyzing the first steps in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin cloning, functional expression in *Pichia pastoris*, and substrate specificity of the isolated recombinant enzymes. *Journal of Biological Chemistry,* 275(3): 1966-1975.

Anestis, A., Karamouzis, M. V., Dalagiorgou, G. & Papavassiliou, A. G. 2015. Is androgen receptor targeting an emerging treatment strategy for triple negative breast cancer? *Cancer Treatment Reviews,* 41(6): 547-553.

Asha, S. & Vidyavathi, M. 2009. Cunninghamella – a microbial model for drug metabolism studies – a review. *Biotechnology Advances,* 27(1): 16-29.

Bankole, S. A. & Mabekoje, O. O. 2004. Mycoflora and occurrence of aflatoxin B_1 in dried yam chips from markets in Ogun and Oyo States, Nigeria. *Mycopathologia,* 157(1): 111-115.

Barclay, M., Hart, A., Knowles, C. J., Meeussen, J. C. & Tett, V. A. 1998. Biodegradation of metal cyanides by mixed and pure cultures of fungi. *Enzyme and Microbial Technology,* 22(4): 223-231.

Barker, A. V. & Pilbeam, D. J. (Eds). 2015. *Handbook of plant nutrition.* Boca Raton, FL: CRC Press.

Baumgarte, S. & Tebbe, C. C. 2005. Field studies on the environmental fate of the Cry1Ab Bt-toxin produced by transgenic maize (MON810) and its effect on bacterial communities in the maize rhizosphere. *Molecular Ecology,* 14(8): 2539-2551.

Baxter, J. & Cummings, S. P. 2006. The impact of bioaugmentation on metal cyanide degradation and soil bacteria community structure. *Biodegradation,* 17(3): 207-217.

Cape Peninsula
University of Technology

Benedetti, R., Nazza, F., Locci, R., & Firrao, G. 2006. Degradation of fumonisin B1 by a bacterial strain isolated from soil. *Biodegradation*, 17(1): 31-38.

Bhat, R., Rai, R. V. & Karim, A. A. 2010. Mycotoxins in food and feed: present status and future concerns. *Comprehensive Reviews in Food Science and Food Safety,* 9(1): 57-81.

Bignell, E. 2010. Aspergillus: Molecular Biology and Genomics. By Machida, M. and Gomi, K. (Eds). *Biotechnology Journal*, 5(3): 336-337.

Bily, A. C., Burt, A. J., Ramputh, A. I., Livesey, J., Regnault-Roger, C., Philogène, B. R. & Arnason, J. T. 2004. HPLC-PAD-APCI/MS assay of phenylpropanoids in cereals. *Phytochemical Analysis*, 15(1): 9-15.

Blackwell, B. A., Gilliam, J. T., Savard, M. E., Miller, J. D. & Duvick, J. P. 1999. Exophiala spinifera. *Natural Toxins,* 7: 31-38.

Borgen, B. H. 2002. Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth. Thesis submitted for the degree of Doctor Scientiarum at the Norwegian University of Science and Technology (NTNU). UNIGEN Center of Molecular Biology and Department of Biology. Faculty of Natural Sciences and Technology.

Bornman, J. F. & Teramura, A. H. 1993. Effects of ultraviolet-B radiation on terrestrial plants. In: Young, A. R., Björn, L. O., Moan, J. & Nultsch, W. (Eds). *Environmental UV Photobiology*. Boston, MA: Springer US. Pages 427-471.

Bosch-Barrera, J. & Menendez, J. A. 2015. Silibinin and STAT3: a natural way of targeting transcription factors for cancer therapy. *Cancer Treatment Reviews,* 41(6): 540-546.

Brakhage, A. A. 1998. Molecular regulation of β-lactam biosynthesis in filamentous fungi. *Microbiology and Molecular Biology Reviews,* 62(3): 547-585.

Brakhage, A. A., Andrianopoulos, A., Kato, M., Steidl, S., Davis, M.A., Tsukagoshi, N. and Hynes, M.J. 1999. HAP-like CCAAT-binding complexes in filamentous fungi: implications for biotechnology. *Fungal Genetics and Biology,* 27(2-3): 243-252.

Brakhage, A. A. 2013. Regulation of fungal secondary metabolism. *Nature Reviews Microbiology,* 11(1): 21-32.

Brakhage, A. A. & Schroeckh, V. 2011. Fungal secondary metabolites - strategies to activate silent gene clusters. *Fungal Genetics and Biology,* 48(1): 15-22.

Breitenbach, M., Crameri, R. & Lehrer, S. B. 2002. (Eds). *Fungal allergy and pathogenicity*. (Vol. 81 of *Chemical Immunology).* Basel, Switzerland: Karger Medical and Scientific Publishers.

Breton, M. L., Bacak, A., Muller, J. B. A., O'shea, S. J., Xiao, P., Ashfold, M. N. R., Cooke, M. C., Batt, R., Shallcross, D. E., Oram, D. E. & Forster, G. 2013. Airborne hydrogen cyanide measurements using a chemical ionisation mass spectrometer for the plume identification of biomass burning forest fires. *Atmospheric Chemistry and Physics,* 13(18): 9217-9232.

Brookes, P. 2001. The soil microbial biomass: concept, measurement and applications in soil ecosystem research. *Microbes and Environments,* 16(3): 131-140.

Budziszewska, J., Boulahdjel, A., Wilk, M. & Wrzosek, M. 2010. Soil zygomycetous fungi in Biebrza National Park (northeast Poland). *Polish Botanical Journal,* 55(2): 391-407.

Camilo, S. B., Ono, C. J., Ueno, Y. & Hirooka, E. Y. 2000. Anti-Fusarium moniliforme activity and fumonisin biodegradation by corn and silage microflora. Brazilian Archives of Biology and Technology, 43(2): 159-164.

Cavender-Bares, J. & Bazzaz, F. A. 2004. From leaves to ecosystems: using chlorophyll fluorescence to assess photosynthesis and plant function in ecological studies. In: Papageorgiou, G. C. & Govindjee, G. (Eds). *Chlorophyll a Fluorescence: a Signature of Photosynthesis.* Dordrecht: Springer Netherlands. Pages 737-755.

Chanchaichaovivat, A., Ruenwongsa, P. & Panijpan, B. 2007. Screening and identification of yeast strains from fruits and vegetables: potential for biological control of postharvest chilli anthracnose (*Colletotrichum capsici*). *Biological Control,* 42(3): 326-335.

Chen, X. & Hu, Q. 2004. Groundwater influences on soil moisture and surface evaporation. *Journal of Hydrology,* 297(1): 285-300.

Chiang, J. C., Kushnir, Y. & Zebiak, S. E. 2000. Interdecadal changes in eastern Pacific ITCZ variability and its influence on the Atlantic ITCZ. *Geophysical Research Letters,* 27(22): 3687-3690.

Chu, F. S. & Li, G. Y. 1994. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. *Applied and Environmental Microbiology,* 60(3): 847-852.

Conn, E. E. 1981. Cyanogenic glycosides. *The Biochemistry of Plants: A Comprehensive Treatise,* 7: 479-500.

Cressey, P., Saunders, D. & Goodman, J. 2013. Cyanogenic glycosides in plant-based foods available in New Zealand. *Food Additives and Contaminants: Part A,* 30(11): 1946-1953.

Cumbana, A., Mirione, E., Cliff, J. & Bradbury, J. H. 2007. Reduction of cyanide content of cassava flour in Mozambique by the wetting method. *Food Chemistry,* 101(3): 894-897.

Dash, R. R., Balomajumder, C. & Kumar, A. 2006. Cyanide removal by combined adsorption and biodegradation process. *Journal of Environmental Health Science and Engineering,* 3(2): 91-96.

Dash, R. R., Dash, R. R. & Balomajumdar, C. 2014. Treatment of cyanide bearing effluents by adsorption, biodegradation and combined processes: effect of process parameters. *Desalination and Water Treatment,* 52(16-18): 3355-3366.

De Ingeniis, J., Raffaelli, N., Ciani, M. & Mannazzu, I. 2009. Pichia anomala DBVPG 3003 secretes a ubiquitin-like protein that has antimicrobial activity. *Applied and Environmental Microbiology,* 75(4): 1129-1134.

Delgado, R. M., Sulyok, M., Jirsa, O., Spitzer, T., Krska, R. & Polišenská, I. 2014. Relationship between lutein and mycotoxin content in durum wheat. *Food Additives and Contaminants: Part A,* 31(7): 1274-1283.

Delong, R. K., Akhtar, U., Sallee, M., Parker, B., Barber, S., Zhang, J., Craig, M., Garrad, R., Hickey, A. J. & Engstrom, E. 2009. Characterization and performance of nucleic acid nanoparticles combined with protamine and gold. *Biomaterials,* 30(32): 6451-6459.

Deshaies, R. J. 1999. SCF and Cullin/Ring H2-based ubiquitin ligases. *Annual Review of Cell and Developmental Biology,* 15(1): 435-467.

Doehlert, D. C., Knutson, C. A. & Vesonder, R. F. 1994. Phytotoxic effects of fumonisin B 1 on maize seedling growth. *Mycopathologia*, 127(2): 117-121.

Draper, J., Enot, D. P., Parker, D., Beckmann, M., Snowdon, S., Lin, W. & Zubair, H. 2009. Metabolite signal identification in accurate mass metabolomics data with MZedDB, an interactive m/z annotation tool utilising predicted ionisation behaviour rules. *BMC Bioinformatics*, 10(1): 1-16.

Dutton, M. F. 1996. Fumonisins, mycotoxins of increasing importance: their nature and their effects. *Pharmacology and Therapeutics,* 70(2): 137-161.

Duvick, J., Rood, T., Maddox, J. & Gilliam, J. 1998. Detoxification of mycotoxins in planta as a strategy for improving grain quality and disease resistance: identification of fumonisin-degrading microbes from maize. In Kohmoto, K & Yoder, O. C. (Eds). Developments in Plant Pathology, Volume 13: *Molecular genetics of host-specific toxins in plant disease*. Dordrecht: Kluwer Academic Publishers (and Springer). Pages 369-382.

Duvick, J., Maddox, J. & Gilliam, J. 2003. Compositions and methods for fumonisin detoxification. U.S. Patent: 6,538,177.

Dzombak, D. A., Ghosh, R. S. & Wong-Chong, G. M. (Eds). 2005. *Cyanide in water and soil: chemistry, risk, and management*. Boca Raton, FL: CRC press.

Ebbs, S. 2004. Biological degradation of cyanide compounds. *Current Opinion in Biotechnology,* 15(3): 231-236.

Ediage, E. N., Hell, K. & De Saeger, S. D. 2014. A Comprehensive study to explore differences in mycotoxin patterns from agro-ecological regions through maize, peanut, and cassava products: a case study, Cameroon. *Journal of Agricultural and Food Chemistry,* 62(20): 4789-4797.

Ellefson, W., Zach, L. & Sullivan, D. (Eds). 2012. *Improving import food safety*. (Vol. 85). Oxford, UK: John Wiley & Sons.

Ernesto, M., Cardoso, A. P., Nicala, D., Mirione, E., Massaza, F., Cliff, J., Haque, M. R. & Bradbury, J. H. 2002. Persistent konzo and cyanogen toxicity from cassava in northern Mozambique. *Acta Tropica,* 82(3): 357-362.

Farag, M. A., Porzel, A. & Wessjohann, L.A., 2012. Comparative metabolite profiling and fingerprinting of medicinal licorice roots using a multiplex approach of GC–MS, LC–MS and 1D NMR techniques. *Phytochemistry*,76: 60-72.

Fasuyi, A. O. 2005. Nutrient composition and processing effects on cassava leaf (*Manihot esculenta*, Crantz) antinutrients. *Pakistan Journal of Nutrition,* 4(1): 37-42.

Cape Peninsula
University of Technology

Fetter, C. W. & Fetter, C. 1999. *Contaminant hydrogeology*. (Vol. 500). New Jersey: Prentice Hall.

Figueira, M. M., Ciminelli, V. S. T. & Linardi, V. R. 1995. Bacterial degradation of metal cyanide complexes. *Biohydrometallurgical Processing, University of Chile, Chile*: 333-339.

Figueira, M. M., Ciminelli, V. S., Andrade, M. C. D. & Linardi, V. R. 1996. Cyanide degradation by an Escherichia coli strain. *Canadian Journal of Microbiology*, 42(5): 519-523.

Frisvad, J. C., Smedsgaard, J., Samson, R. A., Larsen, T. O. & Thrane, U. 2007. Fumonisin B2 production by Aspergillus niger. Journal of Agricultural and Food Chemistry, 55(23): 9727-9732.

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., Asner, G. P., Cleveland, C. C., Green, P. A., Holland, E. & Karl, D. M. 2004. Nitrogen cycles: past, present, and future. *Biogeochemistry,* 70(2): 153-226.

Garibaldi, L. 2012. The FAO global capture production database: a six-decade effort to catch the trend. *Marine Policy,* 36(3): 760-768.

Gatti, L. V., Gloor, M., Miller, J. B., Doughty, C. E., Malhi, Y., Domingues, L. G., Basso, L. S., Martinewski, A., Correia, C. S. C., Borges, V. F. & Freitas, S. 2014. Drought sensitivity of Amazonian carbon balance revealed by atmospheric measurements. *Nature,* 506(7486): 76-80.

Gelderblom, W. C., Marasas, W. F., Vleggaar, R., Thiel, P. G. & Cawood, M. E. 1992. Fumonisins: isolation, chemical characterization and biological effects. *Mycopathologia,* 117(1): 11-16.

Gilham, D. and Lehner, R., 2005. Techniques to measure lipase and esterase activity in vitro. *Methods*, 36(2): 139-147.

Gitelson, A. A. & Merzlyak, M. N. 1997. Remote estimation of chlorophyll content in higher plant leaves. *International Journal of Remote Sensing,* 18(12): 2691-2697.

Gleadow, R. M., Evans, J. R., McCaffery, S. & Cavagnaro, T. R. 2009. Growth and nutritive value of cassava (*Manihot esculenta* Cranz.) are reduced when grown in elevated CO_2. *Plant Biology,* 11(1): 76-82.

Gleadow, R. M. & Woodrow, I. E. 2002. Mini-Review: constraints on effectiveness of cyanogenic glycosides in herbivore defense. *Journal of Chemical Ecology,* 28(7): 1301-1313.

Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M. & Toulmin, C. 2010. Food security: the challenge of feeding 9 billion people. *Science,* 327(5967): 812-818.

Gnonlonfin, G. J. B.; Hell, K.; Fandohan, P.; Siame, A. B. 2008. Mycoflora and natural occurrence of aflatoxins and fumonisin B1 in cassava and yam chips from Benin, West Africa. *Internationl Journal of Food Microbiology*, 122(1): 140-147.

Gregory, I. N. & Healey, R. G. 2007. Historical GIS: structuring, mapping and analysing geographies of the past. *Progress in Human Geography,* 31(5): 638-653.

Guan, S., He, J., Young, J. C., Zhu, H., Li, X.-Z., Ji, C. & Zhou, T. 2009. Transformation of trichothecene mycotoxins by microorganisms from fish digesta. *Aquaculture,* 290(3): 290-295.

Guo, J., Wang, H., Liu, D., Zhang, J. N., Zhao, Y. H., Liu, T. X. & Xin, Z. H. 2015. Isolation of Cunninghamella bigelovii sp. nov. CGMCC 8094 as a new endophytic oleaginous fungus from *Salicornia bigelovii. Mycological Progress,* 14(3): 1-8.

Gurbuz, F., Ciftci, H., Akcil, A. & Karahan, A. G. 2004. Microbial detoxification of cyanide solutions: a new biotechnological approach using algae. *Hydrometallurgy,* 72(1), 167-176.

Ha, H. L., Shin, H. J., Feitelson, M. A. & Yu, D. Y. 2010. Oxidative stress and antioxidants in hepatic pathogenesis. *World Journal of Gastroenterology,* 16(48): 6035-43.

Hansen, J., Ruedy, R., Sato, M. & Lo, K. 2010. Global surface temperature change. *Reviews of Geophysics,* 48(4): 1-29.

Hartinger, D., Schwartz, H., Hametner, C., Schatzmayr, G., Haltrich, D. & Moll, W.-D. 2011. Enzyme characteristics of aminotransferase FumI of Sphingopyxis sp. MTA144 for deamination of hydrolyzed fumonisin B1. *Applied microbiology and biotechnology,* 91**:** 757-768.

Hassan, H. F. & Kassaify, Z. 2014. The risks associated with aflatoxins M1 occurrence in Lebanese dairy products. *Food Control*, 37: 68-72

Heinl, S., Hartinger, D., Thamhesl, M., Schatzmayr, G., Moll, W.-D. & Grabherr, R. 2011. An aminotransferase from bacterium ATCC 55552 deaminates hydrolyzed fumonisin B1. *Biodegradation,* 22(1): 25-30.

Henrikson, J. C., Hoover, A. R., Joyner, P. M. & Cichewicz, R. H. 2009. A chemical epigenetics approach for engineering the in situ biosynthesis of a cryptic natural product from *Aspergillus niger. Organic and Biomolecular Chemistry,* 7(3): 435-438.

Henson, S. & Caswell, J. 1999. Food safety regulation: an overview of contemporary issues. *Food Policy,* 24(6): 589-603.

Hidayat, A., Zuaraida, N., Hanarida, I. & Damardjati, D. S. 2000. Cyanogenic content of cassava root of 179 cultivars grown in Indonesia. *Journal of Food Composition and Analysis,* 13(1): 71-82.

Hidayat, A., Zuraida, N. & Hanarida, I. 2016. The cyanogenic potential of roots and leaves of ninety nine cassava cultivars. *Indonesian Journal of Agricultural Science,* 3(1): 25-32.

Hillocks, R.J., Thresh, J.M. and Bellotti, A. (Eds), 2002. *Cassava: biology, production and utilization*. Wallingford, Oxon: CABI Publishing.

Hodek, P., Trefil, P. & Stiborová, M. 2002. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. *Chemico-Biological Interactions,* 139(1): 1-21.

Cape Peninsula
University of Technology

Hossain, S. M., Das, M., Begum, K. M. M. S. & Anantharaman, N. 2005. Studies on biodegradation of cyanide (AgCN) using Phanerochaete chrysosporium. *Journal Institution Engineers*, 85: 45-49.

Howeler, R.H., 1998. Cassava breeding, agronomy and farmer participatory research in Asia: proceedings. In 5. Regional Workshop, Hainan (China), 3-8 Nov 1996. Centro Internacional de Agricultura Tropical, Regional Cassava Program for Asia.

Howeler, R. H. 2002. Cassava mineral nutrition and fertilization. In: Hillocks, R. J., Thresh, J. M. & Bellotti, A. C. (Eds). *Cassava: biology, production and utilization*. Wallingford, Oxon.: CABI Publishing: 115-147.

Howeler, R. H., Oates, C. G., Allem, A. C., Chuzel, G., Henry, G., Hershey, C. H., Müller-Sämann, K. M., Okogun, A., Sanginga, N., Souza, L. D. S. & Siroth, K. 2001. Strategic environmental assessment, an assessment of the impact of cassava production and processing on the environment and biodiversity. In*: Proceedings of the Validation Forum on the global Cassava Development Strategy* (*Vol. 5).*

Hussein, H. S. & Brasel, J. M. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology,* 167(2): 101-134.

Ibitoye, S. J. 2011. The influence of socio-economic variables of farmers on their choice of cassava varieties in Kogi State, Nigeria. *International Journal of Agricultural Science, Research and Technology,* 1(4): 185-193.

Idowu, M. A., Iroko, K., Adeola, A. A., Oluwalana, I. B., Ayo, J. A. & Ikuomola, D. S. 2015. Quality of wheat-cassava bread as affected by selected improvers. *World Journal of Science, Technology and Sustainable Development,* 12(1): 79-86.

Ismaiel, A. A. & Papenbrock, J. 2015. Mycotoxins: producing fungi and mechanisms of phytotoxicity. *Agriculture,* 5(3): 492-537.

Jansson, C., Westerbergh, A., Zhang, J., Hu, X. & Sun, C. 2009. Cassava, a potential biofuel crop in (the) People's Republic of China. *Applied Energy,* 86: S95-S99.

Jarvis, A., Ramirez-Villegas, J., Campo, B. V. H. & Navarro-Racines, C. 2012. Is cassava the answer to African climate change adaptation? *Tropical Plant Biology,* 5(1): 9-29.

Jenkinson, D. S. 1981. Microbial biomass in soil-measurement and turnover. *Soil Biochemistry,* 6: 415-471.

Jones, D. A. 1998. Why are so many food plants cyanogenic? *Phytochemistry,* 47(2): 155-162.

Kamalu, B. P. 1995. The adverse effects of long-term cassava (Manihot esculenta Crantz) consumption. International Journal of Food Sciences and Nutrition, 46(1): 65-93.

Kanokratana, P., Mhuanthong, W., Laothanachareon, T., Tangphatsornruang, S., Eurwilaichitr, L., Kruetreepradit, T., Mayes, S. & Champreda, V. 2016. Comparative study of bacterial communities in Nepenthes pitchers and their correlation to species and fluid acidity. *Microbial Ecology*, 72(2): 381-393.

Karp, N. A., McCormick, P. S., Russell, M. R. & Lilley, K. S. 2007. Experimental and statistical considerations to avoid false conclusions in proteomics studies using differential in-gel electrophoresis. *Molecular & Cellular Proteomics,* 6(8): 1354-1364.

Kato, M. & Shimizu, S. 1987. Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves; phenolic-dependent peroxidative degradation. *Canadian Journal of Botany,* 65(4): 729-735.

Kim, Y. M., Park, D., Lee, D. S. & Park, J. M. 2008. Inhibitory effects of toxic compounds on nitrification process for cokes wastewater treatment. *Journal of Hazardous Materials,* 152(3): 915-921.

Kim, Y. M., Lee, D. S., Park, C., Park, D. & Park, J. M. 2011. Effects of free cyanide on microbial communities and biological carbon and nitrogen removal performance in the industrial activated sludge process. *Water Research,* 45(3): 1267-1279.

Kimanya, M. E., De Meulenaer, B., Tiisekwa, B., Ndomondo-Sigonda, M., Devlieghere, F., Van Camp, J. & Kolsteren, P. 2008. Co-occurrence of fumonisins with aflatoxins in home-stored maize for human consumption in rural villages of Tanzania. *Food additives and contaminants,* 25 (11): 1353-1364.

Kimura, M., Tokai, T., Takahashi-Ando, N., Ohsato, S. & Fujimura, M. 2007. Molecular and genetic studies of Fusarium trichothecene biosynthesis: Pathways, genes, and evolution. *Bioscience, Biotechnology and Biochemistry*, 71(9): 2105-2123

Kjeldsen, P. 1999. Behaviour of cyanides in soil and groundwater: a review. *Water, Air, and Soil Pollution,* 115(1): 279-308.

Klarić, M. Š., Rašić, D. & Peraica, M. 2013. Deleterious effects of mycotoxin combinations involving ochratoxin A. *Toxins,* 5(11): 1965-1987.

Klich, M. A. 2009. Health effects of Aspergillus in food and air. *Toxicology and Industrial Health,* 25(9-10): 657-667.

Knudsen, G. R. 2006. Bacteria, Fungi, and Soil Health. In Presentation: Idaho Potato Conference, p. 1. Idaho, January 18, 2006.

Knuten, A. P., Bush, R. K., Demain, J. G., Denning, D. W., Dixit, A., Fairs, A., Greenberger, P. A., Kariuki, B., Kita, H., Kurup, V. P. & Moss, R.B. 2012. Fungi and allergic lower respiratory tract diseases. *Journal of Allergy and Clinical Immunology,* 129(2): 280-291.

Kobayashi, D. Y. & Crouch, J. A. 2009. Bacterial/fungal interactions: from pathogens to mutualistic endosymbionts. *Annual Review of Phytopathology,* 47: 63-82.

Koehorst-Van Putten, H. J. J., Sudarmonowati, E., Herman, M., Pereira-Bertram, I. J., Wolters, A. M. A., Meima, H., De Vetten, N., Raemakers, C. J. J. M. & Visser, R. G. F. 2012. Field testing and exploitation of genetically modified cassava with low-amylose or amylose-free starch in Indonesia. *Transgenic Research,* 21(1): 39-50.

Koh, L. P. 2007. Potential habitat and biodiversity losses from intensified biodiesel feedstock production. *Conservation Biology,* 21(5): 1373-1375.

Cape Peninsula
University of Technology

Kohmoto, K & Yoder, O. C. (Eds). 1998. Developments in Plant Pathology, Volume 13: Molecular genetics of host-specific toxins in plant disease. Dordrecht: Kluwer Academic Publishers (and Springer).

Kolmert, Å., Wikström, P. & Hallberg, K. B. 2000. A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. *Journal of Microbiological Methods*, 41(3), 179-184.

Krizek, D. T. 2004. Influence of PAR and UV-A in determining plant sensitivity and photomorphogenic responses to UV-B radiation. *Photochemistry and Photobiology,* 79(4): 307-315.

Kuti, J. O. & Konoru, H. B. 2006. Cyanogenic glycosides content in two edible leaves of tree spinach (*Cnidoscolus* spp.). *Journal of Food Composition and Analysis,* 19(6): 556-561.

Lambert, J. L., Ramasamy, J. & Paukstelis, J.V. 1975. Stable reagents for the calorimetric determination of cyanide by modificatied König reactions. *Analytical Chemistry*, 47(6): 916-918.

Lamptey, J. A., Dwason, E. S., Johnson, P. N. T. & Annor, G. A. 2008. Effects of different processing methods on the nutritional composition and cyanogen content of flour from cassava varieties in Ghana. *Journal of Root Crops,* 32: 157-163.

Lary, D. J. 2005. Halogens and the chemistry of the free troposphere. *Atmospheric Chemistry and Physics,* 5(1): 227-237.

Lary, D. J. 2004. Atmospheric pseudohalogen chemistry. *Atmospheric Chemistry and Physics Discussions,* 4: 5381-5405.

Lategan, M. J., Torpy, F., Newby, S., Stephenson, S. & Hose, G. C. 2012. Fungal diversity of shallow aquifers in southeastern Australia. *Geomicrobiology Journal,* 29,(4):352-361.

Lattanzio, V. M. T., Solfrizzo, M., Powers, S. & Visconti, A. 2007. Simultaneous determination of aflatoxins, ochratoxin A and Fusarium toxins in maize by liquid chromatography/tandem mass spectrometry after multitoxin immunoaffinity cleanup. *Rapid Communications in Mass Spectrometry,* 21(20): 3253-3261.

Lee, L., Zhang, Y., Ozar, B., Sensen, C. W., Schriemer, D. C. 2016. Carnivorous nutrition in pitcher plants (*Nepenthes* spp.) via an unusual complement of endogenous enzymes. *Journal of Proteome Research,* 15(9): 3108-3117.

Li, Y., Liu, X. & Lin, Z. 2012. Recent developments and applications of surface plasmon resonance biosensors for the detection of mycotoxins in foodstuffs. *Food Chemistry,* 132(3): 1549-1554.

Liang, M.C., Heays, A. N., Lewis, B. R., Gibson, S. T. & Yung, Y. L. 2007. Source of nitrogen isotope anomaly in HCN in the atmosphere of Titan. *The Astrophysical Journal Letters,* 664(2): 115-118.

Lilleskov, E. A., Fahey, T. J., Horton, T. R. & Lovett, G. M. 2002. Below ground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology,* 83(1): 104-115.

Lima, D. X., De Azevedo, A. L. C. M. & De Souza-Motta, C. M. 2016. Diversity of *Mucorales* in natural and degraded semi-arid soils. *Brazilian Journal of Botany,* 39(4): 1127-1133.

Liu, D., Coloe, S., Baird, R. & Pedersen, J. 2000. Rapid mini-preparation of fungal DNA for PCR. *Journal of Clinical Microbiology,* 38(1): 471-471.

Liu, J., Zheng, Q., Ma, Q., Gadidasu, K. K. & Zhang, P. 2011. Cassava genetic transformation and its application in breeding. *Journal of Integrative Plant Biology,* 53(7): 552-569.

Liu, G., Lut, M. C., Verberk, J. Q. J. C. & Van Dijk, J. C. 2013. A comparison of additional treatment processes to limit particle accumulation and microbial growth during drinking water distribution. *Water Research,* 47(8): 2719-2728.

Ljungquist, A. & Augustinsson, K. B., 1971. Purification and Properties of Two Carboxylesterases from Rat-Liver Microsomes. *The FEBS Journal*, 23(2): 303-313.

Lund, F. & Frisvad, J. C. 2003. *Penicillium verrucosum* in wheat and barley indicates presence of ochratoxin A. *Journal of Applied Microbiology,* 95(5): 1117-1123.

Mabaya, E., Fulton, J., Simiyu-Wafukho, S. & Nang'ayo, F. 2015. Factors influencing adoption of genetically modified crops in Africa. *Development Southern Africa,* 32(5): 577-591.

Machida, M. & Gomi, K. 2010. *Aspergillus: Molecular Biology and Genomics.* Norfolk, UK: Caister Academic Press.

Magnusson, R., Nyholm, S. & Åstot, C. 2012. Analysis of hydrogen cyanide in air in a case of attempted cyanide poisoning. *Forensic Science International,* 222(1): 7-12.

Malone, B. R., Humphrey, C. W., Romer, T. R. & Richard, J. L. 1998. One-step solid-phase extraction cleanup and fluorometric analysis of deoxynivalenol in grains. *Journal of AOAC International,* 81(2): 448-452.

Manjula, K., Hell, K., Fandohan, P., Abass, A. & Bandyopadhyay, R. 2009. Aflatoxin and fumonisin contamination of cassava products and maize grain from markets in Tanzania and republic of the Congo. *Toxin Reviews,* 28(2-3)**:** 63-69.

Mantle, P., Copetti, M. V., Buddie, A. & Frisvad, J. 2016. Comments on "Mycobiota and Mycotoxins in Traditional Medicinal Seeds from China. Toxins 2015, 7, 3858-3875" - in attributing ochratoxin A biosynthesis within the genus *Penicillium* occurring on natural agricultural produce. *Toxins,* 8(6): 1-4.

Matsumura, M. & Kojima, T. 2003. Elution and decomposition of cyanide in soil contaminated with various cyanocompounds. *Journal of Hazardous Materials,* 97(1): 99-110.

Matumba, L., Sulyok, M., Monjerezi, M., Biswick, T. & Krska, R. 2014. Fungal metabolites diversity in maize and associated human dietary exposures relate to micro-climatic patterns in Malawi. *World Mycotoxin Journal,* 8(3): 269-282.

Cape Peninsula
University of Technology

Mburu, F. W., Swaleh, S. & Njue, W. 2012. Potential toxic levels of cyanide in cassava (*Manihot esculenta* Crantz) grown in Kenya. *African Journal of Food Science,* 6(16): 416-420.

McMahon, J. M., White, W. L. & Sayre, R. T. 1995. Cyanogenesis in cassava (*Manihot esculenta* Crantz). *Journal of Experimental Botany,* 46(7): 731-741.

McQueen, R. D. A. 2015. Annemieke Farenhorst, LA Andronak. *Water Air and Soil Pollution,* 226: 1-11.

Meeussen, J. C., Keizer, M. G., Van Riemsdijk, W. H. & De Haan, F. A. 1994. Solubility of cyanide in contaminated soils. *Journal of Environmental Quality,* 23(4): 785-792.

Méheust, D., Le Cann, P., Reboux, G., Millon, L. & Gangneux, J. P. 2014. Indoor fungal contamination: health risks and measurement methods in hospitals, homes and workplaces. *Critical Reviews in Microbiology,* 40(3): 248-260.

Mehlomakulu, N. N., Setati, M. E. & Divol, B. 2014. Characterization of novel killer toxins secreted by wine-related non-*Saccharomyces* yeasts and their action on Brettanomyces spp. *International journal of Food Microbiology,* 188: 83-91.

Mekuto, L., Jackson, V. A. & Ntwampe, S. K. 2013. Biodegradation of free cyanide using Bacillus sp. consortium dominated by *Bacillus safensis*, *Lichenformis* and *Tequilensis strains*: a bioprocess supported solely with whey. *Environmental Science and Pollution Research,* 22: 10434 – 10443.

Merzlyak, M. N., Gitelson, A. A., Pogosyan, S. I., Chivkunova, O. B., Lehimena, L., Garson, M., Buzulukova, N. P., Shevyreva, V. V. & Rumyantseva, V. B. 1997. Reflectance spectra of leaves and fruits during their development and senescence and under stress. *Russian Journal of Plant Physiology,* 44(5): 614-622.

Minerdi, D., Bianciotto, V. & Bonfante, P. 2002. Endosymbiotic bacteria in mycorrhizal fungi: from their morphology to genomic sequences. In Smith, S. E. & Smith, F. A. (Eds.) *Diversity and Integration in Mycorrhizas: Proceedings of the 3rd International Conference on Mycorrhizas (ICOM3) Adelaide, Australia, 8–13 July 2001.* Dordrecht, Netherlands: Springer. Pages 211-219.

Montagnac, J. A., Davis, C. R. & Tanumihardjo, S. A. 2009. Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. *Comprehensive Reviews in Food Science and Food Safety,* 8(1): 17-27.

Moran, N. A. & Baumann, P. 2000. Bacterial endosymbionts in animals. *Current Opinion in Microbiology,* 3(3): 270-275.

Moran, N. A. & Wernegreen, J. J. 2000. Lifestyle evolution in symbiotic bacteria: insights from genomics. *Trends in Ecology and Evolution,* 15(8): 321-326.

Mousa, W. K. & Raizada, M. N. 2013. The diversity of anti-microbial secondary metabolites produced by fungal endophytes: an interdisciplinary perspective. *Frontiers in Microbiology,* 4: 1-18.

Cape Peninsula
University of Technology

Msangi, S., Ewing, M. & Rosegrant, M. 2010. Biofuels and agricultural growth: challenges for developing agricultural economies and opportunities for investment. *Handbook of Bioenergy Economics and Policy.* New York: Springer: 73-90.

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

Nambisan, B. 1994. Evaluation of the effect of various processing techniques on cyanogen content reduction in cassava.. International Workshop on Cassava Safety. *Acta Horticulture*, 375: 193-202.

Nambisan, B. 2011. Strategies for elimination of cyanogens from cassava for reducing toxicity and improving food safety. *Food and Chemical Toxicology,* 49(3): 690-693.

Nambisan, B. & Sundaresan, S. 1994. Distribution of linamarin and its metabolising enzymes in cassava tissues. *Journal of the Science of Food and Agriculture,* 66(4): 503-507.

Nartey, F. 1968. Studies on cassava, Manihot utilissima Pohl—I. Cyanogenesis: the biosynthesis of linamarin and lotaustralin in etiolated seedlings. *Phytochemistry,* 7(8): 1307-1312.

Nielsen, K. F., Mogensen, J. M., Johansen, M., Larsen, T. O. & Frisvad, J. C. 2009. Review of secondary metabolites and mycotoxins from the Aspergillus niger group. *Analytical and Bioanalytical Chemistry*, 395(5): 1225-1242

National Institute of Standard and Technology (NIST). 2011. Chemistry Web Book NIST Standard Reference Database Number 69, Department of commerce, USA.

O'Donnell, K., Lutzoni, F. M., Ward, T. J. & Benny, G. L. 2001. Evolutionary relationships among mucoralean fungi (Zygomycota): evidence for family polyphyly on a large scale. *Mycologia,* 93: 286-297.

Ojeka, E. O., Adetoyi, I. & Bako, S. S. 2007. Studies on solvent extraction of free hydrogen cyanide from river water. *Bulletin of the Chemical Society of Ethiopia,* 21(3): 305-313.

Okechi, R. N., Ihejirika, C. E., Chiegboka, N. A., Chukwura, E. I. & Ibe, I. J. 2012. Evaluation of the effects of cassava mill effluent on the microbial populations and physicochemical parameters at different soil depths. *International Journal of Biosciences,* 2(12): 139-45.

Okudoh, V., Trois, C., Workneh, T. & Schmidt, S. 2014. The potential of cassava biomass and applicable technologies for sustainable biogas production in South Africa: a review. *Renewable and Sustainable Energy Reviews,* 39: 1035-1052.

Ortin, X., Escoda, L., Llorente, A., Rodriguez, R., Martinez, S., Boixadera, J., Cabezudo, E. & Ugarriza, A. 2004. *Cunninghamella bertholletiae* infection (mucormycosis) in a patient with acute T-cell lymphoblastic leukemia. *Leukemia & Lymphoma,* 45(3): 617-620.

Ottesen, A. R., Magnuson, B. A. 2010. Naturally occurring toxins in plants. *Pathogens and Toxins in Foods*: 301-313. American Society of Microbiology.

Padmaja, G. & Steinkraus, K. H. 1995. Cyanide detoxification in cassava for food and feed uses. *Critical Reviews in Food Science and Nutrition,* 35(4): 299-339.

Papageorgiou, G. C. & Govindjee, G. (Eds). 2004. *Chlorophyll a Fluorescence: a Signature of Photosynthesis.* Dordrecht, Netherlands: Springer.

Partida-Martinez, L. P. & Hertweck, C. 2005. Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature,* 437(7060): 884-888.

Partida-Martinez, L. P., Monajembashi, S., Greulich, K. O. & Hertweck, C. 2007. Endosymbiont-dependent host reproduction maintains bacterial-fungal mutualism. *Current Biology,* 17(9): 773-777.

Pashley, C. H., Fairs, A., Free, R. C. & Wardlaw, A. J. 2012. DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology. *Fungal Biology,* 116(2): 214-224.

Patton, C. J. & Crouch, S. R. 1977. Spectrophotometric and kinetics investigation of the Berthelot reaction for the determination of ammonia. *Analytical Chemistry*, 49(3): 464-469.

Pavlovic, D., Nikolic, B., Djurovic, S., Waisi, H., Andjelkovic, A. & Marisavljevic, D. 2015. Chlorophyll as a measure of plant health: agroecological aspects. *Pesticides and Phytomedicine* 29: 21–34.

Peraica, M. & Domijan, A. M. 2001. Contamination of food with mycotoxins and human health. Arhiv za Higijenu Rada i Toksikologiju, 52(1): 23-35.

Pereira, W. O., Cruz, A. K., Albuquerque, E. M., Santos, E. A., Oliveira, A. S., Sales, M. P. & Oliveira, F. W. 2005. Purification and characterization of a β-Glucuronidase present during embryogenesis of the Mollusk pomacea sp. *Protein and Peptide Letters*, 12(7): 695-700.

Pereira, G.E., Gaudillere, J.P., van Leeuwen, C., Hilbert, G., Maucourt, M., Deborde, C., Moing, A. & Rolin, D., 2006. 1 H NMR metabolite fingerprints of grape berry: comparison of vintage and soil effects in Bordeaux grapevine growing areas. *Analytica Chimica Acta*, 563(1): 346-352.

Perrone, G., Susca, A., Epifani, F. & Mulè, G. 2006. AFLP characterization of Southern Europe population of *Aspergillus* section Nigri from grapes. *International Journal of Food Microbiology,* 111: 22-27.

Perrone, G., Stea, G., Epifani, F., Varga, J., Frisvad, J. C. & Samson, R. A. 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori. Fungal Biology,* 115(11): 1138-1150.

Pestka, J. J. & Smolinski, A. T. 2005. Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Health, Part B,* 8(1): 39-69.

Petraitis, V., Petraitiene, R., Antachopoulos, C., Hughes, J. E., Cotton, M. P., Kasai, M., Harrington, S., Gamaletsou, M. N., Bacher, J. D., Kontoyiannis, D. P. & Roilides, E. 2013. Increased virulence of *Cunninghamella bertholletiae* in experimental pulmonary mucormycosis: correlation with circulating molecular biomarkers, sporangiospore germination and hyphal metabolism. *Medical Mycology,* 51(1): 72-82.

Petre, M. & Petre, V. (Eds). *Environmental Biotechnology - New Approaches and Prospective Applications.* Rijeka, Croatia: INTECH.

Cape Peninsula
University of Technology

Piero, N. M., Joan, M. N., Richard, O. O., Jalemba, M. A., Omwoyo, O. R. & Chelule, C. R. 2015. Determination of cyanogenic compounds content in transgenic acyanogenic Kenyan cassava (*Manihot esculenta* Crantz) genotypes: linking molecular analysis to biochemical analysis. *Journal of Analytical an Bioanalytical Techniques,* 6(5): 1-7.

Pincus, D. H. 2006. Microbial identification using the bioMerieux VITEK® 2 System. *Encyclopedia of Rapid Microbiological Methods,* 1: 1-32.

Plattner, R. D. 1995. Detection of fumonisins produced in *Fusarium moniliforme* cultures by HPLC with electrospray MS and evaporative light scattering detectors. *Natural Toxins,* 3(4): 294-298.

Polthanee, A. & Kotchasatit, A. 1999. Growth, yield and nutrient content of cassava and mungbean grown under intercropping. *Pakistan Journal of Biological Sciences,* 2(3): 871-876.

Prawat, H., Mahidol, C., Ruchirawat, S., Prawat, U., Tuntiwachwuttikul, P., Tooptakong, U., Taylor, W. C., Pakawatchai, C., Skelton, B. W. & White, A. H. 1995. Cyanogenic and non-cyanogenic glycosides from *Manihot esculenta*. *Phytochemistry,* 40(4): 1167-1173.

Reay, D. S., Davidson, E. A., Smith, K. A., Smith, P., Melillo, J. M., Dentener, F. & Crutzen, P. J. 2012. Global agriculture and nitrous oxide emissions. *Nature Climate Change,* 2(6): 410-416.

Rider, B. F. & Mellon, M. G. 1946. Colorimetric determination of nitrites. *Industrial and Engineering Chemistry Analytical Edition*, 18(2): 96-99.

Rickerts, V., Böhme, A., Viertel, A., Behrendt, G., Jacobi, V., Tintelnot, K. & Just-Nübling, G. 2000. Cluster of pulmonary infections caused by *Cunninghamella bertholletiae* in immunocompromised patients. *Clinical Infectious Diseases,* 31(4): 910-913.

Righi, E., Giacomazzi, C. G., Lindstrom, V., Albarello, A., Soro, O., Miglino, M., Perotti, M., Varnier, O. E., Gobbi, M., Viscoli, C. & Bassetti, M. 2008. A Case of *Cunninghamella bertholettiae* rhino-cerebral infection in a leukaemic patient and review of recent published studies. *Mycopathologia,* 165(6): 407-410.

Rillig, M. C. & Mummey, D. L. 2006. Mycorrhizas and soil structure. *New Phytologist,* 171(1)**:** 41-53.

Rotter, B. A. 1996. Invited review: Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health Part A,* 48(1): 1-34.

Rubert, J., Soler, C., Marín, R., James, K. & Mañes, J. 2013. Mass spectrometry strategies for mycotoxins analysis in European beers. *Food Control*, 30: 122-128.

Rui, Y. K., Yi, G. X., Zhao, J., Wang, B. M., Li, Z. H., Zhai, Z. X., He, Z. P. & Li, Q. X. 2005. Changes of Bt toxin in the rhizosphere of transgenic Bt cotton and its influence on soil functional bacteria. *World Journal of Microbiology and Biotechnology,* 21(6): 1279-1284.

Samuel, S. M., Aiko,V., Panda, P.,and Metha, A. 2013. Aflatoxin B1 occurance, biosynthesis and its degradation. *Journal of Pure Applied Microbiology*, 7(2): 965-971.

Schell, W. A., Johnson, M. G., McGinnis, M. R., Weitzman, I. & Crist, M. Y. 1982. Zygomycosis caused by *Cunninghamella bertholletiae*: mycologic aspects. *Archives of Pathology and Laboratory Medicine,* 106(6): 287-291.

Scherm, B., Ortu, G., Muzzu, A., Budroni, M., Arras, G. & Migheli, Q. 2003. Biocontrol activity of antagonistic yeasts against *Penicillium expansum* on apple. *Journal of Plant Pathology*: 205-213.

Schjønning, P., Thomsen, I. K., Møberg, J. P., De Jonge, H., Kristensen, K. & Christensen, B. T. 1999. Turnover of organic matter in differently textured soils: I. Physical characteristics of structurally disturbed and intact soils. *Geoderma,* 89(3): 177-198.

Schomburg, D., Schomburg, I. & Placzek, S., 2013. Brenda - the comprehensive enzyme information system. *Information on EC 2.3. 1.43-phosphatidylcholine-sterol O-acyltransferase*: 1-86

Seipp, M. T., Herrmann, M. & Wittwer, C. T. 2010. Automated DNA extraction, quantification, dilution, and PCR preparation for genotyping by high-resolution melting. *Journal of Biomolecular Techniques,* 21(4): 163.

Selvaraj, J. N., Lu, Z., Yan, W., Zhao, Y. J., Xing, F. G., Dai, X. F. & Yang, L. 2015. Mycotoxin detection - recent trends at global level. *Journal of Integrative Agriculture,* 14(11): 2265-2281.

Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y. J. & Larondelle, Y. 2006. Deoxynivalenol transport across human intestinal CaCO$_2$ cells and its effects on cellular metabolism at realistic intestinal concentrations. *Toxicology Letters,* 164(2): 167-176.

Shein, K. A. 2006. State of the climate in 2005. *Bulletin of the American Meteorological Society,* 87(6): 1-102.

Shifrin, N. S., Beck, B. D., Gauthier, T. D., Chapnick, S. D. & Goodman, G. 1996. Chemistry, toxicology, and human health risk of cyanide compounds in soils at former manufactured gas plant sites. *Regulatory Toxicology and Pharmacology,* 23(2): 106-116.

Silayo, V. C. K., Lazaro, E.L., Yustas, Y. & Laswai, H.S. 2013. Cassava sun drying performance on various surfaces and drying bed depths. *Tanzania Journal of Agricultural Sciences,* 12(1): 31-36.

Siller, H. & Winter, J. 1998. Degradation of cyanide in agroindustrial or industrial wastewater in an acidification reactor or in a single-step methane reactor by bacteria enriched from soil and peels of cassava. *Applied Microbiology and Biotechnology,* 50(3): 384-389.

Sing, N. N., Zulkharnain, A., Roslan, H. A., Assim, Z. & Husaini, A. 2014. Bioremediation of PCP by Trichoderma and Cunninghamella strains isolated from sawdust. *Brazilian Archives of Biology and Technology,* 57(6): 811-820.

Siritunga, D. & Sayre, R. T. 2003. Generation of cyanogen-free transgenic cassava. *Planta,* 217(3): 367-373.

Siritunga, D. & Sayre, R. 2004. Engineering cyanogen synthesis and turnover in cassava (*Manihot esculenta*). *Plant Molecular Biology,* 56(4): 661-669.

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Smith, S. E. & Smith, F. A. (Eds.). 2002. *Diversity and Integration in Mycorrhizas: Proceedings of the 3rd International Conference on Mycorrhizas (ICOM3) Adelaide, Australia, 8–13 July 2001.* Dordrecht, Netherlands: Springer.

Soto-Blanco, B. & Górniak, S. L. 2010. Toxic effects of prolonged administration of leaves of cassava (*Manihot esculenta* crantz) to goats. *Experimental and Toxicologic Pathology,* 62(4): 361-366.

Soto-Blanco, B., Maiorka, P. & Górniak, S. 2002. Neuropathologic study of long term cyanide administration to goats. *Food and Chemical Toxicology,* 40(11): 1693-1698.

Srey, C., Kimanya, M. E., Routledge, M. N., Shirima, C. P. & Gong, Y. Y. 2014. Deoxynivalenol exposure assessment in young children in Tanzania. *Molecular nutrition & food research,* 58 (7): 1574-1580.

Stockmann-Juvala, H. & Savolainen, K. 2008. A review of the toxic effects and mechanisms of action of fumonisin B1. *Human and Experimental Toxicology,* 27(11): 799-809.

Stoilova, I., Krastanov, A., Stanchev, V., Daniel, D., Gerginova, M. & Alexieva, Z. 2006. Biodegradation of high amounts of phenol, catechol, 2, 4-dichlorophenol and 2, 6-dimethoxyphenol by Aspergillus awamori cells. *Enzyme and Microbial Technology, 39(5):* 1036-1041.

Streit, E., Schwab, C., Sulyok, M., Naehrer, K., Krska, R. & Schatzmayr, G. 2013. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins,* 5(3): 504-523.

Su, Y. C., Huang, H., Liu, X. Y. & Zheng, R. Y. 1999. Systematic relationship of several controversial Cunninghamella taxa inferred from sequence comparisons of ITS2 of rDNA. *Mycological Research,* 103(7): 805-810.

Sudakin, D. L. 2003. Trichothecenes in the environment: relevance to human health. *Toxicology Letters*, 143(2): 97-107.

Sulyok, M., Beed, F., Boni, S., Abass, A., Mukunzi, A. & Krska, R. 2015. Quantitation of multiple mycotoxins and cyanogenic glucosides in cassava samples from Tanzania and Rwanda by an LC-MS/MS-based multi-toxin method. *Food Additives and Contaminants*: Part A, 32(4): 488-502.

Sundaresan, S., Nambisan, B. & Amma, C. E. 1987. Bitterness in cassava in relation to cyanoglucoside content. *Indian Journal of Agricultural Sciences,* 57(1): 37-40.

US Environmental Protection Agency (USEPA), 2010. National primary drinking water regulations; announcement of the results of EPA's review of existing drinking water standards and request for public comment and/or information on related issues. *Federal Register,* 75(59): 15500-15572.

Takeshima, H. 2010. Prospects for development of genetically modified cassava in Sub-Saharan Africa. *AgBioForum,* 13: 63-75.

Takeuchi, Y., Salcher, M. M., Ushio, M., Shimizu-Inatsugi, R., Kobayashi, M. J., Diway, B., Von Mering, C., Pernthaler, J. & Shimizu, K. K. 2011. *In-situ* enzyme activity in the dissolved and particulate fraction of the fluid from four pitcher plant species of the genus *Nepenthes*. *PLoS One,* 6(9): 1-9.

Taylor, N. J., Halsey, M., Gaitán-Solís, E., Anderson, P., Gichuki, S., Miano, D., Bua, A., Alicai, T. & Fauquet, C. M. 2012. The VIRCA project: virus resistant cassava for Africa. *GM Crops and Food,* 3(2): 93-103.

Terra, W. R. & Ferreira, C. 1994. Insect digestive enzymes: properties, compartmentalization and function. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* 109(1): 1-62.

Tilman, D., Socolow, R., Foley, J. A., Hill, J., Larson, E., Lynd, L., Pacala, S., Reilly, J., Searchinger, T., Somerville, C. & Williams, R. 2009. Beneficial biofuels—the food, energy, and environment trilemma. *Science,* 325(5938): 270-271.

Tripathi, S. & Mishra, H. N. 2009. Nutritional changes in powdered red pepper upon in vitro infection of *Aspergillus flavus*. *Brazilian Journal of Microbiology,* 40(1): 139-144

Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R. & Keller, N. P. 2005. Three putative oxylipin biosynthetic genes integrate sexual and asexual development in *Aspergillus nidulans*. *Microbiology,* 151(6): 1809-1821.

Ubalua, A. O. 2010. Cyanogenic glycosides and the fate of cyanide in soil. *Australian Journal of Crop Science,* 4(4): 223-238.

Ueno, Y., Iijima, K., Wang, S. D., Sugiura, Y., Sekijima, M., Tanaka, T., Chen, C. & Yu, S. Z. 1997. Fumonisins as a possible contributory risk factor for primary liver cancer: a 3-year study of corn harvested in Haimen, China, by HPLC and ELISA. *Food and Chemical Toxicology,* 35(12): 1143-1150.

Umsza-Guez, M. A., Díaz, A. B., Ory, I. D., Blandino, A., Gomes, E. & Caro, I. 2011. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. *Brazilian Journal of Microbiology,* 42(4): 1585-1597.

Usmani, K. A., Rose, R. L. & Hodgson, E. 2003. Inhibition and activation of the human liver microsomal and human cytochrome P450 3A4 metabolism of testosterone by deployment-related chemicals. *Drug Metabolism and Disposition,* 31(4): 384-391.

Van De Walle, J., Sergent, T., Piront, N., Toussaint, O., Schneider, Y. J. & Larondelle, Y. 2010. Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicology and Applied Pharmacology,* 245(3): 291-298.

Van Der Fels-Klerx, H., Klemsdal, S., Hietaniemi, V., Lindblad, M., Ioannou-Kakouri, E. & Van Asselt, E. 2012. Mycotoxin contamination of cereal grain commodities in relation to climate in North West Europe. *Food Additives & Contaminants:* Part A, 29 (10): 1581-1592.

Van Der Werf, G. R., Morton, D. C., DeFries, R. S., Olivier, J. G., Kasibhatla, P. S., Jackson, R. B., Collatz, G. J. & Randerson, J. T. 2009. CO_2 emissions from forest loss. *Nature Geoscience,* 2(11): 737-738.

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Vanhoutte, I., Audenaert, K. & De Gelder, L. 2016. Biodegradation of mycotoxins: tales from known and unexplored worlds. *Frontiers in Microbiology,* 7: 1-20.

Varga, J., Kocsubé, S., Tóth, B., Frisvad, J. C., Perrone, G., Susca, A., Meijer, M. & Samson, R. A. 2007. *Aspergillus brasiliensis* sp. nov., a biseriate black *Aspergillus* species with world-wide distribution. *International Journal of Systematic and Evolutionary Microbiology,* 57(8)**:** 1925-1932.

Varga, J., Frisvad, J. C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G. & Samson, R. A. 2011. New and revisited species in *Aspergillus* section Nigri. *Studies in Mycology,* 69: 1-17.

Vervier, P., Gibert, J., Marmonier, P. & Dole-Olivier, M.-J. 1992. A perspective on the permeability of the surface freshwater-groundwater ecotone. *Journal of the North American Benthological Society,* 11(1): 93-102.

Vineela, C., Wani, S. P., Srinivasarao, C., Padmaja, B. & Vittal, K. P. R. 2008. Microbial properties of soils as affected by cropping and nutrient management practices in several long-term manurial experiments in the semi-arid tropics of India. *Applied Soil Ecology,* 40(1): 165-173.

Wade, J. W., Omoregie, E. & Ezenwaka, I. 2002. Toxicity of cassava (*Manihot esculenta* Crantz) effluent on the Nile tilapia, *Oreochromis niloticus*(L.) under laboratory conditions. *Journal of Aquatic sciences,* 17(2): 89-94.

Wagacha, J. M. & Muthomi, J. W. 2008. Mycotoxin problem in Africa: current status, implications to food safety and health and possible management strategies. *International Journal of Food Microbiology,* 124(1): 1-12.

Wani, S. P. & Lee, K. K. 1995. Microorganisms as biological inputs for sustainable agriculture. Organic Agriculture (Thampan PK ed.). Cochin, India: Peekay Tree Crops Development Foundation: 39-76.

Warth, B., Petchkongkaew, A., Sulyok, M. & Krska, R. 2014. Utilising an LC-MS/MS-based multi-biomarker approach to assess mycotoxin exposure in the Bangkok metropolitan area and surrounding provinces. *Food Additives and Contaminants*: Part A, 31(12): 2040-2046.

White, T. J., Bruns, T., Lee, S. & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 18, 315-322.

White, C. S. & Markwiese, J. T. 1994. Assessment of the potential for in situ bioremediation of cyanide and nitrate contamination at a heap leach mine in central New Mexico. Soil and Sediment Contamination, 3(3): 271-283.

White, D. M., Pilon, T. A. & Woolard, C. 2000. Biological treatment of cyanide containing wastewater. *Water Research,* 34(7): 2105-2109.

Wheelock, C. E., Severson, T. F. & Hammock, B.D. 2001. Synthesis of new carboxylesterase inhibitors and evaluation of potency and water solubility. *Chemical Research in Toxicology*,14(12): 1563-1572.

Wield, D., Chataway, J. & Bolo, M. 2010. Issues in the political economy of agricultural biotechnology. *Journal of Agrarian Change,* 10(3): 342-366.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M. & Aggarwal, D. 2004. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American Journal of Clinical Nutrition,* 80(5): 1106-1122.

Wu, Q., Jezkova, A., Yuan, Z., Pavlikova, L., Dohnal,V. & Kuca, K. 2009. Biological degradation of aflatoxins. *Drug Metabolism Reviews*, 41(1):1-7.

Yazawa, T., Uesaka, M., Inaoka, Y., Mizutani, T., Sekiguchi, T., Kajitani, T., Kitano, T., Umezawa, A. & Miyamoto, K. 2008. Cyp11b1 is induced in the murine gonad by luteinizing hormone/human chorionic gonadotropin and involved in the production of 11-ketotestosterone, a major fish androgen: conservation and evolution of the androgen metabolic pathway. *Endocrinology,* 149(4): 1786-1792.

Yeoh, H. H. 1989. Kinetic properties of β-glucosidase from cassava. *Phytochemistry,* 28(3): 721-724.

Young, A. R., Björn, L. O., Moan, J. & Nultsch, W. (Eds). 1993. *Environmental UV Photobiology.* Boston, MA: Springer US.

Zhang, R., Zhang, J. W. & Szerlip, H. M. 2002. Endocarditis and hemorrhagic stroke caused by *Cunninghamella bertholletiae* infection after kidney transplantation. *American Journal of Kidney Diseases,* 40(4): 842-846.

Zheng, R. Y. & Chen, G. Q. 2001. A monograph of Cunninghamella. *Mycotaxon,* 80: 1-75.

Zide, D., Fatoki, O., Oputu, O., Opeolu, B., Nelana, S. & Olatuniji, O. 2017. Zeolite 'adsorption' capacities in aqueous acidic media: the role of acid choice and quantification method on ciprofloxacin removal. *Microporous and Mesoporous Materials*, *255*: 226-241.

Zidenga, T. 2011. *Cyanide metabolism, postharvest physiological deterioration and abiotic stress tolerance in cassava (Manihot esculenta* Crantz*).* (Doctoral dissertation, the Ohio State University, USA).

Zidenga, T., Leyva-Guerrero, E., Moon, H., Siritunga, D. & Sayre, R. 2012. Extending cassava root shelf life via reduction of reactive oxygen species production. Plant Physiology, 159(4): 1396-1407.

Zinedine, A., Soriano, J. M., Molto, J. C. & Manes, J. 2007. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology*, 45(1): 1-18.

Zinedine, A. & Mañes, J. 2009. Occurrence and legislation of mycotoxins in food and feed from Morocco. *Food Control*, 20(4): 334-344.

Zuur, A. F., Ieno, E. N. & Elphick, C. S. 2010. A protocol for data exploration to avoid common statistical problems. *Methods in Ecology and Evolution,* 1(1): 3-14.

10. CHAPTER 10

10.1   APPENDICES

APPENDIX

Appendix 1: Used Primers and *C. bertholletiae* accession number and Nucleotide sequences

Primers (reverse and forward) used:

ITS1: 'ITSTCCGTAGGTGAACCTGCGG'

ITS4: ITS 'TCCTCCGCTTATTGATATG'

Fungus *Cunninghamella bertholletiae* accession no: KT275316

Nucleotide sequences submitted on Genbank:

```
tatccacagg  gggaaatgct  tctaacgctt  gtgcctggtt  cagtctagtg  ctgccacttgagtttatcct
taatcaaggg  atctttgggt  agttgttcat  tattttctct  ctctttttag  ggggggggaa  ttaatgatgg
gcacctcttg  taaaggggat  aaattacttt  tattatacta  aattttactg  aactgataac  cataaatcta
tggttgtttt  ttattataat  taaacaaaaa  aacaactttc  agcaatggat  ctctcggctt  tcgtatcgat
aaaaccacaa  atccgatatg  taaggatctg  cctatagtga  atcatcaaat  ctttgaacgc  atcttgcacc
ttatggtatt ccataaggta  cgtctgtttc  agtaccacta  aaaatctctc  tctatccttg  atgata
```

Appendix 2: Fungal species/strains, mycotoxins and affected produce

Species/Strain	Toxins identified	Affected produce	References
Fusarium sp. *Aspergillus* sp.	Fumonisins	Corns, Wheat, Soybeans, Tomatoes, Maize, Cassava, Peanut, Jimsonweed, Malt, Oat, Rice, Potatoes, Carrots, Barley, Rice	Plattner, 1995 Gelderblom *et al.*, 1988; Doehlert *et al.*, 1994; Nielsen *et al.*, 2009; Gnonlonfin *et al.*, 2008; Sulyok *et al.*, 2014; Abbas *et al.*, 1995; Abbas *et al.*, 1998; Mantle *et al.*, 2016; Ediage *et al.*, 2014; Vanhoutte *et al.*, 2016.
Aspergillus sp.	Aflatoxins, Citrinin, Patulin, Ochratoxin, Gliotoxin, Fumonisins	Bean, Cotton, Sorghum, Barley, Wheat, Maize, Cassava, Yam, Almond, Mango, Garlic, Apple, Pineapple, Strawberry, Pistachio, Apricot, Peach, Carrot, Citrus (Citrus sp.), Grape, Raisin, Fig, Milk, Cheese, Date, Maize, Coffee bean, Onion, Red pepper	Wu *et al.*, 2009; Samuel *et al.*, 2013; Hassan & Kassaify, 2014; Gnonlonfin *et al.*, 2008; Ediage *et al.*, (2014); Sulyok *et al.*, 2014; Frisvad *et al.*, 2007; Varga *et al.*, 2007 2010, Tripathi & Mishra, 2009.
Trichothecium sp, *Myrothecium* sp., *Trichoderma* sp.	DON, T2-toxin, HT2-toxin	Wheat, Maize, Cassava, Peanut	Plattner, 1995; Sudakin,2003; Kimura *et al.*, 2007; Li *et al.*, 2011; Ediage *et al.*, 2014; Sulyok *et al.*, 2014.

Cont: Appendix 2: Fungal species/strains, mycotoxins and affected produced

Fusarium sp.	ZEN, zea/F-2 toxin, Moniliformin, Aflatoxins, Fumonisins, Beauvericin, Deoxynivalenol (Vomitoxin), Nivalenol T2-toxin, HT2-toxin,	Wheat, Maize, Cassava, Peanut, Cotton, Spices, Rice, Red pepper	Zinedine *et al.*, 2007; Zinedine & Mañes, 2009; Sudakin, 2003; Kimura *et al.*, 2007; Ediage *et al.*, 2014; Sulyok *et al.*, 2014; Abbas, *et al.*, 1998; Tripathi & Mishra, 2009.
Penicillium sp.	Citrinin, Ochratoxin, Patulin, Penicillic acid, Penitrem A, Roquefortine, Frequentin, Palitantin, Mycophenolic acid, Viomellein, Gliotoxin, Citreoviridin, Rubratoxin B	Maize, Wheat, Barley, Cassava, Peanut, Tabaco, Meat products, Cheese varieties, Grape juice	Ismaiel & Papenbrock, 2015; Ediage *et al.*, 2014; Gnonlonfin *et al.*, 2008; Sulyok *et al.*, 2014; Mantle *et al.*, 2016; Lund & Frisvad, 2003;
Penicillium sp.	Citrinin, Ochratoxin, Patulin, Penicillic acid, Penitrem A, Roquefortine, Frequentin, Palitantin, Mycophenolic acid, Viomellein, Gliotoxin, Citreoviridin, Rubratoxin B	Maize, Wheat, Barley, Cassava, Peanut, Tabaco, Meat products, Cheese varieties, Grape juice	Ismaiel & Papenbrock, 2015; Ediage *et al.*, 2014; Gnonlonfin *et al.*, 2008; Sulyok *et al.*, 2014; Mantle *et al.*, 2016; Lund & Frisvad, 2003;
Monascus sp.	Citrinin	Bean, Cotton, Sorghum, Cassava, Peanut	Ismaiel & Papenbrock, 2015; Ediage *et al.*, 2014; Sulyok *et al.*, 2014.

**Cont: Appendix 2: Fungal species/strains, mycotoxins and affected produced**

|			
Rhizopus oryzae	Fumonisins, Aflatoxins	Cassava, Yam	Gnonlonfin *et al.*, 2008.
Mucor piriformis	Fumonisins, Aflatoxins	Cassava, Yam	Gnonlonfin *et al.*, 2008.
Aspergillus brasiliensis	naphtho-γ-pyrones, tensidol A and B and pyrophen	Grape berries	Varga *et al.*, 2007.

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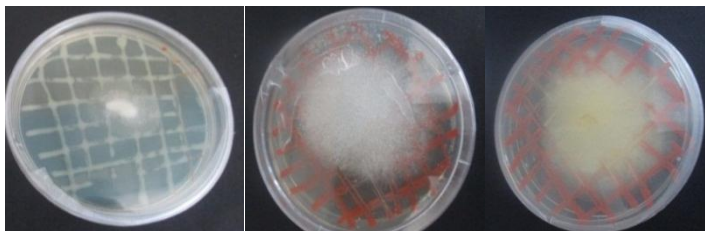**Appendix 3: Decomposing cassava tubers**

Appendix 4: Inoculum of *C. bertolletiae* into nutrient agar and formation of mycelia
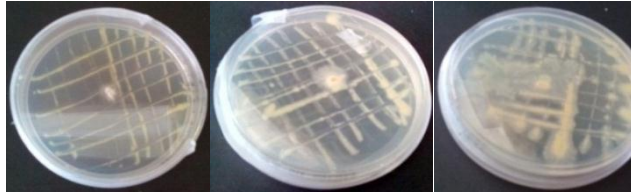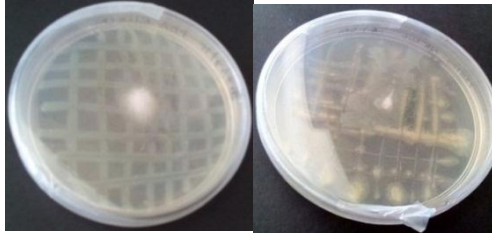
Appendix 5: *C. bertholletiae* pathogenic activity on *Myroides* sp. after 24hr, 48hrs and 120hr
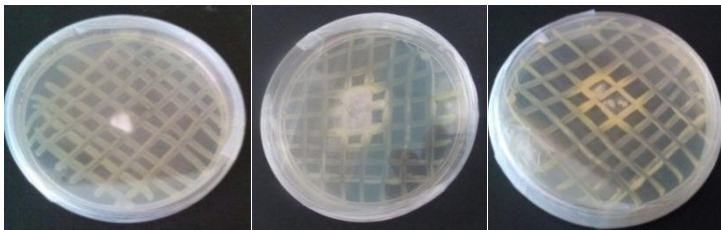

Appendix 6: *C. bertholletiae* pathogenic activity on *Achromobacter denitrificans/Achromobacter xylosoxidans* after 24hr, 48hr and 120hr

Appendix 7: *C. bertholletiae* pathogenic activity on *Methylobacterium* sp. after 24hr, 48hr and 120hr

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Appendix 8: *bertholletiae* **pathogenic activity on**
***Stenotrophomonas maltophilia* after 24hr and 48hr**
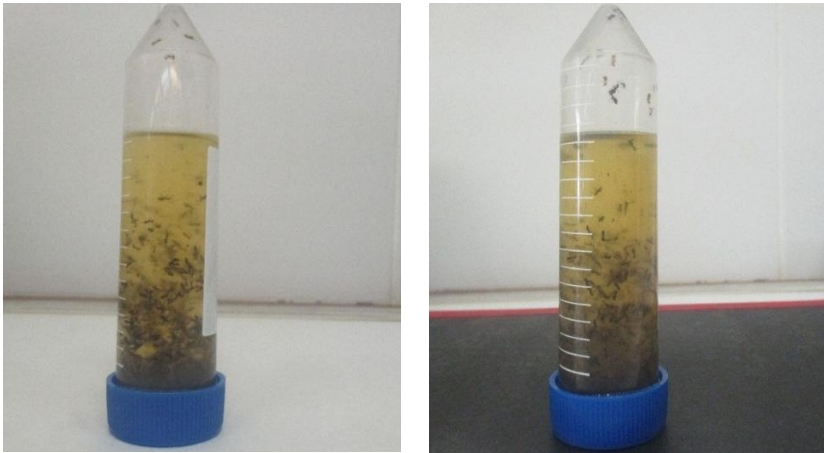
Appendix 9: *Nepenthes mirabilis* **pitcher extracts with prey**

Appendix 10: Calculation of enzyme activity

- The calculation of enzyme activity was done in order to convert the reading units per litre; thus, 1 unit = µmol substrate which is converted in minutes.
- While, the readings of the absorbance is plotted in a graph in which there is time versus absorbance.
- $\dfrac{dA}{dt}$ is an initial slope gradient which determines the value of the reaction's initial rate.
- While, the above ratio is converted into $\dfrac{dc}{dt}$ by dividing it with the extension coefficient (17000 M^{-1}. Cm^{-1} as suggested by Lambert-Beer. Thus, the value will be converted into mol.min. $^{-1}$L $^{-1}$.
- Multiple by 10^6 in order to convert moles into micromoles. Finally, the 1 unit will be micro-moles. min. $^{-1}$L $^{-1}$ with the final answer unit expressed as units per litre.