Diversity of endophytic fungi possessing bioactive compounds isolated from selected medicinal plants

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DECLARATION

I, Madira Coutlyne Manganyi, declare that the thesis entitled **"Diversity of endophytic fungi possessing bioactive compounds isolated from selected medicinal plants"**, hereby submitted for the degree of Doctor of Science in Biology (Molecular Microbiology), has not previously been submitted by me for a degree at this or any other university. I further declare that this is my work in design and execution and that all materials contained herein have been duly acknowledged.

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DEDICATION

I would like to dedicate this PhD thesis to my parents **Fridah** and **John Manganyi** for their endless support, care and love.

Honour Your Father and Mother, so that it may be well with you, and that you may live long on the earth. This is the first commandment with a promise

Ephesians 6:2-3

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Ndza nkhensa Ndzi khense ngopfu Inkomu swinene

'IT TAKES A VILLAGE TO RAISE A CHILD'

May the Almighty GOD continue to BLESS YOU ALL.

"The fear of the LORD is the beginning of knowledge, but fools despise wisdom and instruction."

The Bible, Proverbs 1:7

My Lord, You are my Pinar of strength

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

CDB	: Czapek dox broth
CF	: Colonization Frequency
DPPH	: di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium
Fusarium MLST	: Loci Sampled and Multilocus Sequence Typing
GC-MS	: Gas Chromatography Mass Spectrophotometry
NCBI	: National Center for Biotechnology Information
NMR	: Nuclear Magnetic Resonances
MIC	: Minimum Inhibitory Concentration
MVP	: Mevalonic acid pathway
PCA	: Potato Carrot Agar
PDA	: Potato Dextrose Agar
PPRI	: Plant Protection Research Institute
TLC	: Thin Layer Chromatography
SANBI	: South African National Biodiversity Institute
WA	: Water Agar
WHO	: World Health Organisation

DEFINITION OF CONCEPTS

Antimicrobial properties: Are agent that destroy microorganisms or inhibit their growth

Bioactive compounds: Are compounds or components that consist of chemicals, which have a bioactive effect such as growth promotion on a living organism, tissue /cell.

Endophytes: Are microorganisms that live within the plant tissues.

Epiphytes: Are microorganisms that live on the surface of a plant's leaves.

Ethno-botanical: Study of culture and botany (study of plants) from the word ethnology.

Fungal Diversity: It is a combination of biodiversity, systematic and molecular phylogeny of the fungi, including lichens.

Medicinal plants: Are plants that produce chemical compounds for physiological and ecological functions including defence against insects, fungi, diseases, and herbivorous mammals. Various studies showed the phytochemicals prospective and established biological activities of the medicinal plants.

***Pelargonium sidoides*:** Is a medicinal plant native to South Africa and commonly known as the African geranium. It forms a basal rosette of cordate leaves with a velvet texture and a few short trichomes on long petioles. Its flowers have five dark red to nearly black petals, two of which are sometimes fused.

Primary metabolites: Are those metabolites necessary for the growth of an organism, such as polysaccharides, proteins, fats and nucleic acids.

***Sceletium tortuosum*:** Is a succulent plant commonly known as Kanna, Channa, Kougoed and distributed in South Africa. Plants are climbing or creeping. The slender branches become thick and only slightly woody with age. Water cells are conspicuous on the leaves that have recurved tips and 3 to 5 major veins. The flowers are very shortly pedicellate (almost sessile) and of medium size (20 to 30 mm diameter). Petals are white to pale yellow, pale salmon or pale pink. The calyx has four or five sepals. Fruit are 10 to 15 mm in diameter and open when wet (hygrochastic). The species is readily distinguishable by the imbricate leaves with incurved tips.

Secondary metabolites: include compounds such as terpenes, alkaloids, polyketides and pigments. Although secondary metabolites may not be essential for the growth and health of the organism they often provide the organism with a competitive advantage over other species competing for nutrients by eliciting biological activity.

True fungi: are heterotrophic, filamentous in habit and their cell walls contain chitin.

CONFERENCES AND CONFERENCE PROCEEDINGS

Poster Presentation **1. Madira Coutlyne Manganyi**, Thierry Regnier, Ajay Kumar; Carlos Cornelius Bezuidenhout, Collins Njie Ateba. Biodiversity and preliminary screening of Endophytic fungi associated with indeginoius plants Umckaloaba *Pelagonium sidoides*. *7th Congress of European Microbiologists (FEMS), Valencia – Spain, 9th – 13th July 2017 (FEMS7-1915)*

ARTICLES PUBLISHED AND MANUSCRIPTS SUBMITTED

Proof of articles published and manuscripts summited is in Appendix 1.

1. **Manganyi M.C.**, Regnier T., Kumar A., Bezuidenhout C.C., Ateba C.N. 2018. Biodiversity and antibacterial screening of endophytic fungi isolated from *Pelargonium sidoides*. *South African Journal of Botany* 116: 192–199

2. **Madira Coutlyne Manganyi**, Thierry Regnier, Ajay Kumar; Carlos Cornelius Bezuidenhout, Collins Njie Ateba. Phylogenetic analysis and Diversity of Novel Endophytic fungi isolated from Medicinal Plants of *Sceletium tortuosum*. *Phytochemistry Letters 27 (2018) 36–43 (Accepted with minor Review)*

3. **Madira. C. Manganyi**, Thierry Regnier, Christ-Donald K. Tchatchouang, Carlos C. Bezuidenhout, Collins N. Ateba. Bioactive compound produced by endophytic fungi isolated from Pelargonium sidoides against selected bacteria of clinical importance. *Current Microbiology (CMIC-D-18-00142). (Under review)*

SUMMARY

Throughout history, mankind has used plants as their primary source of sustainability. This includes their use as sources of food (agricultural commodities), production of clothing and fragrances, fertilizers, the enhancement of flavours, and also to provide shelter. The treatment of infections caused by microorganisms is usually achieved through the administration of antibiotics. However, recent studies have indicated a steady increase in antibiotic resistance among bacteria strains and particularly the detection of multiple antibiotic resistant (MAR) isolates. This therefore presents severe public health challenges to both the medical and veterinary professions and resistance has become an important issue of global concern. Against this background, current research has focused on finding alternative agents that could serve as potential treatment options to address the problems associated with the presence of antimicrobial resistance worldwide. Natural products especially those associated with plants are now regarded as potential agents that could address this concern. Despite the fact that a number of studies have assessed different plant species for potential bioactive compounds, very little emphasis has been placed on investigations that are designed to assess the capabilities of endophytes in producing bioactive compounds. Endophytic fungi are the biggest diverse group of microbes that colonise plants tissues without any damage, infections nor symptoms of infections. Endophytic fungi engage in mutualistic relationships that normally benefits both the fungi and the plant. It has also been reported that plant species that harbour some endophytes have displayed enhanced capability to withstand both abiotic and biotic stress. Fungi are able to produce secondary metabolites that possess bioactive properties as well as pigments that have tremendous benefits to mankind. Despite the fact that South Africa is well known for the use of medicinal plants in both primary health care and ethnomedicine, very little information is documented on bioactive compounds produced by fungi that are harboured by commonly utilized indigenous plants.

To our knowledge, this research is the first study on the screening of endophytic fungal diversities present in two medicinal plants *Sceletium tortuosum* and *Pelargonium sidoides* that are indigenous to South Africa. This study encompasses the potential of these fungi to produce potent bioactive compounds with broad-spectrum activities against different resistant pathogenic bacterial strains.

A total of 193 endophytic fungi were successfully isolated and the dominant isolates belonged to phylum Ascomycota with *Fusarium* and *Aspergillus* as the predominant genus. Phylogenetic analysis based on the Internal Transcribed Spacer (ITS) and Transcription Elongation Factor (TEF 1α) regions was used to establish the relationship and diversities of fungi isolated as well as the

evolution time. Cluster analysis produced two-three clusters in each phylogenetic tree and data revealed that three novel fungal isolates that did not clustered with any isolates and were considered to possible new species. Secondary metabolites were extracted from all endophytic fungal isolates and used to assess their potential to inhibit the growth of Gram positive and Gram negative bacteria that comprised environmental strains as well as ATCC strains.

Phenotypic antibiotic resistance assays revealed that *E. faecium* (26%) and *E. gallinarum* (9%) displayed high levels of susceplibility to the fungal extracts. In addition, *E. coli* (ATCC 25922) was most often sensitive to a large proportion (70%) of the fungal extracts tested. With the exception of *Alternaria*, a majority (80%) of the extracts from the fungal species exhibited narrow spectrum activities against the organisms tested. The largest bacterial growth inhibition zone diameter data of 12 mm was produced by an extract obtained from *Alternaria.* Endophytic fungi were assessed for the ability to produce pigments and a total of thirty-one (16%) isolates produced distinct pigments with varied colours ranging from yellow (26%), red (13%), brown (35%) to black (13%). Based on enzyme activity assays large proportions of the isolates produced amylase (61%) and lipase (65%). On the contrary, only a small proportion (13%) of the isolates produced the laccase enzyme.

The GC-MS based metabolite profiling of selected fungal extracts was used to assess the ability of fungi to produce volatile compounds. A total of 106 different volatile compounds were identified. The chemical characterization indicated that 9,12-Octadecadienoic acid (Z,Z) and Cyclodecasiloxane were the predominant compounds in extracts that displayed enhanced microbial activities.

Greenhouse studies were conducted on maize (*Zea mays* L.) using nine extracts from these endophytes that were previously selected according to their ability to produce pigments and their antimicrobial properties. An assessment of plant growth parameters between plants in the treatment groups and control group revealed that the extract from *Fusarium solani* (MHE 55) was the most favourable in enhancing plant growth than the extract from *Alternaria* (MHE 68) than the control.

In conclusion, from 193 isolates only two endophytic fungi namely *Alternaria* sp. (MHE 68) and *F. solani* (MHE 55) have shown to have strong antimicrobial activity, pigment production and are growth promoters. Due to time constraint other biological properties such as antifungal, antiviral, antioxidant, cytotoxicity were not assessed and therefore should be investigated. Furthermore, molecular techniques must be used to determine and confirm the novel fungal isolates and the correlation

between pigments, flavonoids and phenolic compounds using High performance liquid chromatography (HPLC) should be established.

CHAPTER ONE

INTRODUCTION

"If you develop the habits of success,

You will make success a habit" Michael E. Angler

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1. GENERAL INTRODUCTION

1.1 Introduction and problem statement

Throughout history mankind has used plants as a primary source of sustainability. This includes as a source of food (agricultural commodities), a source of clothing and fragrances, as fertilizers, the enhancement of flavours, and also for provision of shelter (Cragg and Newman, 2005). Medicinal plants and their derivatives have also played a crucial role in the treatment of various human ailments or diseases (Koehn and Carter; 2005, Verma and Singh, 2008). According to the World Health Organization (WHO) over 80% of the world's population or 4.3 billion people use medicinal plants as their source of primary health care (Aljaiyash *et al.,* 2014). Currently, there has been an increased global awareness concentrating on the effectiveness of plant-based medicines in treating infections in humans (Kadir Yaakob and Zulkifli, 2013, Kshirsagar *et al.,* 2010).

Conventional antimicrobial agents used today either contain 50–60% of natural products or are synthesized from them, with 10-25% of all prescribed medicines being made up of one or more ingredients derived from plants (Pan *et al.,* 2013). Over the last few decades, these antimicrobial agents have had a dramatic decrease in their efficiency mainly due to the fact that microbes have developed strategies to evade destruction and therefore become resistant to these agents (Carlet *et al.,* 2012). Plant-based medicinal products were the first on the market in the history of drug development and continue to be prominent today (Gurib-Fakim, 2006). Despite the fact that almost 300,000 different plant species exist on earth, only several hundred have been investigated (Verma *et al.,* 2014) and this therefore explains the need to constantly search for new plant-based medicinal products.

Secondary metabolites, produced by distinct endophytic fungi usually present in medicinal plants, are used in the pharmaceutical and agricultural industries (Bhardwaj and Agrawal, 2014). Currently, over one million endophytic fungi that are associated with naturally occurring plant species have been documented (Jena and Tayung, 2013). However, given the constant high demand for

pharmaceutical and agricultural products there is a need to constantly search for microbes displaying the potential to produce these very important environmentally friendly products.

Endophytes are a highly diverse groups of microorganisms either fungi, bacteria or actinomycetes that exist in a symbiotic relationship with plants. Endophytic fungi are known to live and spend either all or part of their life cycle by colonizing the inter-and/or intra-cellular tissues of healthy host plants (Namasivayam, Swetha and Srivatsan, 2014). The implication is that these organisms are able to cause asymptomatic infections in plants (Namasivayam *et al.,* 2014). The first endophytic fungi *Sphaeria typhena* Pers. known as *Epichloë typhina* (Leuchtmann, *et al.,* 2014) was described over 210 years ago by Persoon (1798). In the plant communities, the endophytic fungi are extremely ubiquitous and all vascular plants harbour them. Endophytic fungi are obligate or facultative microorganisms that live in a mutual or antagonistic relationship with its host (Nair and Padmavathy, 2013). In addition, the presence of these fungi may provide several benefits to the plant host including drought tolerance, protection against pathogens, enhanced growth and prevention from destruction by herbivores (Higginbotham *et al.,* 2013).

On the other hand, the endophytic fungi obtain a protected environment in the plant tissues, which play important physiological and ecological roles to the plant. This therefore explains the importance of symbiotic fungi to both the plant and the ecosystem in general (Sandhu *et al.,* 2014). However, the population of endophytic fungi differ among different plants and within species of the same genus. In addition, the occurrences of endophytic fungi are also affected by differences in climatic conditions and therefore vary significantly among different regions (Nair and Padmavathy, 2013).

Studies have been conducted to detect and isolate endophytic fungi that belong to a variety of orders, families, genera and species from plant species (Toju *et al.,* 2013). In a comparison study, Chareprasert *et al.* (2006) reported that matured leaves of teak (*Tectona grandis* L.) and rain tree (*Samanea saman* Merr.) had fungi with great genus and species diversity and the colonization frequency was higher when compared to the young leaves. Moreover, endophytic fungi were most frequently detected in the plants during the rainy season (Chareprasert *et al.*, 2006). These findings clearly revealed that both fungal-specific factors and climatic conditions have significant contributions on the endophytic fungal population in a given area (Chareprasert *et al.*, 2006). Endophytic fungi have been isolated from different parts of plants that include scale primordia, meristem, resin ducts,

leaf segments, roots, stems, bark, leaf blade, petiole, and buds. These fungi have also been isolated from a range of climatic conditions including tropical, aquatic and xerophytic environments (Jalgaonwala, Mohite and Mahajan, 2011).

Over the last two decades, research focused on the search for bioactive compounds from endophytes has increased significantly, since the discovery of *Taxomyces andreanae* in 1993. The identification of paclitaxel (taxol) the "gold" bioactive compound sparked significant interests in the study of fungal endophytes as potential producers of novel biologically active compounds. Medicinal plants serve as hosts to endophytic fungi, known to produce specific secondary metabolites and as stated by Nair and Padmavathy (2013), molecular techniques have been used to study the biodiversity of fungal endophytes in seed and needles of *Pinus monticola*, western white pine (). Hence, it is essential to explore endophytic mycoflora isolated from medicinal plants and screen them for their ability to produce secondary metabolites that will be of benefit to mankind (Gurib-Fakim, 2006).

Secondary metabolites are defined as chemical compounds with relatively low molecular mass and in most cases less than 3 kDa (Vinale *et al.,* 2014). These structural molecules are primarily produced by microorganisms (fungi and bacteria) and plants (Lima and Keller, 2014). Particular metabolites are naturally associated with plants and/or microbes belonging to specific genera, species or strains (Vinale *et al.,* 2014) and biosynthesised from intermediates derived through primary metabolic processes. However, secondary metabolites produced by organisms are not essential for growth and survival mechanisms when compared to the primary metabolites that play essential roles (Mandal and Rath, 2015). In fungi, secondary metabolites either increase the vigour of the producing organism or decrease the fitness of surrounding organisms (Niehaus *et al.,* 2014).

These compounds are also used for morphological differentiation of fungal species and are associated with active growth. Sporulation and elongation of the hyphae have been identified as target specific fungal developments that are associated with the production of secondary metabolites (Vinale *et al.,* 2014). There are five major metabolic pathways used to biosynthesise secondary metabolites, namely amino acid synthesis, the shikimic acid pathway that produces aromatic compounds, the polyketide pathway, the mevalonic acid pathway (MVP), and polysaccharides via

glucose (Dewick, 2002). During the biosynthesis of secondary metabolites some endophytic fungi produce analogues of several metabolites within a single host plant (Selim *et al.,* 2012).

Endophytic fungi have been found to produce a mixture of volatile organic compounds with significant antimicrobial activities against human and plant pathogens (Woropong *et al.,* 2001). The ability of microorganisms to adapt to their habitat and inhibit the growth of competitors is based on their potential to produce chemical signals for communication and therefore significant evolution has occurred among secondary metabolites from the time of identification. This has been proven with well-known antimicrobial agents, such as penicillin and lovastatin, which are metabolites of fungal species (Brakhage, 2013). In the agricultural sector, *Trichoderma* is widely used as a growth promoter hence it acts as a bio-fertilizer. In addition, it is capable of protecting the crop from pest destruction and therefore acts as a bio-pesticide. It is therefore evident that endophytic *Trichoderma* has various potentially effective activities due to its ability to produce a wide variety of secondary metabolites (Vinale *et al.,* 2014). Against this background, it is important to identify secondary metabolites that are produced by an organism and are of ecologic, pharmaceutical and/or agricultural importance. Moreover, an investigation on the interaction between endophytes and their host plants with emphasis on the detection of secondary metabolites that exhibit bioactive potency cannot be underestimated.

In the present study, medicinal plants were selected based on the ethnobotanical history and their traditional usage. The ecological niche and medicinal plants selected based on several points' previously highlighted by Selim *et al.* (2012). *Sceletium tortuosum* and *Pelargonium sidoides* plants adhere to the above mentioned criteria and these plants are prominent, renowned indigenous South African plants that are commercialised for various industrial purposes (Van Wyk, 2011). Therefore, the present study was designed to investigate endophytic fungal species associated with *Sceletium tortuosum* and *Pelargonium sidoides* for their abilities to produce bioactive compounds. A further objective was to identify the fungal species using molecular techniques and also to characterise the bioactive compounds by assessing their antimicrobial potentials. Data generated may provide valuable options for the development of novel antimicrobial agents for pharmaceutical and agricultural industrial applications.

1.2 Research Problem

Not much research has been done on the endophytic fungi isolated from medicinal plants and the biologically active compounds produced (Ribeiro *et al.*, 2012). This can serve as a discovery tool for new, affordable, efficacious antimicrobial compounds for pharmaceutical and agricultural applications.

1.3 Hypotheses

It has been proven throughout history that secondary metabolites produced by fungi possess antimicrobial activities. There is currently a strong correlation between medicinal plants and their fungal isolates It is expected that useful chemical components can be extracted from fungi that reside in medicinal plants and it is anticipated that these secondary metabolites have potent antimicrobial activities.

1.4 Research aim and objectives

1.4.1 Aim

The aim of the study was to investigate the diversity of endophytic fungi from two selected South African medicinal plants; to assess their potential to produce bioactive compounds.

1.4.2 Objectives

The specific objectives of the study were to:

1.4.2.1 isolate and identify endophytic fungi using morphological and molecular techniques from medicinal plants;

1.4.2.2 investigate the biodiversity of the fungi though phylogenetic assessments;

1.4.2.3 determine the potential of endophytes in producing bioactive compounds;

1.4.2.4 characterise the bioactive compounds and the degree of activity;

1.4.2.5 assess if the activity of the bioactive compound is antibacterial;

1.4.2.6 determine the effect of endophytic fungi *in vivo* trials (Greenhouse Trials).

CHAPTER TWO

LITERATURE REVIEW

"A good head and a good heart are always a formidable combination" Nelson Mandela

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The present study was designed to investigate endophytic fungal species isolated from *Sceletium tortuosum* and *Pelargonium sidoides* for their abilities to produce bioactive compounds. A further objective was to identify the fungal species using molecular techniques and also to characterise the bioactive compounds by assessing their antimicrobial potentials. Data generated may provide valuable options for the development of novel antimicrobial agents for pharmaceutical and agricultural industrial applications.

2.2 MEDICINAL PLANTS

It is an indisputable fact that human being rely on plants for their basic survival. Due to the massive increase in population and urbanization, there is a constant increased demand on plants as food. For centuries, Africans have used various parts of the plants to cure aliments and diseases (Mahomoodally, 2013). Medicinal plants were selected based on the ethnobotanical history and their traditional usage. The ecological niche and medicinal plants selected were based on several points' previously highlighted by Selim *et al.* (2012). *Sceletium tortuosum* and *Pelargonium sidoides* plants adhere to the above mentioned criteria and these plants are important indigenous South African plants that have been commercialised for various industrial purposes (Van Wyk, 2011).

2.2.1 *Sceletium tortuosum*

2.2.1.1 Botanical Description

Sceletium tortuosum is a small succulent medicinal plant native to South Africa. It belongs to the Aizoaceae family that is well-known for their dicotyledonous flowering. The taxonomic grouping is illustrated in the below Table 2.1. Its common names include Kanna, Channa, and Kougoed, meaning something to chew or chewable. Kougoed is a traditional concoction prepared from *S.*

emarcidum or *S. tortuosum*, which is used as an intoxicant. In the early 1662 van Riebeeck traded with the local residents in Southern Africa, accepting sheep and 'kanna'. The Europeans referred to this plant as a ginseng-like herb. It was documented in 1685 by van der Stel, the second colonial governor of the Dutch Cape colony, in his journal, Figure 2.1 a,b. It is a perennial, short-lived plant with creeping stems and overlapping pairs of leaves that have glistening water cells (bladder cell idioblasts) on their surfaces.

Figure 2.1: *Sceletium* painting done by Simon van der Stel in 1685 (a), Jan van Riebeek with his Dutch colleagues at the Cape of Good Hope (b) (Scott and Hewett, 2008)

Scientific classification is as follows: Kingdom (Plantae), Angiosperms (unranked): Eudicots (unranked): Core eudicots (unranked), Order (Caryophyllales), Family (Aizoaceae), Subfamily (Mesembryanthemoideae), Genus (*Sceletium*), Species (*S. tortuosum*). "Skeletonised" refers to the skeleton-like structure of the dried out leaves (Figure 2.2a). These persistent leaf veins remain on the plant, hence the generic name *Sceletium* (Latin sceletus).There are eight (8) species belonging to this genus and members are simply recognized by the persistent dry leaves that become skeletonized.

Figure 2.2: *Sceletium tortuosum* dried out leaves (a) and bright yellow flowering plant (b) (Gericke and Viljoen, 2008)

In the dry season, the leaves dry out enclosing the young leaves to protect them against unfavorable environmental conditions. They propagate well in rockeries and pots. The flowering forms are pale to bright yellow (Figure 2.2b) or orange-yellow buds along the branch tips and followed by pale brown, papery capsules containing numerous small, reddish brown, kidney-shaped seeds. Currently, S. tortuosum is well known and used on commercial products.

2.2.1.2 Geographical Distribution

Sceletium tortuosum is indigenous to South Africa and favours the south-western region where the habitat is predominantly dry (Gericke and Viljoen, 2008). The specific areas are in the Karoo district of South Africa. Figure 2.3 maps out the coastal sections where the plant ideally grows.

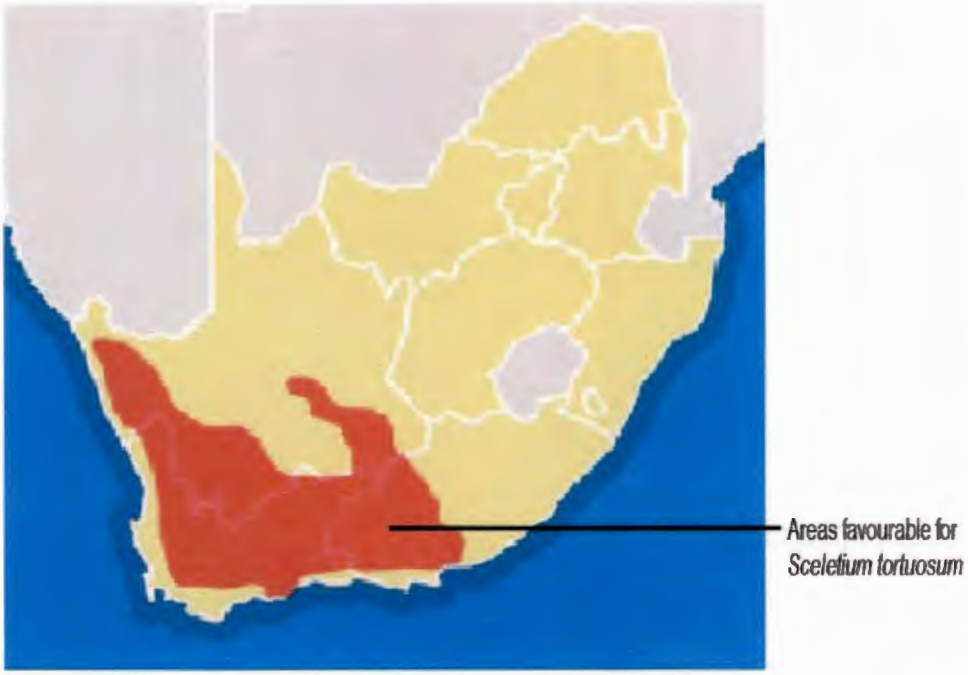


Figure 2.3: South African areas (orange) favourable for *Sceletium tortuosum* (Gericke and Viljoen, 2008)

2.2.1.3 General uses

When the *Sceletium tortuosum* plant is chewed or eaten, it causes a person to gently get into a relaxation mood; the method originates from traditional practices. Tea extracts are useful for alcohol withdrawal. Other dosage forms include gel caps, a snuff and tinctures (Smith *et al.*, 1996).

2.2.1.4 Medicinal features

It can be used as a mild anesthetic in the mouth, if sufficient amount are being chewed. Historically, the San and Khoikhoi tribes used *S. tortuosum* plant for mastication and medicine (Smith *et al*, 1996), although the colonial farmers used it in tincture formula as a psychotropic (Pappe, 1868). *S. tortuosum* has sparked tremendous interest in the last decade due to its capability to relieve stress in healthy individuals, stimulate a sense of wellbeing, hypothesized practices and for treating anxiety and depression in clinically anxious and depressed patients (Gericke and Viljoen, 2008). *In vivo* assays conducted in rats proved that *S. tortuosum* extracts limit induced anxiety (Smith, 2011). Preliminary reports validate the antidepressant and anxiolytic activity in patients anguish from major depression. They were administered *S. tortuosum* tablets pulverized from plant material (Gericke,

2001). Hence, intake of *S. tortuosum* plant may elevate mood and decrease anxiety, stress and tension (Harvey *et al.,* 2011, Gericke and Viljoen, 2008).

2.2.1.5 Side effects

Side effects exhibited from the consumption of *S. tortuosum* are experienced by a small number of individuals. The list is highlighted below[1]:

- Mild headache
- Slight nausea with no vomiting
- Slack stool or stool with no cramping
- Transient increase in anxiety or irritability an hour after initiating treatment, which resolves after an hour or so.
- Insomnia: corrected by lowering the dose or taking the product not later than midday
- A feeling of sedation: corrected by taking the product as a single 50mg dose at night

2.2.2 *Pelargonium sidoides*

2.2.2.1 Botanical Description

Pelargonium sidoides is a herb and medicinal plant used by the Northern and Southern Sotho, the Mfengi, Xhosa and Zulu tribes. The common name is African geranium or South African geranium whereas the native name is Umckaloaba (Timmer *et al.,* 2013). On the market, the plant is known and sold under various names including Umcka, Kaloba or Zucol (Timmer *et al.,* 2013). The plant is geranium like and has heart shaped leaves and a blackish purple flowers; its roots contain medicinal properties. The order is Geraniales and family belongs to Geraniaceae

2.2.2.2 Geographical Distribution

The *P. sidoides* plant is indigenous to South Africa and is widely distributed in the Eastern Cape, Free State, Limpopo, Mpumalanga and Gauteng Provinces at near sea level and also at higher altitudes. It is also endemic to Lesotho at 2746 meters above sea levels (Newton *et al.,* 2013).

[1]http://www.kanna.co.za/

Internationally, the plant has gained wide acknowledgment and large scale cultivation including commercialization in Schwabe, German Pharmaceutical company, in Kenya and Mexico (Van Niekerk and Wynberg, 2012, Van Wyk, 2011).

2.2.2.3 General uses

Historically, *P. sidoides* was used by the British in 1897 for its anti-tuberculotic benefits, until the establishment of antibiotics in the market. Approximately two decades ago, the infusion of liquid alcoholic concoctions made from *P. sidoides* was used to cure acute bronchitis (Drewes, 2012). The droplet formula gained status as the supreme prescribed childhood medication in Germany and other countries. Additional dosage forms such tablets were added in May 2009, subsequently in October 2010 syrups for children to cover the taste were introduced. The United State of America developed and approved lozenges for quick dissolving action. Throughout history, this plant has been used for medicinal proposes and continues to do so today.

2.2.2.4 Medicinal features

Umckaloaba is known to remedy respiratory diseases like tuberculosis, tonsillitis, sore throat, and the common cold. It also has healing effects for dysentery, diarrhea, gonorrhea, and herpes.

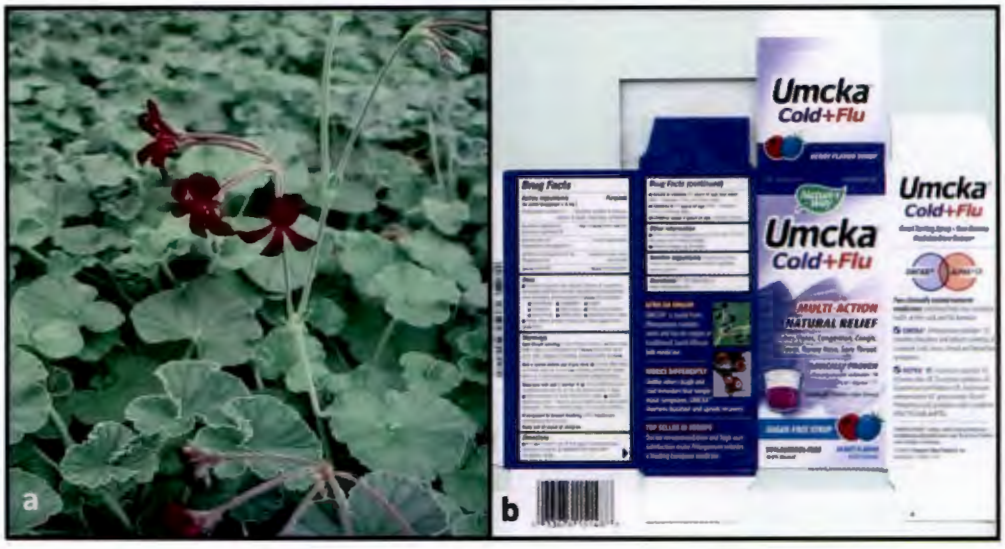


Figure 2.4: (a) *Pelargonium sidoides* in the environment (b) A formulation for colds and flu, *P. sidoides* as its main ingredient; *Pelargonium sidoides* product used for cold symptoms on children

2.2.2.5 Side effects

There are common side effects associated with herbal products, are gastrointestinal problems such as nausea, vomiting, diarrhea and heartburn (Figure 2.5). Some individuals can suffer from itching and hives which is generally classified under skin reactions. A clinical trial reported 13% of the people who took a placebo had side effects. While 18% who used *P. sidoides* experience the same side effects. The German Federal Institute for Drugs and Medical Devices (BfArM) recommended that people must consult with general practitioners if they notice symptoms of liver problems when using the plant extract. Symptoms include yellowing of the skin or eyes, dark urine, severe pain in the upper abdomen, and loss of appetite. Up until June 2012, thirty cases of inflammation of the liver (hepatitis) were reported to be associated with the use of *Pelargonium* (NCBI, 2014).

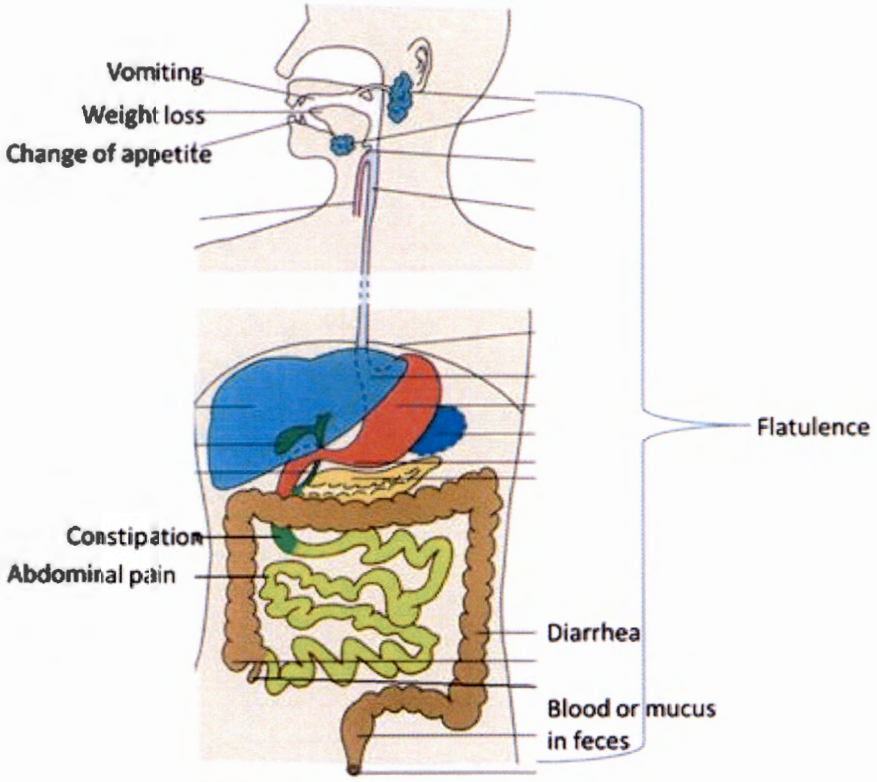


Figure 2.5: Human anatomy of common side effects of *Pelargonium sidoides*

It is crucial to select the host plant meticulously since it will increase the probabilities of isolating novel and beneficial microorganisms.

Selection is based on the following criteria:

- Broad spectrum of biodiversity;
- Untapped habitats;
- Medicinal uses from tradition;
- And high rate of pathogens infestation[2] (Selim *et al.,* 2012).

Endophyte communities are found mainly in untapped habitats such as tropical rainforests with great biodiversity. Therefore, environmental biodiversity of plants growing in an area is directly proportional to house various endophytes (Rai *et al.,* 2012). In a sense, the unique plants that survive in such habitats may produce unique secondary metabolites. Medicinal plants are those that have been used for their medicinal properties. *Sceletium tortuosum* and *Pelargonium sidoides* are medicinal plants, which are indigenous to South Africa. These plants have the ability to yield one or more active constituents for the treatment of various human ailments (Gurib-Fakim, 2006; Mohammad, 2015). Traditional herbal plants such as these have provided scientists with opportunities to explore new therapeutic, more efficacious, more potent, cost-effective and less resistant potential antimicrobial agents (Duval *et al.,* 2014). There are approximately 420,000 plant species on earth (Mukherjee, 2015). Keeping that in mind, the ratio of fungal species to vascular plants is about 1:6, which is estimated at 1.5 million fungal species (Hawksworth, 1991). Medicinal plants provide a distinctive habitation for endophytic organisms more than the non-medicinal plants. Various studies have proven that endophytic fungi isolated from medicinal plants have exhibited novel metabolites that may be used for agricultural and pharmaceutical applications (Kaul *et al.,* 2013). The National Environmental Management: Biodiversity Act (No. 10 of 2004) under the Government Gazette of the Republic of South Africa ensures the protection of species and ecosystems that warrant national protection as well as the sustainable use of indigenous biological resources including plants. The Act is designed to also safeguard the intellectual property of the communities' biological and cultural diversity[3]. Despite the availability of these plant species management policies, some plants including *Sceletium tortuosum* and *Pelargonium sidoides* are on the Red List of South African Plants (SABI) and are endangered which explains the need to generate valuable data outlining their importance.

[2] http://www.superfoods-scientific-research.com/medicinal-herbs/pelargonium-sidoides-side-effects.html

[3] https://www.environment.gov.za/sites/default/files/legislations/nema_amendment_act10.pdf

2.3 ENDOPHYTES

2.3.1 Concept of "Endophytes"

Historically, the word "endophytes" originates from Greek language interpretation states that "endon," means inside or within, and "phyton," which means plant. In 1866, De Bary describes "endophytes" as all organisms colonizing within the plant tissues spending all or part of their life-cycle without causing symptoms of disease to the hosts (Rodrigues, 1996). On the contrary, epiphytes live on plant surfaces. Another definition by Petrini (1991); "*All organisms inhabiting plant organs at some time in their life can colonize internal plant tissues without causing apparent harm to the host*". They inhabit a bulk of the plant parts, including the leaves, stems, and roots. Endophytes are ubiquitously distributed as they have been isolated from virtually every land and marine plant studied (Li *et al.*, 2007). In addition, they cover a broad spectrum and are classified under all phyla.

2.3.2 Categories of endophytes

Fungi, Bacteria, actinomycetes and mycoplasma have been isolated from plants and characterized as endophytes (Kumar *et al.*, 2015; Shekhawat and Shah, 2013).

2.3.3 Diversity of endophytes

2.3.3.1 Host range

Plant-endophytic relationships are ubiquity in nature. They have been identified in wide range of areas including tropical, subtropical, temperate, boreal forests and isolated from herbaceous plants in various territories including extreme arctic, alpine and xeric locations to mesic temperature and tropical forests (Zhang *et al.*, 2006b). Zhang and Yao (2015) isolated 250 fungal strains from high arctic habitats (annual average temperature of -6.0 °C) belonging to major fungal families. The fungi were isolated from four plants growing in those areas (Figure 2.6).

Various investigations have established that endophytes can also be found in marine algae, ferns, lichens, mosses, and vascular plants (Tripathi and Joshi, 2015). Colonization of endophytic fungi have been reported in angiosperms and gymnosperms including tropical palms, broad-leaved trees, estuarine plants, miscellaneous herbaceous annuals, and many deciduous and evergreen perennial host plants (Zabalgogeazcoa, 2008; Zhang *et al.*, 2006).

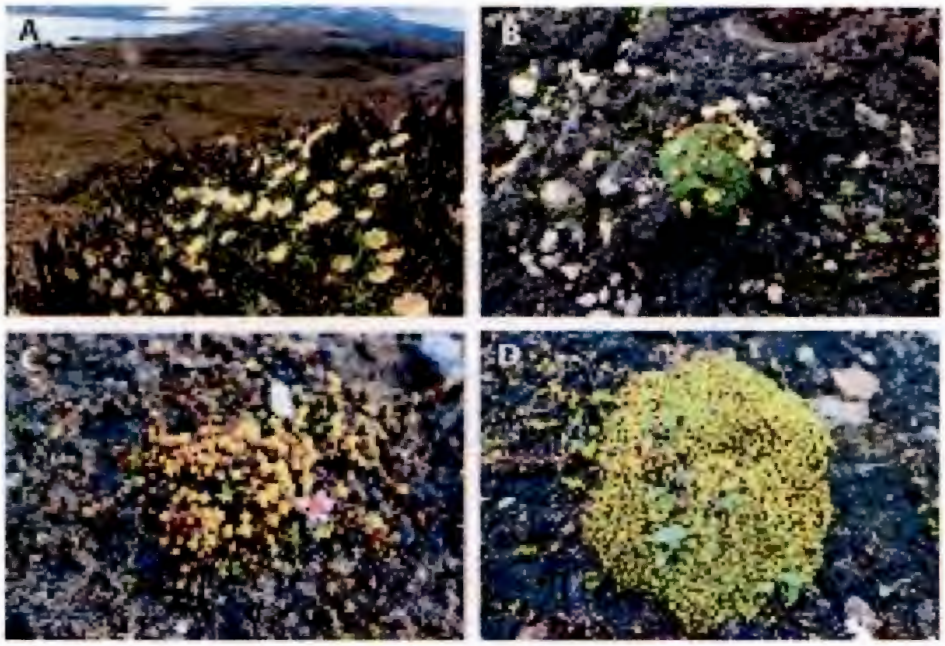


Figure: 2.6: Four plants growing in arctic environments

Medicinal plants provide suitable habitats for endophytes. It has been confirmed that endophytic fungi isolated from medicinal plants possess novel bioactive compounds such as Taxol, which is a multibillion-dollar anticancer drug. Figure 2.7 displays bioactive compound with medicinal potential (Alvin *et al.*, 2014).

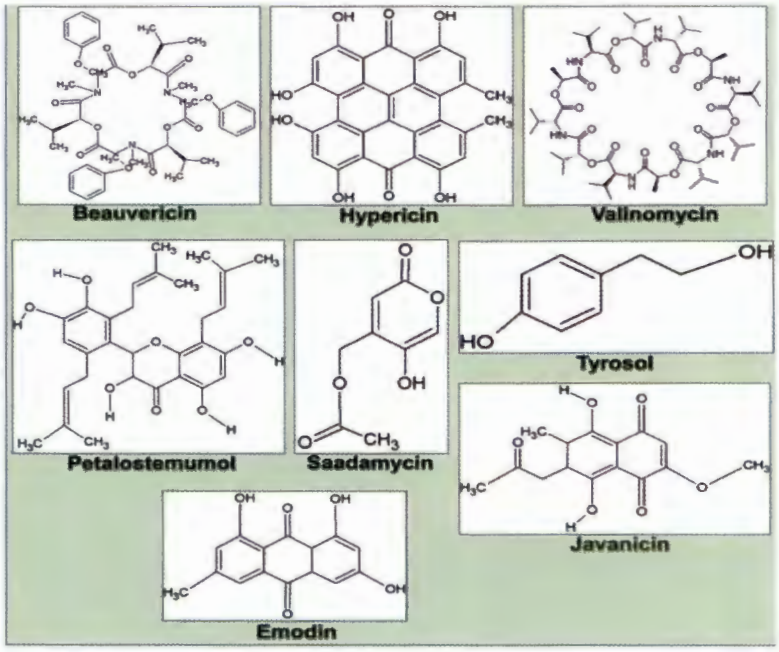


Figure 2.7: Bioactive compounds possessing medicinal properties isolated from endophytes (Alvin *et al.*, 2014)

2.4 RATIONAL OF SELECTING ENDOPHYTIC FUNGI

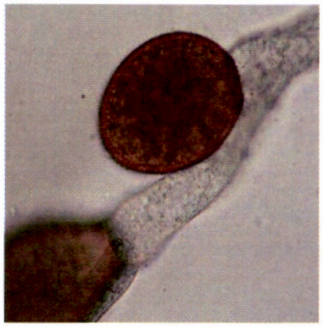
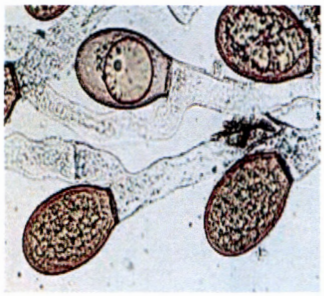
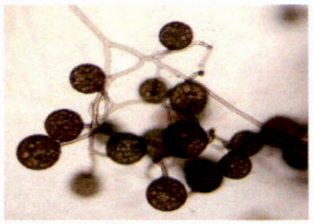
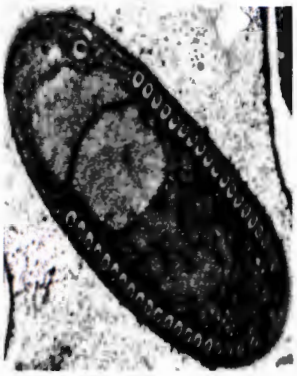
2.4.1 Taxonomy of endophytic fungi

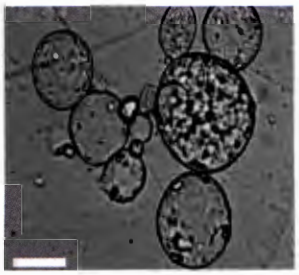
Fungal classification (Class, genus and species) primarily depends on the type of host plant. Ascomycetes and Deuteromycetes are the largest classes of endophytic fungi with plant interaction followed by Basidiomycetes class (Guarro *et al.*, 1999). The phylogenetic classification of fungi divides the kingdom into 7 phyla, 10 subphyla, 35 classes, 12 subclasses, and 129 orders (Zhou *et al.*, 2014).

The seven phyla based on sexual reproductive structures are Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota, and Basidiomycota (Table 2.1).

Table 2.1: Classification of fungi into seven groups (Esser, 2014)

	Phylum Group	General characteristics	Asexual/Sexual Reproduction	Number of Species
	1. Ascomycota	Also referred to as the sac fungi. Morphological diversity of the ascomycota group. Unicellular yeasts to complex cup fungi are also included.	In sexual reproduction, formation of spores called ascospores. Sexual cycle does not form ascospores.	They are approximately over 64,000 species, which makes them the largest phylum group.
	2. Basidiomycota	They are filamentous fungi with hyphae (except for yeasts). Blastocladiomycota are "higher fungi'.	Development of basidia which are specialized club-shaped (Sexual). Basidiospores are formed at asexually reproduction.	This is the second latest phylum group comprising of 31,515 species.

	3. Blastocladiomycota	They have motile spores and gametes. Morphology characteristics differ significantly. Coelomycetaceae are simple, unwalled. The blastocladia are monocentric.	Sexual reproduction occurs by fusion of two sexual gametes, the process is called anisogamy. Zoospores produced though asexual reproduction.	There are about 1,000 species.
	4. Chytridiomycota	"Little pot", refers to the structure containing unreleased zoospores. Motile stages exist. Also, the oldest fossil fungi so far known forms "chytrid-like".	Diploid zoospores are asexual spores And their gametes are the only fungal cells known to have a flagellum.	Approximately 1,000 different chytrid species.
	5. Glomeromycota	Glomeromycota procedure arbuscular mycorrhizas (AMs) with the roots or thalli.	Reproduce only asexually through blastic formation of the hyphal tip to produce spores.	Estimation of 230 described species.
	6. Microsporidia	They are unicellular parasites and can form spores. Known once are protozoans or protists,	Nuclear division takes place in asexual reproduction. Sexual reproduction encompasses autogamous fusion and reorganization of genetic material.	1,500 known species.

	7.Neocallimastigomycota	Anaerobic fungi, which are found in the digestive tracts of herbivores.	Asexual reproduction in the stomach by zoospores that bears a kinetosome. No sexual reproduction.	There are 20 species in six genera.

2.4.2 Latent pathogenicity to mutual association

Microorganisms residing within the host plant may have various interactions, alternating from mutualistic symbiosis to latent pathogenicity (Carroll and Tudzynski, 2012, Schulz and Boyle, 2005). These associations are characterized as plastic because it's dependent on the genetic natures of both partners. Furthermore, the balance depends on microbes\plant development stage, availability of nutrients and environmental factors (Hardoim *et al.,* 2015). Freeman and Rodriguez (1993) confirmed the role of genetic disposition by a single mutation resulting in loss of virulence, hence transforming a pathogenic fungus *Colletotrichum magna* into an endophyte. Another study was conducted using bacterial isolates to prove that the virulence genes (Buonaurio *et al.,* 2015, Schulz *et al.,* 2006, Kobayashi and Palumbo, 2000) influence the balance between pathogenicity and mutualism. With the endophyte-host interaction a balance must be maintained, if disturbed by a decrease in plant defense or an increase in fungal virulence, disease develops in the plant. Therefore, the endophyte will act as an opportunistic pathogen (Singh *et al.,* 2011). Metabolites are synthesized by both the host plant and endophyte, thus toxic to each other. Some metabolites produced by endophyte regulate metabolism of the host, enforcing balance. To establish this, the endophyte must first overcome with the epiphytes then compete with the pathogens to finally colonize the host (Chen *et al.,* 2016). Consequently, the host plant benefit from the mutualistic correlation by gaining extra protection against pathogens and herbivores, and improved growth and competitive advantage over uninfected plants. The host genotype and endophyte population contribute to the host's response to both biotic and abiotic factors (Singh *et al.,* 2011), thus improving the host physiological status.

2.4.3 Physiological and ecological roles

It is apparent that endophytic microorganisms play a significant role in the host plant's physiological and ecological aspects (Tan and Zou, 2001). The endophytes obtain nutrients, protection and propagation opportunities from the host plants (Clay and Schard, 2002). Furthermore, the host plants become more resistant to unfavorable environmental conditions. This will allow them to adapt well against biotic and abiotic stress as compared to endophyte-free counterparts. In nature, constantly plants are being challenged by various attacking agents. The symbiotic relationship improves plant's resistance against insect, pests and herbivores (attacking agents). Other benefits include drought adaptation, increased competitiveness, enhanced photosynthetic rates and tolerance to stressful factors such as heavy metal presence, low pH, high salinity and microbial infections (Zhang *et al.*, 2006; Mandyam and Jumpponen, 2005; Waller *et al.*, 2005, Lewis, 2004). The physiological attributes are elaborated below.

Berg and co-workers (2006) concluded that plants are metaorganism vessels thus comprising of numerous microbial cells as compared to the plant cells. Biotic and abiotic conditions contribute to the structure of the plant. There is a clear association between the metabolisms; plant morphology and their microbiota that are naturally linked to each other. Therefore, the intense connections lead to maintaining the functions of the holobiont (Fig. 2.8).

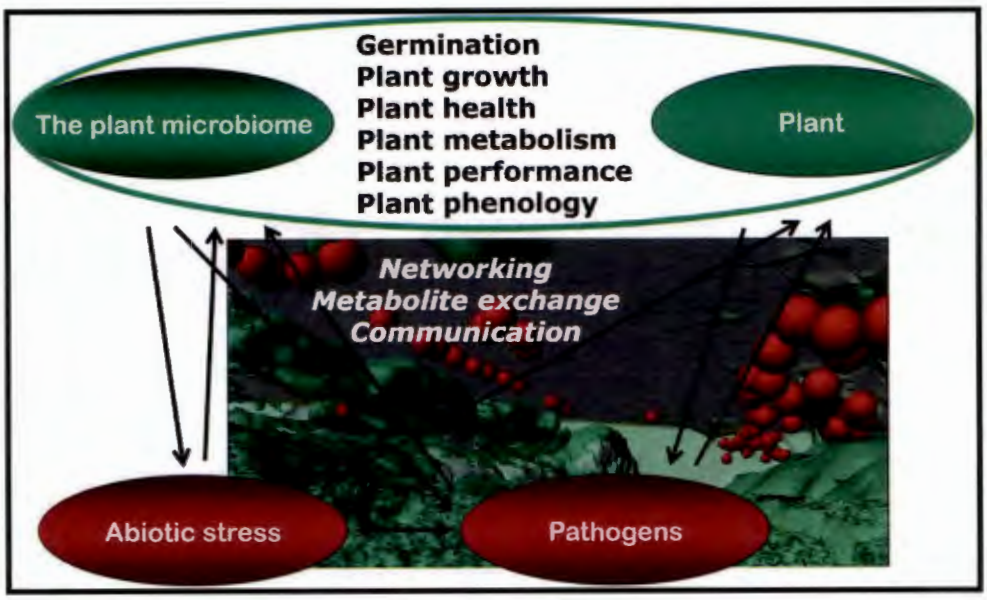


Figure 2.8: Model visualizing the interplay within the plant holobiont

2.4.3.1 Endophytic fungi as a growth promoter

Endophytic *Trichoderma* sp. are some of the most commercially used growth promoters in the agricultural industry (Mukherjee, 2015). These endophytes increase the biomass yield of plants by enhancing their ability to produce or utilize fewer resources (Kauppinen *et al.,* 2016). Peŝkan-Berghőfer *et al.* (2004) found that growth is stimulated when herbivore feeding patterns are altered by producing some secondary metabolites and/or direct interaction with the phyto-hormomes, cytokines as well as other growth-promoting substances. Phyto-hormones responsible for plant growth include indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPYA), cytokines and vitamins (Costacurta and Vanderleyden, 1995).

Various studies have proven, that plants that are infected with endophyte frequently grow faster than non-infected ones (Tan and Zou, 2001). In green house experiments conducted on *Fesuca obtuse* and *Poa sylnestris* (woodland grasses) that were infected with endophytes, they showed more productively than the non-infected plants (Bier, 1995). *Williopsis saturnus* and *Piriformospora indica* endophytes have been proven to improve the growth of many plant species (Sirrenberg *et al.* 2007; Nassar *et al.* 2005). Endophytes also regulate processes such as nitrogen fixation, phosphorus assimilation and the host's uptake of nutritional elements (carbon-nitrogen ratio) (Zhang *et al.,* 2006; Nassar *et al.,* 2005; Waller *et al.,* 2005; Tan and Zou, 2001). A study done by Shahollari *et al.* (2007) re-established that the endophytic fungus (*Piriformospora indica*) contributes to plant growth and higher seed production (*Arabidopsis thaliana*); and a leucine-rich repeat protein was the main cause for this effect. Plant-endophytic relationship assists the host in nitrogen absorption and carbon management by endophytic fungi with photosynthetic cells (Barrow *et al.,* 2007). Glutamine synthetase is an active enzyme responsible for nitrogen utilization. These enzymes dominate in endophyte infected tall fescues as compared to the uninfected ones in low nitrogen soil. It is evident that the endophytic fungi promote the growth of plants by ecological adaptation of their host plants. The physiological structure in terms fitness is increased hence protecting the plants from biotic and abiotic stressful environments (Pandey *et al.,* 2017).

There is a broad spectrum of phytohormones such as auxin (IAA), cytokinins (CKs), abscisic acid (ABA), ethylene (ET), gibberellins (GAs), salicylic acid (SA), brassinosteroids (BRs), and jasmonates (Jas) that plays a vital role in plant development and plastic growth. Abscisic acid (ABA) is responsible for the adaptation of abiotic-stresses. They are referred to as stress hormones and aid in

plant promotion, prevent germination, maintenance of seed dormancy, growth regulation, stomatal closure, fruit abscission, besides mediating abiotic and biotic stress responses (Wani *et al,* 2016).

2.4.3.2 Abiotic stress tolerance

2.4.3.2.1 Heat, low pH, metal and high salinity tolerance

Plants grown naturally are exposed to various physico-chemical stresses, which depend upon the geographical location and type of soil. Plants infected with endophytes have been proven to have more resistance towards heat, low pH, metal and salt content (Pandey *et al.,* 2017). The plant\endophytes interactions increase the heat tolerance (Redman *et al.,* 2002). It was observed that *Dichanthelium lanuginosum* (grass plant) infected with *Curvularia* sp. fungus could help the plants to tolerate high temperature of 65 °C for 10 days. The negative control (non-infected plants) died at this temperature however the infected plants survived. Surface sterilization was performed on the roots and leaves of all the surviving plants hence re-isolation of the fungus. From this it may be concluded that both the fungus and plant were protected from the thermal conditions. The rationale behind this was that the cell-wall melanin pigment embedded in the fungus could disperse heat ray along the hyphae and/or forms a complex with oxygen radicals produced during heating. The endophytic fungus may act as a 'biological trigger' reacting to stress environments more swiftly and strappingly than non-symbiotic plants (Redman *et al.,* 2002). Yamaji *et al.* (2016) investigated the potential for *Clethra barbinervis* Sieb to tolerate high heavy-metal concentrations. Various root endophytic fungi (*P. fortinii, R. veluwensis* and *Rhizoscyphus* sp.) are liable for this. Endophyte-colonized plants exhibit more salt resistance as compared to the non-colonized (Azad and Kaminskyj, 2016).

2.4.3.2.2 Drought tolerance

Over the past few decades' climatic conditions have dramatically become drier and hotter. The environmental dynamics do influence the plants' growth and survival rates. Endophytes place plants at a great advantage in such dry territories. It is reported in many studies that plants infected with endophytes are drought tolerant (Zhang *et al.,* 2006; Tan and Zou; 2001). In a study conducted on tall fescue, endophytes were infested in fledgling merismatic and elongated leaves. This assisted the plant to retain the water and resist the drought by developing low osmotic potential (Elmi *et al.,* 2000). Hesse and co-workers (2005) reported that plants infected with endophytes had increased

drought resistance while improving plant persistence and seed production. It concluded that preservation of low bud growth and enhanced root growth could improve plant survival predominantly in dehydrated regions (Gundel *et al.* 2006) established that the presence of the endophyte may restrict seed germination when water is limiting, reducing the risk of seedling death. West *et al.* (1990) justified and proved the hypothesis that drought tolerance mechanisms due to endophytes is caused by osmotic adjustment, which may aid in plants survival to maintain sufficiently high turgor in growing zones, retard desiccation and allow rapid resumption of leaf growth upon relief of stress. In addition, endophytic fungi having the potential to withstand drought showed other contributing attributes such as storage and secretion of sugars and alcohols that may protect host enzymes and membranes from desiccation damage, leading to the adjustment of leaf structures and a reduction of transpiration losses (Assuero *et al.,* 2006; Zhang *et al.,* 2006).

A study conducted on barley using the fungus *Piriformospora indica* exhibited salt tolerance. The investigated fungus was inoculated in barely seedling for 2 weeks to control salt content (100 mM NaCl). The infected barely formed greater biomass as compared to the non-infected plants under similar conditions. Furthermore, the non-infected plant showed an increase in leaf chlorosis and diminished its growth rate (Waller *et al.,* 2005). Over the last decade, researchers have validated that the host plant increases its resistance against invasion by herbivores, insects, and pathogens (Naidoo *et al.,* 2014; Goggin, 2007; Lambert and Casagrande, 2006) because of the endophytic communities.

2.4.3.3 Endophytic fungi as an Insect repellant

Insect are well-known for their intense feeding habits on plants. In response, plants protect themselves from insects by producing bioactive metabolites (Sánchez-Sánchez and Morquecho-Contreras, 2017; Clay and Schardl, 2002). The elm tree that is inhabited by the fungus *Phomopsis oblonga* is protected from the beetle *Physocnemum brevilineu* (Webber, 1981). The mechanism of repulsion is explained by the ability of the fungus to produce or induce the plant to produce specific metabolites that control the vector. The insect (vector, *P. brevilineu*) was considered a pathogen carrier of *Ceratocystis ulmi* of the elm Dutch disease. There is confirmation that the plant/endophyte relationship reduced the attack occurrence on Argentine stem weevil, *Listronotus bonariensis* in numerous rye grasses (Gaynor and Hunt, 1983). Research has showed a connection between endophytic fungi, the survival rate of the plant, growth rate and feeding pattern of insects. The fungus

Acremonium lolii produced lolitrem B illustrated in Figure 2.9. This metabolite is a powerful toxin, which reduces the insect attacks against *Lolium perenne* infected plant (Pretidge and Gallagher, 1988). N-formilonine and paxilline analogues are secondary metabolites produced by the fungus *Neotyphodium* sp., which are responsible for the killing *L. bonariensis* and other insects. The fungus was isolated from *Echinopogum ovatus* plant (Miles *et al.*, 1998). A similar study showed that heptelidic acid and rugulosine obtained from *Phyllostricta* sp. and *Hormonema dematioides* (endophytes of woody plants) had insecticidal activity (Bills *et al.*, 1992).

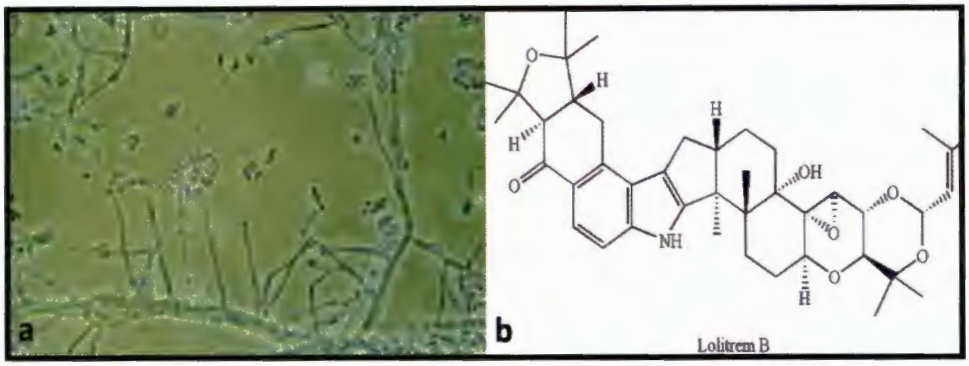


Figure 2.9: Morphological image of *Acremonium lolii* (a) with secondary metabolite lolitrem B (b)

2.4.3.4 Protection from Herbivores

Initially, interest arose from a Bacon study in 1977, where the link between toxicity of an endophytic fungus and herbivorous domestic mammals was formed (Bacon *et al.*, 1977). Recent studies confirmed that endophytic fungi have the ability to protect their host plants from herbivores (Robert and Andrae, 2005). Evidence to this effect was when animals feeding on endophyte infected plants, specifically suffered from a variety of diseases. Tall fescue toxicosis develops in livestock and horse after consumption of *Festuca arundinacea* grass, which was infected with endophytes. Figure 2.10a represents some serious consequences in cattle caused by the *F. arundinacea* grass in Figure 2.9.b.

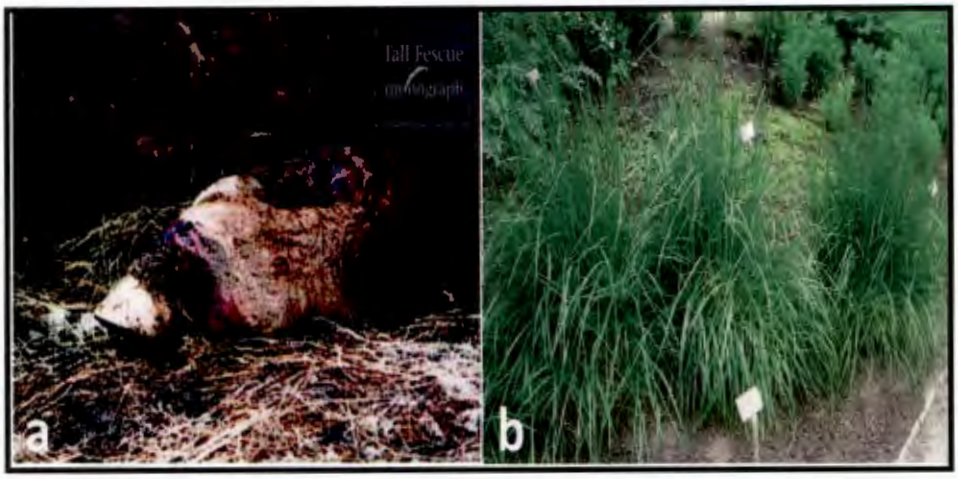


Figure 2.10 :(a)[4]Cattle limb infected with fescue (photo: David Bohnert). (b)[5]*Festuca arundinacea* grass cultivated in Berlin Botanical Garden, Germany

The effects of ingesting endophyte-infected grasses includes high core body temperature, increased respiration, vasoconstriction (narrowing of the blood vessels resulting from contraction of the muscular walls of the vessels), low heart rate, change of metabolism, agalactorrhea (absence of or faulty secretion of breast milk following childbirth), suppression of immune system, decrease in forage intake and weight loss. The most problematic side effects are low pregnancy rate, thickened placenta, dystocia (birthing difficulty) and retained placenta (Robert and Andrae, 2005). The fungus responsible was found to produce metabolites such as alkaloids, lysergic acid amides and ergopeptines. According to Robert and co-workers (2005), high levels of ergot alkaloids were found in infected leaves, grass and seeds.

2.4.3.5 Protection from Pathogens

Since the discovery of antibiotics, fungi have been and still are a rich source of antimicrobial agents. Plant/endophytes association either prompts or triggers the defense mechanisms of plants (Arnold, 2003). This mutual relationship assists the plant's defense system to respond faster than non-symbiotic plants after pathogen invasion (Rodriguez *et al.*, 2004). The defense mechanism is actively provoked and encompasses oxidative eruption, rapid and hypersensitive responses, accumulative phytoalexin and synthesis of pathogenisis-related (PR) proteins. Arnold and co-workers (2003) investigated *Theobroma cacao* tree disease caused by *Phytopthora* sp. (Figure 2.11), a known

[4]http://forages.oregonstate.edu/tallfescuemonograph/Figures/16-3

[5]https://commons.wikimedia.org/wiki/File:Festuca_arundinacea_-_Berlin_Botanical_Garden_-_IMG_8655.JPG

pathogen for black pod disease, and proved that there was a significant decrease in the incidence of the disease. The experiment was conducted using cacao seedlings; the association was formed from the absence or presence of the endophytic fungus cacao and the reduction of the number of incidence and its symptoms. It was stated that the protection was predominantly in the infected tissues. In addition, mature leave appeared to have superior protection against fungal pathogen than young leaves.

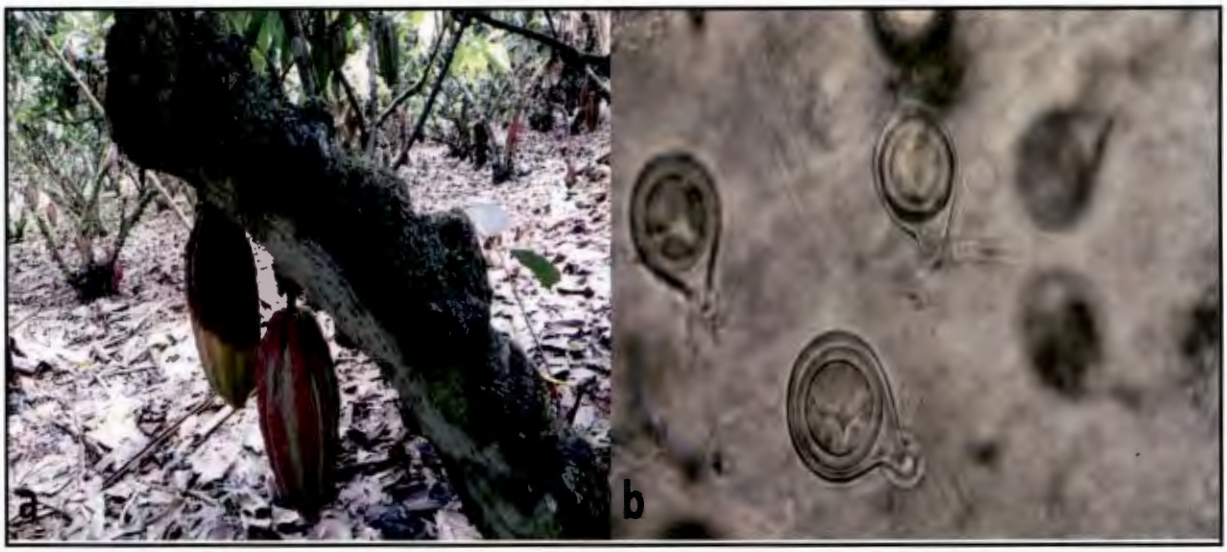


Figure 2.11: (a) *Theobroma cacao* tree disease caused by *Phytopthora* sp, (b) Morphological structure of *Phytopthora* sp.

The study also showed that secondary metabolites secreted by the fungus were primarily responsible for the resistance via hypersensitive reaction, host-cell response and cell-wall-association defense. Waller *et al.* (2005) conducted a similar study using *Piriformospora indica* fungus, which assisted in the resistance to powdery mildew infection by the fungus *Blumeria graminis* f. sp. *Hordei* in barley. This was active plant defence stimulated by the endophyte. Proper classification and identification is a key element of fungal activity.

2.5 PLANTS-ENDOPHYTIC INTERACTIONS

Symbiotic or mutualistic to antagonistic or slightly pathogenic interactions occur between endophytic fungi and their host plants. Scientists now focus on the functional importance of the relationship between host and the endophytes. This emphasizes the capability to adapt stressful environments throughout the evolutionary time (Hopkins *et al.*, 2014). This is also highlighted in section 2.4.2 on the benefits of endophytes in situations of drought, heavy metals, disease, heat, and against

herbivory, and/or promote growth and nutrient acquisition. Mutualistic fungi contribute to the fitness welfare, which is directly proportional to the plants' ability to adapt to stressful conditions. Furthermore, research does not take into account the abundance of the plant-fungi symbolic relationship (Schulz and Boyle, 2005). Research has proven that certain plants lacking endophytic fungi are incapable of fighting off abiotic and biotic stresses (Begum and Tamilselvi, 2016). Microbial metabolites are responsible for a broad range of characteristics including the ones stated in sections 2.4.2 and 2.7.

2.6 IDENTIFICATION OF FUNGAL ISOLATES

In this section, we review the methodology used in this thesis to identify the fungal isolates. Furthermore, the literature will compare the traditional technique with the modern and most preferred method, the molecular markers (DNA).

2.6.1 Fungal Morphology

Historically, identification of fungi relied solely on the morphological structures whether microscopic or macroscopic.

2.6.1.1 Microscopic structures

These structures are not visible with the naked eye. Majority of these fungi consist of filaments, the hyphae; which are tubular, thread-like structures that grow by apical extension. Hyphae can be up to 10 µm in diameter and up to several centimeters in length and are linked end-to-end. In addition, these specialized hyphae are used to absorb water and nutrients from living hosts. Known plant-parasites include numerous parasitic fungi. A network of hyphae will cluster together to form a mycelium. Furthermore, in a laboratory environment when culturing a fungus to form a network of mycelium on solid agar in a circular form, it is referred as colonies. Bead-like asexual structures branching out laterally from the hyphae are called condidiophores. They are arranged similar to beads on a string. The anchor of the hyphae is named stolon. Once unfavourable conditions arise, the fungi reproduce asexually by formation of spores. The spore can be used as an identification tool. On the contrary, not all fungi are microscopic in nature; others like the mushroom are macroscopic with a microscopic stage.

2.6.1.2 Macroscopic structures

The fungal structures are large enough to be seen with the naked eye. These features are unique and are used for diagnostic purposes or classification. Similar structures such as mycelium and hyphae exist but are visible. Reproduction is mainly sexually by using specialized structures especially in ascomycetes and basidiomycetes. The fruiting bodies are cup-shaped enclosing the hymenium in the apothecium species. The cup-shaped feature bears spore cells. A well-known example of a macroscopic fungus is the mushroom (*Armillaria solidipes*). The colony dimensions will be used to estimate the age of *Armillaria solidipes* specie. The oldest specie was 9,000 years which stretched over an area of more than 3.5 square miles, (900 ha). Figure 2.12 illustrates similar structures that found are exhibited by the microscopic and macroscopic fungi.

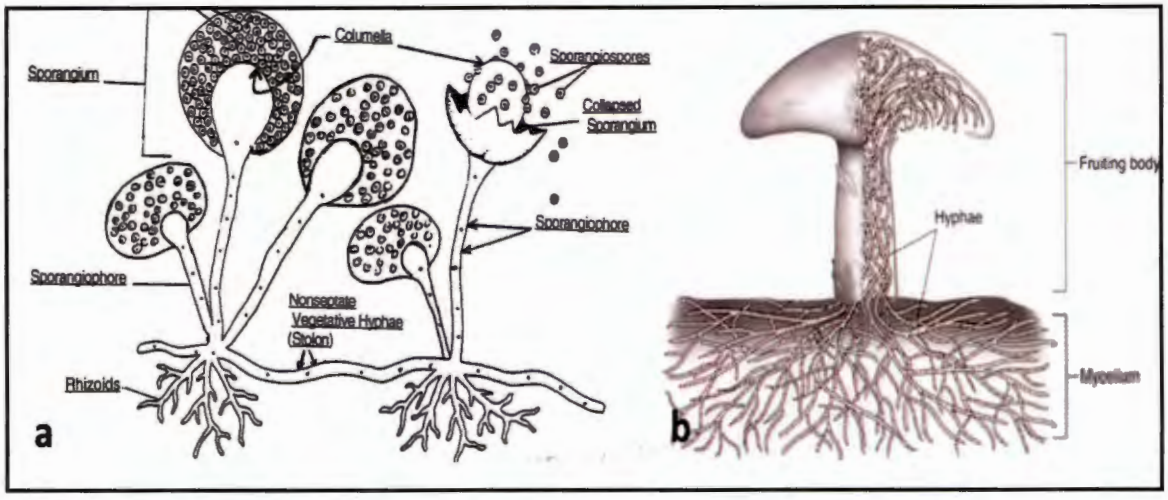


Figure 2.12: Morphological features of the fungi (a) Microscopic features, (b) Macroscopic features

Morphological identification is sometimes difficult to identify isolates to species level. Modern method utilizes genetic identification.

2.6.2 Molecular identification

It is sometimes difficult to identify isolates to species level using morphological identification. For this reason, modern technologies using genetic approaches provide rapid and reliable tools for the identification of the isolates. Molecular techniques have made it possible to identify living things meticulously, to species level (by using a single cell). Polymerase chain reaction (PCR) has become a universal tool to identifying organisms to species as well as strain level (Adeyemo and Onilude, 2014, Shokohi *et al.,* 2010). In the agricultural sector, prompt identification is essential for pathogenic microorganisms, as they must be controlled and if need be quarantined. Furthermore, early detection will place farmers in a better position to save most of their commodities (Tsedaley, 2015). Unlike

conventional procedures, there must be an appropriate understanding of genomic organization and species-specific molecular markers to distinguish the fungi. Schoch *et al.* (2012) found that internal transcribed spacer (ITS) primers are the most suitable for DNA barcode marker. The target area is located between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the transcribed region in the polycistronic rRNA precursor transcript. Upon amplifying this region of the fungal isolates, the primary fungal barcode was established. This method has been used from the 1990s as an evolutionary tool and to establish the phylogenetic relationship (Donoghue and Yang, 2016).

Furthermore, all the above-mentioned activities are due to the ability of the endophytic microorganism to produce metabolites.

2.7 MICROBIAL METABOLITES

Secondary metabolism is primarily responsible for the production of metabolites that benefit the plants and endophytes as well as mankind. Section 2.5, outlined the role and pathways involved.

Metabolism is defined as the overall sum of all the chemical reactions and the day-to-day functioning in a cell (Berne *et al.,* 1998). Cell growth, reproduction, structural maintenance and protection against environmental factors are all due to the metabolic processes. This is critical for all life forms and forms the basis of life as we know it. Metabolic processes may be divided into catabolic and anabolic reactions. Catabolic processes are responsible for a cell's energy yield, such as the breakdown of carbohydrates in cellular respiration.

The following are examples of catabolism of some substances

- Polysaccharides (CHO) breakdown by hydrolysis into
- Monosaccharides are broken down by glycolysis
- Lipids are broken down into Glycerol + Fatty Acids (hydrocarbons; lots of energy)
- Glycerol carbon backbone is broken down through glycolysis

- Fatty Acids broken down by beta oxidation to form two Carbon Fragments (Acetyl CoA) which may join Kreb's Cycle
- Protein uses hydrolysis producing amino acids (contain amino group, NH3) while deamination leads to carboxylic acid which may result in Kreb's Cycle

In contrast, the anabolic processes utilize this energy to generate protein and nucleic acids that are vital components of the cell.

- Anabolism example is Photosynthesis

$6CO_2 + 6H_2O + Energy \rightarrow C_6H12O_6 + 6O_2$

In microbial metabolism, the primary and secondary processes take place that is essential for the living cells. The primary processes include both anabolic and catabolic metabolisms, which are responsible for cell maintenance and proliferation. On the other hand, secondary metabolites are specified as non-essential for cell growth (Saad *et al.,* 2017). Table 2.1 summaries the variance among the two processes. Williams *et al.,* (1989) stated that secondary metabolites might enable the microorganism to compete more efficiently with other forms of life. In 1873, Sachs was the first scientist to identify secondary metabolites, natural products as he referred them. To date, there are approximately 50 000 microbial metabolites known to man.

Table 2.2: Comparison between Primary and Secondary Metabolism

Primary Metabolism	**Secondary Metabolism**
Essential for growth and development	Not essential for growth and development
Uniform	Variable
Constant	Diverse
Constitutive	Adaptive
Relatively simpler structures.	Highly complex structure and a large number of specific enzymatic reactions for synthesis.
Less genetic variation	High genetic variation

The main byproducts from fungal metabolism are the metabolites such as amino acids and fatty acids and peptides. In addition, penicillin, zearalenone, and statins are classified as secondary metabolites (Singh, 2016).

2.7.1 Role of microbial metabolites

Secondary metabolites are produced in nature for various functions. There is still more to discover in the flied of expertise. Essential metabolites such as polysaccharides, proteins, fats and nucleic acids are compulsory for the microbial growth, referred to as the building blocks. Chemotaxonomy is a method of biological classification based on similarities in the structure of certain compounds (secondary metabolites) among the organisms being categorized. Although secondary metabolites are non-essential for growth nevertheless they offer a competitive advantage over other species competing for nutrients (Singh, 2016).

2.7.2 Production of secondary metabolites by fungi

There are strict environmental conditions required for the production of secondary metabolites in both the natural surroundings and in the laboratory. These include temperature, light, carbon, nitrogen levels, pH for normal growth and sporulation of the fungi (Brakhage, 2013). According to Calvo *et al.* (2002), the production of secondary metabolites occurs in the sporulation stage of the fungi. Therefore, in a laboratory situation it is said that the secondary metabolites are formed at the end of the exponential growth phase, where growth is restricted by essential nutrients. Ideally, if there are continuous nutrients, there will be endless production of secondary metabolites (Zimmermann and Entian, 1997). Secondary metabolites help the fungi to survive and reproduce by enhancing their own fitness or decrease a surrounding organism's fitness. As reported by Nűtzmann *et al.* (2011), several regulatory genes or transcription factors are involved in the generation of secondary metabolites (Figure 2.13) and there are a large number of secondary biosynthetic pathways that remain silent or inactive under normal laboratory conditions.

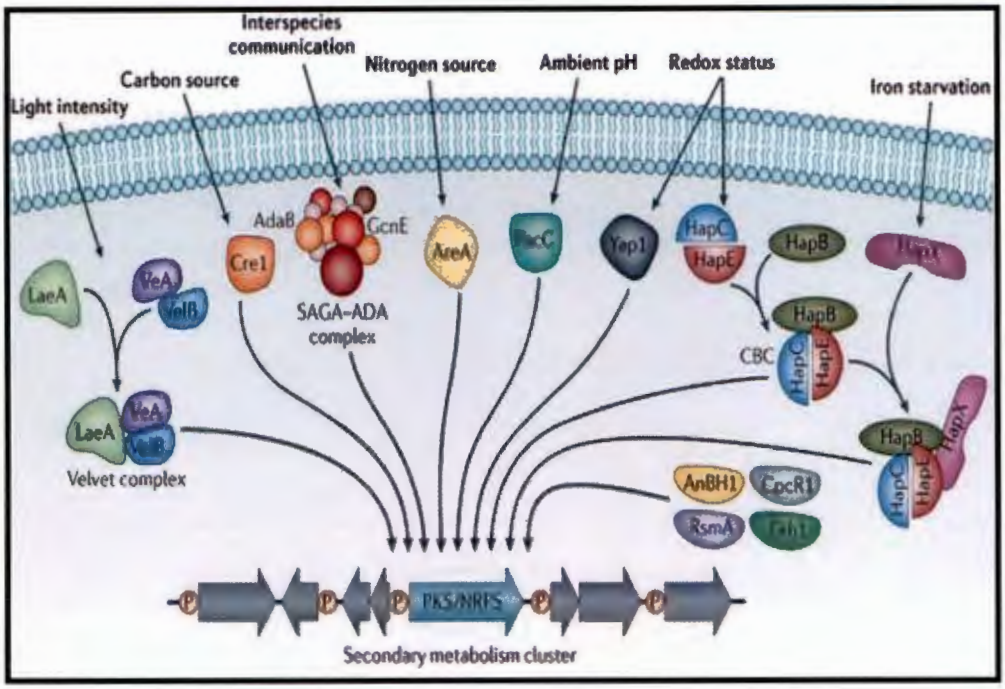


Figure 2.13: Genes involved in the regulation of fungal secondary metabolism[6]

Posttranslational modification is responsible for the regulatory of secondary metabolism and their subsequent synthetic pathways. Histone modification by acetyltransferase of the complexes controls microbial metabolites (Nützmann *et. al.*, 2011). Secondary metabolites are produced by various biosynthetic pathways, which are mentioned below.

2.7.3 Pathway of secondary metabolites

In this section, the literature will focus on the three, well documented and pathways. These biological pathways produce secondary metabolites as a major by-product. The metabolic pathways are illustrated in Figure 2.13.

2.7.3.1 Mevalonic acid pathway

Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the byproducts of this pathway. These molecules are made-up of five-carbon molecular structure, which are the building blocks. The molecules are used to make isoprenoids also called terpenoids, which are organic compounds. The diverse class is over well over 30,000 biomolecules such as cholesterol,

[6] https://microbewiki.kenyon.edu/index.php/Secondary_Metabolites_in_the_*Aspergillus*_Genus

heme, vitamin K, coenzyme Q10, and all steroid hormones. Generally, the precursor used in mevalonic pathway is an acetyl-Co A; another alternative used by certain fungi is leucine (Shidoji and Ogawa, 2004). In the process of forming mevalonic acid, it begins with two acetyl-Co A molecules condensing to yield acetoacetyl-CoA.This is followed by condensation of a third acetyl-Co A to produce HMG-CoA (3-hydroxy-3- methyl-glutaryl-CoA). Reduction occurs when leucine is used as a precursor and converting HMG-CoA to (R)-mevalonate or mevalonic acid.

2.7.3.2 Polyketide pathway

There are several secondary metabolites produced from this biosynthetic pathway as compared to the others (Daley *et al.*, 2017). Polyketide biosynthetic pathway is activated by organic acids. Condensation of a single molecule of acetyl-CoA with at least three malonyl CoA produces Polyketides. Furthermore, the acetyl unit binds to a growing acyl chain resulting in a protein. The by-products of the biosynthesis are a β-ketoacyl thioester, which is further reduced to a β-hydroxyacyl intermediate with a release of carbon dioxide (Caffrey, 2012).

A continuation of this, may lead to a long-chain fatty acid. Such an energy driven process requires active enzymes to catalyze the process. This will include the starter acetyl unit which is thioester-linked to the active site cysteine thiol of the ketosynthase (KS) enzyme. This is followed by acyltransferase (AT) which removes the dicarboxylic acid extender from CoA and places it on the phosphopantetheine thiol of the acyl carrier protein (ACP). A condensation process uses the KS catalysis. Finally, the thioesterase (TE) is catalyzed for chain termination. Polyketide biosynthesis utilizes the equal number of enzymes however producing higher diversity of structures. The metabolites polyketides have added functional benefits (ecological and physiological role) than fatty acids; hence can be used in other reactions.

2.7.3.3 Shikimate-Chorismate pathway

The shikimate chorismate pathway (shikimic acid pathway) is a biosynthesis route for producing aromatic amino acids (phenylalanine, tyrosine, and tryptophan). This process takes place in microorganisms (bacteria, fungi, algae, some protozoan parasites) and plants not animals. Nonetheless, the animals feed on the plants/microbes providing the essential phenylalanine and

tryptophan needed for survival (Herrmann, 1995). Formation of shikimate 3-phosphate is catalyzed by shikimate kinase, which is an ATP-dependent phosphorylation.

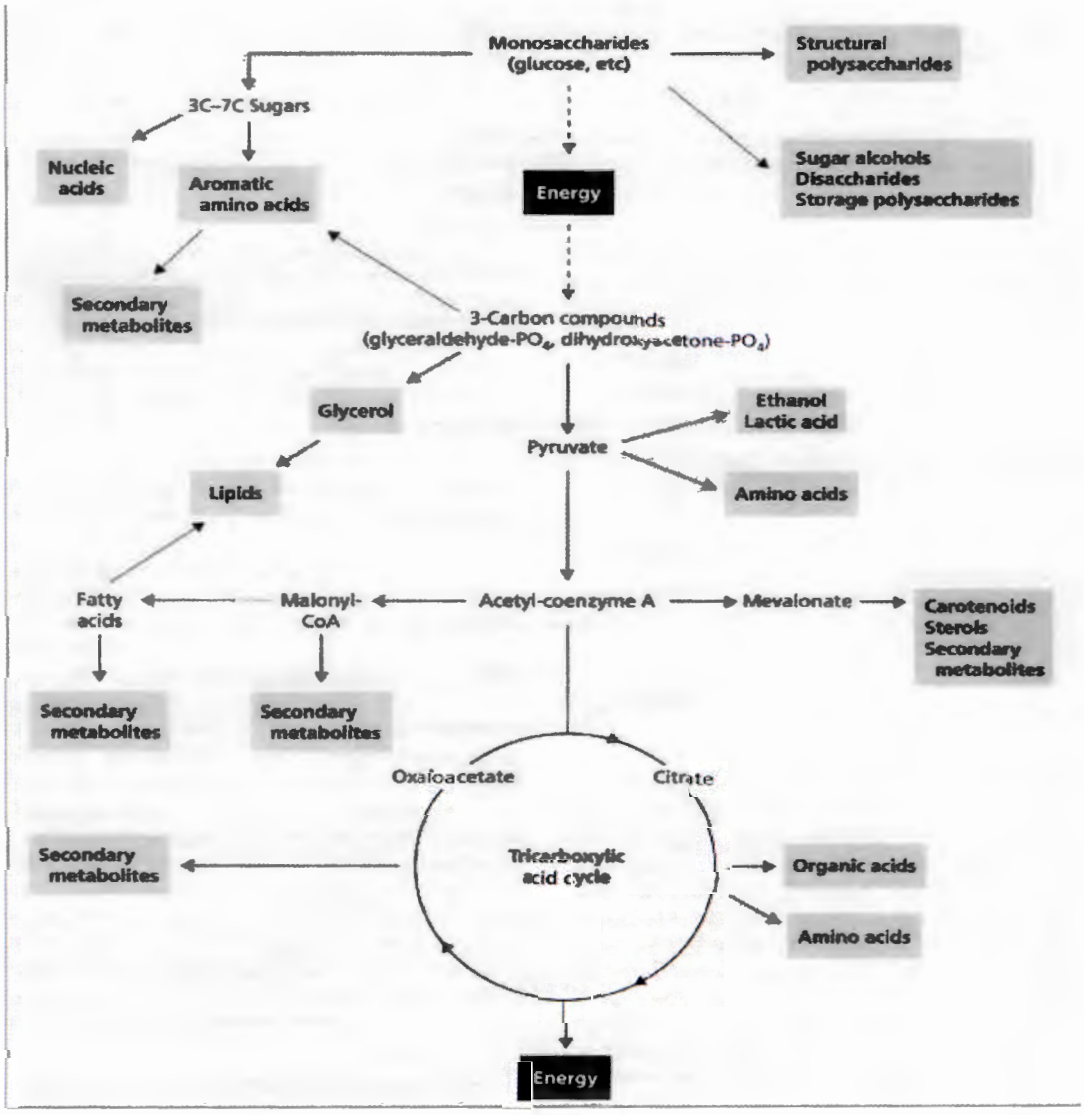


Figure 2.14: Metabolic Pathways comprising of all the individual reactions[7]

Combination of shikimate 3-phosphate with phosphoenol pyruvate to yield 5-enolpyruvylshikimate-3-phosphate is assisted by the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase. Figure 2.14 illustrates the various metabolic pathways involved in producing metabolites. Identification of the metabolites is crucial for large-scale synthesis.

[7] http://archive.bio.ed.ac.uk/jdeacon/FungalBiology/chap7_im.htm

2.7.4 Identification\Characterization of secondary metabolites

Analytical techniques used in the current investigation include Gas chromatography-mass spectrometry (GC-MS). Furthermore, there are other methods that can be utilize to identify the chemical constituents in a concoction. Ultraviolet spectroscopy and infrared system are also highlighted and used for the same purpose. In the present study, GC-MS was used to identify the composition of the fungal exxtracts.

2.7.4.1 Mass spectrometry

Gas-chromatography mass spectrometry is an analytical method used to identify various substances within a test sample. The fundamental principle is based on measuring the mass of a pure compound using mass spectrometry (MS). The gas or liquid chromatography (GC or LC respectively) system is usually coupled with the mass spectrometer. Subsequently, LC system coupled with electrospray ionization (ESI) MS system works on the same principles. Except for sample preparation, the sample must be dissolved in a solvent before introducing it in the LC system. Downstream, the sample is converted into gaseous phase prior to entering the mass analyzer (Kebarle and Verker 2010; Pavia *et al.,* 2009). This analytical technique has dramatically gained popularity because of the high reliability, sensitivity and selectivity. Furthermore, it is high-throughput and automated analysis makes it an ideal way to characterize the fungal metabolomics (Madla *et al.,* 2012; Scalbert *et al.,* 2009).

2.7.4.2 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance spectroscopy utilizes the magnetic characteristics of certain atomic nuclei. Therefore, it analyses the physical and chemical properties of atoms or the molecules in which they are contained. The 13C and 1H NMR outputs support data gathered by mass instruments for appropriately characterizing metabolites. More information may be provided by 135-DEPT spectrum relate proton signals with their corresponding carbon signals, or to determine whether they belong to an amine or hydroxyl group, heteronuclear correlation spectroscopy is employed (Balci, 2005). Association for protons and carbons extrapolated in a two dimensional plot is conducted by Heteronuclear chemical shift correlation (HETCOR), or heteronuclear single-quantum correlation (HSQC). Due to the higher magnetogyric ratio and presence in number, 1H protons are sampler to detect, more resolved spectrum and time safer (Pavia *et al.*, 2009; Balci 2005).

2.7.4.3 Ultraviolet and visible spectroscopy (UV-VIS)

UV-VIS analyses samples by measuring the diminished beam of light after it passes through a sample or after reflection from a sample surface over an extended spectral range. The presence of alkenes, and carbonyls may be detected using UV-VIS. This method has narrow usage in characterizing compounds, unlike infrared spectroscopy and NMR, which will provide structural information (Pavia *et al.* 2009).

2.7.4.4 Infrared radiation (IR)

Infrared radiation (IR) is a type of electromagnetic radiation, which may be used to obtain valuable information about the structure of the compound being analyzed. The instruments have the ability to absorb in the infrared region and provide data. When analyzing the presence of O-H section in the range of 3400-2400 cm^{-1} and a carbonyl section between 1730 and 1700 cm^{-1} indicates the presence of a carboxylic acid. The negative aspect of the technique is that two similar compounds cannot be identified and the identification an unknown metabolite. Nevertheless, IR may be used to confirm the presence of some chemical features (Pavia *et al.*, 2009).

Table 2.3: Summary of the advantages and disadvantages of analytical methods used for secondary metabolites

Method	Advantages	Disadvantages	References
Gas Chromatography (GC)	• High-resolution power compared to other methods. • High sensitivity when coupled with thermal detectors. • Reasonably good accuracy and precision. • Separation and analysis of sample very quickly.	• Only volatile • During injection of the gaseous sample, proper attention is required. • The sample must be thermally stable to prevent degraded when heated.	Barker and Ando (1999)

	• Sample with less quantity is also separated.		
Nuclear magnetic resonance (NMR)	• Noninvasiveness • Lack of Ionizing Radiation. without destroying the sample • Flexibility: variety and versatility	• Sensitivity • Working in a high-Magnetic-Field Surroundings • Motion Sensitivity • Availability and Access to NMR Systems	Chatham and Blackband (2001)
Ultraviolet and visible spectroscopy (UV-VIS)	• Simple • Rapid • UV-Vis spectrometers bring high-tech spectral analysis to the food industry, where they help laboratory technicians study food products • These devices are used in fields as diverse as forensic analysis, research and medicine	• Dust or grim may coat the mirrors • Repair costs • Stray light decreases an instrument's linearity range and reduce the absorbency of the substance it measures • Electronic components in the sample source may also generate noise that decreases measurement accuracy and reduces the device's sensitivity	Sillanpää (2014)
Infrared radiation (IR)	• Free bands • Relatively low cost • Safety restrictions for safe usage	• Directionality: At moderate Distance • Limited Range • Sun Light can interfere with the IR signal	Cummins (2016)

	• Reduce operating range by dust, smoke, rain and fog. • IR systems are not capable of a "maintained link" mode and will continue last command if the transmitter fails. • Night and Low Visibility Operations. • Interference by noise that can be disruptive to IR signals.

Identification of secondary metabolites is essential, especially for large scale production. Furthermore, it is apparent that these metabolites must possess bioactivities (antimicrobial, anticancer, etc) that make them of highly valuable.

2.8 APPLICATION OF BIOACTIVE COMPOUNDS FROM ENDOPHYTIC FUNGI

This section concentrates on the bioassay of the study and the history, literature and potential aspects of future research. Subsequently, our study examines the ability of secondary metabolites produced by endophytic fungi to exhibit antimicrobial activities.

2.8.1 Antimicrobial Assay

The microbial assay in this study was evaluating the bacterial activity on multi resistant Gram negative and positive bacteria.

2.8.1.1. Antibacterial Activity

Currently, the dramatic increase in the ability of microorganisms to develop resistance reduces the efficacy of existing modern medicine. Hence, this will increase prolonged illness and greater risk of death. Antibiotic-resistant microorganisms encourage researchers to find alternatives (Barza and Travers, 2002) cures for infectious conditions.

Microorganisms resistances of are into two types:

- Innate resistance being naturally/innately/ inherently resistant to multiple antibiotics;
- Acquired resistance is a response to mechanisms such as mutation and/or horizontal gene transfer, whereby the microorganisms are provoked by an encounter.

The explanation for this is the ability of microorganisms to acquire resistance against antimicrobial substances such as the emergence of ciprofloxacin resistance Campylobacter (Agunos *et al.*, 2013). Kjer and co-workers (2009) discovered two new secondary metabolites (10-oxo-10H-phenaleno [1,2,3-de] chromene-2-carboxylic acids, xanalteric acids I and II) which were produced by the fungus *Alternaria* sp. housed by the mangrove (*Sonneratia albacollected*) plant located in China. The metabolites exhibited strong antibacterial properties against *E. faecalis, Pseudomonas aeroginosa* and *S. epidermidis.*

2.8.1.2. Antifungal Activity

The theoretical value 6:1 of plant to fungus is still acceptable and practical in this decade (Hawksworth 1991). Soil fungal population was reported to have a greater number of new species; hence, data supported an estimate of 3.5 to 5.1 million species (O'Brien, 2005). This suggests that there may be more fungi interaction whether beneficial pathogenic.

Trichodermin (4β-acetoxy-12,13-epoxy-Δ9-trichothecene) is a secondary metabolite isolated from garlic. It exhibited antifungal activity against *Rhizoctonia solani,* with an EC50 of 0.25 μgmL-1, stronger inhibition against *Botrytis cinerea*, with an EC50 of 2.02 μgmL-1 and *Colletotrichum lindemuthianum* (EC50 = 25.60 μgmL-1) (Shentu *et al.*, 2014).

2.8.2 Anti-cancer Activity (Cytotoxic assay)

Cancer is defined as a malignant growth or tumor causing uncontrolled cells divisions. Well over 60 % of the world's new cancer cases occur in Africa, Asia, and Central and South America; an alarming 70 % of the world's cancer mortality originates in these areas (National Cancer Institute, 2015). Cancers are considered to be at epidemic proportions since more than eight million people worldwide are dying every year (America Cancer Society, 2016). There is an emerging breast cancer pandemic, distressing one in eight American women (Schneider *et al., 2014,* World Health Organization, 2015). Due to the above disquieting facts, research has focused on natural anti-cancer products (Leal *et al.,* 2016, Wu *et al.*, 2015). A "golden" compound, taxol is currently been used as a leading anticancer drug on the market to date. The drug paclitaxel is formulated from 'taxol", the first anticancer drug to generate billions of dollars. The endophytic fungus (*Taxomyces andreanae*) was isolated from the Pacific yew bark (*Taxus brevifolia*) illustrated in Figure 2.15 (a) (Stierle *et al.,* 1993).

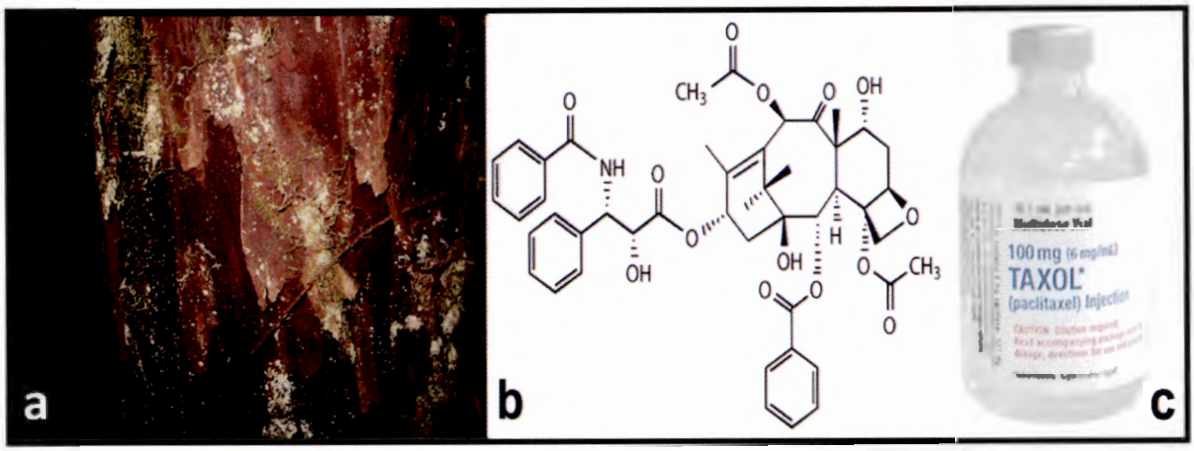


Figure 2.15: (a) Pacific yew bark inhabited by the fungus, Molecular structure of taxol (b) Injectable pacilitaxel, (100 mg)

The paclitaxel drug is used to treat ovarian, breast, lung, pancreatic and other cancers. There are other genera, which were reported to produce the compound taxol, including *Pestalotiopsis microspora, Alternaria alternata, Periconia sp., Pithomyces sp., Chaetomella raphigera, Monochaetia sp.* and *Seimatoantlerium nepalense* (Visalakchi andl Muthumary, 2010). Wani and colleagues (2016), reviewed other endophytic fungi (*Phyllosticta spinarum, Bartalinia robillardoides, Pestalotiopsis terminaliae, Botryodiplodia theobromae*) used against numerous forms of cancer. The endophytic fungus from the Indian medicinal host *Mimusops eleng* was potent against human cervical cancer cells. This was because the fungus produced a secondary metabolite called

ergoflavin a dimeric xanthene (Ganesha *et al.,* 2014). The mechanism of action of paclitaxel (taxol) is inhibition of microtubule assembly. This compound promotes the tubulin polymerization and interferes with normal microtubule breakdown during cell division. Furthermore, metaphase spindle configuration is not completed and blocks the progression of mitosis. Figure 2.15 (b,c), represent the responsible molecular compound and the most commonly used formula of taxol (Mohamed, 2015; Demain and Sanchez, 2009).

2.8.3 Antioxidant Activity

Oxidation is a chemical reaction resulting in the loss of electrons from an atom which may produce free radicals. Free radicals are natural unstable molecules produced during chemical reaction such as digestion. Hence, free radicals may lead to chain reactions that might potentially cause cell damage in the human body (Elochukwu, 2015; Lobo, *et al.,* 2010). Once an atom losses an electron, the cell becomes unbalanced, consequentially having cellular damage. Cellular imbalance is demonstrated in Figure 2.9 (Rahman *et al.,* 2012). The oxidative stress caused may result in various disease states as well as chronic diseases (Rahal *et al.,* 2014). The effects of free radical damage can be observed in our everyday lives, e.g. apples brown and iron rusts due to this. The health implications are tabulated in table 2.2. Antioxidants are molecules that aid in efficiently neutralizing the reaction and enhancing cellular defenses. Neutralization of free radicals is achieved by the radical talking up an electron (Nimse and Palb, 2015). Therefore, preventing and fighting the free radical damage (inhibit cellular damage). Antioxidants can be both non-enzymatic and enzymatic forms and exist in the intracellular and extracellular environment. This is universal. The protection will differ from species to species (Nimse and Palb; 2015; Lobo, *et al.,* 2010).

The main classification of antioxidants is based on their enzymatic activity (enzymatic and nonenzymatic antioxidants). Break down or removal of free radicals is referred to as enzymatic antioxidants. Interference of free radical chain reactions is defined as non-enzymatic antioxidants (e.g. vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione). Using multi-step process, all of the above will convert dangerous oxidative products to hydrogen peroxide (H_2O_2) and then to water, in the presence of cofactors such as copper, zinc, manganese, and iron

Table 2.4: Summarization of diseases and side effect of oxidative stress adapted from Rahman *et al.*, 2010

OXIDATIVE STRESS	
Neurological	**Multi-System Effects**
• Attention-deficit/hyperactivity disorder (ADHD)	• Diabetes
• Alzheimer's Diseases (Smith *et al.*, 2000)	• Cancer (Kinnula and Crapo, 2004)
• Anxiety and Depression	• Inflammation
• Asperger Syndrome	• Fibromyalgia
• Autism	• Lyme Disease
• Multiple Scelerosis	• Chronic Fatigue Syndrome
• Parkinson' Disease	• Metabolic Syndrome
• Multiple Sclerosis	• Anxiety
	• Hyperthyroidism
	• Sleep Apnea
Cardiovascular	**Joints/Skin**
• Cardiovascular Disease (Singh and Jialal, 2006)	• Gout
• Angina Pectoris	• Dermatitis
• Hypertension	• Rheumatoid Arthritis
• Atherosclerosis	• Carpal Tunnel
Gastrointestinal Disorder	**Respiratory**
• Crohn's Disease	• Chronic Obstructive Pulmonary Disease (COPD)
• GERD	• Asthma
• Gastric Ulcers	
• Celiac Disease	
• Functional Dyspepsia	

2.8.4 Anti-biofilm activity

Various studies (Moreau-Marquis *et al.*, 2008) have proved that the potential of most microorganisms to form biofilms contributes to an increase in resistance of the microorganisms to antibiotics. Hence, this leads to the problematic issues of persist resistance, failure of antibiotics and a failure of health care as a whole. Biofilm are sessile communities of microbial cells that are enclosed in a complex extracellular polymeric substance (EPS) or exopolysaccharides while being attached to a surface (biotic or abiotic). Therefore, because of its strong bond, the biofilm is considered as irreversible.

Microorganisms naturally may occur as sessile (free-floating) or planktonic entities (surface-bound) (Van Acker and Coenye; 2016; Harding *et al.*, 2009; Mah and O'Toole, 2001).

It is a known fact that microorganism need nutrients to grow and survive, hence the potential biofilm surface must be preconditioned with nutrients. It is a natural function of microorganisms to colonize and form a community. Most often in nature, the biofilm community may be single population of cells (monospecies), but mixed- culture biofilms exist (Hugo and Russell, 2004). The following are advantages associated with biofilm communities: protection from the environment, nutrient availability, metabolic cooperation, and acquisition of new genetic traits (Jabra-Rizk *et al.*, 2004). Figure 2.16 represents a diagram of the various stages in the biofilm formation. Effective antibiofilm agents are essential to combat these resistant communities.

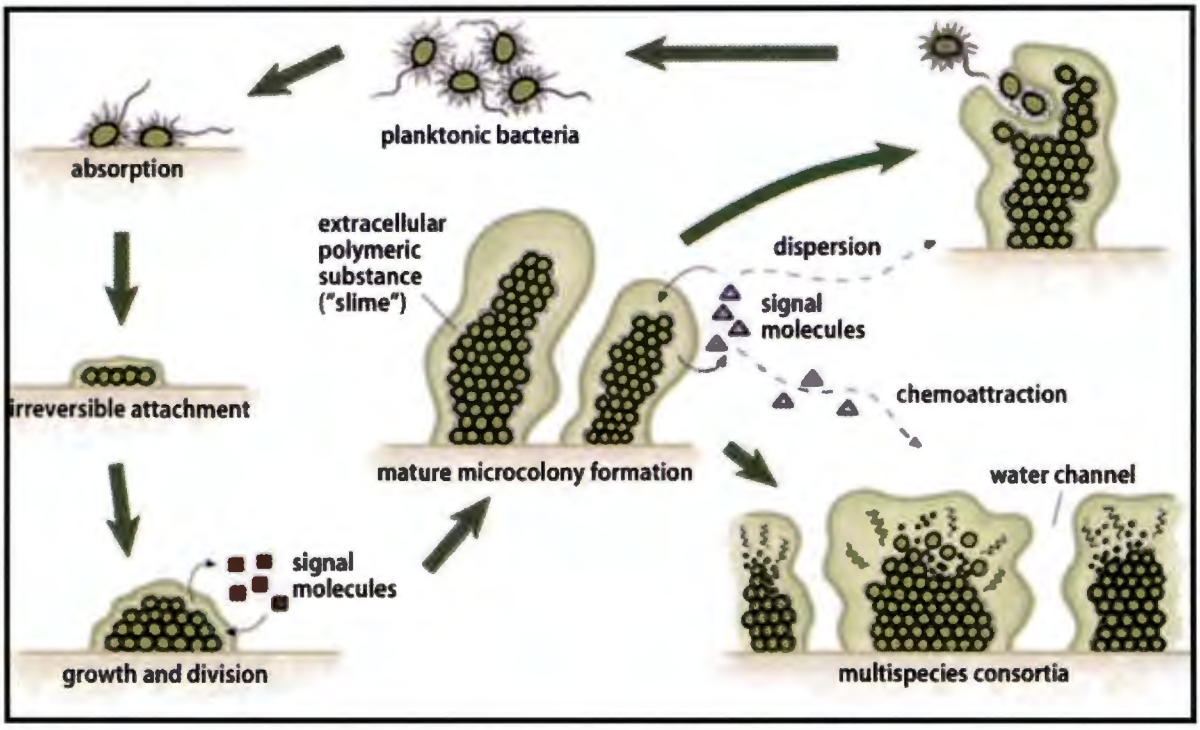


Figure 2.16: Schematic representation of the stages in the development of a microbial biofilm[8]

A summary of some of the investigative studies conducted on endophytic fungi isolated from medicinal plants over the last decade is shown in Table 2.5. This proves that there is a potential to study endophytic fungi as a novel source for new, affordable and efficacious bioactive products.

[8] https://www.researchgate.net/figure/Formation-of-a-biofilm-is-analogous-to-the-development-of-a-multicellular-organism-with_fig2_253263880

Table 2.5: Novel natural compounds isolated from endophytic fungi published in 2007-2017

YEAR	Fungal Species	Host Plants and Part	Bioactivity	Reference
2017	*Syncephalastrum racemosum*	*Markhamia tomentosa*	Antiproliferative a	Ibrahim *et al.*, 2017
2017	*Penicillium sp.*	*Camellia caduca*	Antibacterial and antioxidant activity	Fouda *et al.*, 2014
2016	*Pestaloptiopsis sp.*	*Hugonia mystax L.*	Antioxidant Activity	Abirami and Boominathan, 2016
2015	*Alternaria alternata*	*Asclepias sinaica*	Antibacterial activity	Fouda *et al.*, 2015
2014	*Cladosporium sp.*	*Achyranthes aspera*	Antibacterial activity	Selvi and Balagengatharathilagam, 2014
2013	*Phoma sp.*	*Mitragyna javanica*	Anticancer activities	Pharamat *et al.*, 2013
2012	*Trichoderma citrinoviride*	*Actinidia macrosperma*	Cytotoxic and antitumour activities	Lu *et al.* 2012
2011	*Creosphaeria sassafras*	*Scapania verrucosa*	Antioxidant activity	Zeng *et al.*, 2011
2010	*P. citrinum* and *N. dimidiatum*	*Hyoscyamus muticus*	Antifungal Activity	Abdel-Motaal *et al.*, 2010
2009	*Costus speciosus*	*Ardisia colorata*	Cytotoxic activity	Hazalin *et al.*, 2009
2008	*Viguiera arenaria* and *Tithonia diversifolia*	*Glomerella cingulata*	Cytotoxic activity	Guimaraes *et al.*, 2008
2007	Fungal endophyte (N24)	*Garcinia* plants	Anti-tuberculosis and Anti-Plasmodium falciparum	Phongpaichit *et al.*, 2007

Fungi that possess biological properties have been shown to also produce pigments that are of keen interest to research (Velíšek and Cejpek, 2011).

2.9 PIGMENT-PRODUCTING ENDOPHYTIC FUNGI

For centuries, mankind has been fascinated by colours and their various uses. This section explores the history, chemical composition and application of pigments produced by fungi.

2.9.1 Definition of pigment

Pigments are characterized by their high tinting strength relative to the materials they colour and they are stable in solid form at ambient temperatures. The Latin origin is denoted a colour (in the sense of coloured matter). The reflected or transmitted wavelength light causing colour change. Pigments are basic coloring matter or substance. They can be from living organisms (Natural Pigments) or chemical reagents (Synthetic Pigments). Historic application of pigment goes back to the cave painting using ocher, hematite, brown iron ore and other mineral-based pigments approximately 30,000 years ago (Daniel, 1986).

2.9.2 History of pigment production

Ancient civilizations were utilizing natural earth pigment (ochres) and iron oxides as dyes/pigments. Archaeologists explored caves and discovered that prehistoric cultures used paint for artistic purposes such as body decoration (Meyer, 1991). Ochres were predominantly used in ancient civilizations across the world; this is depicted in Figure 2.17. At this time, there were restrictions on the colour range used for art and decoration. Some pigments originated from botanical materials such as animal waste, insects, and mollusks. Other pigment colours were costly or impossible to obtain. Paucity colours (blue and purple) were associated with royalty (Meyer, 1991).

The developments of synthetic pigments in the early second millennium BCE were inspired and synthesized from ochres and iron oxides. White lead (basic lead carbonate, $(PbCO_3)_2\ Pb(OH)_2$) and blue frit (Egyptian Blue) were the first synthetic pigments. Lead was mixed with vinegar (acetic acid, CH_3COOH) in the presence of CO_2 to create white lead. Glass coloured with a copper ore (malachite) produced and created blue frit, which is calcium copper silicate (Mahapatra, 2016). Natural pigments produced from microorganisms such as yeast, fungi, bacteria and algae mainly consist of anthoquinone, carotenoids, chlorophyll.

2.9.3 Chemical characteristics of fungal pigments

Current research has been focusing on pigment production using filamentous fungi with a broad spectrum of colours. Chemical compositions have a variety such as carotenoids, melanins, flavins, phenazines, quinones, and more specifically monascins, violacein or indigo. The production cycle of pigments depends on acceptability by the consumers, regulatory approval, and the capital investment until the market (Dufosse *et al.,* 2014). Table 2.6, outlines the different filamentous fungi that produce colourful pigments and the chemical component responsible.

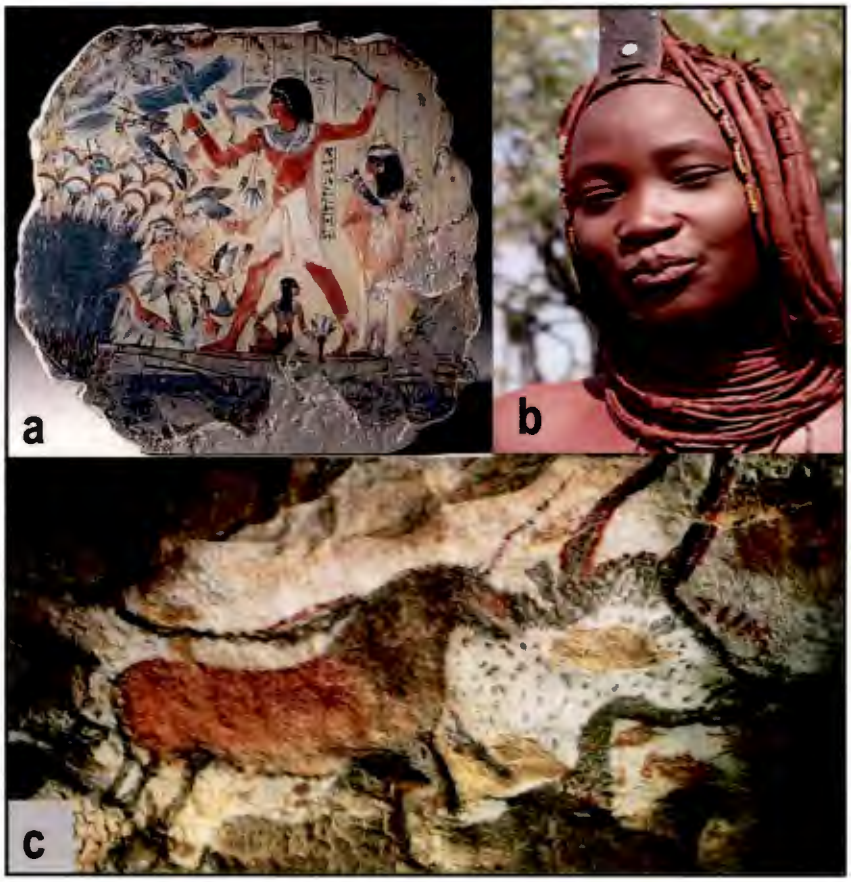


Figure 2.17: Ochre from (a) Egypt, 1350 BCE depicting Tomb of Nebamun (b) Africa, Himba woman ochre-covered (c) France showing Horse and Bull from Lascaux caves

Table 2.6: Microbial production of pigments (already in use as natural food colorants or with high potential in this field) (Liu and Nizet, 2009).

Microorganism (Fungus)	Pigment	Colour	Status
Monascus sp.	Ankaflavin	Yellow	IP
Monascus sp.	Monascorubramin	Red	IP
Penicillium oxalicum	Anthraquinone	Red	IP
Penicillium purpurogenum	Unknown	Red	DS
Phycomyces blakesleeanus	ß-carotene	Yellow-orange	RP
Neurospora crassa	ß-carotene	Yellow-orange	RP
Fusarium sporotrichioides	ß-carotene	Yellow-orange	RP
Blakeslea trispora	ß-carotene	Yellow-orange	IP
Blakeslea trispora	Lycopene	Red	DS
Monascus sp.	Rubropunctatin	Orange	IP
Ashbya gossypi	Riboflavin	Yellow	IP
Cordyceps unilateralis	Naphtoquinone	Deep blood-red	RP
Fusarium sporotrichioides	Lycopene	Red	RP

There are massive environmental implications when using artificial dyes. Azodye, nitrodye and nitroso dye are well known for their toxicity, if unmanageable may lead to pollution. Furthermore, there are serious health hazard associate with them. Some chemical components (azorubin and tartrazine) may cause allergies. While, sunset yellow may result in kidney tumours. Modern research has uncovers particular hair dyes that have caused the development of brain tumours in children. Erythrosine might restrict the absorption of iodine, which may contribute to goitre (Bluhm *et. al.*, 2006). There are major environmental implications when using synthetic dyes for textile and other industrial applications. Hence, it will lead to serious water pollution in the waste water systems. The compounds within the dyes might result in human colloidal caused by toxicity, carcinogenicity and highly resistant to degradation (Chung, 2000). Kamel *et al.* (2005) stated that natural colouring can

result in improved biodegradation and overall higher compatibility with the surroundings. In our current society, people are striving for more natural products, thus the demand of natural colours is escalating day-by-day. The following are motives:

- Health-promoting properties of natural coloured food;
- Consumer priorities natural colours;
- Low-fat content is the objective for many new or improved food formulations, replacing fats with thickeners or other food additives;
- Increased consumer preferences for organic food;
- Variety and internationalisation of food colour and flavours;
- Increasing demand for natural food in comparison to synthetic one (Rymbai *et al.*, 2011)

2.9.4 Biotechnological applications of fungal pigment production

A number of industries such as food production textile manufacturing, paper production, agricultural practices and studies, water science and cosmetics, have utilized both natural and synthetic dyes (Venil *et al.*, 2013). Mapari *et al.* (2010) reported that the international food colorant market is valued at about $1.15 billion USD. The food industries use dyes for various core reasons like preservation, appearance even after processing and storage, color uniformity to prevent a seasonal variation in tone color, to intensify the regular color of the food and to maintain quality, enhancing color ordinary food and therefore to maintain quality, taste and glow. In addition, to safeguard vitamin even when exposed and boost acceptance food as tempting item besides coloring of food (Rymbai *et al.*, 2011). Inclusive specification of specialty industries when choosing Dyes:

- Resistance to heat
- Resistance to weather conditions
- Resistance to ultraviolet light (UV)
- Water soluble
- Conducts electricity
- Contain reinforcing fibers
- Free from heavy metals

2.10 CONCLUSIONS

Endophytic fungi that harbor within the plants have significant benefits. These include protective role, growth promoters and stress tolerance. Regardless, that research will confirm this by in *vivo trial* in the greenhouse. All these are attributed by the production of secondary metabolites with various biological properties. The biological aspects highlighted in this study are exhibited by the endophytic fungi by producing chemical compounds.

CHAPTER THREE

ISOLATION, PRESERVATION AND MORPHOLOGICAL IDENTIFICATION OF ENDOPHYTIC FUNGI FROM TWO INDIGENOUS PLANTS

CHAPTER 3

ISOLATION, PRESERVATION AND MORPHOLOGICAL IDENTIFICATION OF ENDOPHYTIC FUNGI FROM TWO INDIGENOUS PLANTS

ABSTRACT

The objective of this chapter is to investigate the biodiversity of endophytic fungi isolated from indigenous *Pelargonium sidoides* and *Sceletium tortuosum* plants using morphological techniques based on macroscopic and microscopic features. A total of 50 *Pelargonium sidoides* and 50 *Sceletium tortuosum* plants were purchased from eight suppliers around South Africa. Surface disinfection of the leaves and roots were conducted to isolate the endophytic fungi. Colonization factor was calculated to establish preference locations. Successful isolation, preservation and morphological identification of endophytic fungi from two indigenous plants were achieved in this study. The type of classification was based on phylum rankings according to morphological identification. From a total of 100 plants, 193 fungal isolates were successfully isolated from the roots and leaves of the two medicinal plants. And only 160 fungi were morphologically identified which belonged to 32 genera. The phylum grouping determined that Ascomycota was dominating, followed by Basidiomycota and Zygomycota phyla. Conidiophore (54%) was dominated with septate (87%) as hyphae nature. In both plants, the colonization rate was prevalent in leaves. Our date suggests that striving and dominating genus (*Penicillium, Fusaria* sp., *Alternaria* and *Aspergillus*) are established as fast growing and most abundant in nature. In conclusion, to the best of our knowledge, this is the first study in which the diversity of endophytic fungi from *Pelargonium sidoides* and *Sceletium tortuosum* has been assessed and the findings revealed that fungal diversity might provide a baseline study for investigations.

3.1 INTRODUCTION

According to a fungal survey by Sun and co-worker (2012), approximately 1 million species of endophytic fungi have been documented worldwide, and these include species belonging to group known as endophytes. Endophytes are ubiquitous within the plant kingdom and are therefore

isolated from a broad spectrum of hosts including lichens (Sun and Guo, 2012). Early studies on the diversities of fungi indicated that an estimate of 1.5 million fungal species exit (Hawksworth, 1991). However, the number was later reviewed by Blackwell (2011) who reported that up to 5.1 million species occur in nature. Among these reported fungal populations, current studies reveal that between 80,000 - 100,000 fungal species have been identified and fully described (Srinivas *et al.*, 2015). This clearly indicates that investigating fungal diversities and endophytes in particular is a unique area with untapped data needing urgent evaluations.

Endophytic fungi reside asymptomatically inside the living plant tissues due to the fact that they are able to form symbiotic associations with host medicinal plants (Huang *et al.*, 2009; Mitchell *et al.*, 2008). This is based on the observations that medicinal plants provide a unique environmental support to endophytes while the endophytes are known to produce metabolites that are beneficial to the host plant. This fungal-host plant interaction provides a platform for the production of novel metabolites with bioactive properties for medical, agricultural and pharmaceutical applications (Dias *et al.*, 2012, Azman *et al.*, 2015)

The colonization rate and isolation frequencies of various fungal communities are found in different host plants. When a plant is moved from its native environment, it loses endophytes including coevolved endophyte confers on plants. Furthermore, it loses the resistant benefits it had to equally coevolved pathogens (Evans 2002). Prior to the discovery of novel compounds, sampling and characterization of fungal endophyte diversity must take place, which is an emerging task that may lead to new beneficial products (Rodriguez *et al.* 2009; Gazis, 2012). This study focused on diversity of endophytic fungi isolated from *Sceletium tortuosum* and *Pelargonium sidoides* possessing bioactive properties. The purpose of the present chapter investigated the qualitative and quantitative aspects of the endophytic diversity in two indigenous South African medicinal plants using traditional techniques. Classification of endophytic fungi was established by morphological based analysis. Furthermore, diversity was coupled with colonization rate and classification. Although, morphologically-based identification is limited by reliable diagnostic characters. In this case, discernible spores and reproductive structures were utilised for morphology of the fungi (Visagie *et al.*, 2014). Confirmation of identification was done in Chapter four using modern molecular and phylogenetic procedures.

3.2 MATERIALS AND METHODS

All reagents and solvents used in this study were purchased from Sigma Aldrich (South Africa, Johannesburg, Aston Manor) and of analytical grade. All media used for growth and isolation of the fungal isolates were purchased from (South Africa, Midrand, Biolab, Merck). All the preparation and composition of the media and chemicals used in this study are descripted Appendix 2.

3.2.1 Plants material and chemicals

There is a grave concern over-harvesting of medicinal and aromatic plants, worse off the extinction of particular species (Bhattacharya and Mittra, 2002); hence, our study used cultivated plants. *Sceletium tortuosum* and *Pelargonium sidoides* plants was utilised because these plants are endangered. These plants also are incorporated in the Red List of South African Plants (SABI).

Mafikeng, North-West University, South Africa (25° 51' 21.521" S 25° 38' 25.116" E) was the experimental site of the study. Sampling sites and their co-ordinates are summarized in Table 3.1. Mature healthy (no visual disease symptom) *Sceletium tortuosum* and *Pelargonium sidoides* plants were randomly selected by using random sample. A sample specimen was deposited in the National Collection of Fungi, Agricultural Research Council (ARC), Biosystematics, Mycology division, South Africa (PREM collection). The plant material was placed in sterile bags and brought to the laboratory to be processed within 48 hrs after sampling. The sample bags were temporally stored in the cold room at 17 ºC. Plant sample collection was conducted by following strict ethical principles from National Environmental Management Act (Act 107 of 1998) (NEMA) and it has associated Environmental Impact Assessments (EIA) Regulations. Both *Sceletium tortuosum* and *Pelargonium sidoides* plants are on the Red List of South African Plants (SABI), hence endangered, we sampled from cultured plants from nurseries around South Africa. The location of the sample sites, province, geo-locations and the name of the nurseries are included Table 2.1.

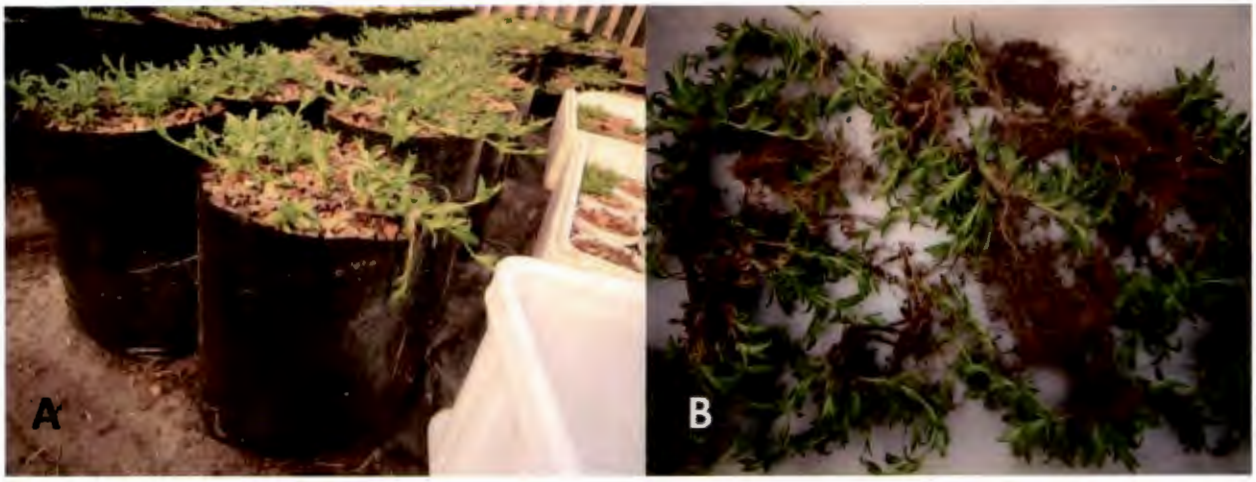


Figure 3.1: *Sceletium tortuosum* medicinal plant (A) *Sceletium tortuosum* plantation (B) Individual *Sceletium tortuosum* with fine roots

Table 3.1: Sampling sites with the geo-locations and number of plants

Pelargonium sidoides				
Province	**City**	**Nursery**	**Geo-location**	**No. of plants**
Gauteng	Pretoria, Hercules	Mountain Herb Estate	25° 43' 0" S, 28° 11' 0" E	5
Western Cape	Cape Town, Klein Karoo	Renu-karoo Nursery	33° 35' 10" S, 21° 00' 16" E	10
Eastern Cape	Port Elizabeth, Uitenhage	Elands Nursery	33. 59' 22" S, 25 37' 04" E	10
Gauteng	Pretoria, Hercules	Mountain Herb Estate	25° 43' 0" S, 28° 11' 0" E	15
Free State	Bloemfontein, Danhof	Pretty Garden	29° 03' 10.7" S, 26° 12' 42.2" E	10
Total Plants				**50**
Sceletium tortuosum				
Province	**City**	**Nursery**	**Geo-location**	**No. of plants**
Gauteng	Johannesburg	Nico Darados	26° 9' 45" S,	12

	Roodepoort		27° 52 '21" E	
Western Cape	Cape town, Sunnydale	Green Guerrillas	34°13' 23" S, 18.37' 37" E	18
Western Cape	Southern Africa, Klein Karoo	Dr. Boxall's	33° 92' 46 58" S, 8°42' 62 29" E	20
Total Plants				**50**

3.2.2 Isolation of Endophytic Fungi (Surface sterilization and Colonizing Frequency)

The plants samples were thoroughly washed under running water to remove dust and debris, and surface sterilised as described by Suryanarayanan *et al.* (2002). Leaves, stems and roots were cut into small pieces and dipped in 70% ethanol for 1 min, sodium hypochlorite solution (2%, household Jik) for 2 min. All samples were further dipped for 20 sec in 70% ethanol and rinsed twice in sterile distilled water as described by Araújo *et al.* (2001). Two to five pieces of the plant material were placed on nutrient-poor media (Potato Carrot Agar plus antibiotics: PCA+N; Selective Fusarium Agar: SFA; Water Agar: WA) (n=5) and the plates were incubated for 7-10 day at 25 °C. Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al.* (2002).

$$\text{Colonization frequency of endophyte} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segment observed}} \times 100$$

3.2.3 Single-Spore

A culture from a single spore was obtained as followed: ten microliter of a spore suspension prepared from a 7-10 day old culture (by dislodging the spores with 10 mL sterile water using a specular) was aseptically transferred to an agar plate and spread before being incubated aerobically while placed at an angle for 16-24 hrs at 24 ± 2 °C. A microscope was used to view the surface of the media to locate germinating spores. A small piece of media with the target spores (±10 spores) was picked up using a sterilized syringe or glass needle, and transferred onto a 60 mm Petri plate containing PDA (n=2). The inoculated plates were then incubated at cultured at 24 ± 2 °C to obtain pure colonies.

3.2.4 Preservation

All isolates identified were deposited, preserved, maintained and stored at Plant Protection Research Institute (PPRI) collection of the National Collections of Fungi, Biosystematics Programme, Agricultural Research Council, South Africa. Each isolate was assigned a unique accession (PPRI) numbers and preserved using four distinct preservation techniques. All the preservation methods performed in this study were in line with the Standard Operating Procedures (SOP) of the laboratory.

3.2.4.1 Water punches

Five plugs of each isolate were obtained from a 7-10 days old culture, transferred into 10 mL of sterile distilled water, and stored at 15-17 ° C until further use.

3.2.4.2 Oil slants

Fungal cultures were aseptically transferred onto PDA and PCA slants in McCartney bottles. The slants were incubated at 24 ºC for 7-10 days. The culture slants were covered with liquid paraffin prior to storage at 15-17 ºC.

3.2.4.3 Freeze-dry

Freeze drying was used for long-term preservation as recommended by the American Type Culture Collection (Ibatsam, Rukhsana and Nasim, 2012). Briefly, the fungal isolates were cultivated for 7-10 days at 24 ºC and approximately 5 mL suspending fluid (meso-inositol 5g, skim milk powder 10g in 100 mL distilled water then autoclaves) was transferred onto the culture media. The mycelia was scraped using a rod and 1 mL of the spore suspension transferred into 4 to 6 tubes. The tubes were placed at -80 ºC overnight and freeze-dried for 24 hrs. The freeze-dry tubes were added to the collection according to the SOPS.

3.2.4.4 Ultralow storage of glycerol suspension

Sterile glycerol (15%, 4-6 mL) was used to remove the mycelium and spores from a 7 -10 days old culture and 1.5 mL of the suspension was aseptically transferred into cryovial tubes. All samples were placed in "Mr Frostie" boxes prior to storage at -70 ºC. All isolated endophytic fungi were maintained and stored in the National Collection of Fungi, Agricultural Research Council (ARC),

Plant Protection Research Institute (PPRI collection) Biosystematics, Mycology division, as stated above. Each isolate was assigned a unique PPRI accession number issued by PPRI.

3.2.5 Morphological Identification

The morphological identification of each isolate obtained from a pure culture was performed using a bright-field and phase-contrast microscope EVOS FL Cell Imaging System (AMEP-VH009, ThermoFisher Scientific, South Africa) with a digital imaging system and the colour, size of the hyphal tip recorded and compared to a colour chart (Flora of British Fungi Colour Identification Chart", CIC) as described by Ihan *et al.,* (2006). Morphology at micro- and macroscopic level of each isolate was observed in cultures grown on Water Agar (WA) and Potato Dextrose Agar (PDA). Pure cultures of each isolate were obtained through hyphal tip or single spore and were grown on PDA under continuous light for 4 to 7 days at 24 ^{0}C ±2 ^{0}C in the lab. A genetic identification of all 193 isolates was done and is reported in details in Chapter 4.

3.3 RESULTS AND DISCUSSION

The two selected plants (*Pelargonium sidoides* and *Sceletium tortuosum)* used in this study are indigenous to South Africa. At the time when this research was initiated only sparks reports existed on the biodiversity of the endophytes from medicinal plants.

3.3.1 Isolation of endophytes (Colonization rate)

There were significant differences between leaves and roots, the CFs in leaves were higher than in the roots. This may be due to the large surface in the leaves than roots. The colonization rates for the leaves from Western Cape site 2 (WC2) were 22%, while those of the roots were 16%. This site generated the highest CF rate as compared to Western Cape site 1 (WC 1) and Gauteng (GP). Similarly, the colonization rate was significantly higher in leaves (20%) compared to roots (13%) in Gauteng site (Figure 3.2). Endophytic fungi were more prevalent on leaf tissue (> 50%) than roots. The results showed that Western Cape site 1 had the lowest CF rate. In a previous study conducted by Naik *et al.* (2006), an overall colonization rate of *Oryza sativa* was observed with an higher CF rate in roots (40.3%) than in leaves (25.83%). As illustrated in Figure 3.3, the overall colonization rate was higher in roots than leave in their respective locations except for Gauteng. Hence, the CF rate of leave was 25% as compared to that of roots (28%).

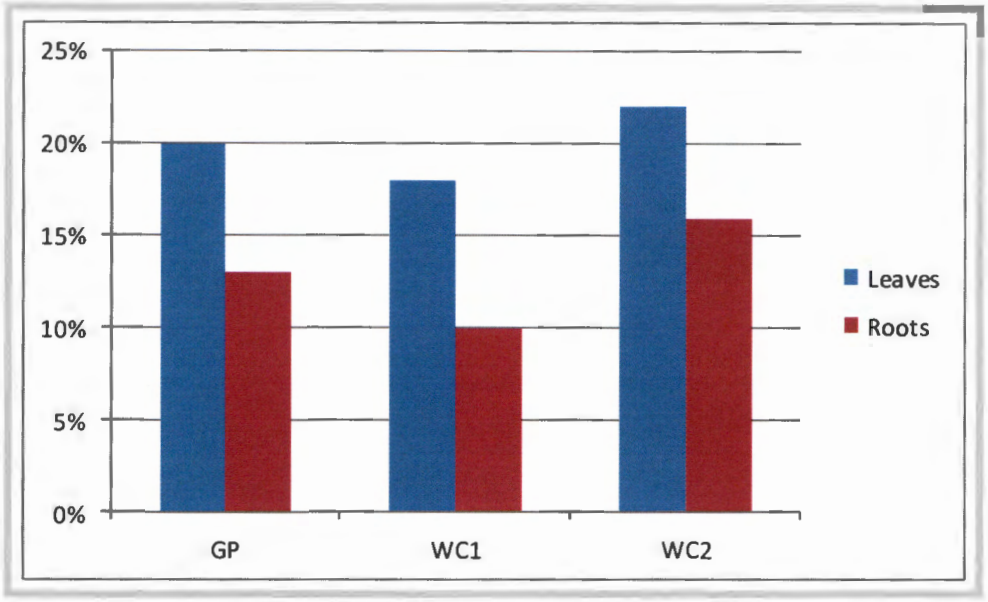


Figure 3.2: Colonization rate of *Sceletium* endophytic fungi isolated in leaves and roots at GP (Gauteng), WC1 (Western Cape site 1), WC2 (Western Cape site 2)

There is a strong correlation between the colonization and isolation rate. This reveals that there are a broader and greater number of endophytic fungi in roots. The CF is high in roots may be due to the large surface area exposed to the surrounding environment. Investigated endophytic fungi isolated from *Melastoma malabathricum* L plant and discovered that the colonization rate was higher in leaf segments (50.76%), followed by root (41.53%) and stem tissues (27.69%).

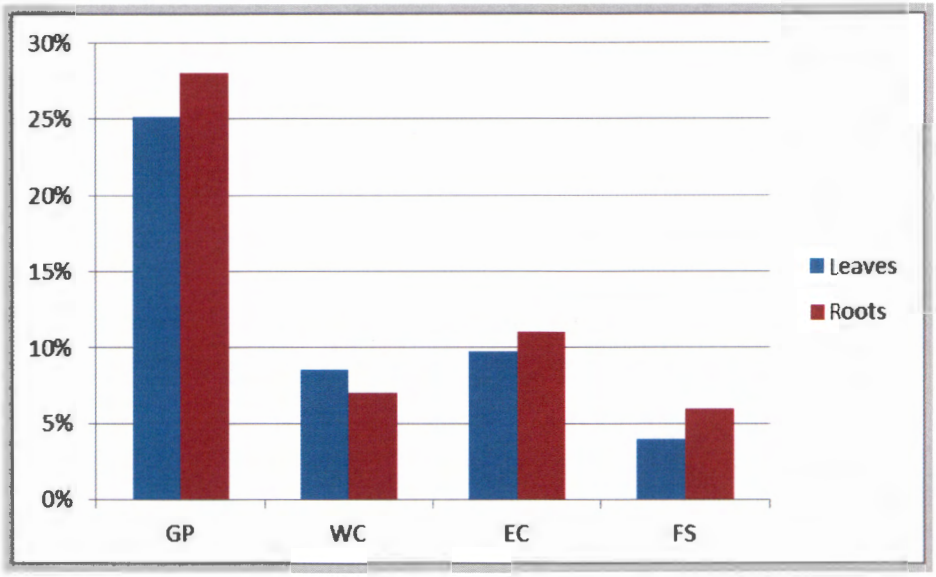


Figure 3.3: Colonization rate of *Pelargonium* endophytic fungi isolated in leaves and roots at GP (Gauteng), WC (Western Cape site 1), EC (Eastern Cape), FS (Free State)

3.3.2 Preservation in the National Collection of Fungi

In the current study, all (193) fungal strains were preserved, maintained and stored in the National Collections of Fungi, Biosystematics Programme, Agricultural Research Council, South Africa. Each isolate was assigned a unique accession (PPRI) number as displayed in Table 3.1.

Table 3.1: Plant Protection Research Institute (PPRI) accession numbers corresponding to sample ID

DR Isolates					
Sample ID	**PPRI no.**	**Sample ID**	**PPRI no.**	**Sample ID**	**PPRI no.**
DB 001	21993	DB 007	21999	DB 013	22005
DB 002	21994	DB 008	22000	DB 014	22006
DB 003	22447	DB 009	22001	DB 015	22007
DB 004	21996	DB 010	22002	DB 016	22008
DB 005	21997	DB 011	22003		
DB 006	21998	DB 012	22004		

The American Type Culture Collection endorses cryopreservation in liquid nitrogen and lyophilization for long-term preservation (American Type Culture Collection, 1991). To achieve in-depth study using fungal isolates, long-term preservation is critical for viability and the stability of living microbes. Freeze-drying or lyphilization, allows microbial cells to stabilize for long periods of time. On the other hand, the process is unwieldy, lengthy and involves expensive equipment (Espinel-Ingroff *et al.*, 2004). Table 3.1 are accession numbers provided by the PPRI, Mycology division for the deposit of fungal isolates, this is the DB isolated from Dr. Boxall site. All the strains were deposited in the National Collection and were allocated unique accession numbers.

3.3.3 Morphological Identification and Diversity of endophytic fungi

Nalini and co-workers (2014), reported that 31 fungal taxa with dominate genera such as *Fusarium*, *Acremonium*, *Colletotrichum, Chaetomium, Myrothecium, Phomopsis,* and *Pestalotiopsis* spp.

A total of 160 fungal isolates were identified using morphology techniques. Successful isolation of endophytes relied primarily on the surface sterilization technique. Epiphytic and other microbes living outside the roots and leaves did not survive this process because of the sterilization. The fungal diversity in both plants were dominated by isolates belonging to the genus *Penicillium*, followed by *Fusarium*, whereas *Alternaria* and *Aspergillus* (Figure 3.4 and 3.5) were in fewer numbers.

3.3.3.1 Ascomycetes

Ascomycota phyla are commonly recognized as the sac fungi or ascomycetes. They comprise of approximately over 64,000 species. Morphological structurale identity is the "ascus" (from Greek: ἀσκός (askos), meaning "sac" or "wineskin"). Ascomycetes reproduce sexually using microscopic structures to produce nonmotile ascospores (Kirk *et al.*, 2008). An enveloping membrane process is carried out by formation of ascospores. Nevertheless, there are certain species that reproduce asexual. Current research focused on utilization of morphological structure like the ascospores, ascus-bearing taxa and molecular analyses of DNA sequences for classification (Lutzoni *et al.*, 2004, James *et al.*, 2006).

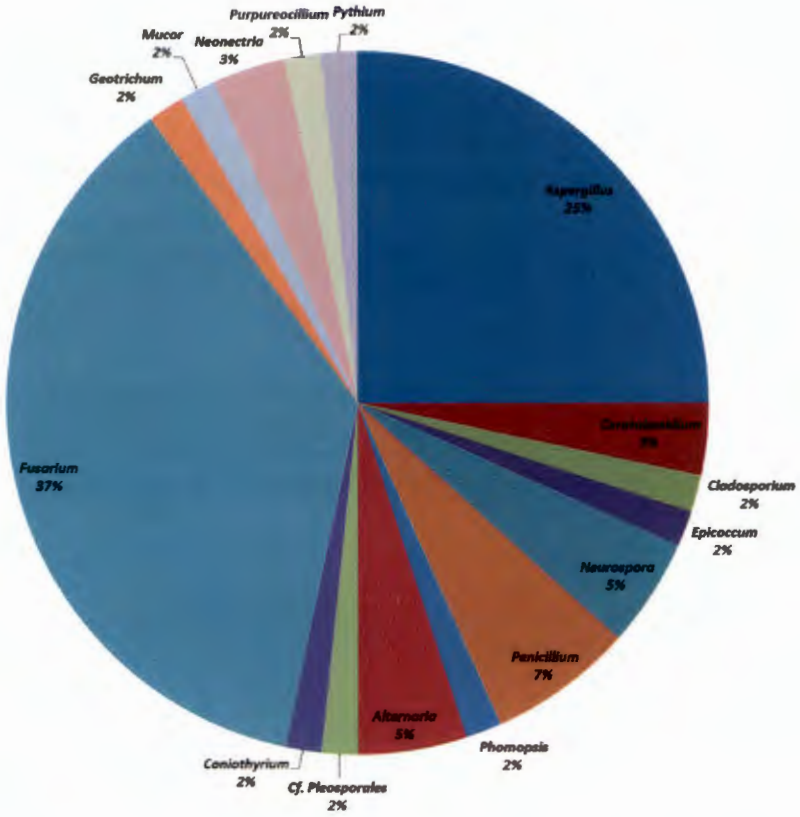


Figure 3.4: Relative abundance (%) of endophytic fungi classified under genus and species of *Sceletium*

3.3.3.1.2 Aspergillus Link

Aspergillus genus has a broad range of environmental conditions and is widely distributed. The first description was by biologist Pier Antonio Micheli, who reported aspergillum as holy water sprinkler, from Latin spargere (to sprinkle) in 1729. Barnett and Hunter (1998) interpreted the genus as "Conidiophores upright, simple, terminating in a globose or clavate swelling, bearing phialides at the apex or radiating from the apex or the entire surface; conidia (phialospores) 1-celled, globose, often variously coloured in mass, in dry basipetal chains".

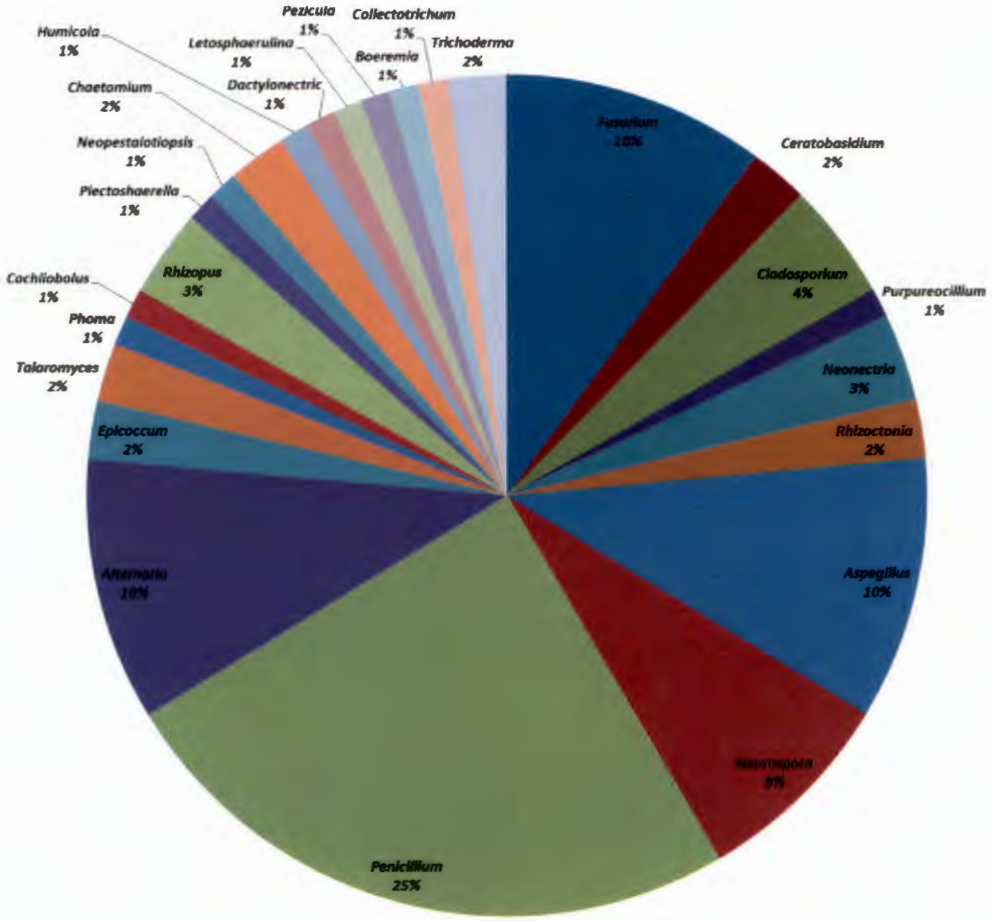


Figure 3.5: Relative frequencies of all the endophytic fungi isolated from *Pelargonium sidoides*

3.3.3.1.3 Phoma Desm.

Phoma genus is commonly known as endophytic fungi. "Like Phyllosticta; parasitic, on various plant parts. Both generic names, *Phoma* and *Phyllosticta*, occur commonly in the literature but morphologically they are alike" is defined by Barnett and Hunter (1998). The fungus produce spores, that are colourless and unicellular while the pycnidia are black and depressed in the tissues of the host. Endophytic *Phoma* isolated from medicinal plants have produced novel secondary metabolites with antimicrobial activities (Borges and Pupo 2006).

3.3.3.1.4 Penicillium

The genera are well-known for agricultural and pharmaceutical importance. It has cemented its place in history as a golden standard for antibiotics. Penicillin will either kills or stops the growth of bacteria. There is nearly 300 species within the genus (Kirk *et al.*, 2008). Colony morphology is brush-like conidiophore, which is asexual fruiting structure.

3.3.3.1.5 Fusarium Link.

Fusarium originates from Latin fusus meaning a spindle. *Fusarium* is a filamentous fungus that is usually distributed in soil and associated with plants. They range from harmless saprobes to pathogenic. Their pathogenic nature is due to the production of mycotoxins in cereal crops that can affect human and animal health. Fumonisins and trichothecenes are the leading toxins formed by these *Fusarium* species. However, there are various novel and bioactive compounds extracted from a diverse pool of endophytic *Fusarium* species (Nascimento *et al.,* 2012). This genus is prevalent in the tropical or subtropical plants (Costa *et al.,* 2012, Zhang and Yao, 2015). "Mycelium extensive and cotton-like in culture, often with some tinge of pink, purple, or yellow in the mycelium on medium; conidiophores variable, slender, and simple, or stout, short, branched irregularly or bearing a whorl of phialides, single or grouped into sporodochia; conidia (phialospores) hyaline, variable, principally of two kinds, often held in small moist heads; macroconidia several-celled, slightly curved or bent at the pointed ends, typically canoe-shaped; microconidia one-celled, ovoid or oblong borne singly or in chains; some conidia intermediate, two- or three-celled, oblong or slightly curved; parasitic on higher plants or saprophytic on decaying plant material. A large and variable genus sometimes placed in the Tuberculariaceae because some species produce sporodochia. Thick walled chlamydospores common in some species" (Barnett and Hunter, 1998).

3.3.3.1.6 Alternaria Nees.

Barnett and Hunter (1998) characterised *Alternaria* as "Conidiophores dark, mostly simple; determinate or sympodial, rather short or elongate; conidia (porospores) dark, typically with both cross and longitudinal septa; variously shaped, obclavate to elliptical or ovoid, frequently borne acropetally in apical simple or branched appendage; parasitic or saprophytic on plant material". Figure 3.6 represent the conidia structure of endophytic *Alternaria* used to identify the genus.

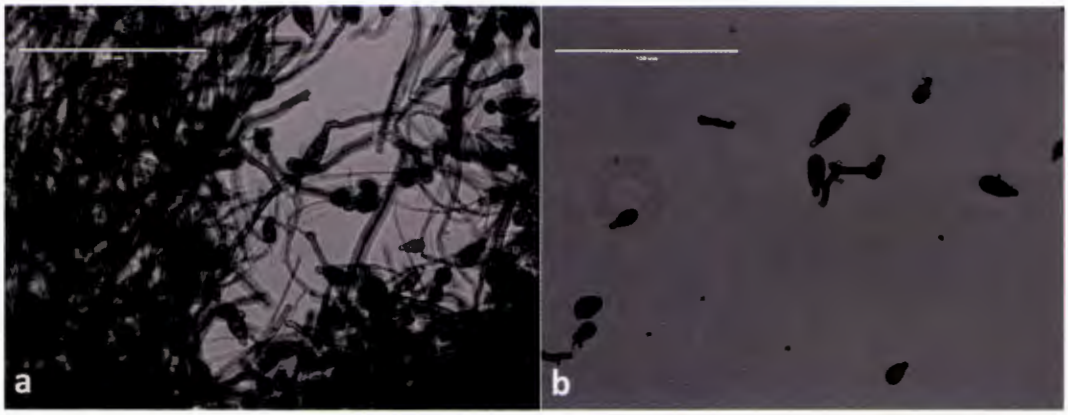


Figure 3.6: Conidia structure of *Alternaria* (a) endophytic fungi MHE 52 (b) endophytic fungi MHE 67

3.3.3.1.7 Trichoderma

Trichoderma is describes as culturable fungi that have loose tufts in shades of green, yellow or less frequently white and named for producing green mold. They dominantly exist in a broad spectrum of environments such as the soil, decaying wood, compost, roots and above ground plant organs. *Trichoderma* species flourishing in the soil deplete nutrients from other competing soil microorganisms. Adaptation of this genus has led to its evolution as an opportunistic, non-pathogen in symbiotic relationship with plants. Endophytic *Trichoderma* have been isolated from healthy South African grapevine wood and have great potential benefits (Halleen *et al.*, 2003). In the agricultural sector, *Trichoderma* is used to protect plant, for resistance to abiotic stresses, and uptake and use of nutrients and as a growth promoter (Harman, 2000; Harman *et al.*, 2004).

3.3.3.1.8 Colletotrichum Corda.

Morphological characterization is "acervuli disc-shaped or cushion-shaped, waxy, subepidermal, typically with dark, sines or setae at the edge or among the conidiophores; conidiophores simple, elongate; conidia hyline, one-celled, ovoid or 4 to8 oblong, to falcatative parasitic; imperfect states of Glomerella. This genus differs from *Gloeosporium* in having spines, which may be absent in some cultures" (Barnett and Hunter, 1998). Territorial habitats of endophytic *Colletotrichum* genera are mostly tropic or temperate plants and have potential to produce various bioactive compounds (Tiwari, 2015; Strobel and Daisy, 2003)

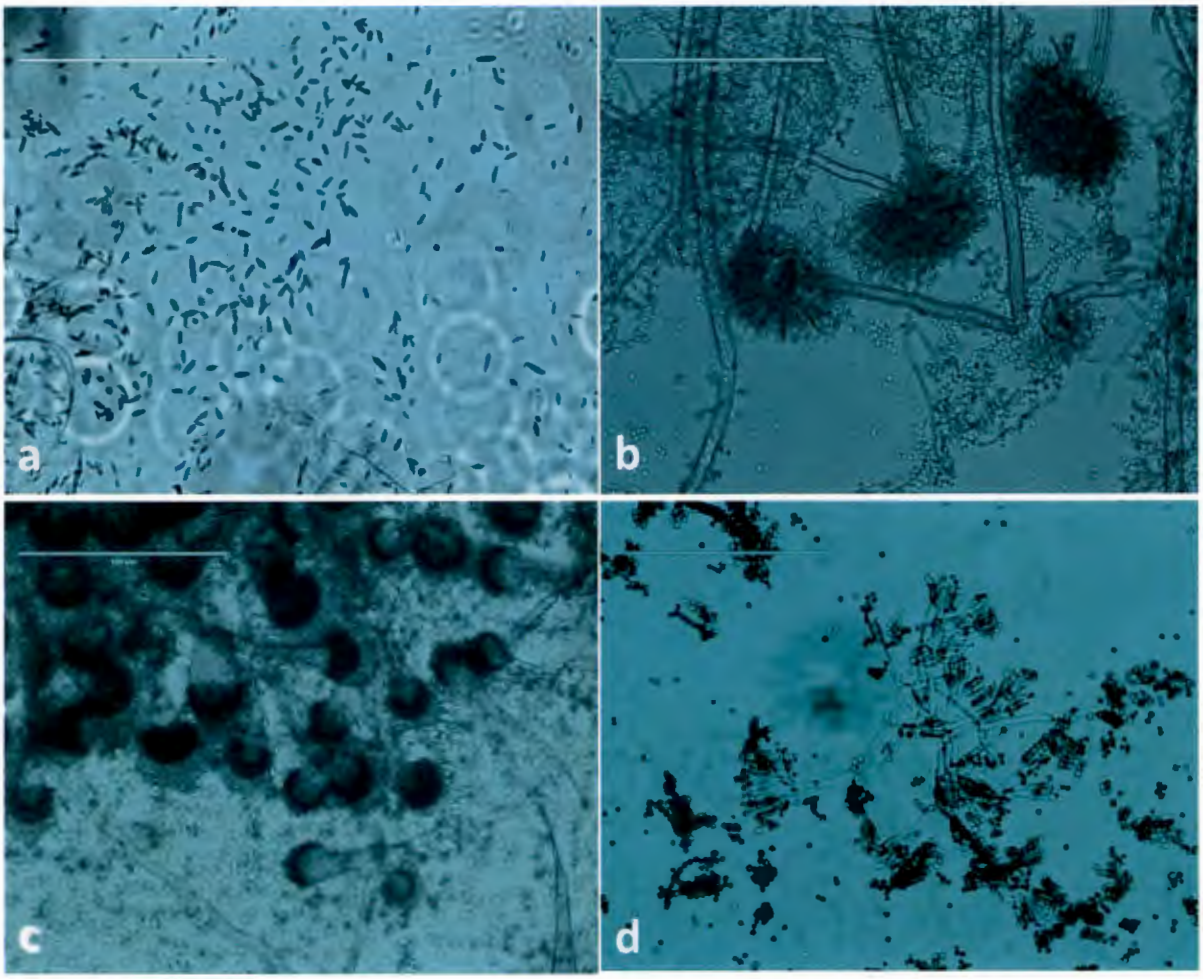


Figure 3.7: Morphological structures used for identification (a) GG 3 *Fusarium* (b) MHE 21 *Aspergillus* (c) MHE 48 (d) *Penicillium* MHE 21

3.3.3.1.9 Chaetomium Kunze.

Fast growing colonies that are cotton white in colour, while becoming grey to olive in their mature stage. Inverse vision of the colony plate is red or brown to black. Morphology identification is septate hyphae, perithecia, asci and ascospores. The perithecia are dark brown to black in colour, breakable and large in size. Furthermore, they have small rounded openings consisting of asci and ascospores. The filamentous fungus is globose to flask shaped and has brown to black appendages (setae). The Asci are tube-shaped and ascospores are discharged in 4s to 8s in number. The ascospores are singular, olive brown in colour, and lemon shaped[9]. Beneficial novel by-products are produced by endophytic Chaetomium (Jiao *et al.*, 2006; Wang *et al.*, 2006).

[9] http://www.doctorfungus.org/thefungi/Chaetomium.htm

3.3.3.1.10 Cladosporium Link.

Barnett and Hunter (1998) defined this genus as has the following "Conidiophores tall, dark, upright, branched variously near the apex, clustered or single; conidia (blastospores) dark, one- or two-celled, variable in shape and size, ovoid to cylindrical and irregular, some typically lemon-shaped; often in simple or branched acropetalous chains; parasitic on higher plants or saprophytic". The endophytic *Cladosporium* was reported to possess active metabolites with cytotoxic and antibacterial potential (Khan *et al.*, 2016).

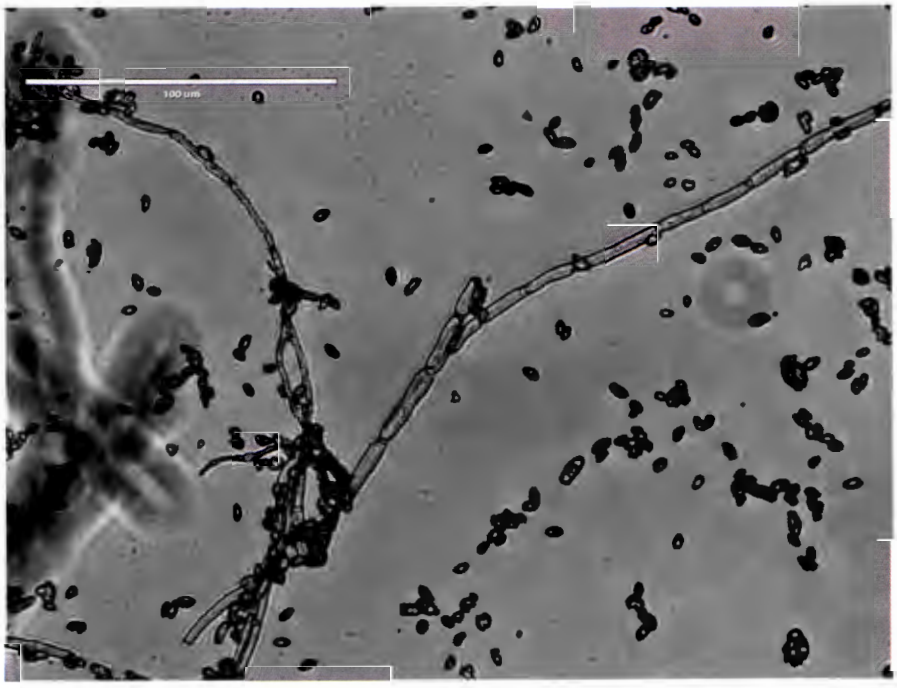


Figure 3.8: *Cladosporium* (DR 16). Macronematous conidiophores and conidial chains. Scale bars = 100 µm.

3.3.3.2 Basidiomycota

Basidiomycota is the second largest phylum group, this correspond to our findings. They division is defined as filamentous fungi with hyphae. Reproduction process is sexual by means of basidia, which are specialized club-shaped end cells. This results in specialized spore formation of basidiospores. Nonetheless, distinctive anatomical, cell wall components are responsible for asexual reproduction of certain *Basidiomycota* (Kües, 2000).

3.3.3.2.1 Rhizoctonia

Rhizoctonia are well-known as "root killer". They are saprotrophic, facultative and might cause diseases to plant/or crops. *Rhizoctonia* are anamorphic fungi, which means they undergo asexual stages hence, no spores are formed. The dominate structures are hyphae and sclerotia (Zhang *et al.,* 2014). Morphological identification of this genus is the number of nuclei present in the young cells (Otero *et al.,* 2002).

3.3.3.3 Zygomycota

Zygote fungi are comprised of nearly 1050 known species. They are mostly terrestrial and symbiotically associated with plants. Morphological identification is by coenocytic, formation septa and gametes found at the wall off dead hyphae. Reproduction is by both sexual and asexual stages using spores production. The exogenous dormancy depends on environmental factors including temperature or nutrient availability. Furthermore, the endogenous dormancy relies on the characteristics of the spore itself (Krogh, 2010).).

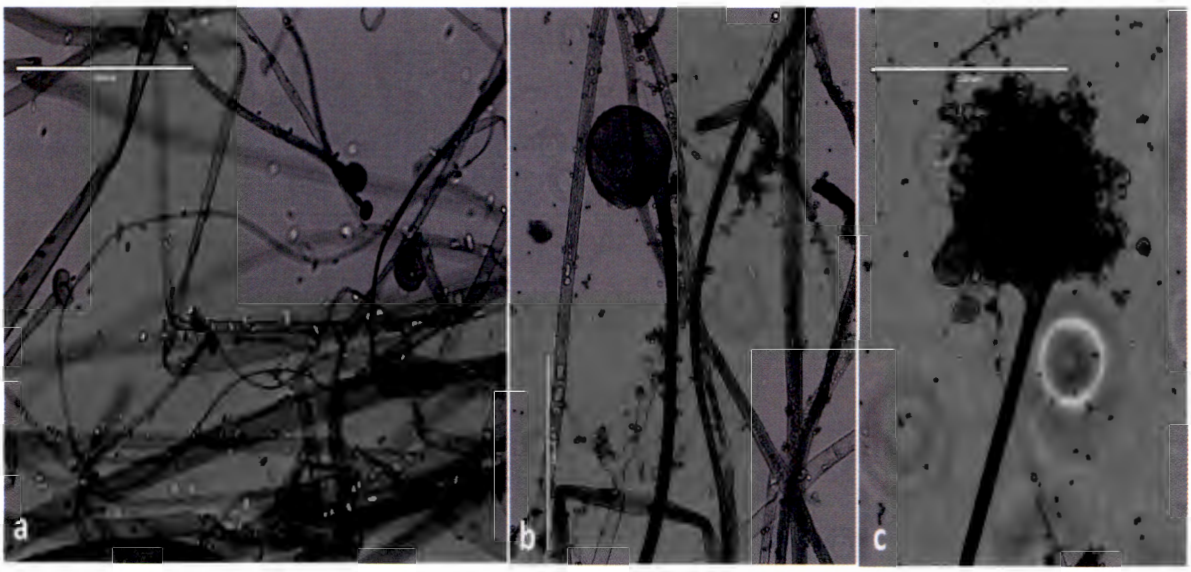


Figure 3.9*:* Macroscopic structures (sporangium) of (a) *Mucor* MHE 58 and (b.c) *Rhizopus* MHE 21

3.2.3.3.1 Rhizopus

Rhizopus is a genus with various functions contingent on the host. For example, they are saprophytic fungi on plants and specialized parasites on animals. *Rhizopus* contains a broad spectrum of organic substrates such as mature fruits and vegetables, jellies, syrups, leather, bread, peanuts, and tobacco

(Kirk *et al.,* 2008). They have an ability to cause human zygomycosis (fungal infection) which may lead to mortality. *Rhizopus* genus is filamentous with branches of hyphae but lack cross-walls (coenocytic). They undergo both asexual and sexual reproduction. Sporangiospores are located in the internal of sporangium during asexual reproduction. Mycelial fusion occurs to form a dark zygospore. Germination forces production of colonies that vary in their gene make-up. Figure 3.9 represents the morphological structures used to identify Zygomycota division.

3.3.3.3.2 Mucor

Mucor is a fast-growing genus that comprises of 6 species. The colony morphology is normally white to beige or grey while becoming grey to brown in colour in the mature stage. Sporangiospores may be artless or branched to produce apical and, globular sporangia, which are sustained and elevated by a column-shaped columella. They form mould with irregular non-septate hyphae branching at wide angles (>90°). Asexual reproduction occurs by the hyphal sporangiophores swelling at the tip to create a globose sporangium that encloses uninucleate, haploid sporangiospores. The columella protrudes which is the extension of the sporangiophore. The sporangium walls will burst to release the spores that eventually germinate to create a new mycelium on appropriate substrates. Gametangia fusion occurs by specialized hyphae (gametangia) during sexual reproduction. Spherical zygosporangium encompassing a single zygospore will develop as result. Nuclear karyogamy and meiosis happens within the zygospores, which are resistant to unfavourable conditions. Germination may result in the formation of hyphae or a sporangium. *Mucor* has both homothallic (self-compatible) and heterothallic species.

In this study, the morphological aspects such as colony colour, hyhae colour, nature of the hyhae and specialised structure were recorded to preliminary distinguish the 193 isolates. This method is currently being used in conjunction with molecular techniques. Figure 3.10 shows colony colour that GG 7 (Fig. 3.10 A) was deep red to black with a black centre. The reverse (Fig. 3.10 B) was black with white outlines. Figure 3.11 C-D was GG 1 front view was white as it mature became black (Fig. 3.10 D) while the front has a strong orange with a yellow outline (Fig. 3.10 C). In Figure 3.11 E-F, front was white (Fig. 3.11 F) and the reverse had the colour red for central region was grey-yellow background (Fig. 3.11 E). The difference in colony colour between the front and back view was also observed in Reddy *et al.* (2010) results.

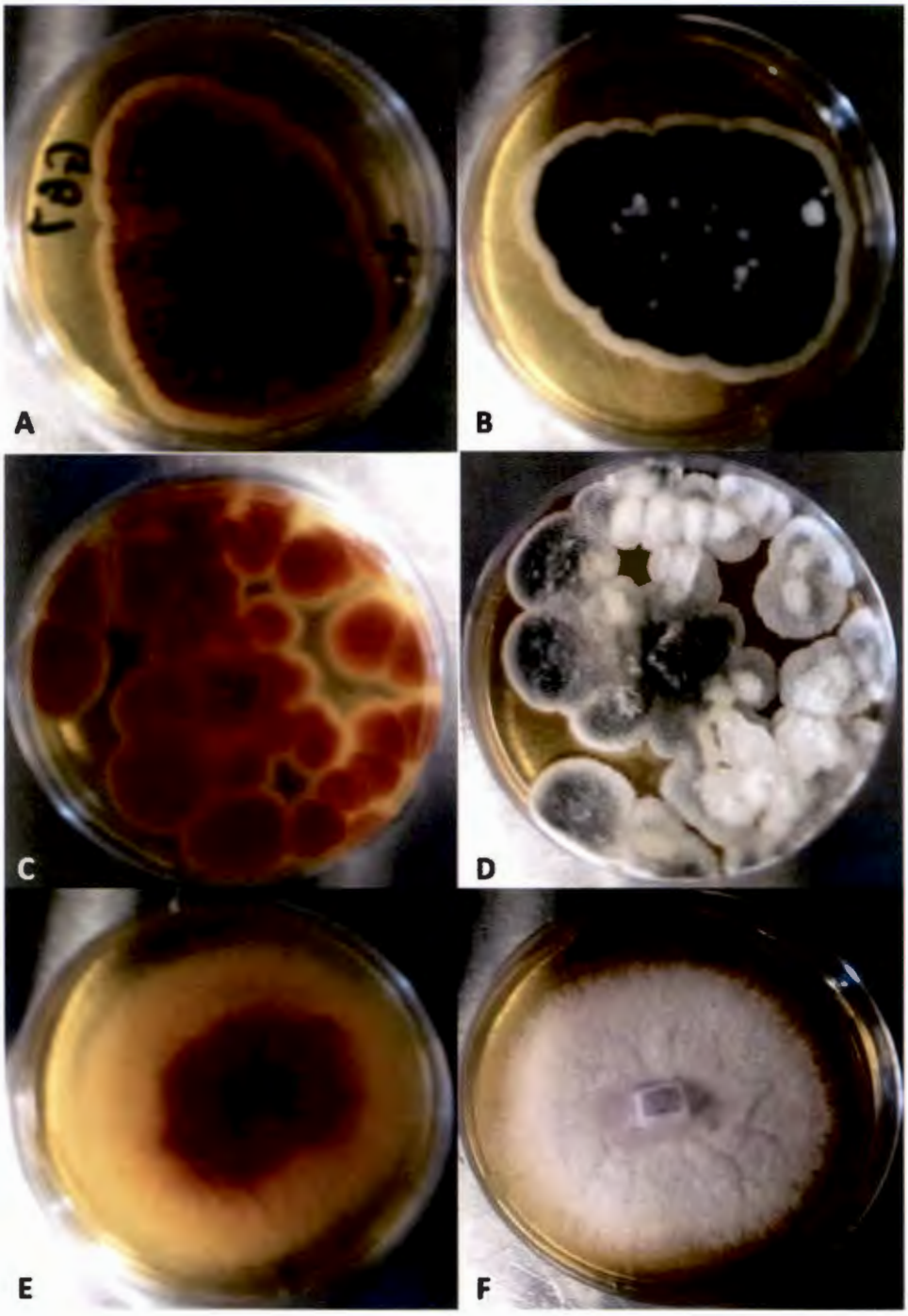


Figure 3.10: Macroscopical characters of endophytic fungi on PDA (A) Front view GG 7 (B) Reverse view GG 7 (C) Reverse view GG 1 (D) Front view GG 1 (E) Reverse view GG 8 (F) Front view GG 8

Table 3.2: Macroscopic and Microscopic features to identify fungi

Sample ID	Probable ID	Macroscopic characteristics		Microscopic characteristics	
		Colony Colour		Nature of Hyhae	Presence of Special Structure
		Top	Bottom		
GG 006	*Ceratobasidium*	Brown	Cream	Septate	Basidia with basidiospores
GG 007	*Alternaria*	Brown	Cream	Septate	Foot cell give rise to conidiophore
GG 008	*Fusarium*	Red	Red	Septate	Foot cell on the. macroconidia
GG 009	*Neurospora*	Orange	Pale Orange	Non-Septate	Foot cell from which the conidiophore grow
ND 4	*Penicillium*	Cream White	Yellow	Septate	Foot cell give rise to conidiophore
ND 5	*Neurospora*	Orange Yellow	Brown	Septate	Foot cell from which the conidiophore grow
ND 6	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
ND 7	*Geotrichum*	White	Cream	Septate with Dichotomous	Initially yeast like metamorphosis into mycelium
ND 12	*Epicoccum*	Yellow	Orange	Septate	Foot cell present
DR 003	*Coniothyrium*	White	Yellow	Septate	Basal foot cell
DR 005	*cf. Pleosporales*	White	Transparent	Septate	Foot cell present
DR 007	*Fusarium*	White	Cream-Pinkish	Septate	Foot cell on the. macroconidia
DR 010	*Phomopsis*	White	Transparent	Septate	Cylindrical asci with a foot cell
DR 011	*Ceratobasidium*	White	Cream	Septate	Basidia with basidiospores

DR 012	*Pythium*	Cream-Brown	Cream	Septate	Foot cell absent
DR 014.1	*Neonectria*	White	Beige	Single Septate	Foot cell on the. macroconidia
DR 015	*Purpureocillium*	Cream	Purplish	Septate	Basal foot cell
DR 016	*Cladosporium*	Olive Brown	Dull Green	Septate	Basal foot cell
DR 019	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
RNK 001	*Talaromyces sp.*	Yellow	Cream	Septate	Foot cells are absent
RNK 005	*Aspergillus*	Brown	Black	Septate	Foot cell present
RNK 013	*Phoma sp.*	White	Cream	Septate	Foot cell absent (conidiogenous cells)
RNK 015	*Cochliobolus sp.*	Cream	Yellow-Reddish	Septate	Foot cell absent
RNK 017	*Fusarium*	Pink	Cream	Septate	Foot cell on the. macroconidia
RNK 023	*Plectosphaerella*	Yellow	Orange	Septate	Curved macroconidia with foot cells
PG 4	*Ceratobasidium*	Cream	Yellow	Septate	Basidia with basidiospores
PG 5	*Rhizoctonia*	White	Cream	Septate	Foot cell present
PG 6	*Colletotrichum*	Brown	Yellow	Septate	Foot cells are absent
PG 7	*Neopestalotiopsis*	White	Cream	Septate	Foot cell- like, with rhizoid
PG 10	*Humicola*	Pink	Deep Pink	Septate	Basal foot cell
PG 11	*Talaromyces*	Brown	Cream	Non-Septate	Foot cells are absent
END 01,2	*Epicoccum*	Yellow-Pinkish	Yellow-Pinkish	Septate	Foot cell present

END 006	*Dactylonectria*	Brown Green	Cream	Septate	Poorly developed foot cell
END 009	*Leptosphaerulina*	Cream	Yellow	Septate	Base of the conidiophore is a foot cell
END 011	*Rhizoctonia*	White Green	Yellow Green	Septate	Foot cell present
END 013	*Pezicula*	Yellow	Yellow	Septate	Prominent foot cell
END 015	*Boeremia*	White Yellowish	Yellow	Septate	Foot cell present
END 017,2	*Alternaria*	Brown	Yellow	Septate	Foot cell give rise to conidiophore
END 023	*Purpureocillium*	Cream	Yellow	Septate	Basal foot cell
MHE 006	*Penicillium*	Brownish	Cream	Septate	Foot cell give rise to conidiophore
MHE 009	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
MHE 016	*Alternaria*	Brownish	Yellow	Septate	Foot cell give rise to conidiophore
MHE 020	*Fusarium*	Yellow Pink	Yellow Orange	Septate	Foot cell on the. macroconidia
MHE 022	*Penicillium*	Deep Yellow	Yellow	Septate	Foot cell give rise to conidiophore
MHE 055	*Fusarium*	Black-Yellowish	Yellow	Septate	Foot cell on the. macroconidia
MHE 057	*Plectosphaerella*	Cream	Yellow	Septate	Curved macroconidia with foot cells
MHE 065	*Mortierella*	Cream	Pinkish	Septate	Rhizoids is produced from the foot cell
MHE 068	*Alternaria*	Cream	Yellow	Septate	Foot cell give rise to conidiophore

Table 3.2 summaries the morphological characteristics of the investigated endophytic fungi. The complete table is listed in Appendix 3. The summarized table was based on the results from Chapter 4, 5, 6 and 7. Furthermore, there were different genus group incorporated in the table.

The diagram below (Figure 3.11) classification of fungi isolates according to their specialized structures. The dominant was the conidiophore, which is a conidium-bearing hypha or filament. Interesting enough, there is yeast like fungi that undergo metamorphosis into mycelium.

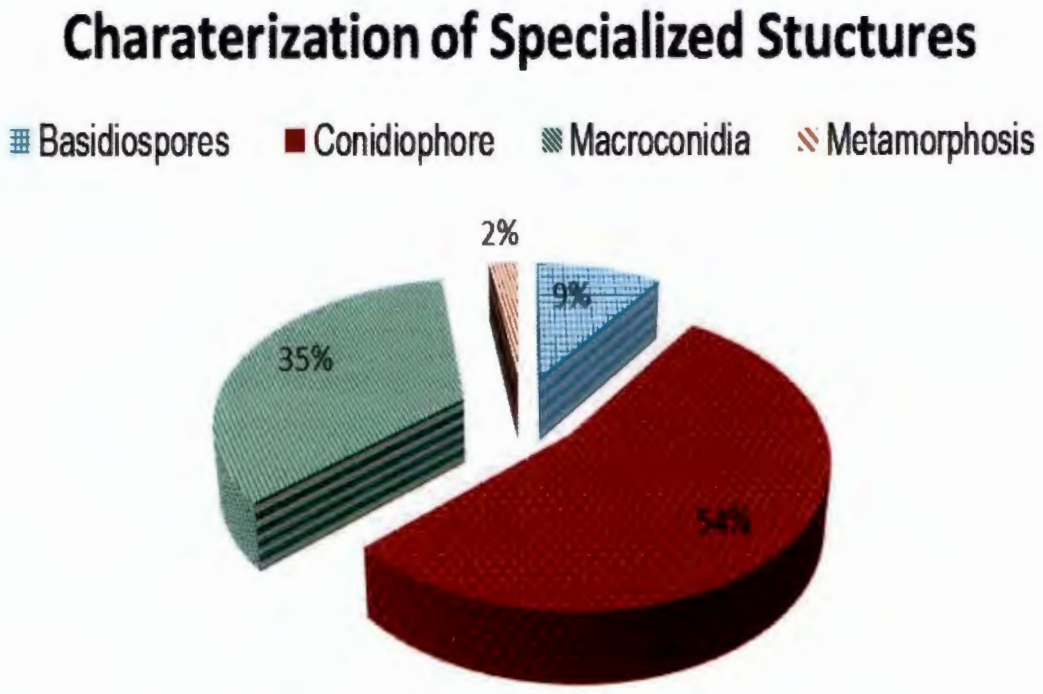


Figure 3.11: Major characterization of Specialized Structures

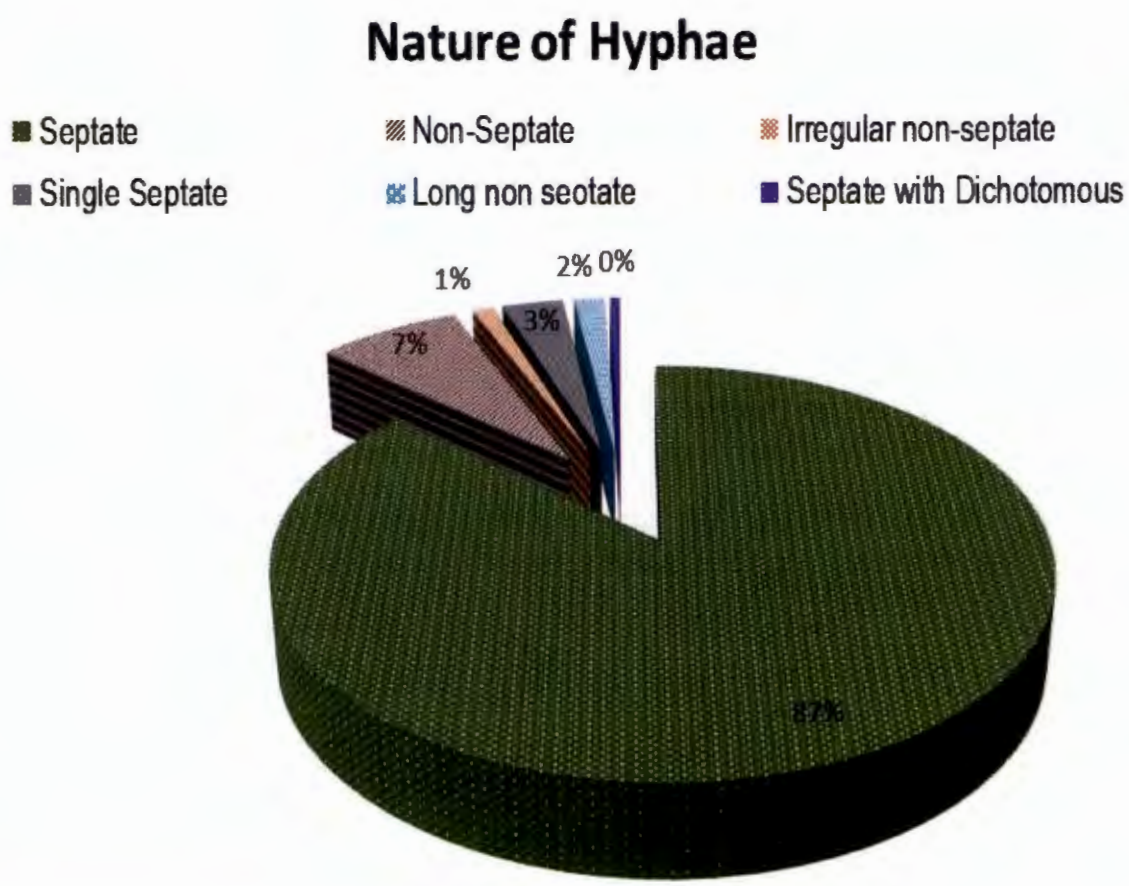


Figure 3.12: Percentages of Nature of the Hyphae under the microscope

Figure 3.12 represent the nature of the hyphae. Approximately, 87% are defined as septate followed 7% are single septate. Only one of them are septate with dichotomous.

3.4 CONCLUSION

Diversity of endophytic fungi isolated from medicinal plants is one of the most understudied field. One-hundred medicinal plants were collected from various locations around South Africa. Endophytic fungi from medicinal plants resulted in a broad spectrum of diversity. In the current study two medicinal plants, *Sceletium tortuosum* and *Pelargonium sidoides* were examined for their endophytic populationS. Surface sterilization of the leaves and roots yielded a total of 193 fungal isolates nonetheless; only 160 of the isolates were identified morphologically. The endophytic isolates were dominated by Ascomycota, followed by Basidiomycota and Zygomycota phyla. Additionally, the predominate genera were *Alternaria, Penicillium, Aspergillus, Neurospora* and *Fusarium*. Although morphological identification requests a skilled person in both Mycology and Taxonomy, it is not the most reliable method of identification. Molecular identification was conducted in chapter 4 to support the morphological results.

CHAPTER FOUR

Phylogenetic analysis and Diversity of Novel Endophytic fungi isolated from Medicinal Plants

CHAPTER 4

PHYLOGENETIC ANALYSIS AND DIVERSITY OF NOVEL ENDOPHYTIC FUNGI ISOLATED FROM MEDICINAL PLANTS

ABSTRACT

One hundred and ninety three (n=193) endophytic fungi were isolated from *Sceletium tortuosum* *Pelargonium sidoides* plants from various sites around South Africa. The fungal internal transcribe spacer (ITS) region (ITS1 and ITS4) and elongation factor (TEF 1 and 2) was used for identification of endophytic fungi to confirm the morphological results. Furthermore, molecular phylogenetic analysis was done using nuclear ribosomal DNA sequences. Predominate genus was *Fusarium*, followed by the genus *Aspergillus* and *Penicillium* which, was identified to species level based from known identities in GenBank. After running the gel electrophoresis, it was confirmed that the TEF region had close to 1000 bp while the ITS region showed a lower size of 700 bp. From the sequence data, three phylogenetic trees were constructed. Based on the sequence clustering, there were only three (DR 10, PG 6, MHE 65) were ungrouped fungal isolates. *Phomopsis columnaris* (DR 10), *Mortierella hyalina* (MHE 65) and *Colletotrichum* sp. (PG 6) were established as novel endophytic fungi in this study. Our results offers basic data on the symbiotic/or mutualistic relationship between medicinal plants and its endophytic fungi, as well as the potential to uncover novel species.

4.1 INTRODUCTION

In diverse sectors such as clinical pathology, biotechnology, environmental industry and pharmaceutics require exact identification of fungi. Traditional techniques are based on spore morphology, fruiting bodies and the development of sexual or asexual reproduction features which are all classified under morphological structures. Furthermore, this was assisted by the usage of identification keys (Kohlmeyer and Volkmann-Kohlmeyer, 1991). Endophytes are commonly referred to as a group of fungi which reside asymptomatically inside the living plant tissues (Hyde and Soytong, 2008; Sánchez Márquez *et al.,* 2007). Previous surveys of diverse host plants have shown that fungal endophytes are ubiquitous in plant species (Huang *et al.*, 2008; Oses *et al.,* 2008; Li *et al.,* 2007; Sánchez Márquez *et al.,* 2007; Zhang *et al.,* 2006). There are about one million species of endophytic fungi worldwide (Ganley *et al.*, 2004), which represent an important genetic resource for biotechnology.

Endophytes have been recognized as potential sources of novel natural products for pharmaceutical, agricultural and industrial uses, especially those secondary metabolites produced by fungal endophytes colonizing medicinal plants. Molecular techniques proved that specialized skills needed for accurately classify fungi at the species level, especially through conventional methods. In addition, due to the increasing population of immunocompromised patients, rapid detection and accurate identification of fungal pathogens are critical for optimal control. Although the study of endophytic fungal communities in plants has gained attention in recent years, little is known about the molecular identification and relatedness diversity of endophytic fungi (González-Teuber *et al.,* 2017). In recent times, molecular techniques are being used extensively in biodiversity studies of endophytes due to their sensitivity and specificity. Further, they are very rapid and economical, the techniques are not affected, or dependent on environmental factors likes culture conditions. Analysis of DNA extracted from fungi has been used widely as a means of identification as well as screening of fungi for their potential to produce certain desired metabolites (Jeewon *et al.,* 2013). Conventional morphological characterization of fungal endophytes has the drawback of difficulty in identifying the species, which have structural similarity. Furthermore, conventional identification is very difficult in fungal isolates that fail to sporulate in culture (Ding *et al.,* 2013).

Molecular methods were successfully utilized in identifying microorganisms at diverse hierarchical taxonomic levels due to their high sensitivity, specificity and quicker procedures. The fact that most of the endophytic fungi can be detected and identified based on comparative analyses of the ribosomal DNA sequences, especially the ITS region amplifies the extensive evaluation of endophytic fungi. The aim of the current segment is to identify the investigative endophytic fungi using modern molecular techniques and the relationship between them.

4.2 MATERIALS AND METHODS

All culture media used in this experiments were purchased from Biolab, Merck (South Africa, Midrand). All reagents, extraction kits were purchased from Inqaba Biotec (Pty.) Ltd in Pretoria, South Africa. All oligonucleotide primers used in this study were synthesized from Inqaba Biotec.

4.2.1 Molecular identification of endophytic fungi isolated from two medicinal plants

Molecular techniques were used in this study to confirm the identity of the isolates.

4.2.1.1 Preparation of fungal material

All 193 fungal strains were isolated from two indigenous medicinal plants of South Africa and were extracted for DNA analysis. All isolates were cultured on PDA or PCA plates until sporulation stage. Fresh mycelia were scraped off the surface of 10-day old cultures with a sterile scalpel. To ensure proper release of the DNA from the fungal cells, one hundred milligrams (100 mg) dried conidia/mycelia were placed in ZR BashingBead™ lysis tube matrix containing extraction buffer. Then tranquilly grinding was conducted using sterilized plastic tissue grinders.

4.2.1.2 Genomic Deoxyribonucleic acid (gDNA) extraction

DNA extraction process was done in an aseptic environment (Laminar flow). The investigative genomic DNA (gDNA) was extracted using the ZR Fungal/Bacterial DNA MiniPrep kit (catalogue number D6005, Inqaba Biotec, Pretoria, South Africa) following the manufacturer's instructions. Figure 4.1 illustrates the different stages in the extraction process when using ZR Fungal/Bacterial DNA MiniPrep kit.

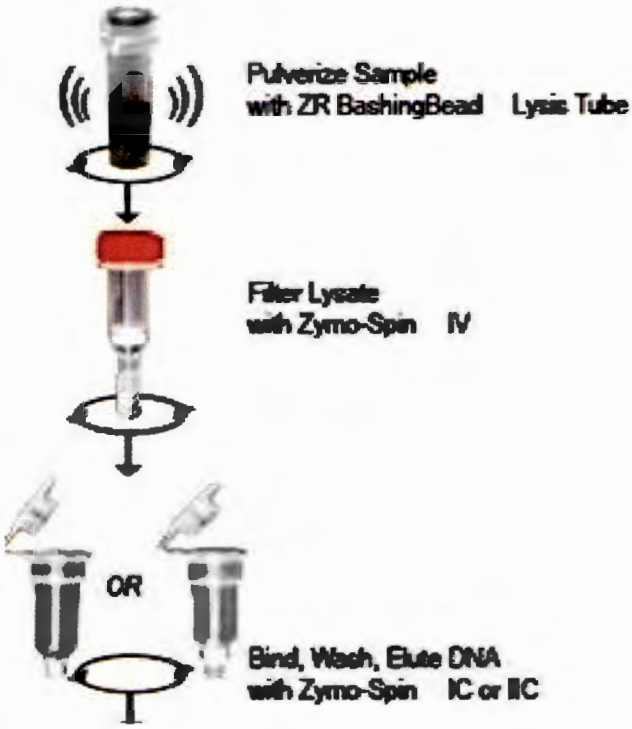


Figure 4.1: Various stages in the extraction process (Image, Funakoshi)

4.2.1.3 PCR identification of fungal species

Amplification of gDNA was done by polymerase chain reaction (PCR). The internal transcribed spacers (ITS1 and ITS 4) regions as well as elongation factor primers (TEF1 and TEF2) are utilized in this study. The TEF primers amplified *Fusarium* sp. and the ITS primers were used on all genus. A PCR reaction was prepared in a final volume of 20 µL in Table 4.1. Tables 4.2 represent the PCR conditions used for the amplification of the targeted genes and the PCR setup per tube (adapted from Khorasani, 2013). Prior to this, the DNA was diluted to 1:10 (1 µL DNA in 9 µL dH_2O). The concentration of the gDNA was measured using a Nano Lite spectrophotometer (Thermo scientific, South Africa).

4.2.1.4 Gel Electrophoresis

The amplified PCR amplicons were examined and visualized using 1.8% (w/v) agarose gel electrophoresis. Ethidium bromide (1 µL) was used as a fluorescent tag (nucleic acid stain) to check the size and purity of the amplicons. The bands were exposed to ultraviolet light by using a UV gel documentation system/Chemi Doc with Universal hood III (Bio Rad Laboratories, South Africa) for visualization (Barril and Nates, 2012). A 100bp DNA Ladder (Fermantas) was included in all gels as

a size marker. Furthermore, a negative control was included, which lacked the gDNA. These results were captured on the UV system.

Table 4.1: PCR reaction PCR reaction setup per tube

Buffer	**2 µl**
dNTPs	2 µl
EF1	0.5 µl
EF2	0.5 µl
Taq	0.2 µl
H20	9.8 µl
Diluted DNA	5 µl
Total reaction volume	20 µl

Table 4.2: PCR programme for ITS and TEF primers

Primers	Sequence (5'-3')	Targeted Species	Cycling conditions	
ITS 1 ITS 4	TCCGTAGGTGAACCTGCGG TCCTCCGCTTATTGATATGC	All genus	94 °C for 30 sec 52 °C for 45 sec 72 °C for 45 sec 72 °C for 7 min	30 Cycles
TEF 1 TEF 2	CGAATCTTTGAACGCACATTG CCGTGTTTCAAGACGGG	*Fusarium* species	94 °C for 30 sec 55 °C for 45 sec 72 °C for 45 sec 72 °C for 7 min	30 Cycles

4.2.1.5 Sequencing and data analysis

The purified PCR products were sent to Inqaba Biotechnologies (Pretoria, South Africa) for sequencing. BioEdit was used to oversee and align both sequences and then the consensus sequences were constructed. Alignments were manually edited where necessary. Identification was

established by BLAST (basic local alignment search tool) NCBI (National Center for Biotechnology Information, *Fusarium* MLST and MycoBank databases.

4.2.1.6 Phylogenetic assay

The consensus sequences were constructed from both the forward and reverse sequences. As stated above the sequences were firstly edited and subjected to BLAST searches to verify identity, taxonomic classification based on sequence similarity measures and phylogenetic interpretation. The investigated sequences were then aligned with other similar sequences downloaded from GenBank using ClustalX, BioEdit and MEGA program. To establish phylogenetic relationships at the genus and species level, the sequences were split into different datasets.

4.2.1.7 National Center for Biotechnology Information (NCBI) submission

All the cleaned sequences were submitted in the National Center for Biotechnology Information (NCBI) GenBank and unique accession numbers were provided.

4.3 RESULTS AND DISCUSSION

4.3.1 Molecular identification

A total of 193 fungal isolates were subjected to DNA extraction and the genomic DNA was used for the amplification of ITS region and TEF region. Amplification was successful in the target genes. After running the gel electrophoresis, it was confirmed that the TEF region had close to 1000 bp while the ITS region showed a lower size of 700 bp.

The Research conducted on fungal barcoding concludes that ITS is considered as the main fungal barcode marker to the Consortium for the Barcode of Life and that nuclear ribosomal small subunit has poor species-level resolution in fungi (Schoch *et al.,* 2016).

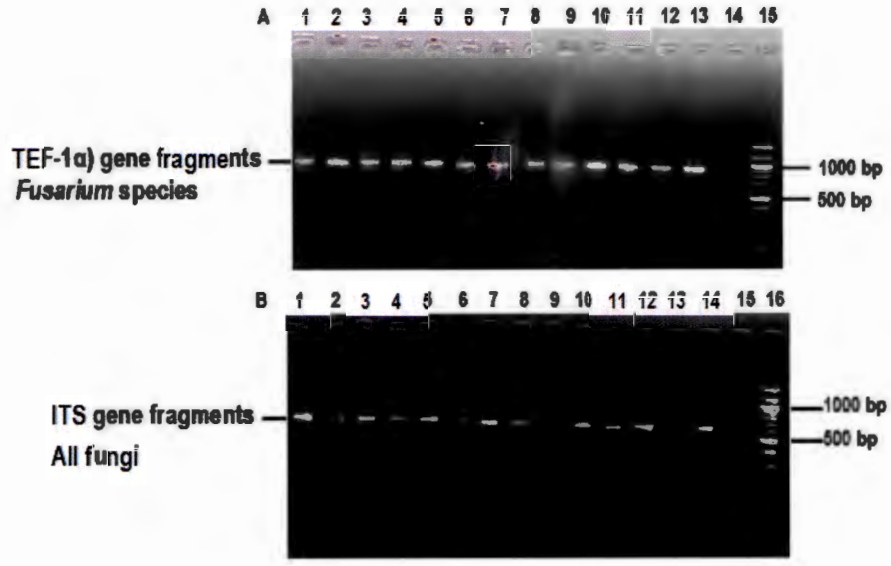


Figure 4.2: Agarose gel electrophoresis of PCR amplicons. Lane 1–14: Positive PCR amplicons. Lane 15: Negative control (water was used insist of DNA) A) TEF gene fragments used for *Fusarium* species B) ITS gene fragments used for all fungal isolates

Phylogenetic studies, maximum parsimony (MP), maximum likelihood (ML) and neighbour joining (NJ) analyses were implemented by using PAUP 4.0 and MEGA. Bootstrapping was done using 1000 replications via. heuristic search option to estimate the reliability of inferred monophyletic groups. This will form branch support of the trees resulting from maximum parsimony (MP) (Liu *et al.*, 2010). Primers (ITS, EF, Beta-tubulin AND RPB2) were used for each isolate to confirm the taxonomic classification and relationship at the familial, genetic, or species level, depending on the fungi of interest (Manitchotpisit *et al.*, 2009).

Table 4.3: Morphological identification, GenBank accession numbers and their top BLAST match sequences of the fungal isolates isolated from *Sceletium tortuosum*

Sceletium tortuosum					
Sample ID	**Closest related species**		**GenBank Best BLAST Match**		
			Accession No.	**No. coverage**	**Max iden**
GG 001	*Aspergillus*	sp.	KR154911.1	100%	98%
GG 002	*Mucor*	*circinelloides*	DQ118990.1	99%	99%
GG 003	*Fusarium*	*phaseoli*	KF717534.1	99%	98%
GG 004	*Penicillium*	*janthinellum*	KM268704.1	99%	99%
GG 005	*Fusarium*	*solani*	KP784419.1	100%	99%
GG 006	*Ceratobasidium*	sp.	KT265713.1	100%	99%
GG 007	*Alternaria*	sp.	KX270745.1	99%	100%
GG 008	*Fusarium*	*oxysporum*	KJ774041.1	100%	100%
GG 009	*Neurospora*	*sp.*	KT844666.1	100%	99%
GG 010	*Aspergillus*	*terreus*	KJ685810.1	100%	98%
GG 011	*Fusarium*	*solani*	KX349467.1	100%	99%
GG 012	*Fusarium*	*oxysporum*	JX840353.1	87%	90%
GG 013	*Aspergillus*	*fumigatus*	HE864321.1	100%	97%
GG 014	*Aspergillus*	*niger*	KP172477.1	98%	99%
GG 015.1	*Aspergillus*	sp.	KP881423.1	98%	99%
GG 015.2	*Aspergillus*	sp.	KM924435.1	99%	99%
GG 016	*Aspergillus*	*niger*	KM460938.1	95%	99%
ND 1	*Aspergillus*	sp.	KM924435.1	100%	99%
ND 2	*Aspergillus*	*fumigatus*	KF305755.1	98%	98%
ND 3	*Penicillium*	*brevicompactum*	KR912330.1	98%	98%
ND 4	*Penicillium*	*echinulatum*	KP411588.1	100%	98%

ND 5	*Neurospora*		KT844664.1	92%	99%
ND 6	*Penicillium*	sp.	KT192314.1	100%	99%
ND 7	*Geotrichum*	sp.	KU571521.1	97%	97%
ND 8	*Alternaria*	sp.	KP027305.1	98%	98%
ND 9	*Aspergillus*	sp.	KP881423.1	98%	99%
ND 10	*Alternaria*	sp.	KR094438.1	96%	100%
ND 12	*Epicoccum*	*nigrum*	KP721576.1	99%	95%
ND 13	*Fusarium*	*oxysporum f.* sp. *ciceris*	JN400682.1	100%	98%
ND 14	*Aspergillus*	*fumigatus*	JQ776545.1	99%	99%
ND 15	*Aspergillus*	*niger*	KJ881376.1	99%	98%
ND 16	*Aspergillus*	*niger*	JQ929761.1	99%	99%
ND 17	*Aspergillus*	sp.	KF305740.1	99%	99%
ND 18	*Neurospora*		KT844666.1	99%	99%
ND 19	*Fusarium*	*oxysporum f.* sp. *lycopersici*	KC478629.1	99%	97%
DR 001	*Aspergillus*	*niger*	KP748369.1	100%	100%
DR 002	*Fusarium*	*oxysporum*	EF495235.1	100%	99%
DR 003	*Coniothyrium*	*aleuritis*	KP749188.1	98%	97%
DR 004	*Fusarium*	*oxysporum f.* sp. *ciceris*	KU097318.1	98%	99%
DR 005	*cf. Pleosporales*	sp.	HM596868.1	98%	97%
DR 006	*Fusarium*	*equiseti*	KU715166.1	97%	94%
DR 007	*Fusarium*	*oxysporum f.* sp. *lycopersici*	KC478629.1	97%	96%
DR 008	*Fusarium*	sp.	DQ446211.2	99%	100%
DR 009	*Fusarium*	*equseti*	KU715166.1	99%	97%
DR 010	*Phomopsis*	*columnaris*	GU934561.1	99%	92%
DR 011	*Ceratobasidium*	sp.	KT428729.1	99%	83%
DR 012	*Pythium*	*heterothallicum*	KU210972.1	99%	98%

DR 013	*Fusarium*	*equiseti*	JQ412109.1	99%	100%
DR 014.1	*Neonectria*	sp.	HQ731630.1	99%	99%
DR 014.2	*Neonectria*	sp.	HQ731630.1	86%	99%
DR 015	*Purpureocillium*	sp	KJ935014.1	100%	99%
DR 016	*Cladosporium*	sp	KF976501.1	99%	99%
DR 017	*Fusarium*	*solani*	KP784419.1	99%	99%
DR 018	*Fusarium*	*dimerum*	JQ434586.1	98%	87%
DR 019	*Fusarium*	*penzigii*	NR_137707.1	83%	98%
DR 020	*Fusarium*	*subglutinans*	KU715164.1	97%	96%
DR 021	*Fusarium*	*equiseti*	JQ412109.1	99%	99%
DR 022	*Fusarium*	sp.	DQ446211.2	100%	99%
DR 023	*Fusarium*	*oxysporum*	KU746660.1	100%	99%
DR 024	*Fusarium*	sp	DQ446211.2	99%	99%

Table 4.4: Morphological identification, GenBank accession numbers and their top BLAST match sequences of the fungal isolates isolated from *Pelargonium sidoides*

Pelargonium sidoides					
Sample ID	**Closest related species**		**GenBank Best BLAST Match**		
			Accession No.	**No. coverage**	**Max iden**
RNK 001	*Talaromyces*	sp.	LT558971.1	87%	99%
RNK 002.1	*Penicillium*	*commune*	KP411582.1	99%	98%
RNK 002.2	*Fusarium*	*solani*	LN828155.1	99%	99%
RNK 003	*Aspergillus*	*niger*	KJ101598.1	24%	89%
RNK 004	*Penicillium*	*glabrum*	LT558918.1	99%	99%
RNK 005	*Aspergillus*	*tubingensis*	KX015988.1	100%	99%

RNK 006	*Penicillium*	*adametzioides*	LT558904.1	100%	99%
RNK 007	*Penicillium*	*pancosmium*	KU686681.1	99%	99%
RNK 008	*Penicillium*	*adametzioides*	KT717073.1	93%	96%
RNK 009	*Penicillium*	*glabrum*	JF327812.1	98%	99%
RNK 010	*Penicillium*	sp.	LC133853.1	100%	99%
RNK 011	*Penicillium*	*adametzioides*	LT558904.1	39%	95%
RNK 012	*Alternaria*	*brassicae*	JF439443.1	99%	100%
RNK 013	*Phoma*	sp.	AJ972865.1	100%	99%
RNK 014	*Aspergillus*	*niger*	KP172477.1	97%	99%
RNK 015	*Cochliobolus*	sp.	HQ174562.1	99%	99%
RNK 016	*Alternaria*	*tenuissima*	KF941273.1	90%	93%
RNK 017	*Fusarium*	*fujikuroi*	KJ000430.1	100%	99%
RNK 018	*Alternaria*	sp.	KU571545.1	100%	99%
RNK 019	*Alternaria*	*alternata*	KT192214.1	99%	99%
RNK 020	*Rhizopus*	*oryzae*	KX957745.1	100%	95%
RNK 021	*Rhizopus*	*oryzae*	KX957745.1	79%	92%
RNK 022	*Penicillium*	*thomii*	KU293591.1	100%	99%
RNK 023	*Plectosphaerella*	*oligotrophica*	KX446769.1	100%	99%
PG 1	*Neurospora*	*tetrasperma*	KU821473.1	52%	81%
PG 2	*Neurospora*		KT844677.1	100%	99%
PG 3	*Aspergillus*	*fumigatus*	KX610742.1	99%	100%
PG 4	*Ceratobasidium*	sp.	KM386636.1	99%	99%
PG 5	*Rhizoctonia*	*solani*	HF948013.1	100%	96%
PG 6	*Colletotrichum*	sp.	KM036382.1	99%	99%
PG 7	*Neopestalotiopsis*	*clavispora*	KR052094.1	99%	99%
PG 8	*Alternaria*	sp.	KU377235.1	100%	100%
PG 9	*Chaetomium*	*subaffine*	JN209929.1	99%	99%

PG 10	*Humicola*	sp.	KR259874.1	99%	99%
PG 11	*Talaromyces*	*funiculosus*	LT558961.1	98%	99%
PG 12	*Alternaria*	*alternata*	KX015999.1	99%	99%
END 01,1	*Epicoccum*	*nigrum*	KP721576.1	100%	96%
END 01,2	*Epicoccum*	*nigrum*	KU204774.1	100%	100%
END 001	*Penicillium*	*glabrum*	LT558918.1	99%	99%
END 002	*Aspergillus*	sp.	KP881422.1	100%	99%
END 03,1	*Cladosporium*	sp.	KF976501.1	99%	99%
END 03,2	*Cladosporium*	sp.	KF976501.1	100%	99%
END 04,1	*Cladosporium*	*cladosporioides*	KX664373.1	99%	99%
END 04,2	*Cladosporium*	sp.	KF976501.1	99%	99%
END 005	*Neonectria*	sp.	KF428653.1	95%	96%
END 006	*Dactylonectria*	*anthuriicola*	NR_121494.1	89%	99%
END 007	*Neonectria*	sp.	HQ731630.1	100%	99%
END 008	*Trichoderma*	*asperellum*	KC479809.1	99%	99%
END 009	*Leptosphaerulina*	*chartarum*	KM979510.1	87%	91%
END 010	*Penicillium*	*glabrum*	F327812.1	99%	98%
END 011	*Rhizoctonia*	sp.	HQ713496.1	95%	93%
END 012	*Aspergillus*	*niger*	KP172477.1	98%	98%
END 013	*Pezicula*	*rhizophila*	KR859245.1	100%	99%
END 014	*Penicillium*	*commune*	KP411582.1	99%	98%
END 015	*Boeremia*	*exigua var. pseudolilacis*	KT193802.1	91%	87%
END 016	*Alternaria*	*tenuissima*	KP278184.1	100%	99%
END 017,1	*Penicillium*	sp.	KJ935029.1	98%	99%
END 017,2	*Alternaria*	sp.	KT192334.1	99%	100%

END 018,1	*Aspergillus*	*niger*	KP172477.1	98%	99%
END 018,2	*Aspergillus*	sp.	KP881422.1	100%	99%
END 019	*Penicillium*	*chrysogenum*	KP216888.1	100%	98%
END 020,1	*Penicillium*	*commune*	KP411582.1	99%	98%
END 020,2	*Rhizopus*	sp.	KM401403.1	99%	99%
END 021	*Penicillium*	*commune*	KP411582.1	99%	98%
END 022	*Penicillium*	*commune*	KP411582.1	97%	98%
END 023	*Purpureocillium*	*lilacinum*	KC478538.1	100%	99%
END 024	*Penicillium*	*ludwigii*	KU933447.1	99%	99%
END 031	*Neonectria*	sp. *papochf*	HQ731630.1	96%	100%
MHE 001	*Fusarium*	*solani*	AB470903.1	99%	99%
MHE 002	*Fusarium*	*solani*	EF152426.1	99%	100%
MHE 003	*Trichoderma*	*asperellum*	KU171009.1	100%	86%
MHE 004	*Fusarium*	sp.	KT831452.1	98%	99%
MHE 005	*Ceratobasidium*	sp.	DQ102424.1	99%	99%
MHE 006	*Penicillium*	*rubens*	LC105692.1	100%	98%
MHE 007	*Penicillium*	*rubens*	LC105692.2	100%	100%
MHE 008	*Fusarium*	*solani*	AB518683.1	98%	99%
MHE 009	*Fusarium*	*nematophilum*	HQ897786.1	98%	99%
MHE 010	*Neurospora*	*crassa*	KR399979.1	99%	100%
MHE 011	*Penicillium*	sp.	LT558877.1	99%	99%
MHE 012	*Chaetomium*	sp.	KX345371.1	100%	99%
MHE 014	*Neurospora*		KT844666.1	70%	86%
MHE 015	*Fusarium*	*solani*	KP992930.1	91%	92%
MHE 016	*Alternaria*	*alternata*	KT192214.1	99%	99%

MHE 017	*Neurospora*		KT844677.1	99%	99%
MHE 018	*Neurospora*		KT844675.1	81%	99%
MHE 019	*Neurospora*		KT844666.1	99%	96%
MHE 020	*Fusarium*	sp.	GQ505449.1	99%	93%
MHE 021	*Aspergillus*	*sclerotiorum*	AY373866.1	100%	96%
MHE 022	*Penicillium*	*ruben*	LC105692.1	100%	80%
MHE 023	*Penicillium*	*chrysogenum*	KR233468.1	100%	98%
MHE 024	*Aspergillus*	*fumigatus*	KX610742.1	99%	100%
MHE 025	*Penicillium*	*chrysogenum*	JF834167.1	96%	97%
MHE 026	*Penicillium*	*chrysogenum*	KF039676.1	100%	99%
MHE 027	*Talaromyces*	*funiculosus*	LT558961.1	99%	99%
MHE 028	*Albifimbria*	*viridis*	KU845899.1	99%	98%
MHE 029	*Penicillium*	sp.	KC871050.1	77%	80%
MHE 030	*Undifilum*	*oxytropis*	HM588132.1	6%	97%
MHE 031	*Penicillium*	*adametzioides*	LT558904.1	86%	98%
MHE 032	*Penicillium*	*rubens*	KX011023.1	96%	97%
MHE 033	*Aspergillus*	sp.	KM458796.1	80%	84%
MHE 034	*Penicillium*	*rubens*	KX011023.1	99%	98%
MHE 035	*Penicillium*	*chrysogenum*	GU985086.1	99%	99%
MHE 036	*Penicillium*	*expansum*	LC015096.1	91%	96%
MHE 040	*Fusarium*	*nematophilum*	HQ897786.1	98%	99%
MHE 041	*Neurospora*		KT844672.1	100%	99%
MHE 042	*Neurospora*		KT844674.1	95%	80%
MHE 043	*Geotrichum*	*candidum strain*	KT921198.1	80%	89%
MHE 044	*Fusarium*	*solani*	KU382607.1	100%	100%
MHE 045	*Fusarium*	sp.	KR093179.1	97%	92%
MHE 046	*Fusarium*	*solani*	KT211516.1	100%	99%

MHE 047	*Trichoderma*	*asperellum*	JF501661.1	99%	99%
MHE 047,2	*Trichoderma*	*asperellum*	KC479809.1	99%	97%
MHE 048	*Neocosmospora*	*rubicola*	KU059906.1	100%	97%
MHE 049	*Fusarium*	*solani*	KP784419.1	100%	99%
MHE 050	*Aspergillus*	*fumigatus*	KM491894.1	99%	99%
MHE 051	*Neurospora*	*tetrasperma*	KX247295.1	100%	83%
MHE 052	*Alternaria*	*alternate*	KX015999.1	100%	99%
MHE 053	*Epicoccum*	*nigrum*	FN868456.1	98%	99%
MHE 054	*Aspergillus*	*fumigatus*	KX610742.1	98%	97%
MHE 055	*Fusarium*	*solani*	KX349467.1	93%	85%
MHE 056	*Fusarium*	sp.	KU974306.1	72%	77%
MHE 057	*Plectosphaerella*	*oligotrophica*	KX446769.1	100%	99%
MHE 058	*Mucor*	*nidicola*	KM923759.1	35%	91%
MHE 059	*Geotrichum*	*candidum strain*	KT921198.1	100%	91%
MHE 060	*Aspergillus*	*fumigatus*	JQ764801.1	53%	78%
MHE 061	*Aspergillus*	*fumigatus*	LT596575.1	93%	86%
MHE 062	*Alternaria*	*alternata*	KX015999.1	100%	99%
MHE 063	*Phomopsis*	sp.	KT192253.1	31%	92%
MHE 064	*Alternaria*	sp.	GU004277.1	99%	89%
MHE 065	*Mortierella*	*hyalina* strain	JN943801.1	98%	98%
MHE 066	*Alternaria*	sp.	KT192334.1	100%	99%
MHE 067	*Alternaria*	*alternata*	KX015999.1	91%	89%
MHE 068	*Alternaria*	sp.	KX011019.1	98%	99%

4.3.2 Phylogenetic Relationship

All the consensus sequences were aligned using Multiple Alignment using Fast Fourier Transform (MAFF) program and construction of the phylogenetic tree was based on the neighbor joining method consisting of 1000 bootstrap replications associated taxa clustered (Fig. 4.3). Phylogenetic analysis using internal transcribed spacer (ITS) and translation elongation factor regions of endophytic fungi distributed isolates into three Clusters, which are illustrated in Fig. 4.3. Cluster 2a showed a significant weakness in the bootstrap support, which was less than 50%. This means weak relatedness to each other. Further, interpretation revealed that all clusters (Cl 1-3) have strains belonging to Division ascomycota while the Division basidiomycota was located in clusters 1 and 2. Nevertheless, the genus *Ceratobasidium* (GG 6, DR 11) is clustered under different taxa groups 1b and 3b. Thought-provoking strains were situated in cluster 1 but in different sub-clusters (DR 12, GG2) belonged to the division Oomycota and Zygomycota. Ascomycota dominate the scientific classification with an average of 81% in the phylogenetic tree. Strong phylogenetic relationships/homology (95-100%) was shown with symbols. On the other hand, 50% and less were not displayed in the phylogenetic tree. This data corresponds with Miguel *et al.* (2017), Basidiomycota and Ascomycota were the dominate phyla isolated from eucalyptus leaves.

Table 4.3 shows all the investigative fungal isolates utilized in the BLAST search with the closest related species. A 100% maximum identity was established for the *following Fusarium equiseti, Alternaria sp. Fusarium oxysporum, Alternaria sp. Aspergillus niger, Fusarium sp. Fusarium equiseti* (DR 13, GG 7, GG 8, ND 10, DR 1, DR 8, DR 13). The consensus sequences were submitted in NCBI database, GenBank bank it, and the accession numbers were issued as supportive documentation.

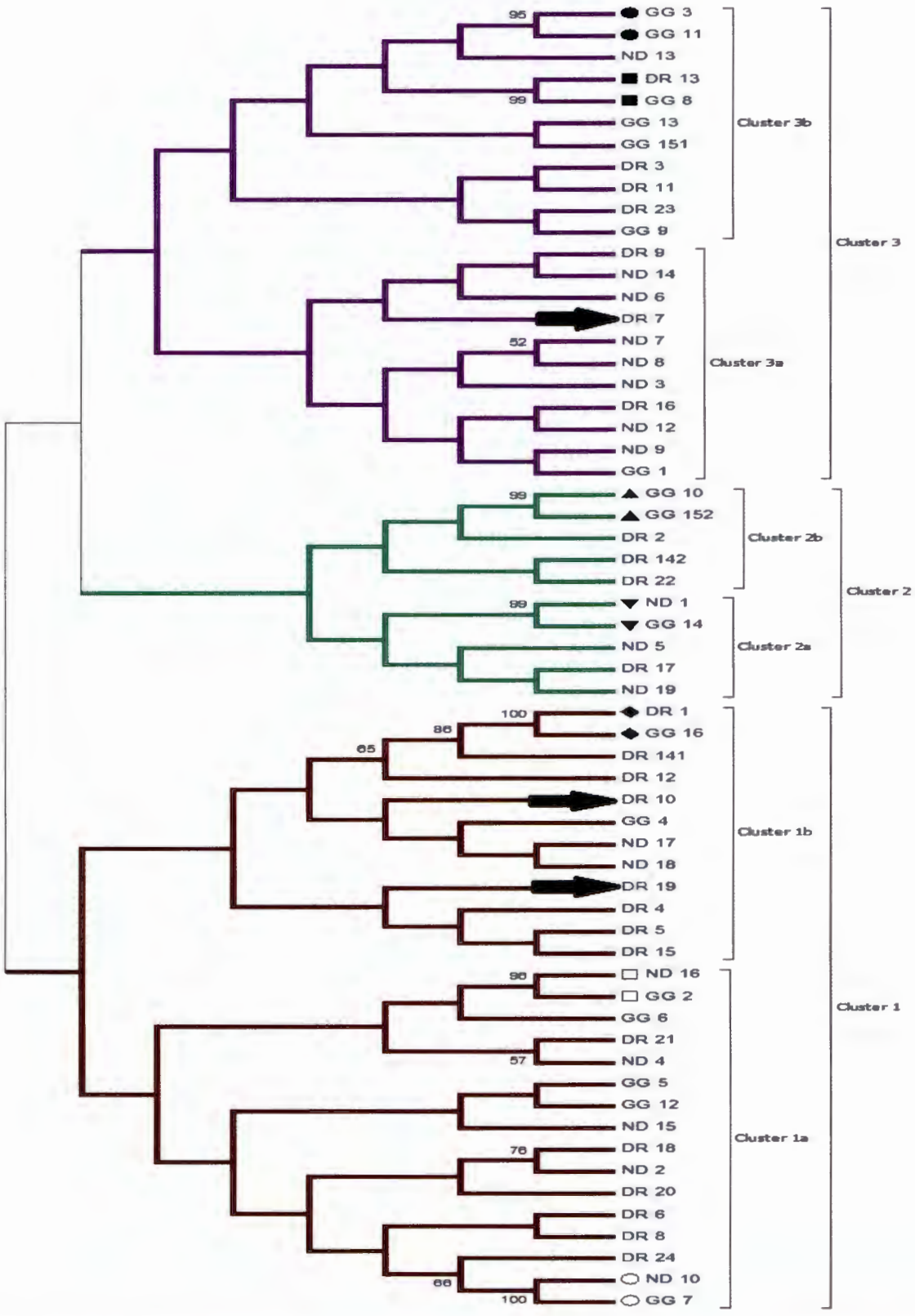
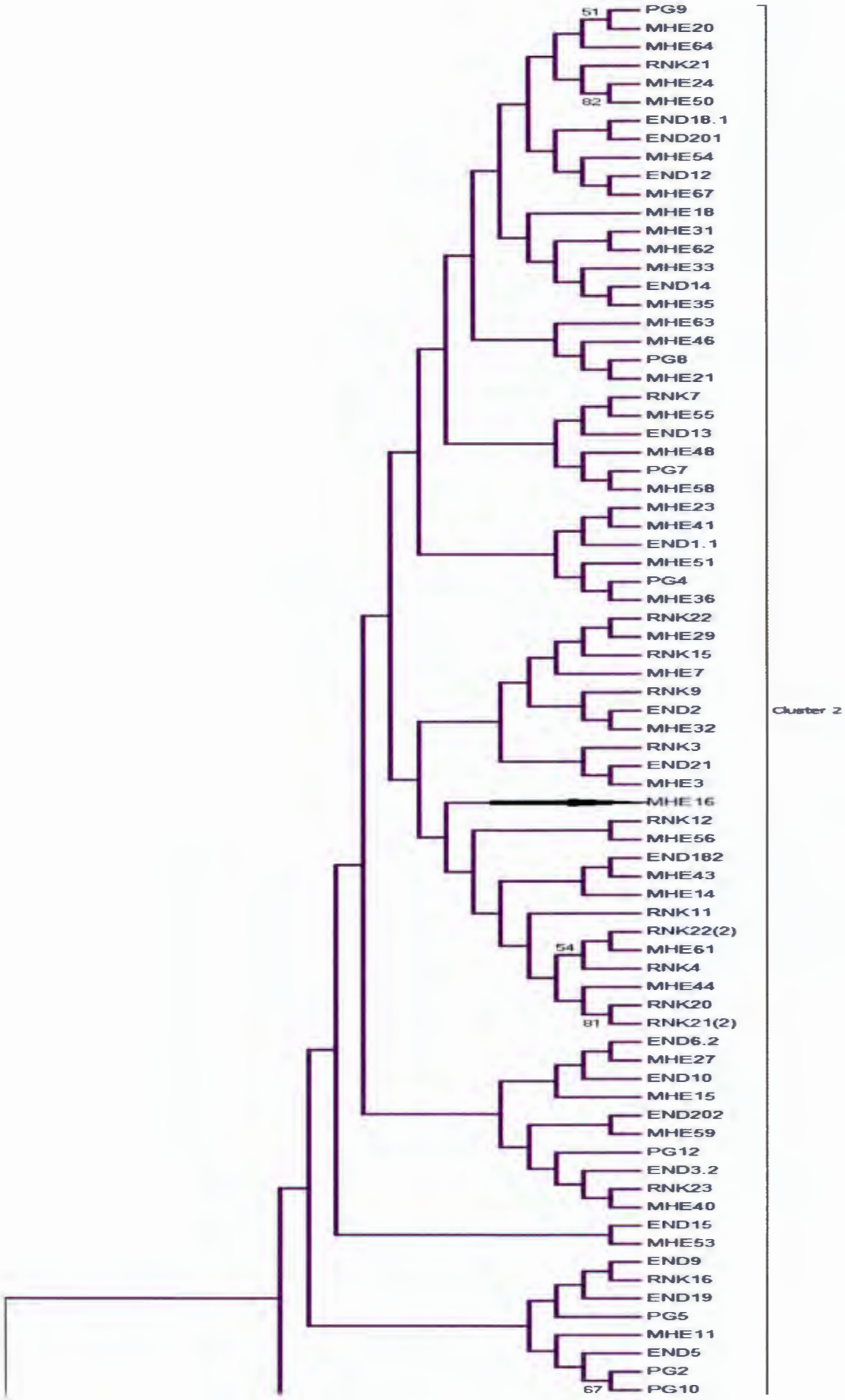


Figure 4.3: Phylogenetic tree constructed by neighbour joining method using ITS sequences of 60 fungal strains. Bootstrap values are based on 1,000 replicates while above 50% are indicated on the branches.


51
PG9
MHE20
MHE64
RNK21
MHE24
82
MHE50
END18.1
END201
MHE54
END12
MHE67
MHE18
MHE31
MHE62
MHE33
END14
MHE35
MHE63
MHE46
PG8
MHE21
RNK7
MHE55
END13
MHE48
PG7
MHE58
MHE23
MHE41
END1.1
MHE51
PG4
MHE36
RNK22
MHE29
RNK15
MHE7
RNK9
END2
MHE32
RNK3
END21
MHE3
MHE16
RNK12
MHE56
END182
MHE43
MHE14
RNK11
RNK22(2)
54
MHE61
RNK4
MHE44
RNK20
81
RNK21(2)
END6.2
MHE27
END10
MHE15
END202
MHE59
PG12
END3.2
RNK23
MHE40
END15
MHE53
END9
RNK16
END19
PG5
MHE11
END5
PG2
67
PG10
Cluster 2

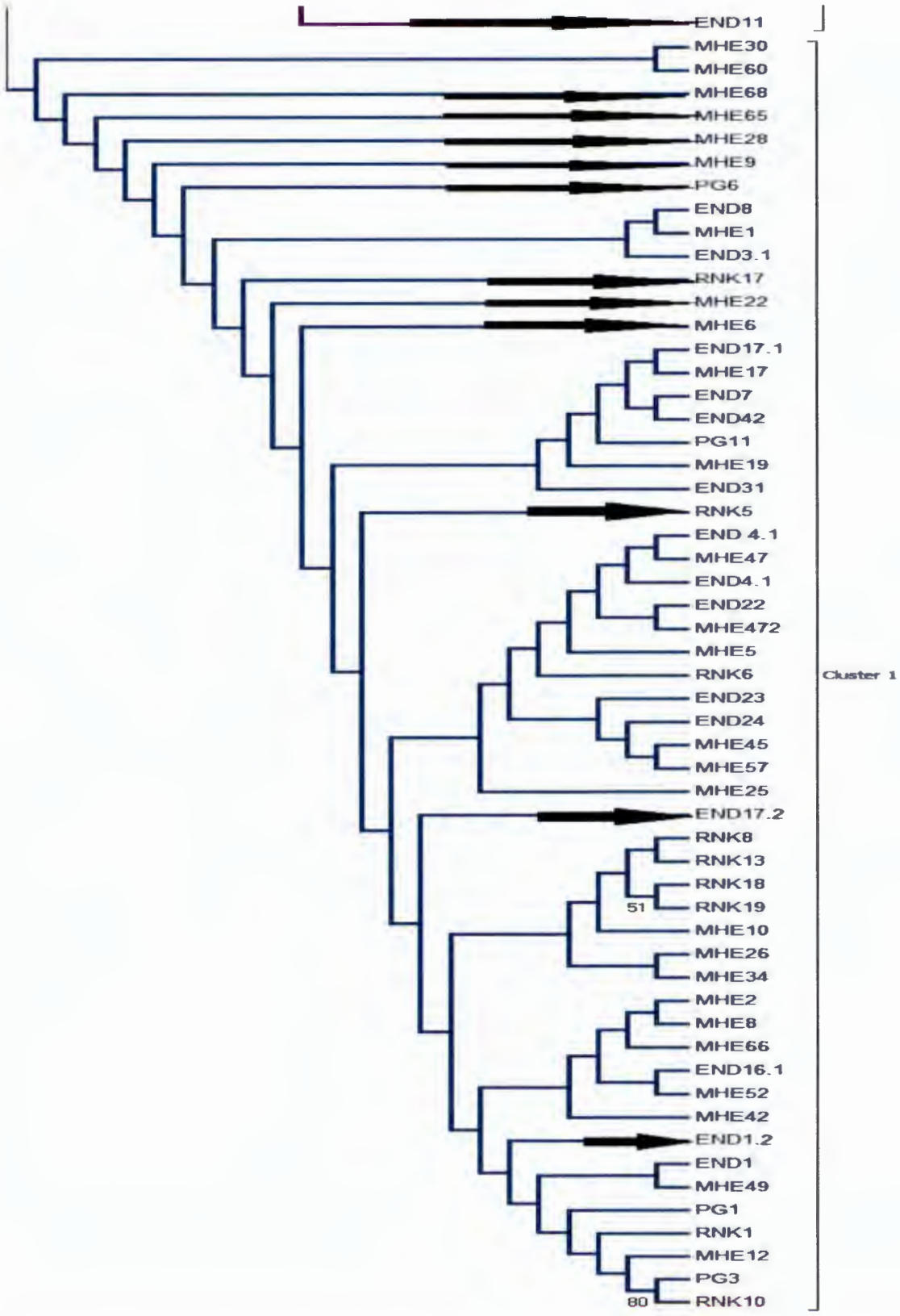


Figure 4.4: Phylogenetic tree constructed by neighbour joining method using ITS sequences of 60 fungal strains. Bootstrap values are based on 1,000 replicates while above 50% are indicated on the branches.

The reconstruction of the tree was carried out to determine the evolutionary and phylogenetic relationships or close relatives. The sequences generated from the isolates were compared to reference fungal taxa in the database. The sum of branch length was calculated as 156.35442900 and the final dataset total was 226 positions. This was extracted from 60 nucleotide sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). Using phylogenetic trees, the clades were grouped and clustered, in accordance there are novel endophytic isolates represented by a black arrow in both the trees.

Table 4.4: Uncluttered strains as possible novelty

No.	Sample ID	Genus	Species	Subspecies
1	DR 007	*Fusarium*	*oxysporum*	f. sp. *lycopersici*
2	DR 010	*Phomopsis*	*columnaris*	
3	DR 019	*Fusarium*	*penzigii*	
4	MHE 016	*Alternaria*	*alternata*	
5	MHE 006	*Penicillium*	*rubens*	
6	MHE 009	*Fusarium*	*nematophilum*	
7	MHE 020	*Fusarium*	sp.	
8	MHE 022	*Penicillium*	*ruben*	
9	MHE 065	*Mortierella*	*hyalina*	
10	MHE 068	*Alternaria*	sp.	
11	END 01,2	*Epicoccum*	*nigrum*	
12	END 011	*Rhizoctonia*	sp.	
13	END 017,2	*Alternaria*	sp.	
14	RNK 005	*Aspergillus*	*tubingensis*	
15	RNK 017	*Fusarium*	*fujikuroi*	
16	PG 6	*Colletotrichum*	sp.	

The novel isolates clustered with the broad range of species including *Fusarium, Aspergillus, Phomopsis* and others, which are listed in Table 4.5. Interestingly, all novel isolates have been reported to have latent pathogenicity to mutual association.

4.4. NOVEL ENDOPHYTIC FUNGAL ISOLATES

Due to the divergent in allocation and ungrouped species indicated in table 4.3 and Figure 4.5, they were novel in this study. Figure 4.5 was constructed using novel 16 fungal strains. The phylogenetic tree demonstrates clades/groupings. However, three strains (DR 10., MHE 65, PG 6) were separated from the clades and are represented with a red dot. In this section, these strains are described and justification for their novelty.

4.4.1 *Phomopsis columnaris* (DR 10)

In the last decade, *Phomopsis columnaris* was identified as a pathogen due to the fact that it was linked to an outbreak of dieback and fruit rot of blueberry and cranberry (*Vaccinium* spp.) in Oregon (Farr *et al.*, 2002). However, in the current study, this endophytic fungus can be regarded as beneficial to the plant because it does not display any pathogenic traits. This demonstrates that the balance of mutualistic\pathogenic relationship can vary depending on the host and the environmental conditions. In addition, symbolic relationships between this fungus and its host benefits all parties involved (Yokoya *et al.*, 2017). As indicated in table 4.3, the isolate DR 10 identified as *Phomopsis columnaris* revealed a 92% sequence identity similarity to a previously deposited sequence in GenBank (GU934561.1) and was considered a novel endophyte isolated from *Sceletium tortuosum.*

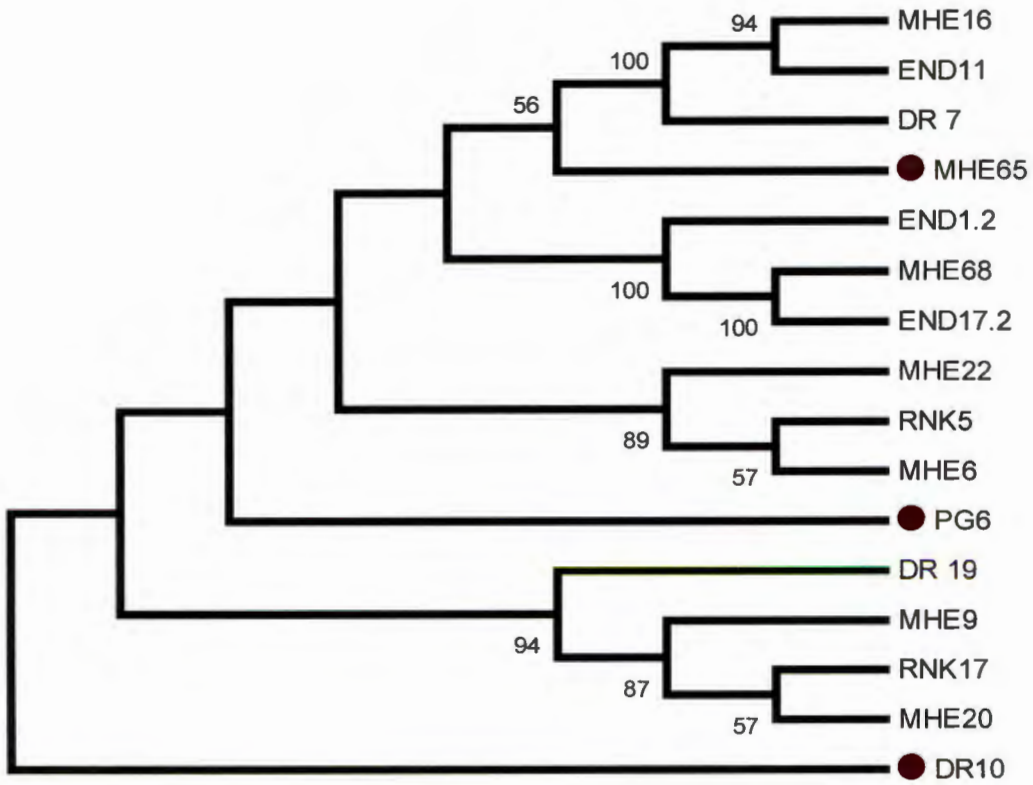


Figure 4.5: Phylogenetic tree constructed by neighbor joining method using ITS sequences of novel 16 fungal strains. Bootstrap values is based on 1,000 replicates while above 50% are indicated on the branches.

4.4.2 *Mortierella hyalina* (MHE 65)

They are regarded as non-pathogenic to plants, animals and humans. The full name of isolate MHE 65 is *Mortierella hyalina* (Harz) W. Gams, titled in 1970. *Mortierella hyalina* clusters with MHE 16, END 11 and DR 7 supported 56 % bootstrap. Even though they are clustered, MHE 65 is still distinct. Sherameti *et al.* (2014) refer to *Mortierella hyalina* as beneficial root-colonizing fungus. MHE 65 showed 98% identity with *Mortierella hyalina* (JN943801.1) from the database of GenBank.

4.4.3 *Colletotrichum* sp. (PG 6)

Colletotrichum sp. (PG 6) is very distinct, because it does not form part of a clade. The genus exists predominantly as endophytes or phytopathogens in a symbolism with plants. Nevertheless, other species within the genus are plant pathogens. Mutualistic relationship with hosts also occur (Rodriguez and Redman, 2008). PG 6 strain showed 99% sequence similarities with *Colletotrichum* sp. (KM036382.1) according to the GenBank database.

4.5 UNIQUE ACCESSION NUMBERS FROM GENBANK

The National Center for Biotechnology Information (NCBI) is progressive information site that provides the science and health community with access to biomedical and genomic information. Furthermore, the GenBank tools assist with genetic sequence database, an annotated collection of all publicly available DNA sequences. Table 4.6 is examples of submitted and published sequences from this study.

Table 4.6: Published sequences submitted to the NCBI

gb|KX840132| AG at 425	gb|KX840075| AG at 424	gb|KX840076| AG at 424
gb|KX840134| AG at 425	gb|KX840077| AG at 424	gb|KX840078| AG at 424
gb|KX840136| AG at 425	gb|KX840079| AG at 424	gb|KX840080| AG at 424
gb|KX840138| AG at 425	gb|KX840081| AG at 424	gb|KX840082| AG at 424
gb|KX840140| AG at 425	gb|KX840083| AG at 424	gb|KX840084| AG at 424
gb|KX840142| AG at 425	gb|KX840085| AG at 424	gb|KX840086| AG at 424
gb|KX840144| AG at 400	gb|KX840087| AG at 424	gb|KX840088| AG at 424
gb|KX840146| AG at 425	gb|KX840089| AG at 234	gb|KX840089| AG at 424
gb|KX840148| AG at 400	gb|KX840090| AG at 424	gb|KX840091| AG at 424
gb|KX840150| AG at 425	gb|KX840092| AG at 424	gb|KX840093| AG at 408
gb|KX840152| AG at 425	gb|KX840094| AG at 425	gb|KX840095| AG at 425
gb|KX840154| AG at 425	gb|KX840096| AG at 425	gb|KX840097| AG at 425
gb|KX840156| AG at 425	gb|KX840098| AG at 425	gb|KX840099| AG at 425
gb|KX840158| AG at 425	gb|KX840100| AG at 425	gb|KX840101| AG at 425
gb|KX840160| AG at 425	gb|KX840102| AG at 425	gb|KX840103| AG at 394
gb|KX840162| AG at 425	gb|KX840104| AG at 425	gb|KX840105| AG at 425
gb|KX840164| AG at 425	gb|KX840106| AG at 425	gb|KX840107| AG at 425
gb|KX840166| AG at 425	gb|KX840108| AG at 425	gb|KX840109| AG at 400
gb|KX840168| AG at 425	gb|KX840110| AG at 425	gb|KX840111| AG at 425
gb|KX840170| AG at 425	gb|KX840112| AG at 425	gb|KX840113| AG at 425
gb|KX840172| AG at 425	gb|KX840114| AG at 425	gb|KX840115| AG at 425
gb|KX840174| AG at 425	gb|KX840116| AG at 425	gb|KX840117| AG at 425
gb|KX840176| AG at 425	gb|KX840118| AG at 425	gb|KX840119| AG at 425
gb|KX840167| AG at 425	gb|KX840120| AG at 425	gb|KX840121| AG at 425
gb|KX840169| AG at 425	gb|KX840122| AG at 425	gb|KX840123| AG at 425
gb|KX840171| AG at 425	gb|KX840124| AG at 425	gb|KX840125| AG at 425
gb|KX840173| AG at 425	gb|KX840126| AG at 425	gb|KX840127| AG at 425
gb|KX840175| AG at 425	gb|KX840128| AG at 425	gb|KX840129| AG at 425
gb|KX840177| AG at 425	gb|KX840130| AG at 425	gb|KX840131| AG at 425
gb|KX840157| AG at 425	gb|KX840145| AG at 425	gb|KX840133| AG at 425
gb|KX840159| AG at 425	gb|KX840147| AG at 400	gb|KX840135| AG at 425
gb|KX840161| AG at 425	gb|KX840149| AG at 425	gb|KX840137| AG at 425
gb|KX840163| AG at 425	gb|KX840151| AG at 425	gb|KX840139| AG at 425

gb|KX840165| AG at 425	gb|KX840153| AG at 425	gb|KX840141| AG at 425
gb|KX840143| AG at 394	gb|KX840155| AG at 425	

4.6 CONCLUSION

The endophytic fungi were identified using molecular techniques and their relationships were established using phylogenetic analysis. Our findings indicate sixteen (16) novel endophytic fungi isolated from two indigenous medicinal plants. However, they were clustered together and only three (DR 10, PG 6, MHE 65) were ungrouped. The clustering of the endophytic fungi is in agreement with other studies, thereby supporting the hypothesis that plant growth and fitness are inhibited by a diverse group of genus and species. Finally, as demonstrated in this study, novel species might be isolated from indigenous plants in Africa. Such untapped environment could be a novel source of endophytes with growth promoting properties.

CHAPTER FIVE

Antimicrobial Activity and Characterization of Secondary Metabolites Extracted FROM Endophytic Fungi

CHAPTER 5

ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF SECONDARY METABOLITES EXTRACTED FROM ENDOPHYTIC FUNGI

ABSTRACT

Endophytic fungi have the ability to live inside the host plant tissues without causing neither symptoms of diseases/ or harm. The study was aimed to examine the antimicrobial activity of endophytic fungi isolated from native South Africa medicinal plants. A total of 193 endophytic fungi were test against Gram positive and negative bacteria. Out of 193 isolates only 24 (12%) inhibited the growth of selected bacteria. Majority of fungal extracts exhibited activity against *Escherichia coli* (ATCC 25922). In contrast, *Enterococcus faecium* (ATCC 700221), *E. gallinarum* (ATCC 700425) were the most resistant pathogens. *Alternaria* endophytic fungus was responsible for a broad spectrum of activities and the highest. GC-MS identified the compound primary liable for the activity, which were identified as 9,12-Octadecadienoic acid (Z,Z) and Cyclodecasiloxane. In conclusion, our study showed that endophytic fungi possess bioactive compound that directly benefits the host plants. This is the first study conducted on fungal isolates isolated from *Sceletium tortuosum* L. and *Pelargonium sidoides* DC plants.

CHAPTER 5

ANTIMICROBIAL ACTIVITY OF SECONDARY METABOLITES EXTRACTED FROM ENDOPHYTIC FUNGI

5.1 INTRODUCTION

Pharmaceutical and agricultural products are currently under threat from resistance by microorganisms thus limiting therapeutic options as well as agricultural crop production. This has resulted in renewed efforts to search for new products with enhanced antimicrobial properties (Schulz *et al.*, 2002). Given that, it has been reported that natural products are adapted to a specific function in nature, this therefore implies that efforts to search for novel secondary metabolites should concentrate on organisms such as endophytic fungi that inhabit unusual unique biotopes. Despite this, in South Africa there is an abundance of untapped wealth from medicinal and aromatic plants because these plants occur naturally in mountainous landscapes. Against this background, some native tribes in Southern Africa have used the flora and fauna available to their communities in the generation of indigenous knowledge practices and the generation of diverse natural resources for the benefit of humankind (Willis, 2006). This therefore explains why some native tribes in South Africa have used the indigenous medicinal plants (*Sceletium tortuosum* L. and *Pelargonium sidoides* DC) for primary health care purposes (Department of Agriculture, Forestry and Fisherie, 2013).

Sceletium tortuosum has been used traditionally as a hypnotic, a sleep-inducing drug and or as a sedative (a drug that calms patients or ease agitation) (van Wyk, 2008). However, *Sceletium tortuosum* has been used in recent years in the reduction of stress, improving wellbeing, as well as treating anxiety and depression in clinically anxious and depressed patients (Gericke and Viljoen, 2008). On the contrary, *Pelargonium sidoides* is mainly used for the treatment of respiratory diseases such as tuberculosis, tonsillitis, sore throat, and the common cold (van Wyk, 2008).

Secondary metabolites (SMs) are small organic molecules that produced by an organism and are not essential for their growth, development and reproduction. A wide variety of medicinal plants is capable of producing secondary metabolites; and these plants have been reported to harbour endophytic fungi that inhabit these natural biotopes. There is thus a positive correlation of endophytic fungi possessing the same or even more enhanced metabolites producing properties than their hosts. There is a possible that the host plants have the medicinal properties because of the

endophytes (Blackwell, 2011). This explains why there is a huge variance between plant-to-fungal diversity in nature which currently stands at 1:6 (Blackwell, 2011), thereby increasing the probabilities of discovering novel secondary metabolites within the fungal community. Endophytic fungi also play an essential role in the physiological and ecological adaptation of plants to the constantly changing environmental conditions (Thatoi *et al.*, 2013) and thus serve as growth promoters, and also facilitate resistance to both biotic and abiotic stress, tolerance to drought and as natural replants of insects and herbivores (Meena *et al.*, 2017). Given the challenges caused by the constant detection and presence of antibiotic resistant bacterial strains, fungi that produce secondary metabolites with enhanced antimicrobial properties were not just the first to be discovered but continue to dominate in both antimicrobial research as well as the market (Makut and Owolewa, 2011).

Current research in the area therefore is centred on the continual search for new antimicrobial products for pharmaceutical and agricultural purposes and thus focuses on medicinal plants and their endophytic fungi which have been under-exploited due to their geographical locations (Kumar, 2010). This is aimed at discovering novel and affordable active compounds with high pharmaceutical efficacies against resistant strains thus reducing the public health implications of resistant bacteria to humans (Kumar, 2010). In this chapter, the potential role of endophytic fungi from two indeginous plants; *Sceletium tortuosum* L. and *Pelargonium sidoides* in the production of biologically active secondary metabolites at optimum temperature, time and ideal media were investigated. This is motivated from the fact that secondary metabolites have been reported to provide most of the therapeutic activities of both medicinal plants and fungi (Yarnell, 2003). A further objective was to assess the potential of the fungal extracts in inhibiting the growth of bacterial strains and also identify the bioactive compounds in the extracts using the Gas Chromatography Mass Spectrophotometry (GC-MS).

5.2 MATERIALS AND METHODS

5.2.1 Bacteria strains

Fungal isolates were obtained from indigenous medicinal plants *Sceletium tortuosum* L. and *Pelargonium sidoides* as indicated in Sections 3.2.2 (Chapter 3) and their identities confirmed as shown in Sections 4.3.1 (Chapter 4). In order to assess the potential of fungal isolates as a source of

natural bioactive products all the 193 endophytes were subjected to laboratory protocols designed to screen for the production of biologically active secondary metabolites as well as an antimicrobial phenotypic assay adapted from the Kirby-Bauer technique. Bacterial test strains that were used in the antibiotic assay comprised control strains purchased from the American Type Culture Collection (ATCC) and environmental pathogenic strains previously isolated either from animals or water and identities confirmed by PCR. The environmental pathogenic strains were thus obtained from the bacterial culture collection of the Molecular Microbiology Research Laboratory in the Department of Microbiology, North West University and the data in Table 5.1 indicates the genera of the bacterial isolates, their origins identities and accession numbers.

Table 5.1: Target bacteria used in the analysis of secondary metabolite production and antimicrobial assays.

Target Bacteria	Accession no.	Origin
E. coli	ATCC 25922	ATCC Collection
E. coli	O177	Environmental isolate from cattle faeces
B. cereus	ATCC 10876	ATCC Collection
E. faecalis	ATCC S1299	Environmental isolate from ground water
E. faecium	ATCC 700221	Environmental isolate from ground water
E. gallinarum	ATCC 700425	ATCC Collection

5.2.2 Growth and production of secondary metabolites/fungal extracts

Preserved component fungal cells were grown on Potato Dextrose agar (PDA) (Merck, Darmstadt, Germany) and aerobically incubated at 25 °C for 10 days. Pure cultures from the single spore method were assessed for the potential to produce secondary metabolites using the fermentation method (Szijártó, 2007). In order to achieve this, the mycelia of fungal isolates were inoculated into 250 mL Erlenmeyer flask containing 50 mL of Malt Extract broth (MEB) (Merck, Darmstadt, Germany) and incubated aerobically while shaking on a rotary shaker (Labcon 3081U, Gauteng, South Africa) at 150 rpm for 5 days at 25 °C. The spore suspensions were withdrawn using sterile syringes and filtered through 0.25 mm, 0.45 µm PALL Sterile AcrodiscSyringe Filters (Separations, South Africa) in order to remove fungal cell mass. The fungal extracts that may or may not possess

secondary metabolites were transferred to clean sterile flasks and stored 4 °C in the cold room and used for further analysis (Premjanu and Jaynthy, 2015).

5.2.3 Screening for Bioactive properties

The fungal extracts were subjected to *in-vitro* antimicrobial assessment of their potential to produce biologically active compounds as indicated in Section 5.2.3.1. Screening for antibiotic activity against selected bacterial strains was performed using an adaptation of the Kirby-Bauer agar disc diffusion method (CLSI, 2010) while the broth microdilution technique was used to determine the minimum inhibitory concentrations (MICs).

5.2.3.1 Agar Disc Diffusion Assay (Antibacterial activity)

The antibacterial activity assays of the fungal extracts (secondary metabolites) were evaluated in triplicates using the agar disc diffusion method (Ahmad *et al.*, 2013). The discs were prepared by punching Whatman No. 3 filter paper (Separations, South Africa) and sterilizing the discs twice using an autoclave to ensure that they are completely free of microbial contaminants and were used in this experiment. Bacterial cultures of test strains that appear in Table 5.1 were sub-cultured on Nutrient agar and plates were incubated aerobically at 37 °C for 24 hours. Bacterial suspensions were prepared at concentrations of $1x10^7$ cells/mL. A bacteria lawn was prepared by spread-plating aliquots of 100 µL of bacteria suspensions on Muller Hinton agar. The discs were soaked in fungal extracts for 10 minutes and placed on the inoculated plates using a sterile inoculating needle. The inoculated plates were incubated aerobically at 37 °C for 24 hrs. Biological activity was identified based on the presence of a zone of clearing referred to as bacterial growth inhibition zone diameter which was measured in millimeters (mm).

5.2.4 Characterization of bioactive compounds by Gas-Chromatography Mass Spectrometry

In order to avoid bias all the fungal extracts were subjected to the Gas-Chromatography Mass Spectrometry (GC-MS) to facilitate identification and confirmation of the presence of biologically active compounds using the Shimadzu GC-MS-TQ8050 with Multifunctional Autosampler AOC-6000.

In the analysis a capillary column with a RTX-5 (60 m × 0.25 mm × 0.25 µm) in JNU, New Delhi was used. The initial temperature of 70 °C was used in the analysis and the temperature was maintained for 2 minutes. The temperature in the oven program was at 180 °C for two minutes, and elevated to 310 °C for 10 min and later increased by 1 °C per minute. The injection port temperature was kept as 250 °C and Helium flow rate was 1 mL per minute while the ionization voltage was 70 eV. In addition, the mass spectral scan range was set at 45–450 (m/z) and the samples were injected using a split mode of 10:1. The results were compared with those in the database from NIST05 (National Institute of Standards and Technology, US), WILEY 8, and FFNSC1.3 (Flavour and Fragrance Natural and Synthetic Compounds) libraries. The identities of the bioactive compounds in the extracts as well as their retention time molecular weights and structures were retrieved (Sharma *et al.,* 2016).

5.3 RESULTS AND DISCUSSION

Currently natural products remain an essential source of secondary metabolites and present a renewed hope for the search of novel compounds with enhanced antimicrobial properties to solve the problem of bacterial resistance (Deshmukh *et al.,* 2015). This explains why numerous studies are currently being designed to assess natural biotopes such as plants, microbes particularly endophytes and marine environments are constantly evaluated to determine their potential in producing secondary metabolites and hence considered as sources for the synthesis of bioactive compounds (Fráková *et al.,* 2007). Production of secondary metabolites is based on the characteristic that microorganisms can grow and significantly increase their cellular mass or cell numbers within a short time.

5.3.1 Antibacterial activity of extracts isolated from endophytes

Using the test bacterial strains *E. coli* (ATCC 25922), *E. coli* (ATCC 0177), *B. cereus* (ATCC 10876), *E. faecalis* (ATCC S1299), *E. faecium* (ATCC 700221) *and E. gallinarum* (ATCC 700425) in the agar diffusion assay, a total of 24 out of the 193 (12%) secondary metabolites or extracts exhibited significant antibacterial activities against both Gram negative and Gram positive bacteria. Data of the extracts that produced activities against the test bacterial strains are shown in Tables 5.2, 5.3 and Figure 5.1 while detailed data sets have been presented in Appendix 4. Despite the fact that a small proportion (12%) of the fungal extracts possessed bioactive potentials against the bacteria isolates,

biologically active extracts were produced by endophytes belonging to different genera and species isolated from both host plants. A large proportion (n=10; 43%) of the fungal extracts with bioactive potentials were produced by *Fusarium* species when compared to *Penicillium* (n=4; 17%) and *Alternaria* (n=3; 13%) species. These findings are similar to those reported by Ratnaweera *et al.* (2015) in which *Fusarium* species were the most bioactive fungus. On the contrary, the present findings revealed that *Aspergillus* species did not produce biologically activities against the bacterial strains investigated when compared to their results (Ratnaweera *et al.*, 2015). Interestingly, a large proportion (n=8; 25%) of the fungal extracts produced biological activity against an environmental *E. coli* O177 strain that possessed both multi-drug resistance and shiga-toxigenic virulence gene determinants.

In addition, the present findings indicate that only one endophytic fungus (MHE 068) belonging to the genus *Alternaria* produced biological activity against *E. gallinarum* (ATCC 700425) by exhibiting a growth inhibition zone of 12 mm in diameter. This fungus exhibited the highest activity and produced broad spectrum activity against the test strains indicated by a growth inhibition zone diameter data of 8 mm and 11 mm against *E. coli* (ATCC 10876) and *E. faecium* (ATCC 700221) respectively. In general, bacterial species *E. gallinarum* and *E. faecium* were most often resistant to the extracts followed by *E. faecium* (ATCC 700221). The secondary metabolites (MHE 68) produced by fermentation shown highest zone of inhibition 8 mm against *B. cereus*, 11 mm against *E. faecium* (ATCC 700221) and 12 mm against *E. gallinarum*, respectively. In accordance with Sadrati and co-workers (2013), reported similar results about *Alternaria* sp. that exhibited significant antimicrobial activity against *Candida albicans* and *Escherichia coli* by producing secondary metabolites with bioactive properties.

Table 5.2: Antimicrobial activity of extracts produced by endophytic fungal isolated from *Sceletium tortuosum*

S. No	Sample ID	Probable Name	Zone of Inhibition (mm)			
			E. coli ATCC 25922	*E. coli* O177	*B. cereus* ATCC 10876	*E. faecalis* ATCC S1299
1	GG 008	*Fusarium*	—	+ (7)	—	—
2	GG 009	*Neurospora*	—	—	+ (9)	—
3	GG 012	*Fusarium*	—	+ (7)	—	—
4	GG 013	*Aspergillus*	—	—	+ (7)	—
5	ND 19	*Fusarium*	—	+ (8)	—	—
6	DR 006	*Fusarium*	+ (7)	—	—	—
7	DR 017	*Fusarium*	—	—	+ (2)	—
8	DR 020	*Fusarium*	+ (8)	—	—	—
9	DR 023	*Fusarium*	—	—	+ (9)	—

-: no activity; +: slight activity (5—10 mm); ++: good activity (11—19 mm); +++: very good activity (20 mm); PC: positive control; ATCC: microt

5.3.2 Structure elucidation of biologically active compounds in extracts by Gas Chromatography-Mass Spectrophotometry

The endophytic fungal extracts were subjected to GC-MS analysis for structure elucidation of active compounds and detailed data of the chromatograms (*Figure* 5.3). The chemical profiles obtained gave the details of both the active and inactive extracts are shown in Appendix 5. The extract from *Alternaria* sp. (MHE 68) produced a total of 20 peaks, with the highest height of 304562 mm eluting at retention time 26.676 minutes with an area of 563966 mm^2. The chemical profile of the extract from *Alternaria* sp. (MHE 68) indicated that 9, 12-Octadecadienoic acid (Z,Z) and Cyclodecasiloxane, eicosamethyl were the major compounds and the chemical structures of these main compounds are shown in Figure 5.2. These finding are similar to those reported by previous studies (Devi and Singh, 2013; Parthipan *et al.*, 2015.

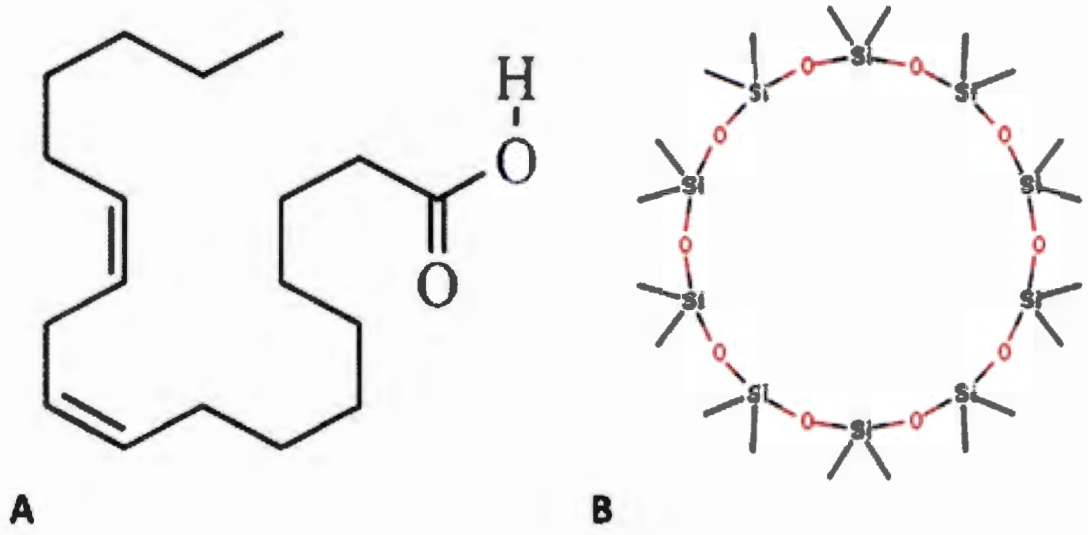


Figure 5.1: Chemical structures of (A) 9,12-Octadecadienoic acid (Z,Z) (B) Cyclodecasiloxane

Data generated by the GC-MS protocol was further analysed to determine the overall composition of compounds that were dominant in the fungal extracts from the endophytes obtained in this study and the results are shown in Figures 5.4 and 5.5. Despite the fact that there are a majority of compounds that are reoccurring in both active and inactive fungal extracts, Dodecanoic acid was identified as a major compound in all the extracts. Although, the compound 4 H Pyran-4-one was detected only in

extracts with no biologically activity, it has also been previously detected in extracts from the endophyte *Colletotrichum gloeosporioides* isolated from *Phlogacanthus thyrsiflorus* (Murugan *et al.*, 2017). From these it is suggested that synergistic as well as antagonistic interactions between the different compounds in both the active and inactive extracts might be responsible for the activities observed against bacterial tests strains. Similar suggestions were obtained by Freeman *et al.* (2010), who investigated the activities of different combinations of phenolic compounds against microbes. Despite the fact some of the fungal extracts did not produce any biological activities against the test organisms which were selected Gram negative and Gram positive bacteria; their potential to inhibit the growth of other microorganisms such fungi, viruses, and protozoans cannot be ruled out. Inactive fungal extracts in the present study may serve as potential antiviral, antifungal and antiprotozoan agents given the fact that there was an overlap in the occurrence of compounds between the active and inactive extracts based on GC-MS data.

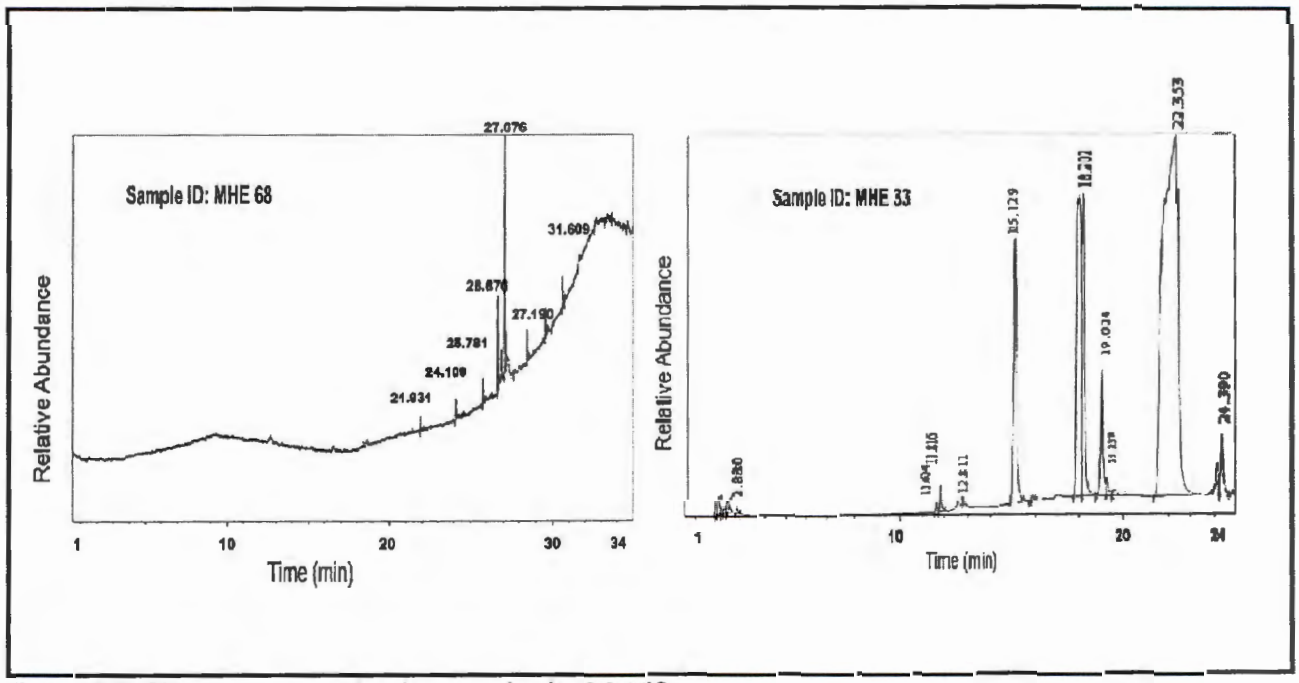
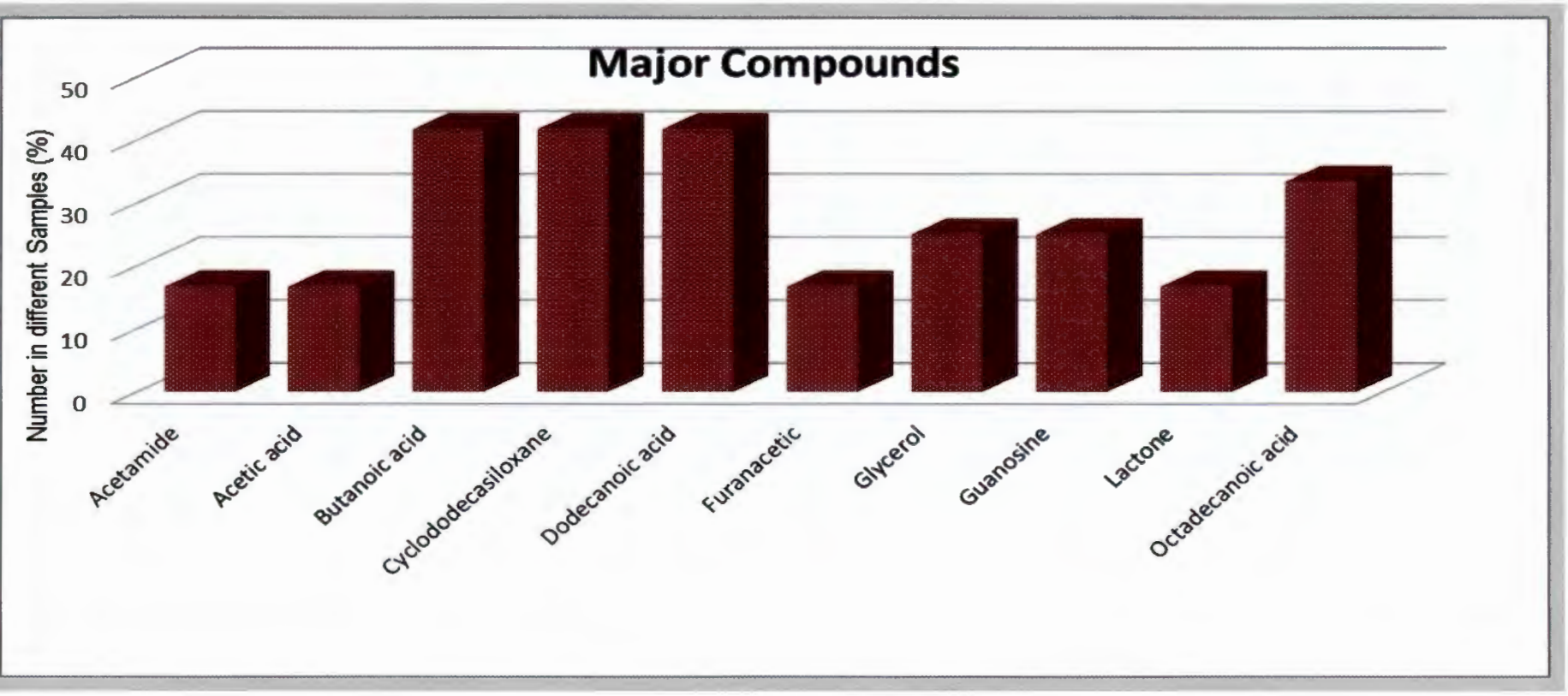


Figure 5.2: Chromatograms of active samples by GC-MS

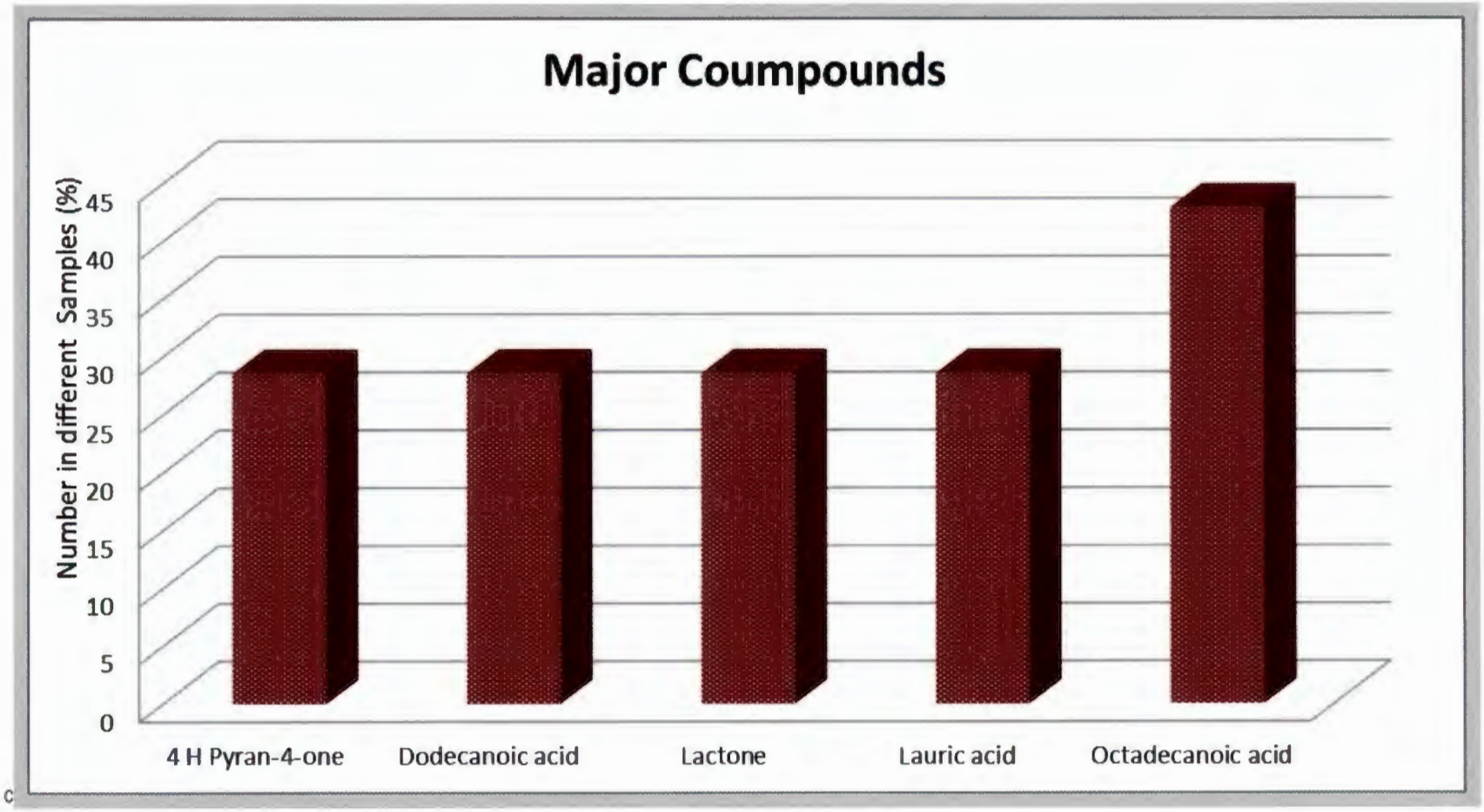
Table 5.4: Chemical composition of secondary metabolites produced by *Alternaria* (MHE 68)

Peak	R. Time	Area	Height	Name
1	21.931	107935	49846	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexasiloxane
2	24.109	134156	70746	Hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-Dode
3	25.781	146379	84122	Cyclododecasiloxane,Tetracos
4	26.644	271510	178016	9,12-Octadecadienoicacid(Z,Z)-,M
5	26.676	593966	304562	9,12-Octadecadienoicacid(Z,Z)-,M
6	26.730	340337	172773	9,12-Octadecadienoicacid(Z,Z)-,M
7	26.917	287016	96415	9,12-Octadecadienoicacid(Z,Z)-,Methylest
8	27.076	1597988	779997	9,12-Octadecadienoicacid(Z,Z)-,M
9	27.190	250984	114397	Cyclodecasiloxane,Eicosamethyl-
10	27.601	68648	22586	1h-Purin-6-Amine,[(2-Fluoropheny
11	28.437	292869	108477	Cyclodecasiloxane,Eicosamethyl-
12	29.575	272854	99113	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18
13	29.937	78786	13195	1h-Purin-6-Amine,[(2-Fluoropheny
14	30.628	244851	102788	Cyclodecasiloxane,Eicosamethyl-
15	30.750	61412	23772	1h-Purin-6-Amine,[(2-Fluoropheny
16	31.609	107960	50978	Cyclodecasiloxane,Eicosamethyl-
17	32.568	64355	26327	Propanoicacid,3-[[Bis[(Trimethyl
18	33.220	84775	19795	2,3,4-Tri-O-Acetyl-6-Deoxy-6-Iodoh
19	33.687	94106	25879	1,2-Benzenediol,3,5-Bis(1,1-Dimeth
20	34.636	107673	25345	D-Mannitol,1-Decylsulfonyl-

The GC-MS data is represented by retention time, area and height. The active compounds of *Alternaria* (MHE 68) are presented in Table 5.4. Appendix 5 illustrates the chromatograms and the list of compounds identified using GC-MS of both the active and inactive secondary metabolites.



gure 5.3: Major compounds identified by GC-MS in Active samples



gure 5.4: Major compounds identified by GC-MS in Inactive samples

5.4 CONCLUSION

The results of the present chapter indicate that the indigenous medicinal plants (*Sceletium tortuosum* L. and *Pelargonium sidoides*) did not only harbour several endophytic fungi but the endophytes were capable of producing secondary metabolites or antimicrobial substances with varied or selective antibacterial properties. Through the production of biologically active antibacterial secondary metabolites these fungi could serve as a potential source for the isolation and purification of novel antimicrobial agents that will assist in addressing the problem of bacterial resistance that presents severe challenges to both the medical and veterinary professions and public health in general. Despite that a large proportion of the bioactive extracts were obtained from *Fusarium* species, an extract with broad-spectrum activity was produced by the endophyte *Alternaria* from *Pelargonium sidoides*. Chemical characterization and structural elucidation of compounds in the extracts indicated that 9,12-Octadecadienoic acid (Z,Z) and Cyclodecasiloxane were dominant and these compounds may have acted synergistically and produced the antibacterial profiles of the fungal extracts.

CHAPTER SIX

PIGMENT PRODUCTION, CHEMICAL CHARACTERISATION AND ENZYMATIC PROPERTIES OF ENDOPHYTIC FUNGI FOR BENEFICIAL TRAITS

CHAPTER 6

Pigment Production, Chemical Characterisation and Enzymatic Properties of Endophytic Fungi for Beneficial Traits

ABSTRACT

Endophytes are organisms that inhabit plants' organs at some stage in their life cycle and their symbiotic relationships with their host plants provide unique biochemical pathways that facilitate the production of biologically active metabolites. Among the metabolites produced by fungi, pigments have received a lot of attention owing to their biological activity. Pigments are used as colouring additives in various essential and profitable industries in the production of textile, pharmaceutical, food, photographic and cosmetics products as well as plastics, paint, ink, and paper. In the present study, a total of 193 fungal isolates were screened for the ability to produce pigments and 31 (16.1%) of the isolates produced pigments with a broad spectrum of colours. Replication of cells during pigment production revealed that a large proportion of the endophytes, 68%, had high cell concentration while 29% had moderate cell concentration and only a small proportion (3%) with low cell numbers. When subjected to enzymatic assays large proportions of the endophytes produced lipase (65%) and amylase (61%) respectively. On the contrary, only a small proportion (12.9%) of the endophytes produced the enzyme laccase that was identified by the presence of red colonies. These enzymes are protein products that assist endophytes in the degradation of polysaccharides. These results indicate that these fungal strains can serve as novel sources of pigments that have important industrial and commercial applications.

CHAPTER 6

PIGMENT PRODUCTION, CHEMICAL CHARACTERISATION AND ENZYMATIC PROPERTIES OF ENDOPHYTIC FUNGI FOR BENEFICIAL TRAITS

6.1 INTRODUCTION

Endophytes are organisms that inhabit plants organs at some stage in their life cycle and their symbiotic relationships with their host plants provide unique biochemical pathways that facilitate the production of biologically active metabolites (Strobel and Daisy, 2003). Despite the fact that endophytic fungi are capable of producing a wide variety of secondary metabolites ranging from antimicrobial and anticancer agents, pigments have received a lot of attention owing to their biological activity (Celestino *et al.,* 2014). Pigments are known to be chemical compounds that absorb light at various wavelengths of visible light and reflect the colours of photons that are not absorbed (Delgado-Vargas *et al.,* 2000; Thesaurus, 2002). This explains why pigments are defined as substances that are characterized by colour, all pigments are generally classified based on their origin, and three main categories that include natural, synthetic and inorganic pigments have been identified and documented (Delgado-Vargas *et al.,* 2000). Pigment production may also serve as a beneficial survival mechanism within a given ecological niche due to the relatively highly competitive processes that exist among microorganisms and therefore natural pigments are those produced by microorganisms (Rajagopal *et al.,* 1997).

The commercial production of pigment is an industry that has received increasing revenue growth as well as huge expansion in many countries worldwide over the past decades (Scott and Bristow, 2002). Pigments derived from natural sources (Pandey *at el.*, 2001, Tibor, 2007) such as fungi, algae and plants have been commercially available in the market. The pigments are used in various industries such as the food and paper production facilities, textile industries, agricultural companies as well as water science and technology facilities that utilize both natural dyes and synthetic dyes (Saxena and Raja, 2014; Panesar *et al.,* 2015). The potential of microorganisms to produce natural pigments gives them the ability to adapt to various stressful situations such as camouflage for protection, mimicry, warning strategy, mating selection, photosynthesis in plants and countless other biological purposes (Bonansea *et al.,* 2017). The manufacture and distribution of natural pigments is very selective due to their costs and hence, they are limited and hard-to-find thus frequently used by fine artists and decorators (Yusuf *et al.,* 2017). Despite this, there is constant growing interest in natural pigments when compared to synthetic pigments because they are environmentally friendly due to their

biodegradable nature (Tuli *et al.*, 2015). Against this background, natural pigments are currently regarded as valuable replacements and alternatives to synthetic pigments that pose severe health complications to consumers due to their high toxicity as well as their mutagenic and carcinogenic capabilities (Lopes *et al.*, 2013). Natural pigments most often do not produce any irritation to the skin and are also currently utilized on a variety of natural fibers (Prasad, 2015, Działo *et al.*, 2016, Alves *et al.*, 2017).

Despite the fact that variety of fungal species that include *Monascus purpureus, Isaria farinosa, Emericella nidulans, Fusarium verticillioides, Purpureus, Isaria* spp., *Emericella* spp. and *Fusarium* species have been reported to produce a variety of coloured pigments (Velmurugan *et al.*, 2010a, 2010b), to the best of our knowledge no study has assessed the potential of endophytes from the plants *Sceletium tortuosum* and *Pelargonium sidoides* that are indigenous to South Africa in producing natural pigments. This chapter therefore was designed to explore the potential of endophytes previously isolated and identified as potential source for natural pigments. A further objective of the chapter was to assess the enzymatic potential of the fungi based on the degradation of compounds in selective media.

6.2 MATERIALS AND METHODS

6.2.1 Fungal strains

A total of 193 fungal strains isolated from indigenous medicinal plants *Sceletium tortuosum* and *Pelargonium sidoides* were assayed to assess their potential for pigment production, chemical characterisation and enzymatic properties. The identities of the fungal isolates were confirmed using macro or colonial and microscopic characteristics as well as PCR through amplification and sequence analysis of the genus specific ITS and TEF gene fragments as shown in Section 4.2.1.3 in Chapter 4.

6.2.2 Cultivation and growth selective fungal isolates

Competent fungal cells were cultured on Potato Dextrose Agar and incubated aerobically at room temperature (25 °C) for 10 days. Pure fungal colonies were used in the fermentation tests designed to assess for production of pigments.

6.2.3 Pigment Production and Extraction

Fungal plugs from pure cultures were prepared using standard procedures and these were inoculated into 50 mL Malt extract broth (MEB; Merck, Darmstadt, Germany) placed in 250 mL Erlenmeyer flasks. Three parameters that include fermentation length, medium composition and aeration were optimally adjusted since they are essential to achieve optimum production of secondary metabolites and pigments. The inoculated Malt extract broths were incubated at 25 °C for 5 days while shaking at 150 rpm using a shaking incubator (Labcon 3081U, Gauteng, South Africa). After incubation, the broth cultures were filtered using sterilized Acrodisc® Premium 25 mm Syringe Filter with 0.45 µm GHP Membrane (Separation, South Africa) in order to remove the fungal cell mass. The filtrates were stored at 4 °C until further analysis. The experiment was performed in triplicate as previously indicated by Kumar *et al.,* (2010).

6.2.4 Quantitative analysis of Pigment Production

Pigment production was quantified using a spectrophotometer, Hexios, Thermo Spectronics (Merck, Darmstadt, Germany) using the protocol previously described by Da Costa Souza *et al.,* (2016). The quantification of pigments produced was based on the absorbance units obtained. The wavelengths (λ) used in the assay ranged from 400 to 700 nm. However, the detection and quantification of the different pigments was done at specific wavelengths (Da Costa Souza *et al.,* 2016).

6.2.5 Screening of enzymatic assay

6.2.5.1 Amylase activity

Glucose yeast extract peptone agar (GYP) (Glucose 10.0 g/L; Yeast extract 0.1 g/L, Peptone 0.5 g/L; Soluble Starch 2.0 g/L; Agar 16.0 g/L) was used to assess the potential of fungal strains to digest carbohydrates. After autoclaving, the pH of GYP media was adjusted to 6.0. Plugs of pure cultures of the endophytic fungi were inoculated on the GYP media and incubated aerobically at 25 °C for 3-5 days. Plates with pure cultures were flooded with 1% (v/v) iodine. A clear zone around the colonies was indicative of a positive result for amylase production (Toghueo *et al.,* 2017).

6.2.5.2 Lipase activity

Lipases are enzymes that catalyse the hydrolysis or breakdown of fats and are therefore essential digestive enzymes. In the present study analysis of endophytes for lipase production capabilities was done using Peptone agar medium (Peptone 10.0 g/L; NaCl 5.0 g/L; $CaCl_2.2H_2O$ 0.1 g/L; Agar 16.0 g/L) (Company, Country) based on a standard protocol (Toghueo *et al.,* 2017). After autoclaving the final pH of the medium was adjusted to 6.0 and the media was later supplemented with 1% (v/v) filter-sterilized Tween 20 (Company, Country). Isolates that were positive for the lipase enzyme produced a clear zone around fungi colony and vice versa (Toghueo *et al.,* 2017). Plates were evaluated and results recorded.

6.2.5.3 Protease activity

Proteases are proteolytic enzymes that catalyze the catabolism of proteins through hydrolysis of the peptide bonds. In order to assess for protease activity, fungal strains were cultured on Glucose yeast extract peptone agar (GYP) (Glucose 1.0 g/L; Yeast extract 0.1 g/L; Peptone 0.5 g/L; Agar 16.0 g/L). After autoclaving, the pH of GYP was adjusted 6.0 by supplementing with 0.4% (v/v) gelatin (CAS 9000-70-8, Merck Millipore, Darmstadt, Germany). Plates were grown at 25 °C for 5 days. Pure culture in plates were treated with saturated ammonium sulphate $(NH_4)_2SO_4$), (CAS No. 7783-20-2, Merck Millipore, Darmstadt, Germany) and the identification of a clear zone around the colonies was indicative of a positive test (Patil *et al.,* 2015).

6.2.5.4 Laccase assay

Laccase are copper-containing oxidase enzymes that are found in a variety of plants, fungi and microorganisms and act on phenols and related molecules where they reduce oxygen to produce water. In order to assess the endophytes for laccase production, isolates were cultured on Potato dextrose agar (PDA) (Potatoes infusion 200.0 g/L; Dextrose 20.0 g/L; Agar 15.0 g/L) (Merck, Darmstadt, Germany) that was prepared according to manufacturer's instructions. The media was autoclaved, held at 50 °C and later supplemented with 0.04% (v/v) guaiacol (Inqaba Biotechnical Industries, South Africa) and 0.01% (w/v) chloramphenicol to inhibit bacterial contamination. The final pH of the medium was 5.5. The PDA plates were inoculated with plugs from pure fungal colonies and incubated at 28 °C for 72 hours. The formation of reddish brown zones around the fungal colonies was recorded as positive result (Abd El Monssef *et al.,* 2016).

6.3 RESULTS AND DISCUSSION

6.3.1 Screening of pigment-production and Quantitative analysis

Wavelength is the distance between particles in a medium and optical density is also referred to as an index of refraction is the absorbance that is normalized to the optical pass length. Optical density is therefore measured by the light spectrum scattering in a sample using a spectrophotometer but at a particular wavelength (Coselli and LeMaire, 2009). In microbiology, the wavelength value is indirectly proportional to the number of microbial cells present in the sample and thus the lower the wavelength number, the higher the microbial cells.

In the present study a large proportion (21/31; 68%) of the fungal isolates showed very high concentration of cells, 29% was moderate and only 3% revealed low concentration of cells (Figure 6.1). Detailed data on the potential to produce pigments, the intensity of the pigments produced as well as the different colours of pigments that were identified are shown in Table 6.4. The different wavelengths used in the detection of the different coloured pigments have also been indicated. As shown in Table 6.1 *Alternaria* (MHE 16) recorded the highest pigment production at 25 °C (OD_{550} = 0.018) that was green in colour. These samples also revealed the highest cell concentration when compared to the others. Similarly, isolates END 1, MHE 48 and GG 3 that were identified as *Epicoccum*, *Cladosporium*, *Neocosmospora* and *Fusarium* species also revealed significantly higher pigment production potentials with brown (OD_{600} = 0.035), red (OD_{600} = 0.067), yellow-clear (OD_{600} = 0.069) and light red (OD_{600} = 0.044) pigments detected respectively. On the contrary, *Fusarium* (MHE 56) revealed the lowest pigment production potential (OD_{600} = 0.708) and produced a black pigment after analysis. This endophyte (MHE 56) also had the lowest cell concentration when compared with other isolates. Figure 6.3 shows a representation of some of the pigments produced by endophytic fungi after 5 days of incubation in Malt Extract broth at 25 °C while Figure 6.4 reveals pigmentation on agar plates by endophytic fungi DR32, MHE4, MHE16, MHE55 and MHE68 respectively. In this study, endophytes produced pigments with varying colours and this indicated that these isolates can serve as a potential source for natural pigments and dyes thus making them important isolates for numerous biotechnological applications (Geweely, 2011; Premalatha *et al.*, 2012; Telxeira *et al.*, 2012).

It has been reported that fungi are able to adapt and change the colour of pigments produced by sensing changes in environmental conditions (Liu and Nizet, 2009). Thus, changes in environmental conditions resulting from the exposure of fungi to ultraviolet radiation, oxidants, extreme heat and cold,

protection against natural antimicrobial compounds produced by other microbes, acquisition of nutrients, such as iron have been reported to significantly contribute to the nature and colour of the produced pigment by the fungi (Liu and Nizet, 2009). A report by Pradeep and Pradeep (2013) revealed that *Fusarium moniliforme* (KUMBF1201) produced maximum pigment (OD_{500} = 1.363) which is far higher/more than that recorded in the present study. However, the present finding also reveals that a *Fusarium* species possessed the highest pigment producing capability. Despite this, the potential of an endophyte to produce pigments cannot be assumed based on its genetic identity without laboratory investigations.

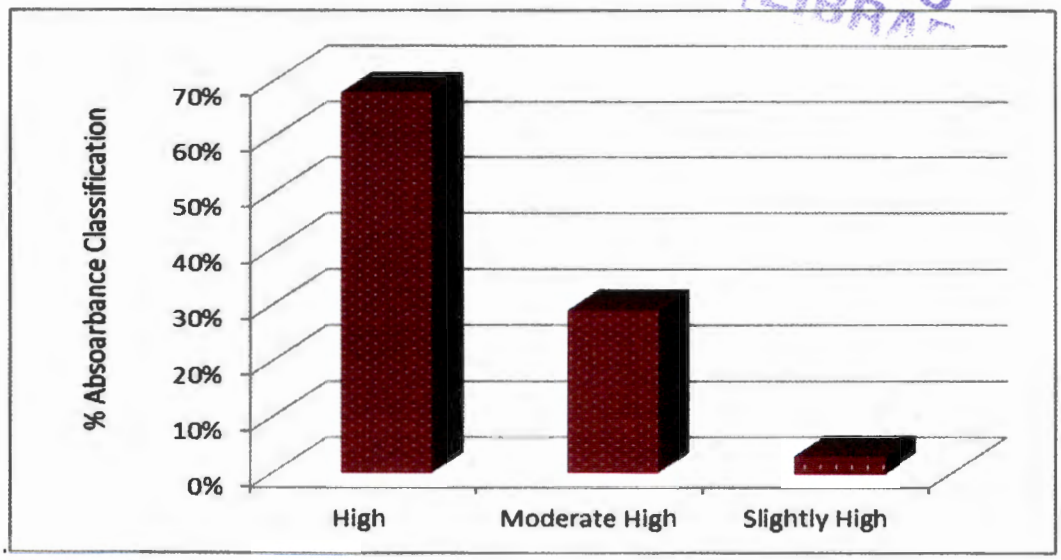
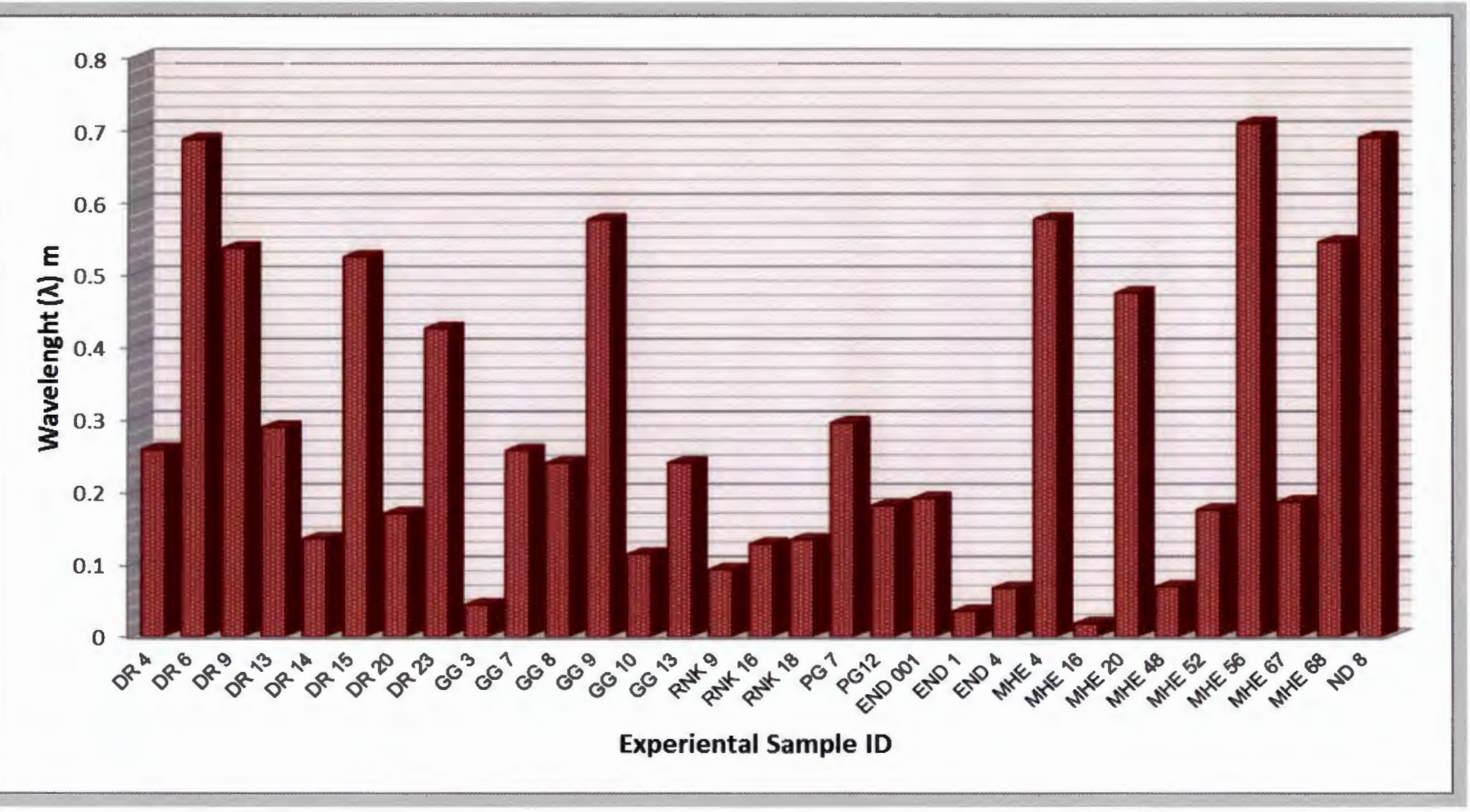


Figure 6.1: Absorbance classification of cell concentration producing pigments

Table 6.1: Pigment production of fungal isolates and their quantities

No.	Sample ID	Genus Name	Pigment Colour	Wavelength λ (nm)		
				Absorbance	Measure	Intensity
1	DR 4	*Fusarium*	Light Brown	600 nm	0.258	+++
2	DR 6	*Fusarium*	Black	600 nm	0.686	++
3	DR 9	*Fusarium*	Yellow-Clear	600 nm	0.536	++
4	DR 13	*Fusarium*	Deep Red	600 nm	0.289	+++
5	DR 14	*Neonectria*	Brown-Gold	600 nm	0.135	+++
6	DR 15	*Neonectria*	Light Brown	600 nm	0.524	++
7	DR 20	*Fusarium*	Brown-Yellowish	600 nm	0.170	+++
8	DR 23	*Fusarium*	Deep Red	700 nm	0.425	++
9	GG 3[a]	*Fusarium*	Light Red	680 nm	0.044	+++
10	GG 7	*Alternaria*	Brown	600 nm	0.257	+++
11	GG 8	*Fusarium*	Yellow-Brown	600 nm	0.240	+++
12	GG 9	*Neurospora*	Brown-Yellowish	600 nm	0.575	++
13	GG 10	*Aspergillus*	Yellow	600 nm	0.114	+++
14	GG 13	*Aspergillus*	Deep Yellow	640 nm	0.240	+++
15	RNK 9[a]	*Penicillium*	Deep Yellow	640 nm	0.093	+++
16	RNK 16	*Alternaria*	Black	600 nm	0.128	+++
17	RNK 18	*Alternaria*	Deep Yellow	640 nm	0.134	+++
18	PG 7	*Neopestalotiopsis*	Light Green	600 nm	0.295	+++
19	PG12	*Alternaria*	Yellow-Clear	600 nm	0.181	+++
20	END 001	*Penicillium*	Deep Yellow	640 nm	0.191	+++
21	END 1[a]	*Epicoccum*	Brown	600 nm	0.035	+++
22	END 4[a]	*Cladosporium*	Red	680 nm	0.067	+++
23	MHE 4	*Fusarium*	Reddish-Yellow	650 nm	0.576	++
24	MHE 16[a]	*Alternaria*	Green	550 nm	0.018	+++
25	MHE 20	*Fusarium*	Brown-Yellowish	600 nm	0.474	++
26	MHE 48[a]	*Neocosmospora*	Yellow-Clear	600 nm	0.069	+++
27	MHE 52	*Alternaria*	Brown	600 nm	0.175	+++
28	MHE 56[b]	*Fusarium*	Black	600 nm	0.708	+
29	MHE 67	*Plectosphaerella*	Brown	600 nm	0.186	+++
30	MHE 68	*Alternaria*	Brown	600 nm	0.544	++
31	ND 8	*Alternaria*	Black	600 nm	0.688	++

The range value indicates cell concentration (cells/mL) of fungi, 0.7-1.0 (+) slightly high, 0.4-0.6 (++) moderately high, 0.01-0.3 (+++) very high. Isolates with superscripts [a] indicates those with high pigment producing capabilities while the isolates with the superscripts [b] had a low pigment producing capability.



gure 6.2: Measurement of the absorbance for pigment production

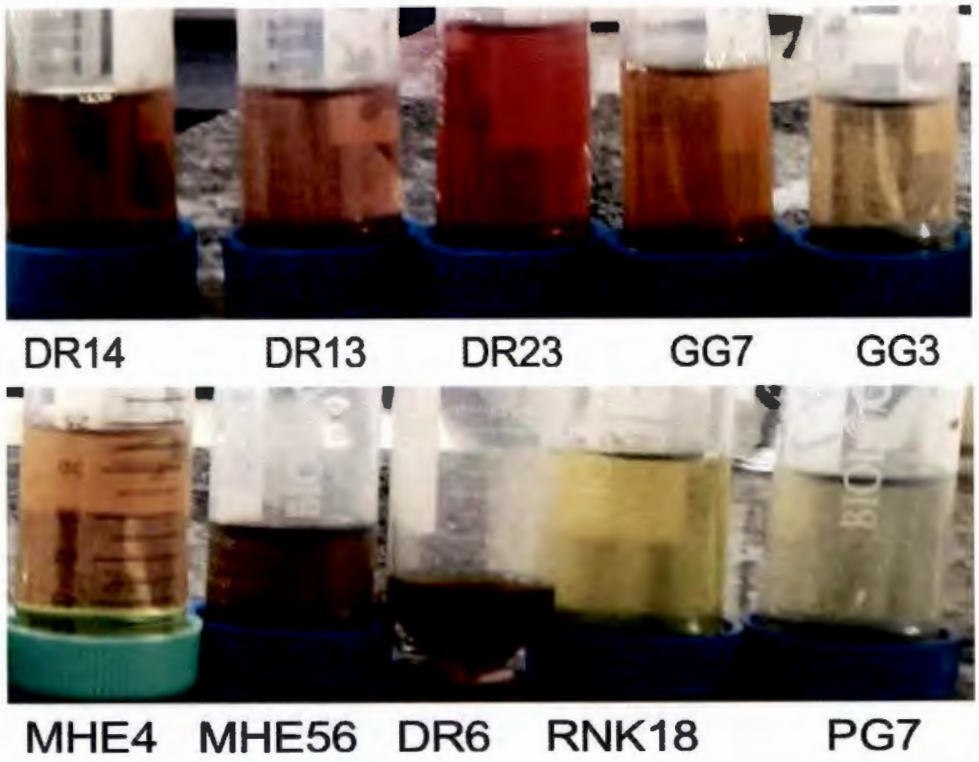


Figure 6.3: Pigments extracts of endophytic fungi after 5 days of incubation in Malt Extract broth at 25 °C

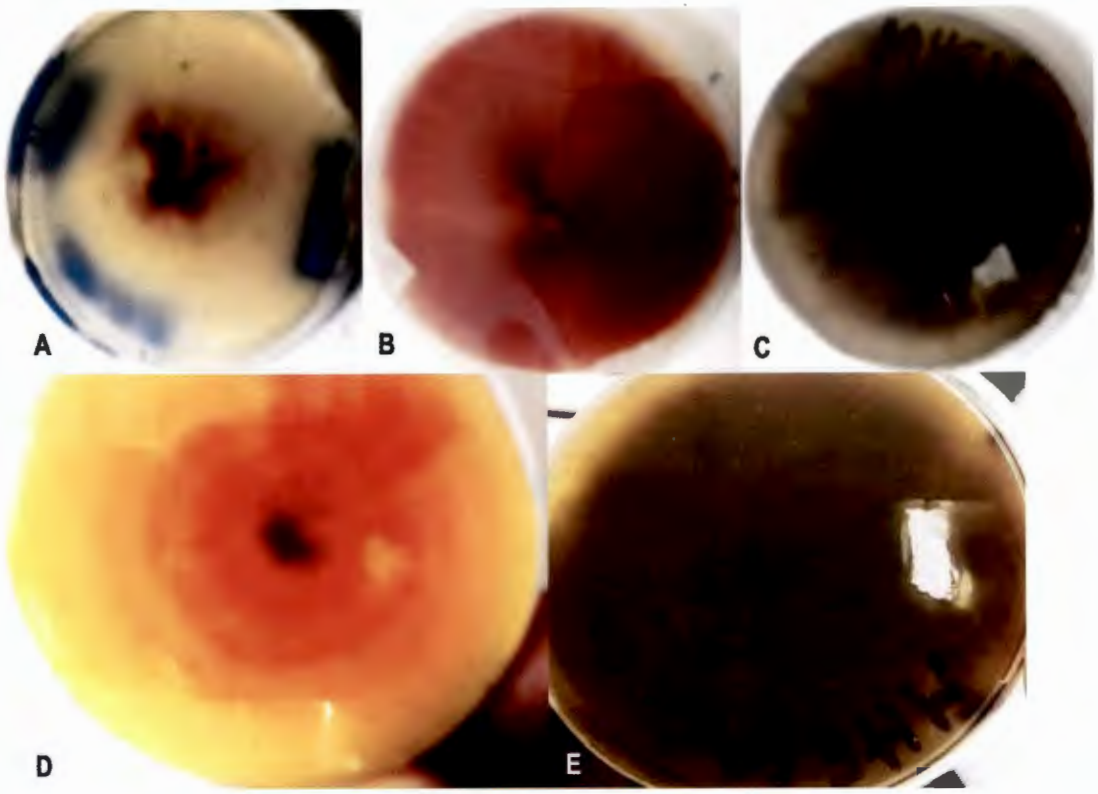


Figure 6.4: Pigmentation in plate of endophytic fungus (A) DR32 (B) MHE4 (C) MHE16 (D) MHE55 (E) MHE68

6.3.2 Qualitative analysis of enzymes

Fungi have over the past decades been known as invaluable sources of natural products that are essential for industrial and biomedical purposes and thus secrete a wide range of extracellular enzymes (Turner and Aldridge, 1983). These extracellular enzymes also referred to as exoenzymes are secreted and their key functions include the regulation of vital components of numerous biological processes within the cell. Enzyme activity is achieved using nutrients from the environment surrounding the cell (Esteves *et al.,* 2014). In the present study, hydrolytic enzymes protease, amylase and lipase that are associated with pathogenicity by enhancing penetration of cellular barriers by fungal host strains were investigated. In addition, laccase activity that correlates with pigment production was also determined.

Data indicating the proportion of isolates that were positive for the different enzymes are shown in Figure 6.5 while Table 6.2 provides a detailed summary of the enzymatic potentials of the different isolates. Figure 6.6 shows representative culture plates of isolates that were negative and positive for the target enzymes. Thirty-one endophytic fungi belonging to different genera and species that produced pigments of different colours were subjected to enzyme activity assays. Large proportions of the isolates were positive for lipase (20; 65%) and amylase (19; 61%) enzymes, respectively. Despite the fact that only a small proportion (4; 13%) of these isolates produced the laccase enzyme a significantly larger proportion (16; 52%) of the isolates were positive for the protease enzyme. The results reveal that the enzymes amylase and lipase were dominant metabolites produced by endophytes in the present study. Individual isolates were assessed for the potential to produce these target enzymes and despite the fact that the endophyte (GG 008) previously identified as *Fusarium oxysporum* (Accession no: KJ774041.1) was negative for all the enzymes, isolate MHE 001 a *Fusarium solani* (Accession no: AB470903.1) produced all the four target enzymes (Table 6.2).

Earlier studies conducted by Pavithra *et al.* (2012) to assess the potential of endophytic fungi from Tulsi to produce amylase and protease results indicated that 50% of the isolates were positive for these enzymes. In addition, another study by Sunitha *et al.* (2013) revealed that 62%, 50%, 30% and 28% of the endophytes were positive for amylase, lipase, laccase and protease activities. The findings of the present study are in agreement with these studies in which the enzymes amylase and lipase have been the most prominent enzymes produced by endophytes (Pavithra *et al.,* 2012; Sunitha *et al.*, 2013; Uzma *et al.* (2016). In addition, the findings of the previous studies are similar to those reported herein where very small proportions of the isolates were positive for laccase activity.

Laccases are enzymes that belong to the group of oxidases and they catalyse the oxidation of a range of phenolic compounds, diamines and aromatic amines. Recently, laccase has received a lot of attention due to its importance in a variety of industrial applications such as dye decolourization, waste detoxifications and bioremediation applications (Monssef *et al.,* 2015). Controlled substrate concentration designed to enhance high enzymatic activity especially the improvement laccase production by endophytes over a wide range of pH and temperature is of great importance (Mohajershojaei *et al.,* 2015). This is motivated from the fact that the use of laccase as a catalyst in different industrial applications such as textile dye bleaching, pulp bleaching, bioremediation and biotransformation provides very unique advantages over the use of synthetic chemicals that are not environmentally friendly (Alfarra *et al.,* 2013).

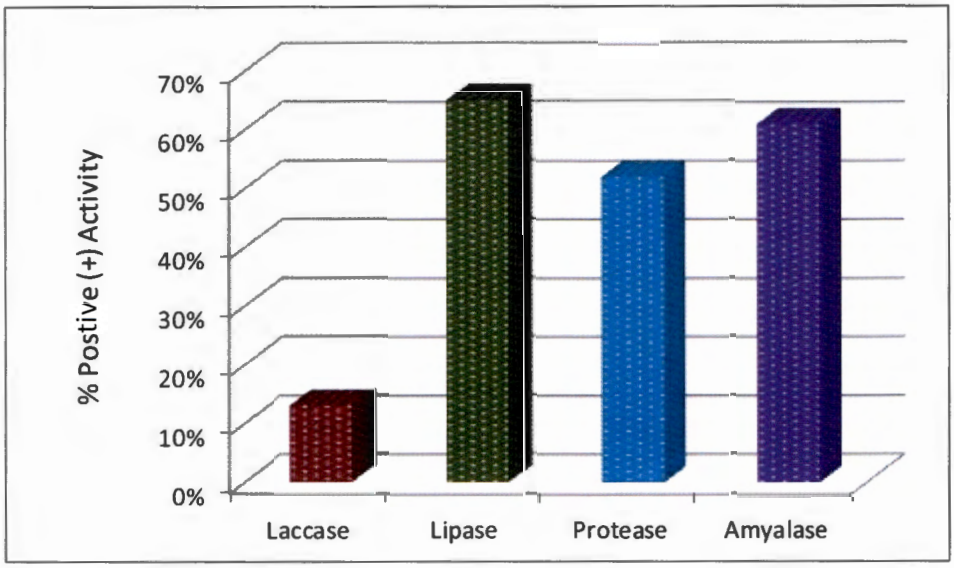


Figure 6.5: Comparative analysis of positive enzymatic activity

Table 6.2: Production of extracellular enzymes by pigment-producing fungi

No.	Sample ID	Amylase activity	Proteolytic activity	Lipase activity	Laccase activity
1	DR 4	+	–	+	–
2	DR 6	–	+	+	–
3	DR 9	+	+	+	–
4	DR 13	+	+	–	–
5	DR 14	+	+	–	–
6	DR15	+	–	–	–
7	DR20	+	+	–	–
8	DR 23	–	–	+	–
9	END 001	–	+	+	+
10	END 1	+	+	+	+
11	END 4	–	+	+	–
12	GG 3	–	–	+	–
13	GG 7	+	+	+	–
14	GG 8	–	–	–	–
15	GG 9	+	–	–	–
16	GG 10	–	–	–	+
17	GG13	–	+	–	–
18	RNK 9	–	+	–	–
19	RNK 16	–	+	+	–
20	RNK 18	+	+	+	–
21	PG 7	–	–	+	–
22	PG 12	+	–	+	–
23	MHE 4	+	+	+	–
24	MHE 16	+	–	+	+
25	MHE 20	–	+	–	–
26	MHE 48	+	–	–	–
27	MHE 52	+	+	+	–
28	MHE 56	+	–	+	–
29	MHE 67	+	–	+	–
30	MHE 68	+	–	+	–
31	ND 8	+	–	+	–

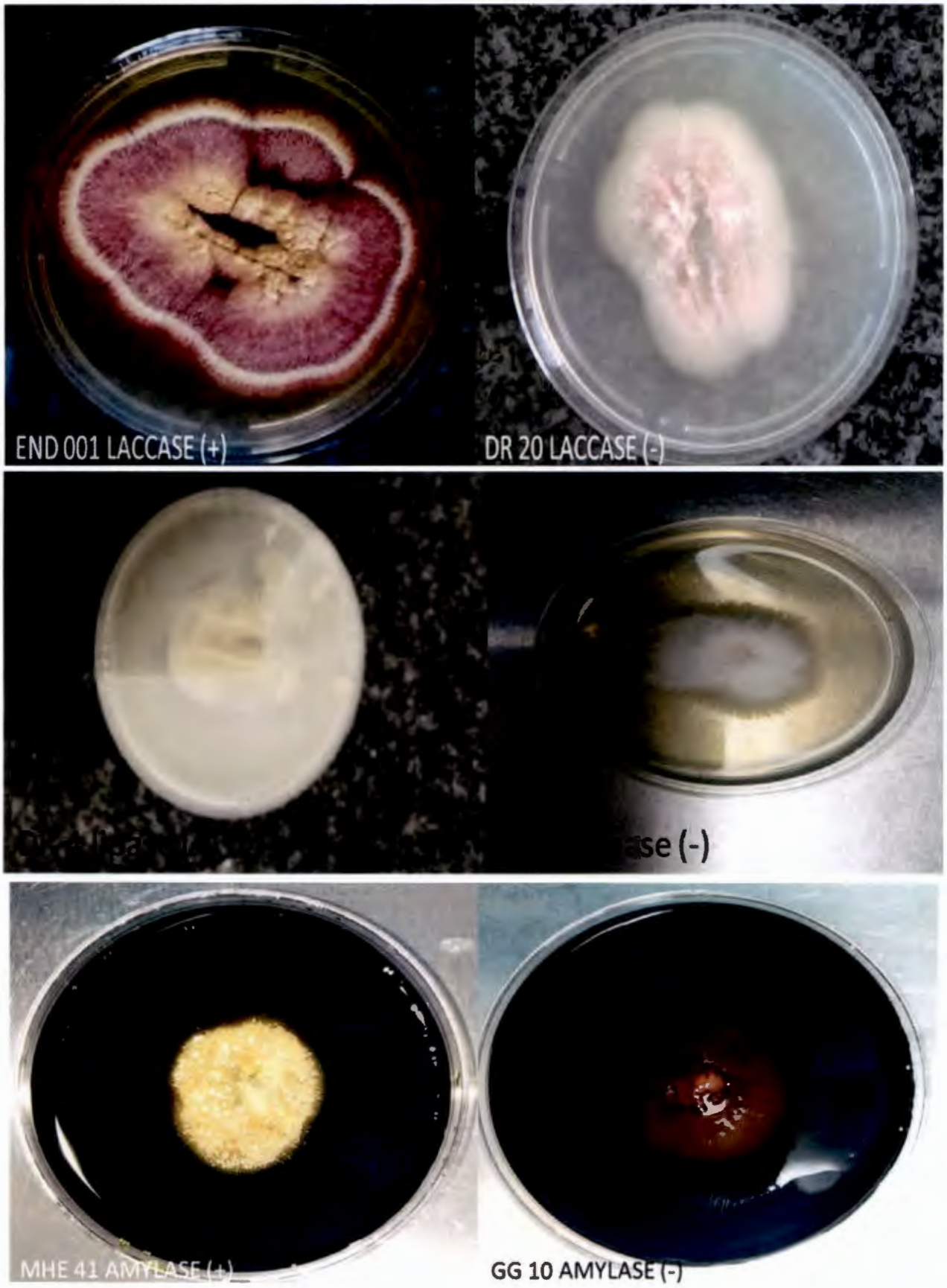


Figure 6.6: Enzymatic activity of endophytic fungi

6.4 CONCLUSION

Initially a total of one hundred and ninety-three (n=193) endophytic fungi were screened for pigment production and the presence of extracellular enzymes. Results indicated that a very small proportion (31; 16%) of the isolates were capable of producing pigments. In addition, a large proportion (68%) of the endophytic fungi produced very high cell concentrations. In addition, isolates in the present study produced pigments with a varied or broad range of colours. All but one of the endophytes produced enzymatic activities on selective media while large proportions of the isolates produced amylase, lipase and protease. One of the endophytes (END 1) was positive for all the enzymes targeted. These results indicate that these isolates may serve as potential sources for natural dyes and hence may be very useful in some agricultural, textile, pharmaceutical, cosmetics, food and biotechnology processes.

CHAPTER SEVEN

IN VIVO PLANT GROWTH PROMOTING ACTIVITIES OF SELECTED ENDOPHYTIC FUNGI FROM *SCELETIUM TORTUOSUM* L. AND *PELARGONIUM SIDOIDES* ON *ZEA MAYS* L. SPECIES

CHAPTER 7

IN VIVO PLANT GROWTH PROMOTING ACTIVITIES OF SELECTED ENDOPHYTIC FUNGI FROM *SCELETIUM TORTUOSUM* L. AND *PELARGONIUM SIDOIDES* ON *ZEA MAYS* L. SPECIES

ABSTRACT

Zea mays (L.) is considered as a staple food source in many countries throughout the world including South Africa. New technological approach uses endophytic fungi as growth promoters and their secondary metabolism for protecting the plant against pathogens. In the current study, nine (n=9) endophytic fungi were inoculated in the maize seeds prior to plantation. The outcome of the greenhouse experiments were that *F. solani* (MHE 55) showed significant plant height, root weight, fresh weight, leaf size and high survival rate. In contrast with *Chaetomium* (PG 9) which had low leaf size, weight and survival rate. *Alternaria* (MHE 68) had moderate growth when compared with *F. solani* (MHE 55). Natural growth promoters are essential due to a number of benefits. Endophytic fungi can be used as growth promoters without causing any disease or symptoms of diseases. The results will be valuable in the agricultural and food industries. This also contributes to crop production and protection; hence more food availability.

CHAPTER 7

IN VIVO PLANT GROWTH PROMOTING ACTIVITIES OF SELECTED ENDOPHYTIC FUNGI FROM *SCELETIUM TORTUOSUM* L. AND *PELARGONIUM SIDOIDES* ON *ZEA MAYS* L. SPECIES

7.1 INTRODUCTION

Food security is a global problem, especially when referring to the massive reduction in productivity of *Zea mays* L. resulting from infection of the plants with pathogens (Kumar and Kalita, 2017). Recent reports from the Food and Agricultural Organisation (FAO) of the United Nations indicate that one in four individuals within the African continent are unable to live an active and healthy life as a result of inavailability of adequate food supply (Bremner, 2012; FAO, 2015). While some African countries can be commended for the implementation of strategies to reduce the total number of undernourished people, and thus achieving the Millenium Development Goals targets, the ever increasing population also presents a severe challenge to many (FAO, 2015). In addition, the constant contamination of crops particularly maize in farmlands with usually diverse group of pathogens including viruses, bacteria, nematodes, fungi, and mycoplasmas present an even more severe challenge to the agricultural sector (Roberge, 2015) due to a reduction in productivity.

Maize can be contaminated during storage where kernels are easily susceptible to a number of toxigenic fungi (Cardwell *et al.,* 2000). Pathogenic fungi belonging to the genera *Aspergillus, Penicillium* and *Fusarium* have very high capabilities to produce the aflatoxins, fumonisins as well as other mycotoxins. The presence of pathogenic microbes has been reported to affect the quality and quantities of grains produced and thus present serious health risks to humans and animals (Bradley *et al.,* 2001). It is against this reason that seed dressings are now considered among the most effective ways to reduce deterioration of seeds during storage (Adebisi *et al.,* 2004). In addition, the treatment of seeds is currently used to enhance productivity of plants in the field.

Plants are known to exhibit intrinsic mutualistic relationships with endophytic fungi that colonise their tissues (Qin *et al.,* 2011) and reside in these tissues for at least some part of their life cycle without causing any signs and symptoms of disease (Qin *et al.,* 2011). When present in their plant hosts endophytes provide a wide variety of benefits that include growth promotion, protection against pathogenic bacteria and fungi and resistance to drought (Hardoim *et al.,* 2015). These beneficial traits in the host plants are enhanced through the production of bioactive compounds by endophytes.

Zea mays (L.) is the botanical name for maize or corn that belongs to the family *Poaceae* (Inglett, 1970; Hall, 2002). Maize a plant that originates from Southern Mexico is one of the largest grain plants that is widely cultivated globally (Farnham *et al.,* 2003) because it is considered as a staple food for a large proportion of individuals. Global consumption of maize amounts to 94% when compared to all cereals currently available. This may also be motivated by the fact that it is a valuable source of energy (density of 365 Kcal/100) and comprises 72% starch, 10% protein, and 4% fat. Furthermore, maize is rich in essential minerals, B vitamins and fibre and these are easily retained in the human body after consumption of maize or its associated food products (Ranum *et al.,* 2014). This therefore implies that the evaluation of ideal cultivation conditions for maize that may also involve the screening of potential endophytic fungi for growth promoting traits cannot be underestimated. The present chapter was therefore designed to functionally characterise endophytes from selected medicinal plants *Sceletium tortuosum* L. and *Pelargonium sidoides* and assess their potential use for growth promotion of *Zea mays* (L.) individually in a greenhouse experiment by measuring plant height, root weight, fresh weight, leaf size and high survival rate. Results obtained were aimed at providing options for the use of these endophytes in the development of microbial formulations to enhance plant growth and *Zea mays* (L.) in particular, thus increasing crop productivity.

7.2 MATERIALS AND METHODS

7.2.1 Endophytic fungal strains

The rational for the selection of endophytic fungi that were utilized in the greenhouse experiment was based on data generated from the *in-vitro* antimicrobial activities outlined in Chapter 5. A total of nine fungal isolates with significant bioactive properties were selected for the greenhouse experiments.

Table 7.1: Fungal strains that were used in the greenhouse experiments

Sample ID	Genus specie	Positive Activity	Highest Activity (mm)
END 15	*Boeremia exigua* var. *pseudolilacis*	+	11
GG 8	*Fusarium oxysporum*	++	9
GG 9	*Neurospora sp.*	+	9
GG 13	*Aspergillus fumigatus*	+	7
MHE 10	*Neurospora crassa*	++	9
MHE 55	*Fusarium solani*	+	12
MHE 68	*Alternaria sp.*	+++	12
PG 9	*Chaetomium subaffine*	++	9
RNK 4	*Penicillium glabrum*	+	11

7.2.2 Greenhouse experimental site

The greenhouse field trials were carried out at North-West University, Mafikeng, Mmabatho located at 25° 38' 39.16" E and -25° 51' 54.79" S.

7.2.3 Greenhouse (*In vivo*) studies

7.2.3.1 Preparation of the endophytic fungal cultures

Endophytic fungi previously isolated from *Sceletium tortuosum* and *Pelargonium sidoides* plants (Chapter 3) and their identities confirmed using morphological and genus specific PCR (Chapter 4) were used in this *in-vivo* greenhouse experiments. The identities of the endophytes are *Boeremia exigua* var. *pseudolilacis*, *Fusarium oxysporum, Neurospora* sp., *Aspergillus fumigatus, Neurospora crassa, Fusarium solani, Alternaria* sp., *Chaetomium subaffine, Penicillium glabrum.* All the fungi were revived by culturing on Potato Dextrose agar (PDA) (Potatoes infusion 200.0 g/L; Dextrose 20.0 g/L; Agar 15.0 g/L) (Merck, Darmstadt, Germany) and plates were incubated aerobically at 25°C in a 12 hours day/night cycle for seven days.

7.2.3.2 Surface disinfection and inoculation of the pasteurized soil

The maize *Zea mays* (L.) seeds (BG5685B) obtained from Pannar Seed Company from Mpumalanga, Republic of South Africa were used in the greenhouse experiments based on a standard *in vivo* technique (Orole and Adejumo, 2009). The seeds and seedling trays were sterilized with 1% (v/v) sodium hypochlorite (NaClO) for 5 minutes and further rinsed with distilled water to remove the residues. Figures 7.1a and 7.1b show maize seeds after rinsing with sodium hypochlorite and chemical treated seeds respectively.

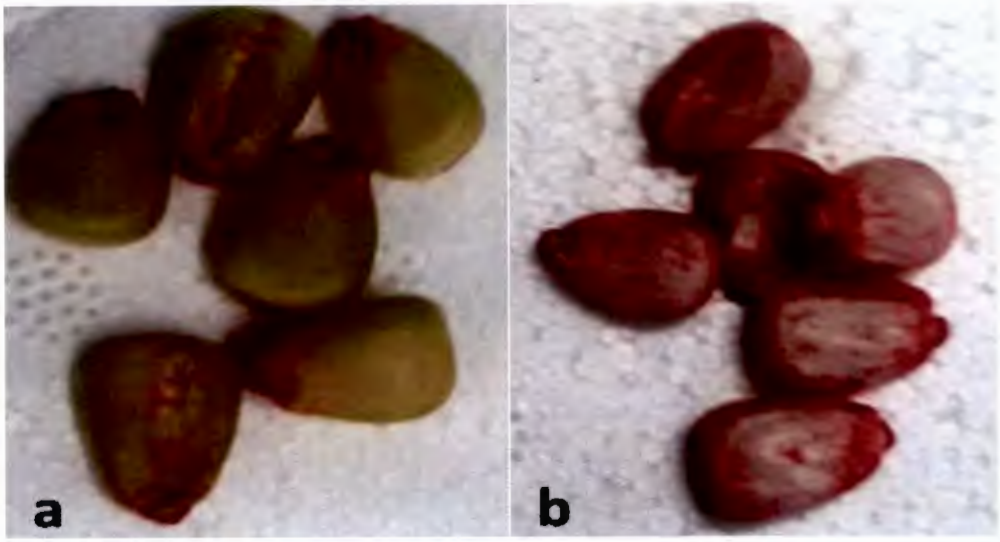


Figure 7.1: Maize seeds (a) after rinsing with sodium hypochlorite (b) chemical treated seeds

The seeds and seedling trays were kept at room temperature for 24 hours to air dry. Spore suspensions of the endophytes were prepared by inoculating a plug of the fungal mycelia into 300 mL of Malt extract broth that was placed in a 500 mL beaker. The final concentration of fungal spore suspension in the broth was adjusted to 1 X 10^6 cfu/mL using a Neubauer heamacytometer. Figure 7.2 shows fungal spore suspension in Malt extract broth.

Artificial wounds were created in the seeds using a sterile scalpel blade and seeds were soaked in the fungal suspension for 48 hours. Negative control seeds were soaked in 300 mL of distilled water for 48 hours. Ten replicates of five (5) seeds were prepared and used in this experiment. The soil was autoclaved twice before transferring it into the trays and planting pots. The temperature within the greenhouse ranged from 25-30°C. Seeds were sowed on the trays and six days after sowing, the seedlings were transplanted into the planting pots. Plants were consistently monitored for signs of leaf chlorosis, browning of stem and leaves, wilting and cracking every 24 hours. Table 7.2 below shows the scoring system or disease severity scale that was used to classify both treatment and control plants in the study. The experiments were terminated four weeks after planting.

Table 7.2: Disease severity scale used in the classification of plants in the study

Assigned Number	Meaning
1	No symptom of disease (healthy plants)
2	Yellowing and wilting of 1 leaflet
3	Yellowing and wilting of 2 leaflets
4	Yellowing and wilting of 3 leaflets
5	Yellowing and wilting of 4 leaflets

Figure 7.2: Fungal spore suspension in Malt extract broth

7.2.4 Statistical analysis

A randomized design was used in this experiment and each endophytic fungus was assessed in replicates of ten. Data were analyzed using Genstat (R) Statistical Analysis Software (Genstat, 2006).

7.3 RESULTS AND DISCUSSION

The maize seeds were placed, for absorption, in the various treatments highlighted in table 7.1. The average weight gained by the seeds was calculated as:

$$\%\text{Weight increase} = \frac{\text{Weight increase} - \text{Weight before}}{\text{Weight before}} \text{x} 100\%$$

Sample GG 13 had the highest (58.4%) weight gain, followed by the control gaining 57.5%. Other samples of significant weight gain were MHE 10, GG 8 and MHE 68. This is due to the absorption of moisture content within the seeds. Table 7.3 listed the initial weight and final weight to calculate the

moisture content. De Fátima Ferreira *et al.* (2013) supported our findings by reporting a 40-50% moisture content increase in maize seeds in Brazil.

Table 7.3: Average weight increase of five seeds before and after inoculation with the endophytic fungi

No.	Sample ID	Average Weight (g)	Weight after inoculation	Percentage of weight gained
1	END 15	1,576	2,291	45, 4%
2	GG 8	1,634	2,427	48,5%
3	GG 9	1,728	2,539	46.9%
4	GG 13	1,520	2,408	58,4%
5	MHE 10	1,636	2,448	49,6%
6	MHE 55	1,622	2,365	45.8%
7	MHE 68	1,558	2,308	48.1%
8	PG 9	1,636	2,348	43.5%
9	RNK 4	1,542	2,233	44.8%
10	Control	1, 532	2,413	57.5%

7.3.1 Seed germination after inoculation

Seed germination rate of 90% was observed in this trial. The control (water) seeds germinated faster and adequately as compared to the fungal treated seeds as shown in Figure 7.3 (a, b). This may be due to the dense spore suspension and thick mycelia utilized in this study. Furthermore, this could have influenced the low or non-geminated seeds, illustrated in Figure 7.3 (c) by blocking the incision portion. Maraghni *et al.* (2010) used deionized water as a control which was reported to have the highest and fastest germination in *Ziziphus lotus* (L.) Lam shrub. These results supports our finding in this study.

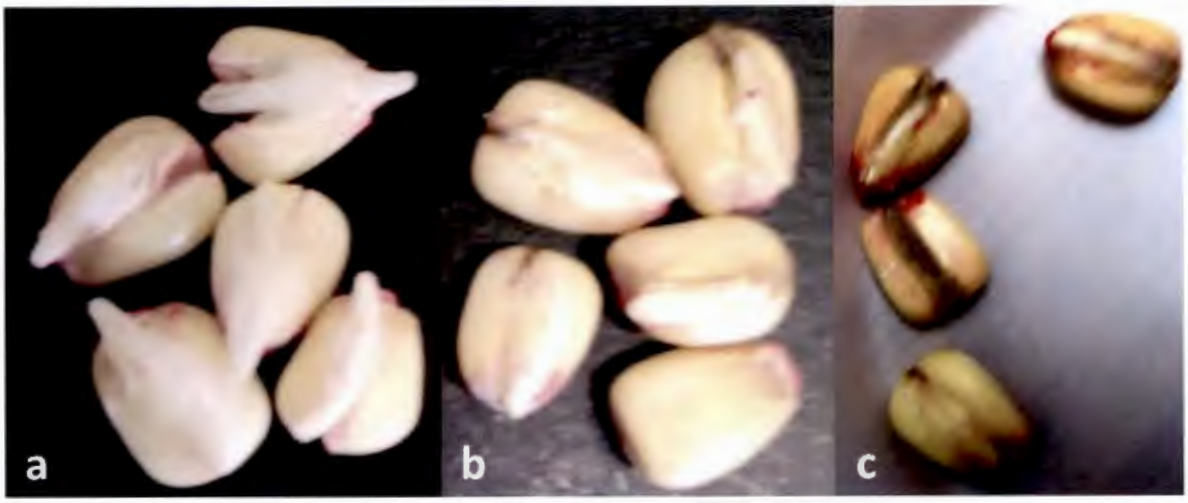


Figure 7.3: Seed germination (a) control treatment (b) fungal treatments (c) low germinated seeds

7.3.2 Effect of endophytic isolates on maize plant in the greenhouse

7.3.2.1 Growth parameters

The growth parameters were checked every seven days until the 28th day from the planting stage.

7.3.2.1.1 Plant height

Table 7.4 listed the maize height with an interval of seven days for twenty-eight days. The growth height was consistent throughout the greenhouse experiments. This was demonstrated in a schematic line graph in Figure 7.4.

Table 7.4: Effect on endophytic fungi on the plant height (cm) over 28 days

Plant age (days)	Treatments (Plant height cm)									
	Control	END 15	GG 8	GG 9	GG 13	MHE 10	MHE 55	MHE 68	PG 9	RNK 4
7	11	8	13	10	6	9	11	8	9	9
14	19	17	25	23	14	17	21	17	15	18
21	33	29	34	34	21	26	31	25	22	26
28	41	37	46	42	30	36	40	36	28	35

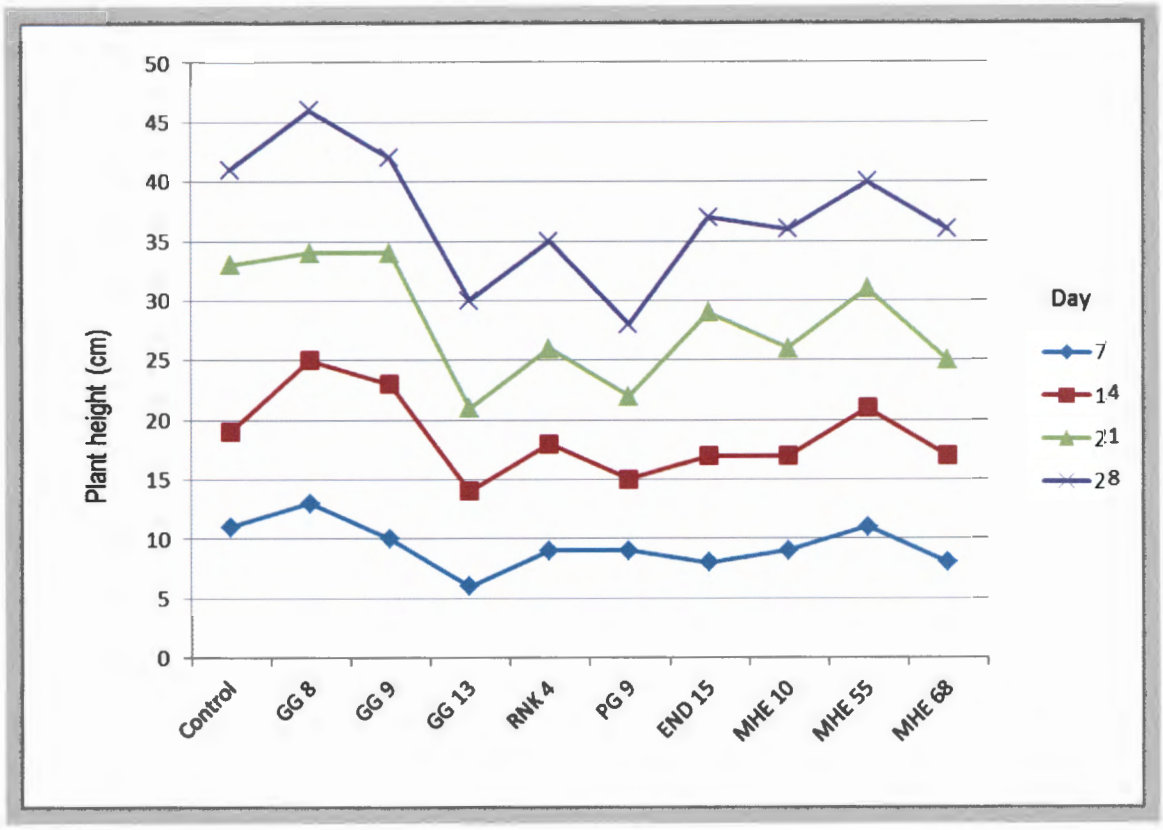
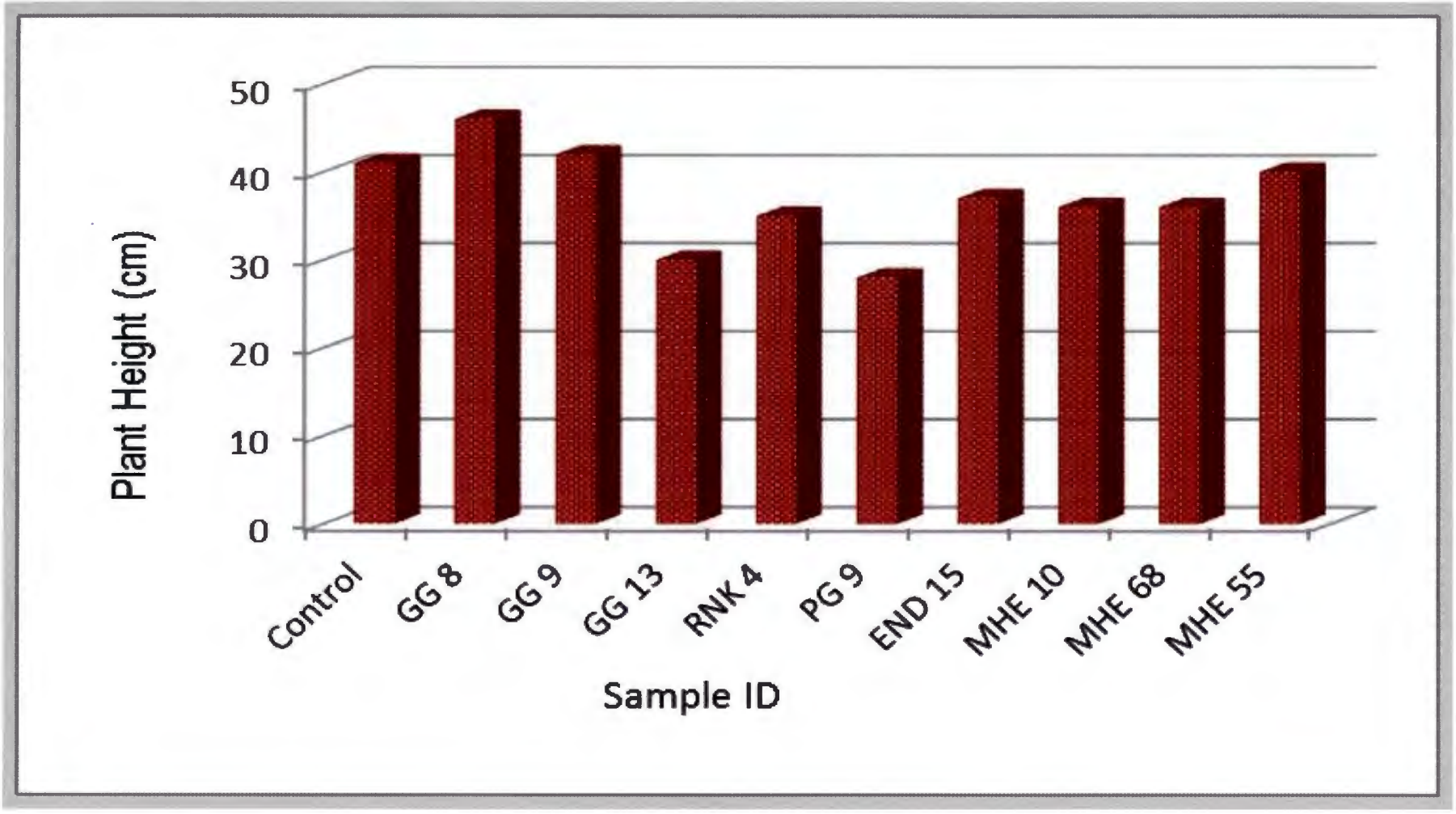


Figure 7.4: Schematic diagram of consistent increase in plant height

The data (Table 7.4 and Figure 7.4) reveals that the average height of the control is 41 cm. The highest plant height was observed in treatment which was inoculated with *Fusarium oxysporum* (GG 8) followed by *Fusarium solani* (MHE 55), reaching 46 cm and 40 cm respectively. The MHE 55 inoculated plants were greater than the controls. In contrast, maize plants inoculated with endophytic fungi, GG 13, RNK 4 and PG 9 gave the lowest plant height (30, 35, 28 cm), respectively as compared to the control (41 cm) table (7.4). Moreover, this suggests that the endophytic fungi enhanced the plant height. The greenhouse trials conducted by Machungo *et al.* (2009) in Uganda using banana plants supported our findings. In the experiments, endophytic *F. oxysporum* isolates (V5W2, Emb2.4o and Eny7.11o) showed a significant increase of 11.3% in plant height.

The maize plants that were treated with *Chaetomium subaffine* (PG 9) were the shortest plants as compared to the control and all other investigated endophytic fungi (Figure 7.5). Hence, *Chaetomium subaffine* (PG 9) suppressed the growth of the maize plants. *Fusaria* are well known as pathogens that mainly suppress the infected growth of plants. In this current study, this is totally the opposite due the abiotic and biotic stresses. The plant-host interaction contributes as well as the microbial interaction within the plants; this is highlighted in chapter 2.



gure 7.5: Effect on Maize plant height after inoculation with endophytic fungi isolates

7.3.2.1.2 Fresh weight

The results of the fresh weigh recorded after 28 days of plantation are presented in Figure 7.7. The control plants were for baseline comparison (Figure 7.6). The average weight of the control plants were 8.1 g. The most weight gain of 44% was recorded in *Boeremia* (END 15) treated seeds. The second was in *Fusarium* (MHE 55) treated seed with 40% increase resulting in a total weight of 11.3 g. Maximum fresh weight was observed with END 15, MHE 55 and MHE 10 measuring an average of 11.7 g, 11.3 g, and 9.7 g respectively.

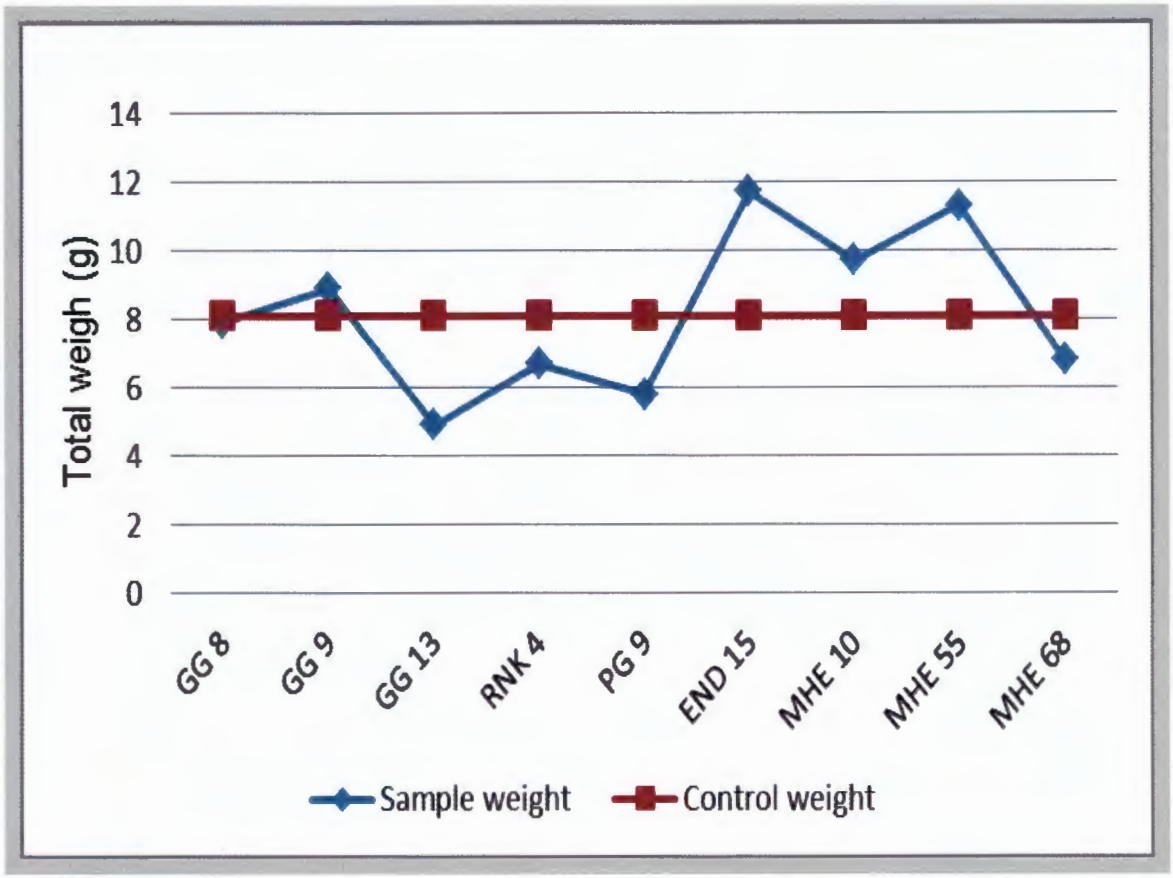
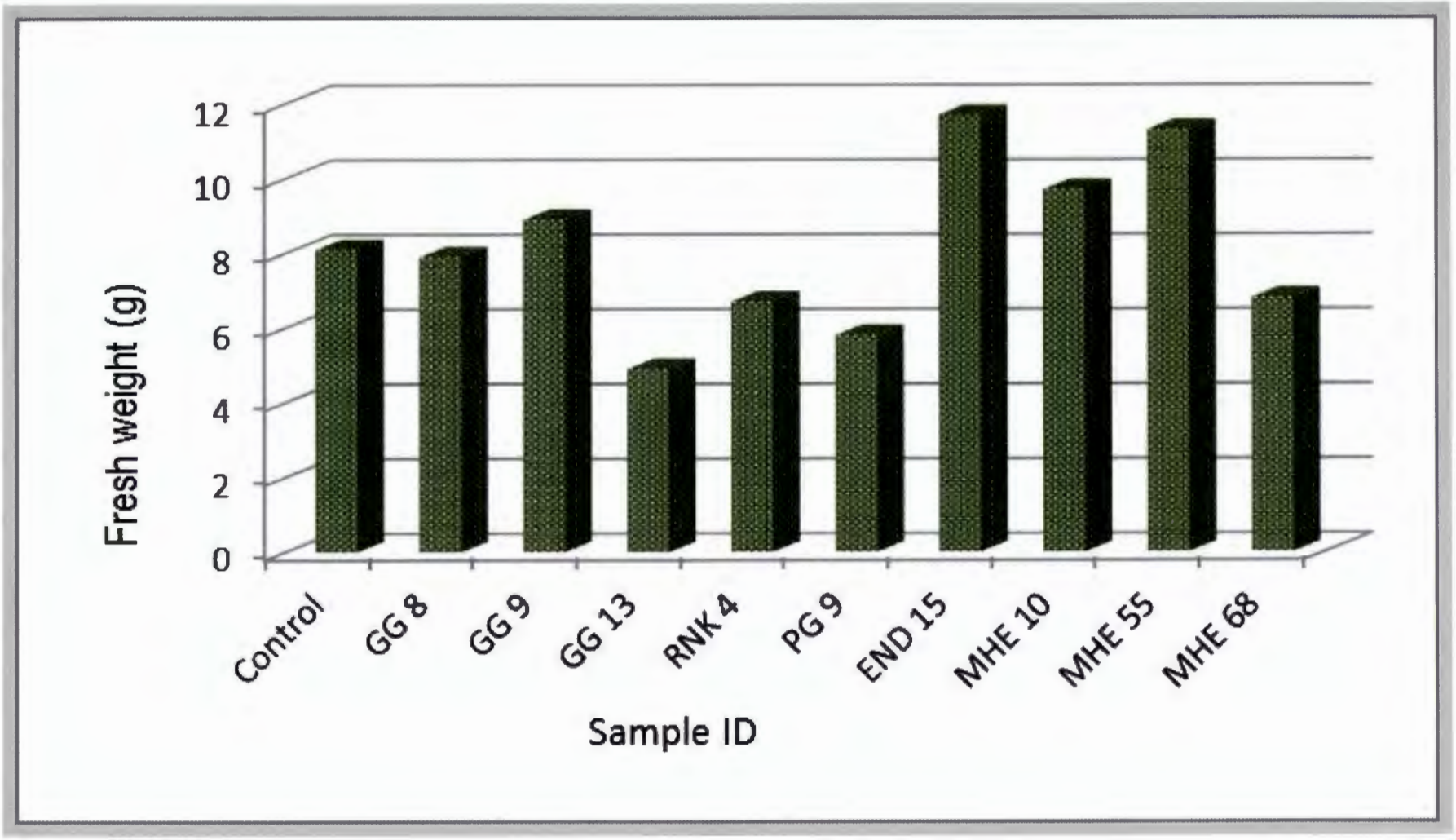


Figure 7.6: Line graph of total weight gained by the plants and baseline comparison with control.

The lowest weight was observed in *Aspergillus* (GG 13) and *Chaetomium* (PG 9) treated seeds, having a total weight of 4.9 g and 5.8 g respectively. *Salvia miltiorrhiza* seedlings were inoculated with *Alternaria* sp. A13 that exhibited significant increase in fresh weight, dry weight, and total phenolic acid (Zhou *et al.*, 2018).



gure 7.7: Effect of inoculation of endophytic fungi on the fresh plant weight

7.3.2.1.3 Root weight

The maize plant has fibre-like, thin, clustered roots. They are a fibrous root systems. The roots are of short lifespans and are replaced easily in the system. The heaviest root formation was observed in *Boeremia* (END 15) treated seeds followed by *Fusarium* (MHE 55) and *Fusarium* (GG 8) treatment. *Aspergillus* (GG 13, 1.6 g) treated seeds showed lowest root weight after *Chaetomium* (PG 9, 2.4 g) and *Penicillium* (RNK 4, 2.4 g). Inoculums (GG 13, PG 9, RNK 4) suppress the growth of the maize plants. Figure 7.8 clearly shows that three fungal inoculated seeds showed lower root weight as compared to the controls. The data revealed in Figure 7.9 summarised all the results.

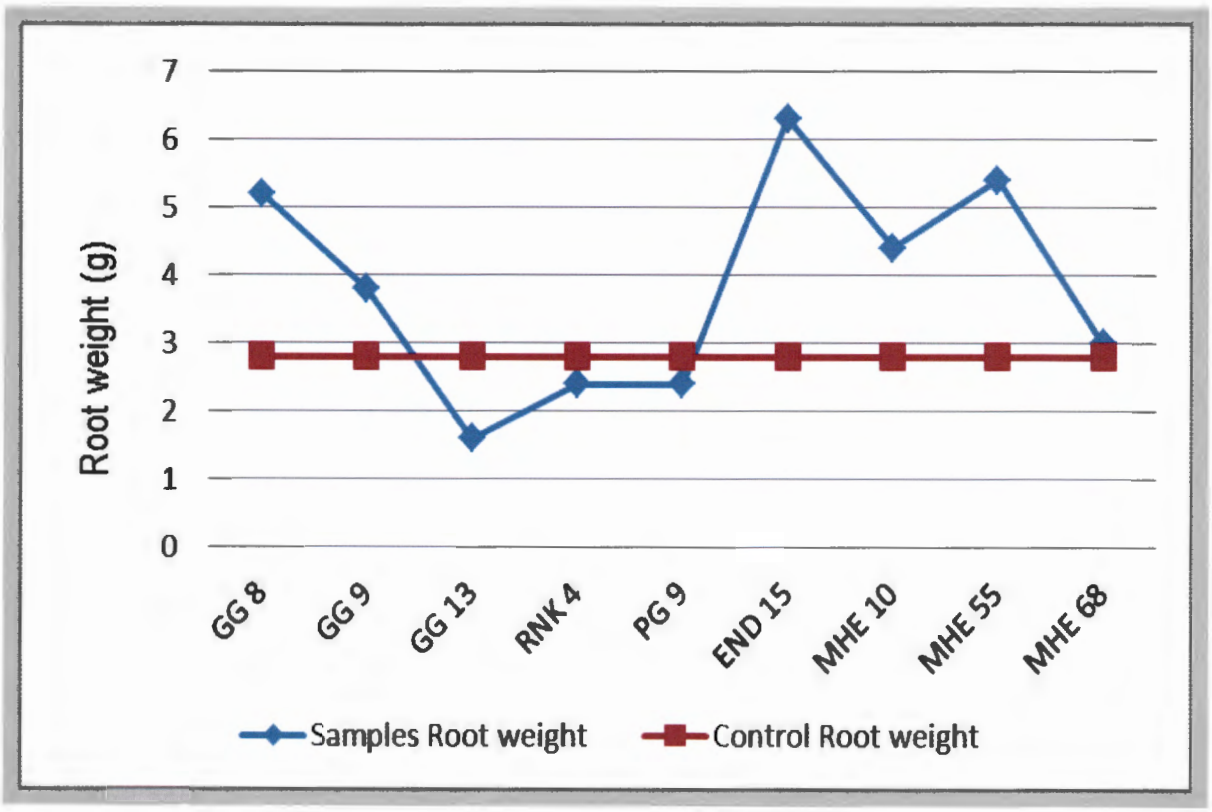
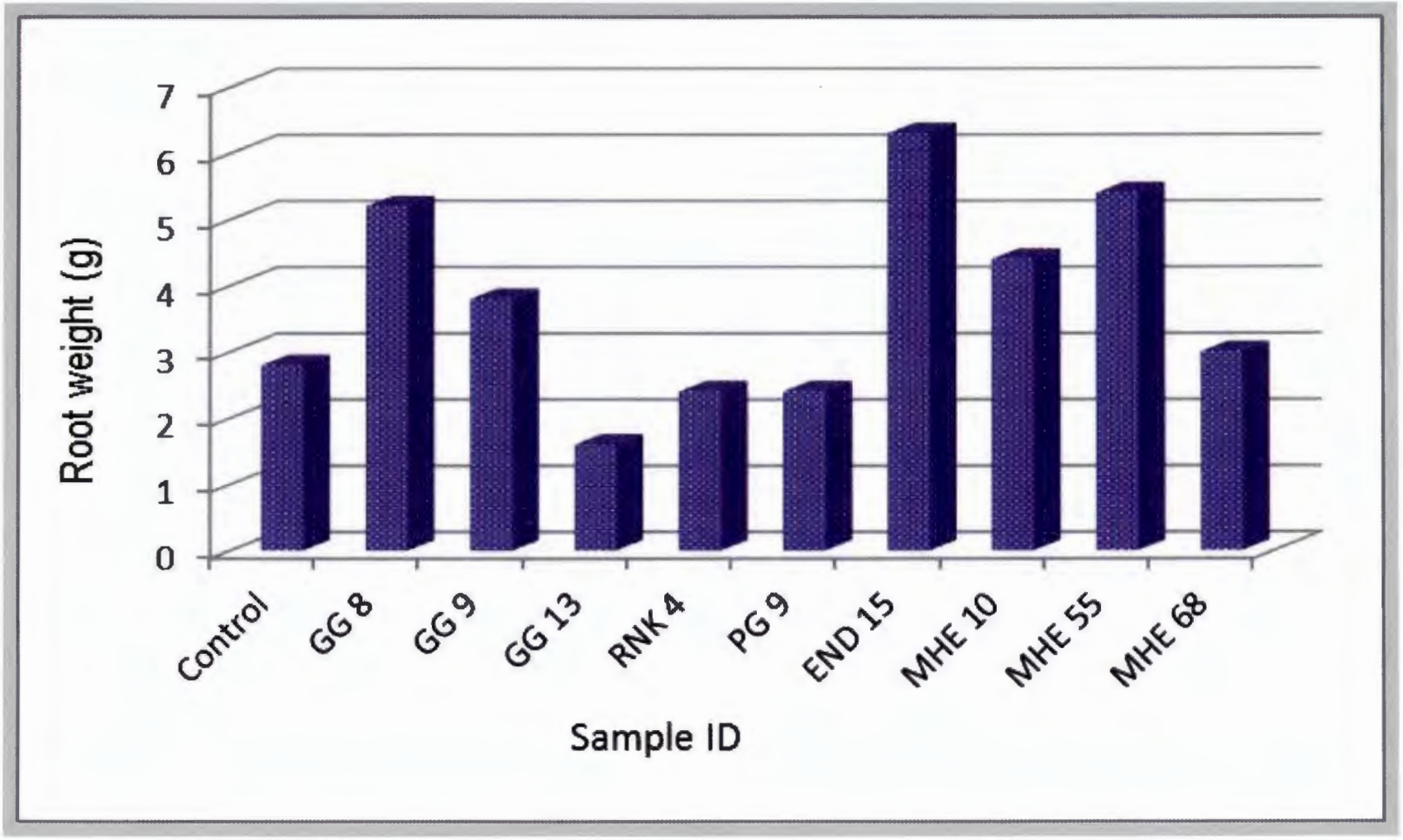


Figure 7.8: Effect on the root weight against the control



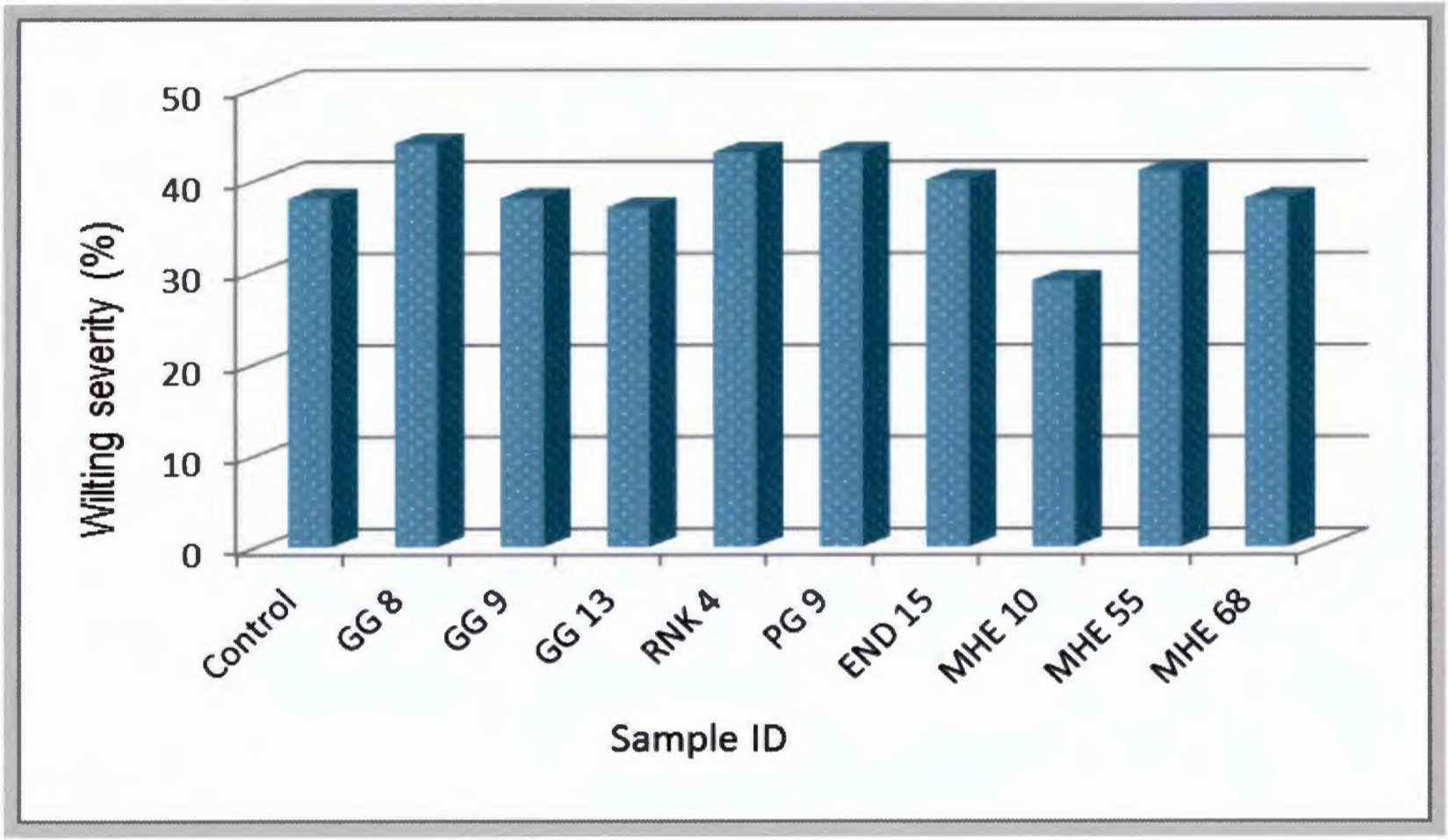
gure 7.9: Effect on the average root weight after inoculation of endophytic fungi

7.3.2.1.4 Wilting severity

Wilting is when a leaf losses their flexibility and turn from green to yellow or brown. Loss of water can result into wilting. Table 7.1 was used to categorize the severity of wilting. The results revealed that wilting severity for the majority of plants were moderate but in the case of *Penicillium* (RNK 4) treated plants they showed very high wilting as displayed in Table 7.5. Data retrieved from the number of leaves compared against the wilting leaves are shown in Figure 7.10. The control was at 38% and the highest was *Fusarium* (GG 8) at 44%. *Neurospora* (MHE 10) plant samples had the lowest wilt rate of 29% (Table 7.9). The ability of endophytes to promoter plant health and improve growth by acting as biological control agents is mainly attributed to the production of bioactive metabolites (Orole and Adejumo, 2009). Hence, volatile organic chemicals (VOCs) can aid in reduction of wilting in plants.

Table 7.5: Wilting severity scale outcomes

No. of leaves (wilting)	Treatments (Scale)									
	Control	END 15	GG 8	GG 9	GG 13	MHE 10	MHE 55	MHE 68	PG 9	RNK 4
0										
1										
2					X=3	X=3				
3	X=4	X=4		X=4				X=4	X=4	
4			X=5				X=5			
5										X=MORE

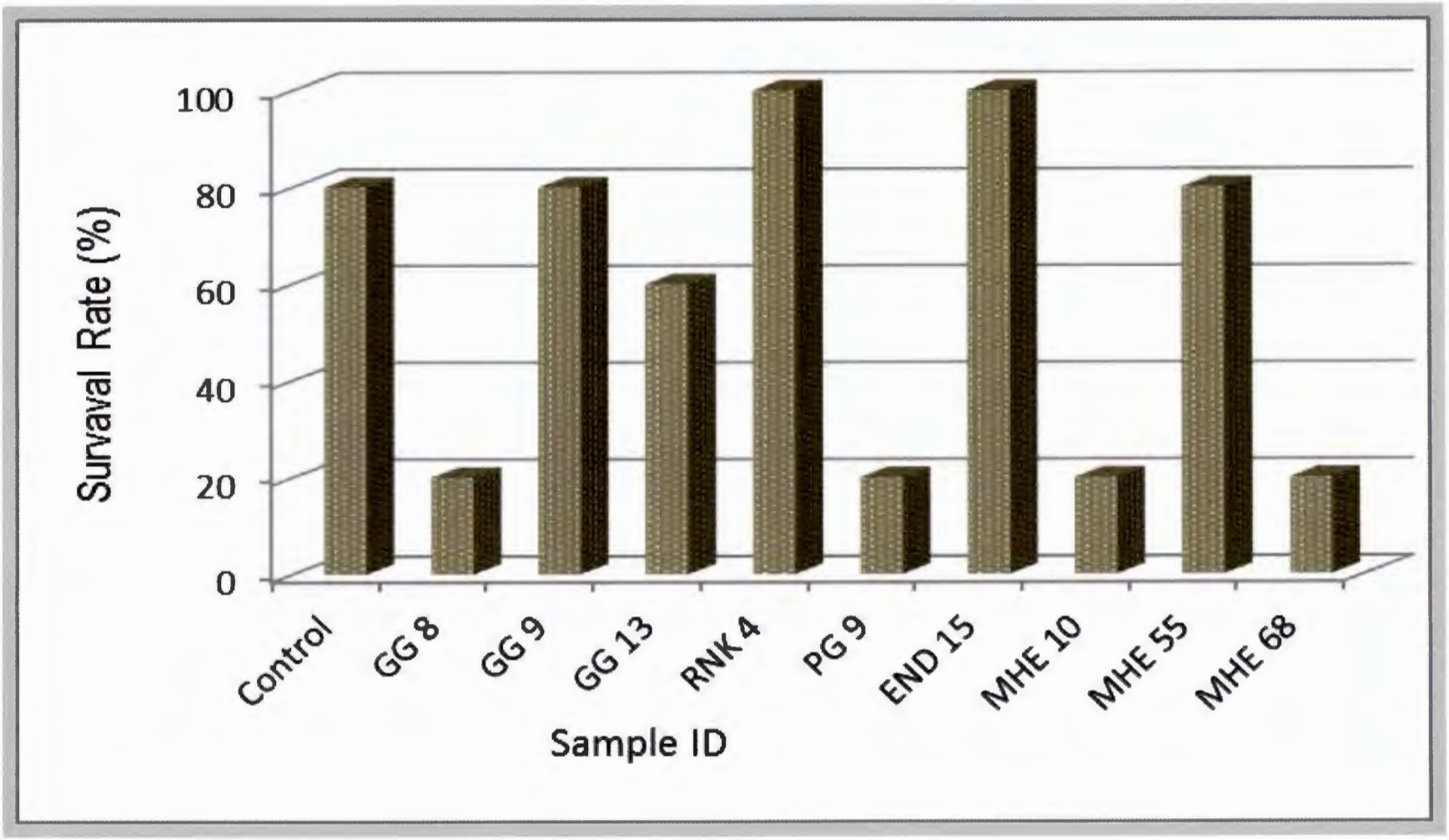


gure 7.10: Wilt symptoms on maize leaves after inoculation of endophytic fungi

7.3.2.1.5 Survival Rate

Survival Rate is termed in this study as the number of surviving plants as compared to the seeds planted. The effect of endophytic fungal extract treatments on survival plants (%) was summarized in Figure 7.11. The highest percentage of survival plants was from 80-100%. The bar graph (Figure 7.11) established *Boeremia* (END 15) and *Penicillium* (RNK 4) treated seeds had 100% survival rate. Furthermore, the control seeds showed 80% survival rate. The lowest percentage of survival of plants were found in *Fusarium* (GG 8), *Chaetomium* (PG 9) and *Neurospora* (MHE 10) treated seeds all having 20% survival percentages.

Current research has shown that the mutualistic relationship between the host plant and their endophytic fungi significantly reduces plant survival, nonetheless increasing population growth and regeneration. Furthermore, the benefits of this association overcome the negative aspects (Rudgers *et al.*, 2012).



gure 7.11: Survival rate of the maize plants after inoculation of endophytic fungi

7.3.2.1.6 Leaf Size

The largest leaves were measured and compared. Figure 7.13 shows that the largest (43 cm) leaf was from the control samples. This is followed by *Fusarium* (MHE 55) having 42 cm. The smallest leaf was from the *Chaetomium* (PG 9) group which had 19 cm. Endophytic fungi colonize the plant and contribute to the plant health, protection against herbivorous insects and plant pathogens. Therefore, the growth of the plant will be increased (Tefera and Vidal, 2009).

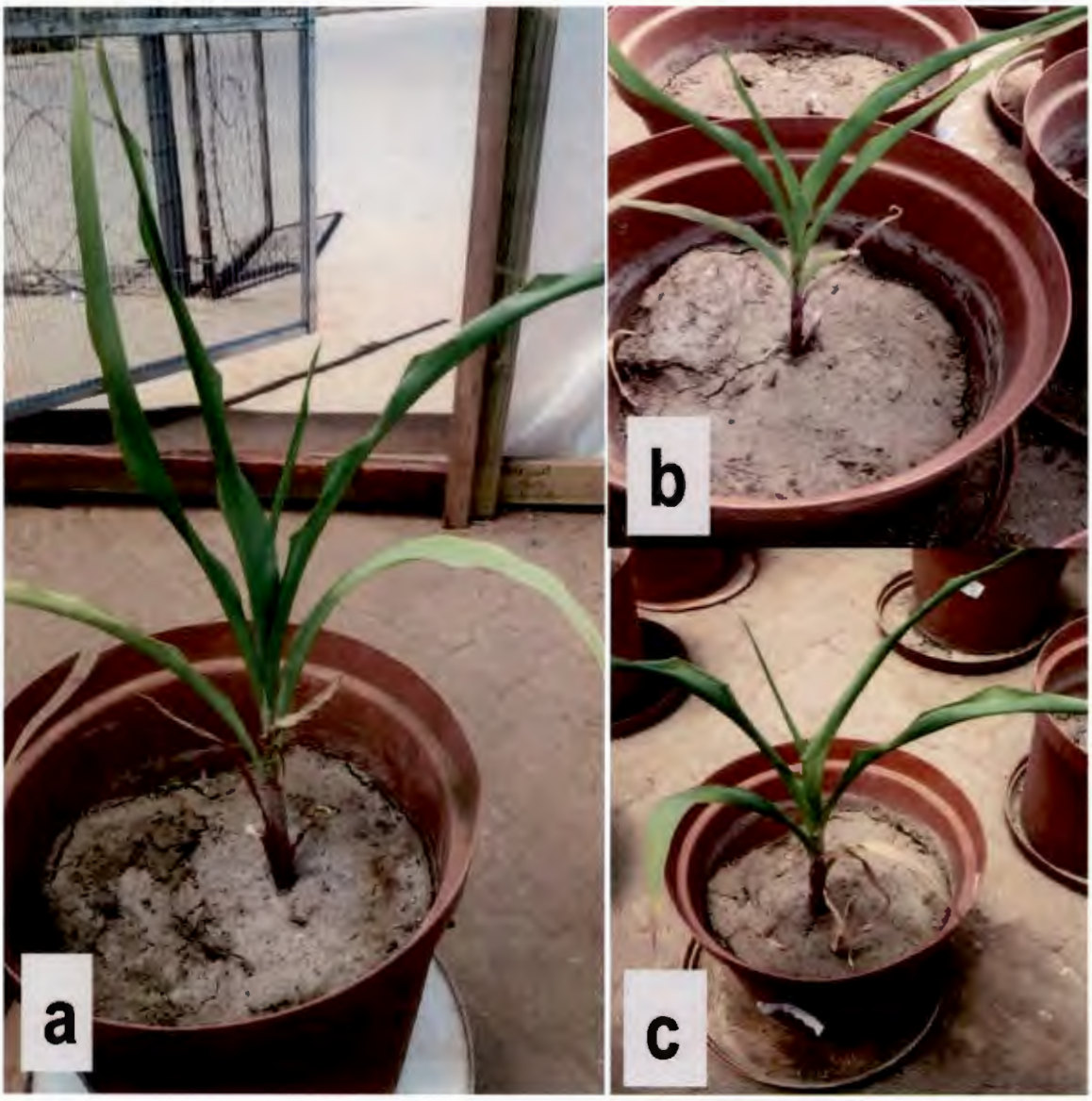
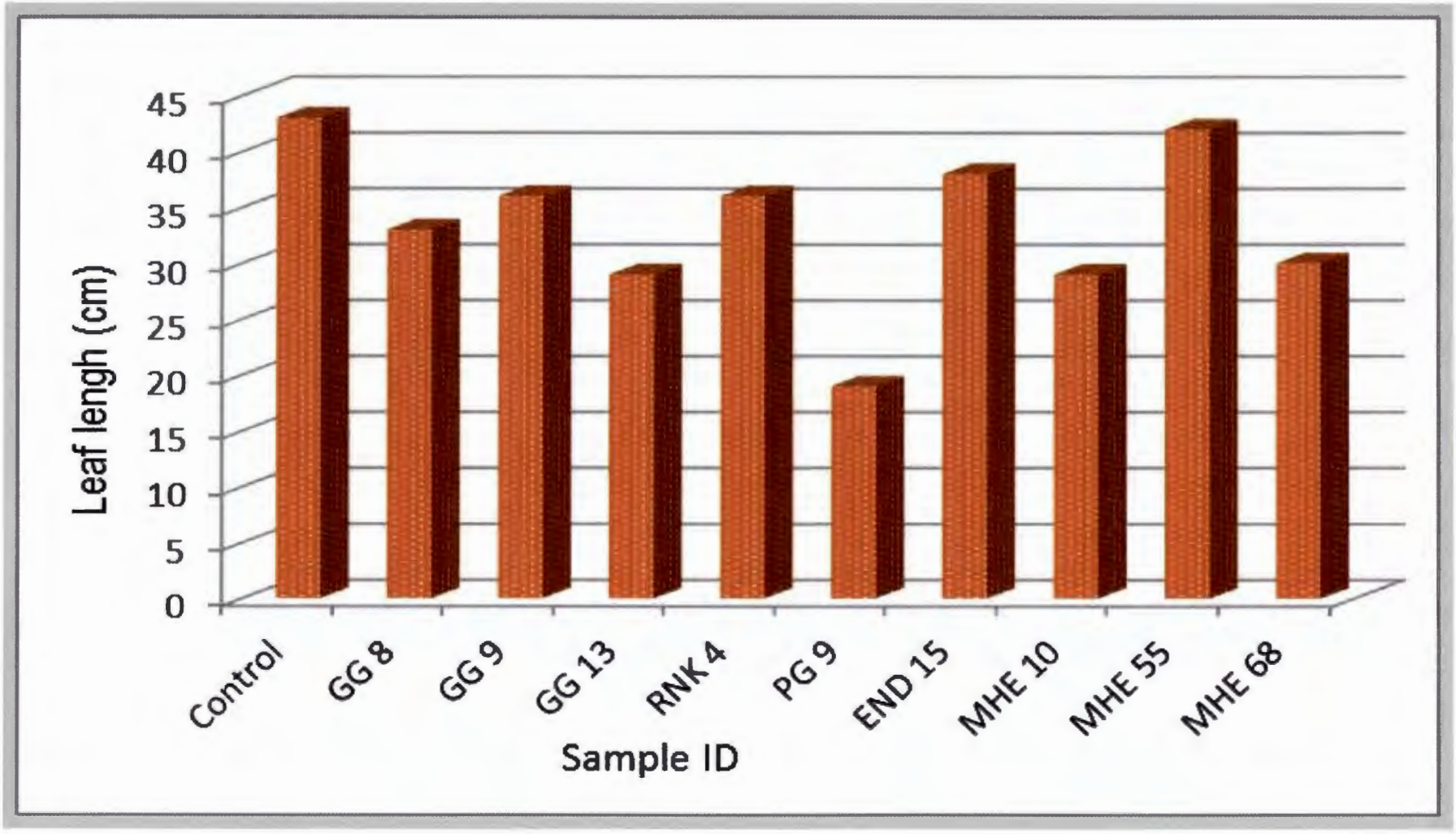


Figure 7.12: Maize plants used for different growth parameter (a) biggest plant (b) smallest plant (c) wilting symptoms



gure 7.13: Length of the largest leaf from maize plants

Figure 7.12 is a photographic illustration of experimental maize samples after 28 days. The largest plant in terms of height was *Fusarium* (GG 8, Figure 7.12 a) and Figure 7.12 b is smallest plant inoculated by *Chaetomium* (PG 9). Figure 7.11 c shows the wilting of the maize leaves.

7.4 CONCLUSION

A total of nine (n=9) fungal isolates were selected for the greenhouse experiments. The maize plants inoculated with *F. solani* (MHE 55) exhibited significant plant height, root weight, fresh weight, leaf size and high survival rate. In contrast with *Chaetomium* (PG 9) that had low leaf size, weight and survival rate. *Alternaria* (MHE 68) had moderate effect on growth as compared to *F. solani* (MHE 55). Endophytic fungi can be used as growth promoters without causing any disease or symptoms of diseases. These findings can be beneficial in the agricultural industries specifically to biofertilizer industry. Hence, it will increase the production and help in the protection of crops resulting in more food availability.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

CHAPTER 8

GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

8.1 INTRODUCTION

Drug discovery and development remains an essential aspect of human survival due to a massive increase in antimicrobial resistance of microbial pathogens to the currently recommended antibiotics. Over a period of decades, human have relied on natural products especially in developing countries as their alternative agents for the treatment of diseases and alignments. Findings of previous studies reveal that medicinal plants have received significant attention resulting from their extensive use by traditional knowledge holders especially in rural communities within developing countries (Selim *et al.*, 2012).). Some phytochemical studies have revealed the presence of bioactive compounds from plant extracts (Nair and Padmavathy, 2013). However, it also been reported that plants including medicinal plants harbour endophytic fungi within their tissues and these fungi can contribute to the phytochemical properties of the plants and therefore make them valuable agents for the search of bioactive compounds with enhanced antimicrobial properties. Moreover, endophytes are capable of producing other metabolites that can be used in a variety of applications to produce products that are of benefit to humans. The overall aim of the study was to determine the diversity of endophytic fungi possessing bioactive compounds isolated from selected medicinal plants (*Sceletium tortuosum* and *Pelargonium sidoides*) that are indigenous to South Africa. In order to achieve this aim specific objectives were set and addressed as individual chapters that are outlined in Section 8.2.

8.2 KEY FINDINGS AND SIGNIFICANCE

Generally, the aim and objectives of the study were achieved and findings revealed the presence of endophytic fungi in plants that were analysed.

8.2.1 Chapter 3: Biodiversity of endophytic fungi isolated from native medicinal plants (*Sceletium tortuosum* and *Pelargonium sidoides*) using morphological characteristics

The findings of this chapter revealed that a total of hundred and ninety-three (193) endophytic fungal isolates were obtained from the two native medicinal plants. The make-up of this was 60 and 133 fungal endophytes from *Sceletium tortuosum* and *Pelargonium sidoides* respectively. It was also revealed that in *Sceletium tortuosum* the leaves possessed largest proportion of endophytes when compared to the roots. On the contrary, the roots of *Pelargonium sidoides* harboured a large proportion of the fungi than the leaves. In this chapter, it was identified that both medicinal plants harboured a wide variety of endophytic fungi that belong to different genera and species. The distribution of fungi in these plants were dominated by isolates belonging to the genus *Fusarium, Aspergillus, Neurospora, Penicillium* and *Alternaria* thus indicating a highly diverse fungal population. Although morphological identification has been used for preliminary identification of fungal isolates, this procedure requires highly skilled personnel thus affecting its reliability. Against this backdrop, isolates were subjected to molecular identification using PCR as indicated in chapter 4.

8.2.2 Chapter 4: Phylogenic analysis and potential novel endophytic fungi

This chapter was designed to confirm the identities of all 193 endophytes obtained in Chapter 3 using ITS and TEF sequence specific PCR, for all fungal species and *Fusarium* species respectively. A phylogenetic tree was conducted using data obtained from ITS and TEF sequence analysis revealed that a total of 133 isolates from *Pelargonium sidoides* clustered into two major groups that were further divided into seven sub-clusters. Based on the sequence data for ITS and TEF sequence analysis for 60 isolates from *Sceletium tortuosum* a phylogenetic tree produced three major clusters. In addition, three isolates from this study did not cluster with others in the trees and these uncluttered isolates were considered as potential novel fungal endophytes. These potential novel endophytic fungi were MHE 65, DR 10 and PG 6 identified as *Mortierella hyalina*, *Phomopsis columnaris* and *Colletotrichum* sp. respectively. These novel fungal species may be very useful in the development of products in both pharmaceutical industries in order to address the public health threats posed by resistant microorganisms as well as in the enhancement of crop productivity in the agricultural sector. The findings herein also reveal the need to increase the search for naturally occurring bioactive compounds from South African medicinal and aromatic plants that are currently considered to be under-exploited.

8.2.3 Chapter 5: Antimicrobial activity exhibited by secondary metabolites and characterization

All the 193 endophytic fungi were assessed for their ability produce bioactive secondary metabolites with enhanced antimicrobial activities. This was motivated by the constant increase in the detection of antimicrobial resistant isolates that pose severe public health threats humans even in countries with advanced public health facilities. The findings of this chapter indicated that the phytochemical properties were of 193 endophytic fungi were investigated and the secondary metabolites or fungal extracts of all the isolates were screened against pathogenic enviromental as well as ATCC control bacteria strains for antimicrobial activities. The composition of bioactive compounds in the fungal extracts was determined using GC-MS. A total of 24 fungal extracts exhibited antibacterial activity against the bacteria strains and *E. faecium* as well as *E. gallinarum* displayed the highest levels of resistance to the fungal extracts. On the contrary, *E. coli* (ATCC 25922) was most often sensitive to a large proportion of the fungal extracts tested in this study. Individual comparison of the fungal extracts revealed that (MHE 65) exhibited a broad-spectrum activity against the isolates when compared to the others. Chemical characterization of the extracts revealed that the compounds 9,12-Octadecadienoic acid (Z,Z) and Cyclodecasiloxane were dominant in both active and inactive fungal extracts. This study also revealed that endophytic fungi from South African medicinal plants *Sceletium tortuosum* and *Pelargonium sidoides* are a potential source of bioactive compounds that may be very useful in the possible control of diseases in humans.

8.2.4 Chapter 6: Fungal pigment production, characterization and the enzymes responsible

Endophytes inhabit plant tissues in symbiotic relationships that provide unique biochemical pathways resulting in the production of biologically active metabolites. Among the metabolites produced by fungi, pigments have received a lot of attention due to their biological activity that include their use as colouring additives in various essential and profitable industries in the production of textile, pharmaceutical, food, photographic and cosmetics products as well as plastics, paint, ink, and paper. In our attempt to gain insight on pigment production and also assess the correlation of this trait with the production of enzymes as well as bioactive antimicrobial compounds, a total of 193 fungal isolates were analysed in this chapter. Despite the fact that only 31 (16.1%) of the fungal isolates were able to produce pigments with high a number of cell concentrations. The pigments exhibited a broad spectrum of colours. Analytical analysis also revealed that some pigments had more than one colour. Enzymatic assays revealed that lipase, amylase, laccase and protease. Given that these

enzymes are protein products that assist endophytes in the degradation of polysaccharides, these results indicate that the fungal strains obtained in this study can potentially serve as novel sources for pigments that have important industrial and commercial applications.

8.2.5 Chapter 7: Inoculum response of fungal extract using the greenhouse trials

Food security is a global problem and the massive reduction in productivity of *Zea mays* L. resulting from infection of the plants with pathogens (Kumar and Kalita, 2017) is known to be the most important contributing factor. In addition, the use of endophytic fungi to enhance plant growth has been documented due to their beneficial growth promoting traits. Despite this, no study has assessed the contribution of endophytes from the indigenous plants *Sceletium tortuosum* and *Pelargonium sidoides*. Nine endophytic fungi were used to determine their contribution to plant growth on maize plants. The findings of this chapter revealed thatthe extract from the endophytes MHE 55 identified as *F. solani* was the most effective in enhancing the growth of plants and this was followed by *Alternaria* (MHE 68). These findings indicate that these endophytes may be very useful in agricultural applications.

8.3 RECOMMENDATIONS AND FUTURE DIRECTIONS

Based on the outcomes of the study, *Alternaria* sp. (MHE 68) and *F. solani* (MHE 55) possessed very strong antimicrobial activities, and were also capable of producing a wide variety of pigments coupled with their potential to serve as very active growth promoting agents in maize plants. Hence based on our observations, the following recommendations are made:

- A comprehensive study needs to be undertaken using the same samples to explore other biological properties such as antifungal, antiviral, antioxidant andalso assess the cytotoxicity of the metabolites.
- The establishment of the correlation between pigments, flavonoids and phenolic compounds using High performance liquid chromatography (HPLC).
- *Alternaria* sp. (MHE 68) and *F. solani* (MHE 55) must undergo several greenhouse trials in which the sample size must be increased over all seasons and until mature stage of the maize plant is achieved.

- In addition, combinations of fungal extracts must be assessed for antagonistic or synergistic activities when compared to their individual antimicrobial profiles. Furthermore, these combinations should also be assessed for growth promotion in maize.

8.4 CONCLUSIONS

To the best of our knowledge and based on current literature the findings of this study is the first that has been documented on the diversity of endophytes from the medicinal plants *Sceletium tortuosum* and *Pelargonium sidoides* in South Africa. In addition, these findings present a novel source of biologically active secondary metabolites that have broad-spectrum antimicrobial activities and also prove to be a potential source of a variety of pigments and enzymes. Moreover, these fungi also possess plant growth promoting traits thus confirming their potential to form symbiotic relationships with their host plants and in this specific case *Sceletium tortuosum* and *Pelargonium sidoides*. The findings presented in this thesis cements and increase emphasis on exploiting endophytic fungi for agricultural, textile, pharmaceutical, cosmetics, food, beverage, oil recovery, paper, biomaterial, waste management and biotechnological processes.

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APPENDICES

APPENDIX 1

ARTICLES PUBLISHED AND MANUSCRIPTS UNDER REVIEW FROM THE STUDY

A1. ARTICLE PUBLISHED

South African Journal of Botany 115 (2018) 193–199

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Biodiversity and antibacterial screening of endophytic fungi isolated from *Pelargonium sidoides*

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ABSTRACT

To investigate the biodiversity of endophytic fungi isolated from indigenous *Pelargonium sidoides* plants using morphological and molecular techniques and also screen isolates for antibacterial activities.
In the present study, a total of 50 *Pelargonium sidoides* plants were collected and the roots were analysed for the presence of endophytic fungi. The fungi were initially sorted according to morphological characteristics. Fungal identities were further confirmed through amplification of sequences encoding Translation Elongation Factor-1α (TEF-1α) gene for *Fusarium* species and Internal Transcribed Spacer (ITS) regions for all other fungi. Fungi were screened for antibacterial activity by adopting the standard Kirby-Bauer agar disc diffusion method using a pathogenic *Escherichia coli* strain (Code from the collection) previously isolated from cattle. A total of 133 fungi belonging to 32 genera were successfully isolated and identified in the study based morphological analyses. Fungi belonging to the genus *Penicillium* were dominant (23%) among the isolates when compared with *Fusarium* species (12%) as well as *Alternaria* and *Aspergillus* that were detected at 11% respectively. Antibacterial agent producing data revealed that *Aspergillus* sp. (KM487961), *Penicillium expansum* (LC015085.1) and *Aspergillus niger* (KP172477.1) produced the strongest bactericidal activity against the *E. coli* strain with growth inhibition zone diameter data of 11 mm, 5 mm and 4 mm respectively. To the best of our knowledge, this is the first study in which the diversity of fungi from *Pelargonium sidoides* has been assessed and the findings revealed that fungal diversity is directly related to their potential to produce bioactive compounds.

1. Introduction

Based on an estimate, up to 400,000 different plant species occur worldwide and majority are used in treating different ailments (Bussmann et al., 2007, 2008; Abbasi et al., 2010; Jeppa et al., 2010). In addition, it is also reported that approximately 80% of the world's population and especially those who live in developing countries depend on herbal medicines to address primary health complications (World Health Organization 2003). This is based on the premise that a number of pharmaceutical products used in modern day health care systems are obtained from plants (Newman and Cragg, 2016). On the contrary, some of these plants may be colonised by endophytic fungi (Rodriguez et al., 2009). Endophytes are microorganisms that colonize internal plant tissues and therefore spend all or part of their life within the host but without causing disease-like symptoms (Rodriguez et al., 2009). Given that the relationship between the plant and endophytes is symbiotic (Nair and Padmavathy, 2014), the fungi receive shelter and nutrients from the plant while plant is protected from attack by pathogens and herbivores (Singh et al., 2011; Higginbotham et al., 2013) as well as increased resistance to factors that are responsible for abiotic stress and toxicity to high concentrations of heavy metals (Khan et al., 2014).

Endophytic fungi have also been reported to possess bioactive secondary metabolites and enzymes that may be valuable for the production of pharmaceutical products (Zou et al., 2000; Strobel et al., 2004; Krishnamurthy et al., 2008). Endophytic fungi also have the ability to produce a mixture of volatile organic compounds (hydrocarbons, heterocycles, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives) with significant antimicrobial activities against some human and plant pathogens (Worapong et al., 2001). In the last few decades, broad spectrum well-known antimicrobial agents such as penicillin and lovastatin have been isolated from endophytic fungi (Brakhage, 2013). Furthermore, *Trichoderma* species are currently utilized as growth promoters or bio-fertilizers in the agricultural industry (Vinale et al., 2014).

In addition, endophytic fungi isolated from marine environments produced secondary metabolites with broad spectrum biological

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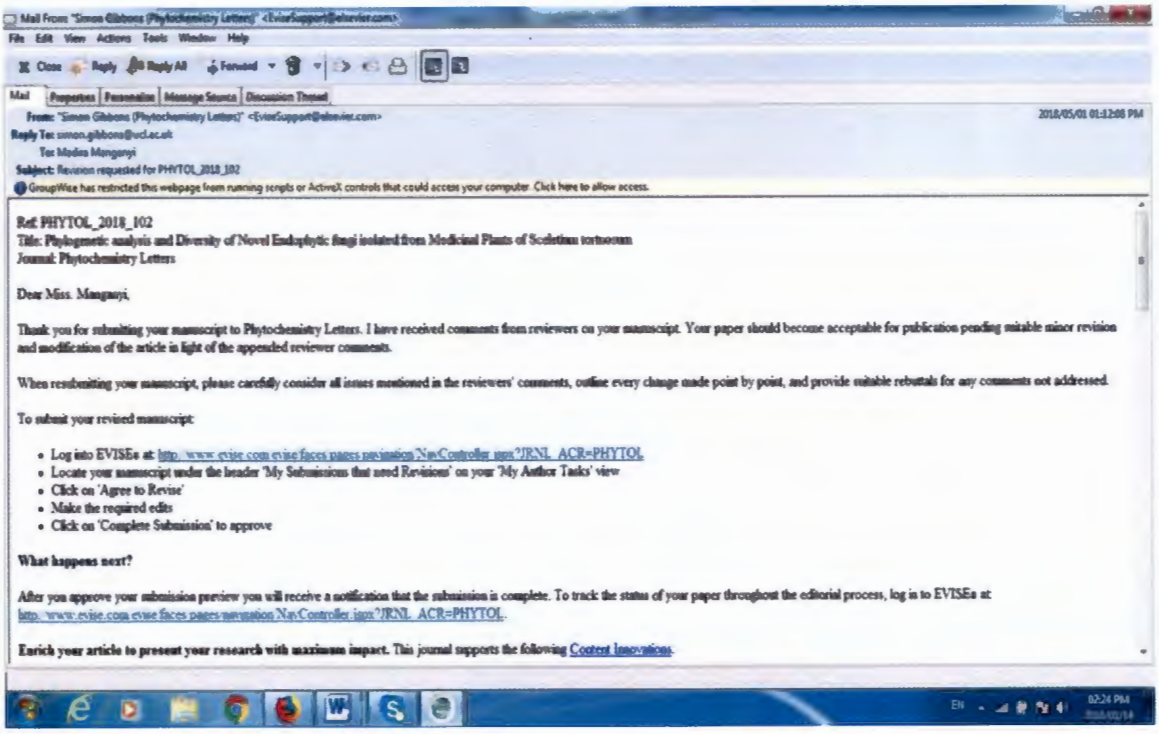
0254-6299/

A2. ARTICLE ACCEPTED

Phylogenetic analysis and Diversity of Novel Endophytic fungi isolated from Medicinal Plants Sceletium tortuosum, Phytochemistry Letters (PHYTOL_2018_102 R1)



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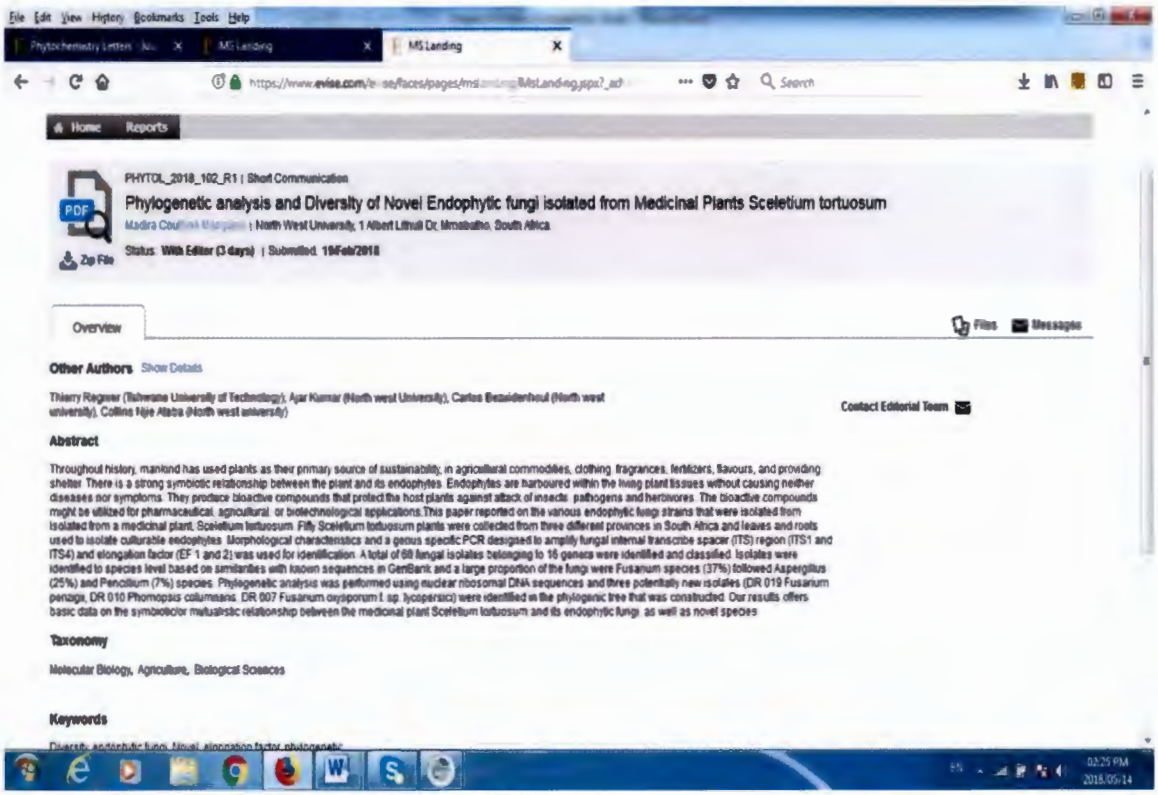
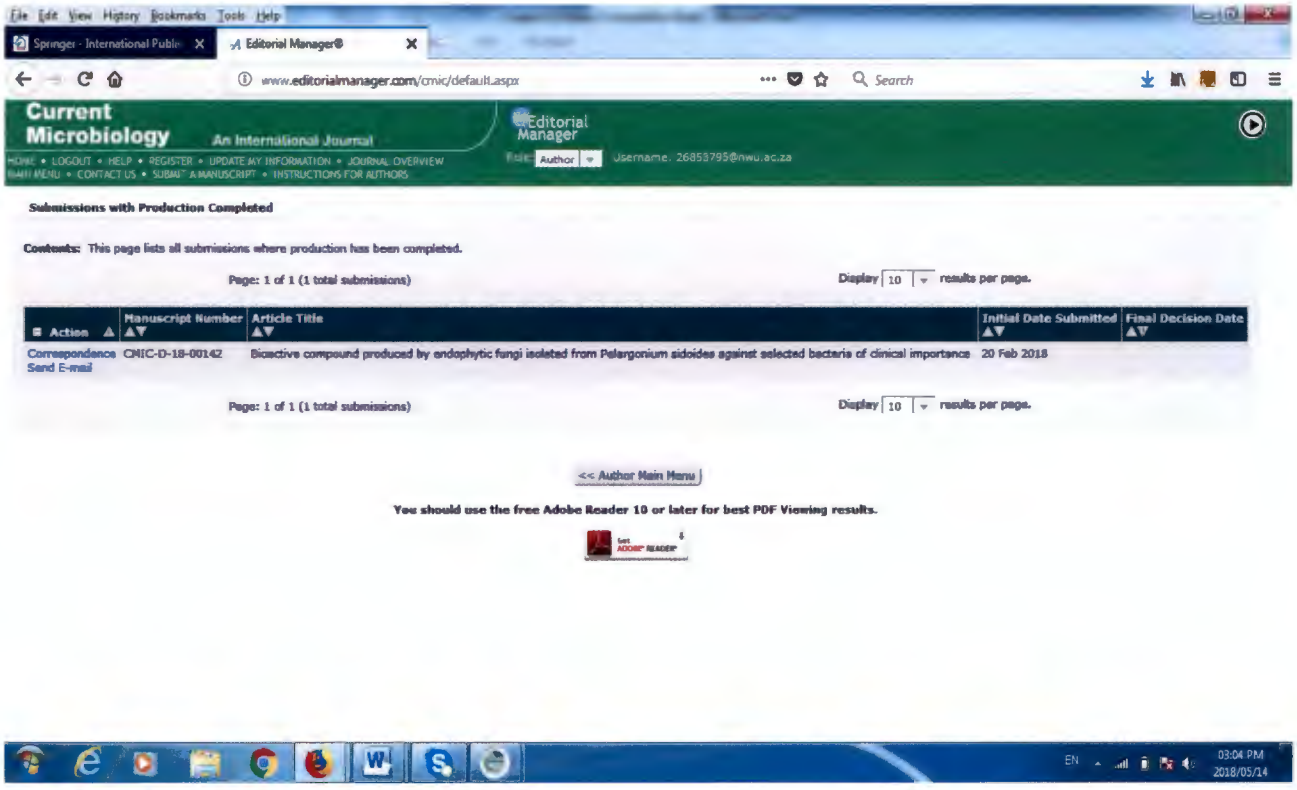


A3. MANUSCRIPTS UNDER REVIEW

Bioactive compound produced by endophytic fungi isolated from Pelargonium sidoides against selected bacteria of clinical importance, Current Microbiology (CMIC-D-18-00142)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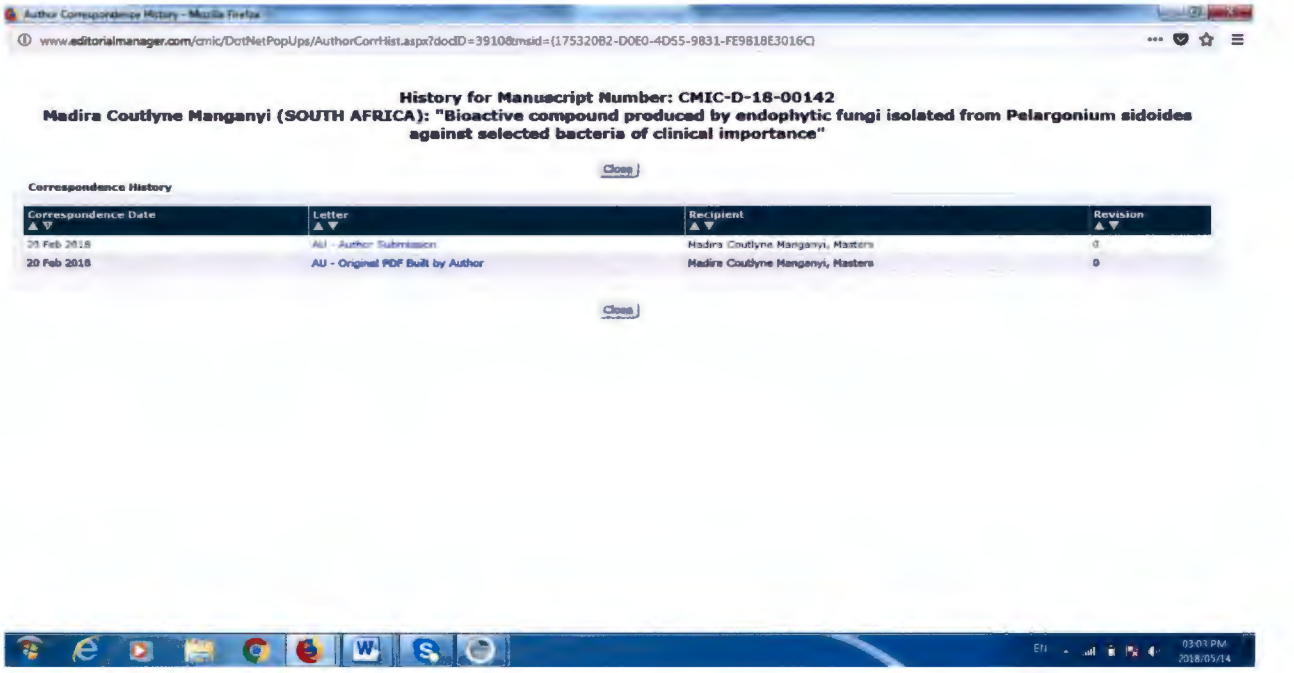
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Madira Coutlyne Manganyi (SOUTH AFRICA): "Bioactive compound produced by endophytic fungi isolated from Pelargonium sidoides against selected bacteria of clinical importance"
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APPENDIX 2

PREPARTION OF CULTURE MEDIA AND REAGENTS

A2.1 Potato Carrot Agar (PCA):

Chopped carrot	20 g
Chopped potatoes	20 g
Agar	20 g
Distilled water	1000 ml

Lightly boil carrot and potatoes for 1 hour, strain through cheesecloth. Discard the carrot and potato pieces. Add 20g agar and dissolve. Add distilled water to reach 1000 ml and autoclave for 15 min.

A2.2 Potato Dextrose Agar (PDA):

Potato (peeled and diced)	200 g
Dextrose (glucose)	20 g
Distilled water	1000 ml

Rinse potato under running water, and then add to water. Boil for 1 hour. Filter through a cloth, squeezing through as much pulp as possible. Autoclave at 15 psi for 30 minutes.

A2.3 Malt Extract Agar (MEA):

Malt extract	25 g
Agar	15 g
Water	1000 ml

Add the malt extract in water until dissolved. Add agar and dissolve. Fill up the liquid with distilled water to 1 liter. Adjust the medium to a final pH of 6.5 by NaOH.

A2.4 Water Agar:

Agar	15 g
Distilled water	1000 ml

Add the agar into the 1000ml water, mix thoroughly and bring to a boil. Sterilize by autoclaving.

Selective Fusarium agar (SFA):

Peptone	15 g
KH_2PO_4	1 g
$MgSO_47H2O$	0.5 g
Pentachloronitrobenzene (PCNB)	1 g
Agar	20 g
Distilled Water	1000 ml

Adjust the pH to 5.5-6.5 using 1M NaOH and autoclave the solution. Add 200 ml of antibiotics such as streptomycin sulphate to the media before pouring the plates.

Nutrient Agar:

Meat extract	1.0g
Yeast extract	2.0 g
Peptone	5.0 g
Sodium chloride	5.0g
Agar	15.0g
Water	1000 ml

Mix the required amount of the powdered medium in 1 liter distilled water. Autoclave for 15 min.

Sabouraud's Dextrose Agar (SDA):

Dextrose	40 g
Peptone	10 g
Agar	20 g

Mix the ingredients, boil to melt the agar, adjust pH to 5.6 and sterilize. Autoclave for 10 min.

1 M Hydrochloric acid solution:

Hydrochloric acid (37 %)	1 ml
Distilled water	10 ml

Dilute 1 ml of 37% of Hydrochloric acid in 10 ml of distilled water. Mix well and storage at room temperature.

1M Sodium hydroxide solution:

Sodium hydroxide pellets	50 g
Distilled water	10 ml

Weigh 50 g of sodium hydroxide pellets and dissolved in 1 litre of water to create 1 M solution of sodium hydroxide. Both 1 M hydrochloric acid and sodium hydroxide solution were used for adjusting the pH of media. Storage was at room temperature

3% Sodium hypochlorite solution:

Sodium hypochlorite (3.5%)	257 ml
Distilled water	43 ml

Dilute 3.5% of Sodium hypochlorite with 43 ml of distilled water. Mix well and storage at room temperature. To make 300 ml of 3% sodium hypochlorite.

APPENDIX 3

Macroscopic and Microscopic features to identify fungi

Sample ID	Probable ID	Macroscopic characteristics		Microscopic characteristics	
		Colony Colour		Nature of Hyhae	Presence of Special Structure
		Top	Bottom		
GG 001	*Aspergillus*	Brown	Yellow	Septate	Foot cell present
GG 002	*Mucor*	Cream-black	Cream white	Irregular non-septate	Foot cell give rise to conidiophore
GG 003	*Fusarium*	Pink	Pink	Septate	Foot cell on the. macroconidia
GG 004	*Penicillium*	Green	Yellow	Septate	Foot cell give rise to conidiophore
GG 005	*Fusarium*	Pink	Deep Pink	Septate	Foot cell on the. macroconidia
GG 006	*Ceratobasidium*	Brown	Cream	Septate	Basidia with basidiospores
GG 007	*Alternaria*	Brown	Cream	Septate	Foot cell give rise to conidiophore
GG 008	*Fusarium*	Red	Red	Septate	Foot cell on the. macroconidia
GG 009	*Neurospora*	Orange	Pale Orange	Non-Septate	Foot cell from which the conidiophore grow
GG 010	*Aspergillus*	Cream white	Brown	Septate	Foot cell present
GG 011	*Fusarium*	White Pink	Pink	Septate	Foot cell on the. macroconidia
GG 012	*Fusarium*	White Pink	Pinkish	Septate	Foot cell on the. macroconidia
GG 013	*Aspergillus*	Deep Green	Green Yellow	Septate	Foot cell present
GG 014	*Aspergillus*	Black	White	Septate	Foot cell present
GG 015.1	*Aspergillus*	Deep Green	Green Yellow	Septate	Foot cell present
GG 015.2	*Aspergillus*	Deep Green	Green Yellow	Septate	Foot cell present

GG 016	*Aspergillus*	Yellowish-Brown	Yellow	Septate	Foot cell present
ND 1	*Aspergillus*	Deep Green	Green Yellow	Septate	Foot cell present
ND 2	*Aspergillus*	Deep Green	Green Yellow	Septate	Foot cell present
ND 3	*Penicillium*	Cream White	Yellow	Septate	Foot cell give rise to conidiophore
ND 4	*Penicillium*	Cream White	Yellow	Septate	Foot cell give rise to conidiophore
ND 5	*Neurospora*	Orange Yellow	Brown	Septate	Foot cell from which the conidiophore grow
ND 6	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
ND 7	*Geotrichum*	White	Cream	Septate with Dichotomous	Initially yeast like metamorphosis into mycelium
ND 8	*Alternaria*	Brown	Black	Septate	Foot cell give rise to conidiophore
ND 9	*Aspergillus*	Green	Green	Septate	Foot cell present
ND 10	*Alternaria*	White	Cream	Septate	Foot cell give rise to conidiophore
ND 12	*Epicoccum*	Yellow	Orange	Septate	Foot cell present
ND 13	*Fusarium*	White Pink	Pink Yellow	Septate	Foot cell on the. macroconidia
ND 14	*Aspergillus*	Green	Pale Green	Septate	Foot cell present
ND 15	*Aspergillus*	Green	Pale Green	Septate	Foot cell present
ND 16	*Aspergillus*	Cream	Yellow	Septate	Foot cell present
ND 17	*Aspergillus*	Black	White Black	Septate	Foot cell present
ND 18	*Neurospora*	Yellow	Orange-Yellowish	Non-Septate	Foot cell from which the conidiophore grow
ND 19	*Fusarium*	Cream Pink	Yellow Pink	Septate	Foot cell on the. macroconidia
DR 001	*Aspergillus*	Cream white	Cream	Septate	Foot cell present

DR 002	*Fusarium*	Red	Deep Red	Septate	Foot cell on the. macroconidia
DR 003	*Coniothyrium*	White	Yellow	Septate	Basal foot cell
DR 004	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
DR 005	*Cf. Pleosporales*	White	Transparent	Septate	Foot cell present
DR 006	*Fusarium*	Cream-Pinkish	Deep Red	Septate	Foot cell on the. macroconidia
DR 007	*Fusarium*	White	Cream-Pinkish	Septate	Foot cell on the. macroconidia
DR 008	*Fusarium*	Cream-Pinkish	Cream-Reddish	Septate	Foot cell on the. macroconidia
DR 009	*Fusarium*	Cream	Yellow	Septate	Foot cell on the. macroconidia
DR 010	*Phomopsis*	White	Transparent	Septate	Cylindrical asci with a foot cell
DR 011	*Ceratobasidium*	White	Cream	Septate	Basidia with basidiospores
DR 012	*Pythium*	Cream-Brown	Cream	Septate	Foot cell absent
DR 013	*Fusarium*	Pink	Orange	Septate	Foot cell on the. macroconidia
DR 014.1	*Neonectria*	White	Beige	Single Septate	Foot cell on the. macroconidia
DR 014.2	*Neonectria*	White	Beige	Single Septate	Foot cell on the. macroconidia
DR 015	*Purpureocillium*	Cream	Purplish	Septate	Basal foot cell
DR 016	*Cladosporium*	Olive Brown	Dull Green	Septate	Basal foot cell
DR 017	*Fusarium*	White-Pinkish	Yellow-Orange	Septate	Foot cell on the macroconidia
DR 018	*Fusarium*	Orange	Light Orange	Septate	Foot cell on the. macroconidia
DR 019	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
DR 020	*Fusarium*	White-Purplish	Cream	Septate	Foot cell on the. macroconidia

DR 021	*Fusarium*	Purple	Deep Red	Septate	Foot cell on the. macroconidia
DR 022	*Fusarium*	Pale Purple	Yellow	Septate	Foot cell on the. macroconidia
DR 023	*Fusarium*	Cream	Yellow Purple	Septate	Foot cell on the. macroconidia
DR 024	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
RNK 001	*Talaromyces sp.*	Yellow	Cream	Septate	Foot cells are absent
RNK 002.1	*Penicillium*	Green	Yellow Green	Septate	Foot cell give rise to conidiophore
RNK 002.2	*Fusarium*	Orange	Orange	Septate	Foot cell on the. macroconidia
RNK 003	*Aspergillus*	Black-yellowish	Cream	Septate	Foot cell present
RNK 004	*Penicillium*	Cream	Yellow	Septate	Foot cell give rise to conidiophore
RNK 005	*Aspergillus*	Brown	Black	Septate	Foot cell present
RNK 006	*Penicillium*	Green	Yellow	Septate	Foot cell give rise to conidiophore
RNK 007	*Penicillium*	Green	Yellow	Septate	Foot cell give rise to conidiophore
RNK 008	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
RNK 009	*Penicillium*	Green	Pale Green	Septate	Foot cell give rise to conidiophore
RNK 010	*Penicillium sp.*	White	Cream	Septate	Foot cell give rise to conidiophore
RNK 011	*Penicillium*	Green-Brownish	Yellow	Septate	Foot cell give rise to conidiophore
RNK 012	*Alternaria*	Yellow-Blackish	Yellow	Septate	Foot cell give rise to conidiophore
RNK 013	*Phoma sp.*	White	Cream	Septate	Foot cell absent (conidiogenous cells)
RNK 014	*Aspergillus*	Cream	Yellow	Septate	Foot cell present
RNK 015	*Cochliobolus sp.*	Cream	Yellow-Reddish	Septate	Foot cell absent

RNK 016	*Alternaria*	Brown	Black	Septate	Foot cell give rise to conidiophore
RNK 017	*Fusarium*	Pink	Cream	Septate	Foot cell on the. macroconidia
RNK 018	*Alternaria sp.*	White-Orange	White-Orange	Septate	Foot cell give rise to conidiophore
RNK 019	*Alternaria*	White-Orange	White-Orange	Septate	Foot cell give rise to conidiophore
RNK 020	*Rhizopus*	White Black	Clear White	Long non seotate	Rhizoid stolon columnal
RNK 021	*Rhizopus*	White Black	Clear White	Long non seotate	Rhizoid stolon columnal
RNK 022	*Penicillium*	Brown	Yellow	Septate	Foot cell give rise to conidiophore
RNK 023	*Plectosphaerella*	Yellow	Orange	Septate	Curved macroconidia with foot cells
PG 1	*Neurospora*	Light Yellow	Cream	Non-Septate	Foot cell from which the conidiophore grow
PG 2	*Neurospora*	Light Yellow	Cream	Non-Septate	Foot cell from which the conidiophore grow
PG 3	*Aspergillus*	Brown	Cream	Septate	Foot cell present
PG 4	*Ceratobasidium*	Cream	Yellow	Septate	Basidia with basidiospores
PG 5	*Rhizoctonia*	White	Cream	Septate	Foot cell present
PG 6	*Colletotrichum*	Brown	Yellow	Septate	Foot cells are absent
PG 7	*Neopestalotiopsis*	White	Cream	Septate	Foot cell- like, with rhizoid
PG 8	*Alternaria*	Cream-Pinkish	Yellow	Septate	Foot cell give rise to conidiophore
PG 9	*Chaetomium*	Cream	Reddish	Septate	Foot cell present
PG 10	*Humicola*	Pink	Deep Pink	Septate	Basal foot cell
PG 11	*Talaromyces*	Brown	Cream	Non-Septate	Foot cells are absent
PG 12	*Alternaria*	Cream	Yellow	Septate	Foot cell give rise to conidiophore

END 01,1	*Epicoccum*	Yellow-Pinkish	Yellow-Pinkish	Septate	Foot cell present
END 01,2	*Epicoccum*	Yellow-Pinkish	Yellow-Pinkish	Septate	Foot cell present
END 001	*Penicillium*	Yellow-Green	Pale Green	Septate	Foot cell give rise to conidiophore
END 002	*Aspergillus*	Yellow Brown	Cream White	Septate	Foot cell present
END 03,1	*Cladosporium*	Cream-Yellowish	Yellow	Septate	Basal foot cell
END 03,2	*Cladosporium*	Cream-Yellowish	Yellow	Septate	Basal foot cell
END 04,1	*Cladosporium*	Olive Brown	Dull Green	Septate	Basal foot cell
END 04,2	*Cladosporium*	Olive Brown	Dull Green	Septate	Basal foot cell
END 005	*Neonectria*	Yellow-Brown	Deep Red	Single Septate	Foot cell on the. macroconidia
END 006	*Dactylonectria*	Brown Green	Cream	Septate	Poorly developed foot cell
END 007	*Neonectria*	White	White	Single Septate	Foot cell on the. macroconidia
END 008	*Trichoderma*	White Yellowish	Yellow	Septate	T' or 'L' shaped 'foot cells' that produce conidiophore
END 009	*Leptosphaerulina*	Cream	Yellow	Septate	Base of the conidiophore is a foot cell
END 010	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
END 011	*Rhizoctonia*	White Green	Yellow Green	Septate	Foot cell present
END 012	*Aspergillus*	White-Brownish	Yellow	Septate	Foot cell present
END 013	*Pezicula*	Yellow	Yellow	Septate	Prominent foot cell
END 014	*Penicillium*	Green	Cream Yellow	Septate	Foot cell give rise to conidiophore
END 015	*Boeremia*	White Yellowish	Yellow	Septate	Foot cell present
END 016	*Alternaria*	Brown	Black	Septate	Foot cell give rise to conidiophore

END 017,1	*Penicillium*	Green	Pale Green	Septate	Foot cell give rise to conidiophore
END 017,2	*Alternaria*	Brown	Yellow	Septate	Foot cell give rise to conidiophore
END 018,1	*Aspergillus*	Yellow- Black	Cream	Septate	Foot cell present
END 018,2	*Aspergillus*	Yellow- Black	Cream	Septate	Foot cell present
END 019	*Penicillium*	Cream	Cream-Brownish	Septate	Foot cell give rise to conidiophore
END 020,1	*Penicillium*	Yellow	Yellow	Septate	Foot cell give rise to conidiophore
END 020,2	*Rhizopus*	Greyish Brown	White	Long non seotate	Rhizoid stolon columnal
END 021	*Penicillium*	Yellow-Brownish	Cream	Septate	Foot cell give rise to conidiophore
END 022	*Penicillium*	Brown	Yellow	Septate	Foot cell give rise to conidiophore
END 023	*Purpureocilliu m*	Cream	Yellow	Septate	Basal foot cell
END 024	*Penicillium*	Cream	Yellow	Septate	Foot cell give rise to conidiophore
END 031	*Neonectria*	White	Beige	Single Septate	Foot cell on the. macroconidia
MHE 001	*Fusarium*	White-Pink	Pink	Septate	Foot cell on the. macroconidia
MHE 002	*Fusarium*	Yellow- Black	Cream	Septate	Foot cell on the. macroconidia
MHE 003	*Trichoderma*	Yellow-Pinkish	Yellow	Septate	T' or 'L' shaped 'foot cells' that produce conidiophore
MHE 004	*Fusarium*	White	Red	Septate	Foot cell on the. macroconidia
MHE 005	*Ceratobasidiu m*	Yellow-Brownish	Cream	Septate	Basidia with basidiospores
MHE 006	*Penicillium*	Brownish	Cream	Septate	Foot cell give rise to conidiophore
MHE 007	*Penicillium*	Brownish	Cream	Septate	Foot cell give rise to conidiophore
MHE 008	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia

MHE 009	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
MHE 010	*Neurospora*	Cream-Reddish	Yellow-Reddish	Non-Septate	Foot cell from which the conidiophore grow
MHE 011	*Penicillium*	White	Orange	Septate	Foot cell give rise to conidiophore
MHE 012	*Chaetomium*	Yellow-Brownish	Deep Red	Single Septate	Foot cell present
MHE 014	*Neurospora*	Yellow	Yellow	Non-Septate	Foot cell from which the conidiophore grow
MHE 015	*Fusarium*	Cream	Orange	Septate	Foot cell on the. macroconidia
MHE 016	*Alternaria*	Brownish	Yellow	Septate	Foot cell give rise to conidiophore
MHE 017	*Neurospora*	White	Cream	Non-Septate	Foot cell from which the conidiophore grow
MHE 018	*Neurospora*	Brownish	Cream	Non-Septate	Foot cell from which the conidiophore grow
MHE 019	*Neurospora*	White	Cream	Non-Septate	Foot cell from which the conidiophore grow
MHE 020	*Fusarium*	Yellow Pink	Yellow Orange	Septate	Foot cell on the. macroconidia
MHE 021	*Aspergillus*	White	Cream	Septate	Foot cell present
MHE 022	*Penicillium*	Deep Yellow	Yellow	Septate	Foot cell give rise to conidiophore
MHE 023	*Penicillium*	Yellow Green	Cream	Septate	Foot cell give rise to conidiophore
MHE 024	*Aspergillus*	White	Cream	Septate	Foot cell present
MHE 025	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
MHE 026	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
MHE 027	*Talaromyces*	Pink	Red	Non-Septate	Foot cells are absent
MHE 028	*Albifimbria*	White	Cream	Septate	Foot cell present
MHE 029	*Penicillium*	Greenish	Pinkish	Septate	Foot cell give rise to conidiophore

MHE 030	*Undifilum*	Brownish	Cream	Septate	Foot cell present
MHE 031	*Penicillium*	Brown	Cream	Septate	Foot cell give rise to conidiophore
MHE 032	*Penicillium*	Brown	Cream	Septate	Foot cell give rise to conidiophore
MHE 033	*Aspergillus*	White	Cream	Septate	Foot cell present
MHE 034	*Penicillium*	Brown Green	Cream	Septate	Foot cell give rise to conidiophore
MHE 035	*Penicillium*	Brown	Cream	Septate	Foot cell give rise to conidiophore
MHE 036	*Penicillium*	White	Cream	Septate	Foot cell give rise to conidiophore
MHE 040	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
MHE 041	*Neurospora*	Pale White	White	Non-Septate	Foot cell from which the conidiophore grow
MHE 042	*Neurospora*	White	Cream	Non-Septate	Foot cell from which the conidiophore grow
MHE 043	*Geotrichum*	White-Yellowish	Cream	Septate	Initially yeast like metamorphosis into mycelium
MHE 044	*Fusarium*	White	Cream	Septate	Foot cell on the. macroconidia
MHE 045	*Fusarium*	Pink	White	Septate	Foot cell on the. macroconidia
MHE 046	*Fusarium*	Pink	White	Septate	Foot cell on the. macroconidia
MHE 047	*Trichoderma*	Yellow Greenish	Yellow	Septate	T' or 'L' shaped 'foot cells' that produce conidiophore
MHE 047,2	*Trichoderma*	Yellow Greenish	Yellow	Septate	'T' or 'L' shaped 'foot cells' that produce conidiophore
MHE 048	*Neocosmospora*	Cream	Yellow	Septate	Poorly developed foot cell
MHE 049	*Fusarium*	Reddish	Deep Red	Septate	Foot cell on the. macroconidia
MHE 050	*Aspergillus*	Cream	Yellow	Septate	Foot cell present
MHE 051	*Neurospora*	Yellow	Yellow	Non-Septate	Foot cell from which the conidiophore grow

MHE 052	*Alternaria*	Pink	Pink	Septate	Foot cell give rise to conidiophore
MHE 053	*Epicoccum*	White	Cream	Septate	Foot cell present
MHE 054	*Aspergillus*	Cream Brownish	Yellow	Septate	Foot cell present
MHE 055	*Fusarium*	Black-Yellowish	Yellow	Septate	Foot cell on the. macroconidia
MHE 056	*Fusarium*	Cream-Reddish	Yellow-Orange	Septate	Foot cell on the. macroconidia
MHE 057	*Plectosphaerella*	Cream	Yellow	Septate	Curved macroconidia with foot cells
MHE 058	*Mucor*	Cream	Cream	Irregular non-septate	Foot cell give rise to conidiophore
MHE 059	*Geotrichum*	White	Cream	Septate	Initially yeast like metamorphosis into mycelium
MHE 060	*Aspergillus*	Brownish	Cream	Septate	Foot cell present
MHE 061	*Aspergillus*	White	Cream	Septate	Foot cell present
MHE 062	*Alternaria*	White	Cream	Septate	Foot cell give rise to conidiophore
MHE 063	*Phomopsis*	Cream	Cream	Septate	Cylindrical asci with a foot cell
MHE 064	*Alternaria*	White	White transparent	Septate	Foot cell give rise to conidiophore
MHE 065	*Mortierella*	Cream	Pinkish	Septate	Rhizoids is produced from the foot cell
MHE 066	*Alternaria*	Brown	Black	Septate	Foot cell give rise to conidiophore
MHE 067	*Alternaria*	Cream	Cream	Septate	Foot cell give rise to conidiophore
MHE 068	*Alternaria*	Cream	Yellow	Septate	Foot cell give rise to conidiophore

APPENDIX 4

Antimicrobial Assay

S. No	Sample ID	Probable Name	Zone of Inhibition (mm)			
			E. coli ATCC 25922	*E. coli* ATCC 0177	*B. cereus* ATCC 10876	*E. faecalis* ATCC S1299
1	GG 001	*Aspergillus*	—	—	—	—
2	GG 002	*Mucor*	—	—	—	—
3	GG 003	*Fusarium*	—	—	—	—
4	GG 004	*Penicillium*	—	—	—	—
5	GG 005	*Fusarium*	—	—	—	—
6	GG 006	*Ceratobasidium*	—	—	—	—
7	GG 007	*Alternaria*	—	—	—	—
8	GG 008	*Fusarium*	—	+ (7)	—	—
9	GG 009	*Neurospora*	—	—	+ (9)	—
10	GG 010	*Aspergillus*	—	—	—	—
11	GG 011	*Fusarium*	—	—	—	—
12	GG 012	*Fusarium*	—	+ (7)	—	—
13	GG 013	*Aspergillus*	—	—	+ (7)	—
14	GG 014	*Aspergillus*	—	—	—	—
15	GG 015.1	*Aspergillus*	—	—	—	—
16	GG 015.2	*Aspergillus*	—	—	—	—
17	GG 016	*Aspergillus*	—	—	—	—
18	ND 1	*Aspergillus*	—	—	—	—
19	ND 2	*Aspergillus*	—	—	—	—
20	ND 3	*Penicillium*	—	—	—	—
21	ND 4	*Penicillium*	—	—	—	—
22	ND 5	*Neurospora*	—	—	—	—
23	ND 6	*Penicillium*	—	—	—	—
24	ND 7	*Geotrichum*	—	—	—	—

APPENDIX 4

Antimicrobial Assay

Sample ID	Probable Name	Zone of Inhibition (mm)					
		E. coli ATCC 25922	*E. coli* ATCC 0177	*B. cereus* ATCC 10876	*E. faecalis* ATCC S1299	*E. faecium* ATCC 700221	*E. gallinarum* ATCC 700425
GG 001	*Aspergillus*	—	—	—	—	—	—
GG 002	*Mucor*	—	—	—	—	—	—
GG 003	*Fusarium*	—	—	—	—	—	—
GG 004	*Penicillium*	—	—	—	—	—	—
GG 005	*Fusarium*	—	—	—	—	—	—
GG 006	*Ceratobasidium*	—	—	—	—	—	—
GG 007	*Alternaria*	—	—	—	—	—	—
GG 008	*Fusarium*	—	+ (7)	—	—	+ (9)	—
GG 009	*Neurospora*	—	—	+ (9)	—	—	—
GG 010	*Aspergillus*	—	—	—	—	—	—
GG 011	*Fusarium*	—	—	—	—	—	—
GG 012	*Fusarium*	—	+ (7)	—	—	—	—
GG 013	*Aspergillus*	—	—	+ (7)	—	—	—
GG 014	*Aspergillus*	—	—	—	—	—	—
GG 015.1	*Aspergillus*	—	—	—	—	—	—
GG 015.2	*Aspergillus*	—	—	—	—	—	—
GG 016	*Aspergillus*	—	—	—	—	—	—
ND 1	*Aspergillus*	—	—	—	—	—	—
ND 2	*Aspergillus*	—	—	—	—	—	—
ND 3	*Penicillium*	—	—	—	—	—	—
ND 4	*Penicillium*	—	—	—	—	—	—
ND 5	*Neurospora*	—	—	—	—	—	—
ND 6	*Penicillium*	—	—	—	—	—	—
ND 7	*Geotrichum*	—	—	—	—	—	—

	ND 8	*Alternaria*	—	—	—	—	—	—
	ND 9	*Aspergillus*	—	—	—	—	—	—
	ND 10	*Alternaria*	—	—	—	—	—	—
	ND 12	*Epicoccum*	—	—	—	—	—	—
	ND 13	*Fusarium*	—	—	—	—	—	—
	ND 14	*Aspergillus*	—	—	—	—	—	—
	ND 15	*Aspergillus*	—	—	—	—	—	—
	ND 16	*Aspergillus*	—	—	—	—	—	—
	ND 17	*Aspergillus*	—	—	—	—	—	—
	ND 18	*Neurospora*	—	—	—	—	—	—
	ND 19	*Fusarium*	—	+ (8)	—	—	—	—
	DR 001	*Aspergillus*	—	—	—	—	—	—
	DR 002	*Fusarium*	—	—	—	—	—	—
	DR 003	*Coniothyrium*	—	—	—	—	—	—
	DR 004	*Fusarium*	—	—	—	—	—	—
	DR 005	*Cf. Pleosporales*	—	—	—	—	—	—
	DR 006	*Fusarium*	+ (7)	—	—	—	—	—
	DR 007	*Fusarium*	—	—	—	—	—	—
	DR 008	*Fusarium*	—	—	—	—	—	—
	DR 009	*Fusarium*	—	—	—	—	—	—
	DR 010	*Phomopsis*	—	—	—	—	—	—
	DR 011	*Ceratobasidium*	—	—	—	—	—	—
	DR 012	*Pythium*	—	—	—	—	—	—
	DR 013	*Fusarium*	—	—	—	—	—	—
	DR 014.1	*Neonectria*	—	—	—	—	—	—
	DR 014.2	*Neonectria*	—	—	—	—	—	—
	DR 015	*Purpureocillium*	—	—	—	—	—	—
	DR 016	*Cladosporium*	—	—	—	—	—	—
	DR 017	*Fusarium*	—	—	+ (2)	—	—	—
	DR 018	*Fusarium*	—	—	—	—	—	—
	DR 019	*Fusarium*	—	—	—	—	—	—
	DR 020	*Fusarium*	+ (8)	—	—	—	—	—
	DR 021	*Fusarium*	—	—	—	—	—	—
	DR 022	*Fusarium*	—	—	—	—	—	—
	DR 023	*Fusarium*	—	—	+ (9)	—	—	—

	DR 024	*Fusarium*	—	—	—	—	—	—

	Sample ID	Probable Name	Zone of Inhibition (mm)					
			E. coli ATCC 25922	*E. coli* O177 (Environmental isolate)	*B. cereus* ATCC 10876	*E. faecalis* ATCC S1299	*E. faecium* ATCC 700221	*E. gallinarum* ATCC 700425
	RNK 001	*Talaromyces sp.*	+ (9)	—	—	—	+ (6)	—
	RNK 002.1	*Penicillium*	—	—	—	—	—	—
	RNK 002.2	*Fusarium*	—	—	—	—	—	—
	RNK 003	*Aspergillus*	—	—	—	—	—	—
	RNK 004	*Penicillium*	++ (11)	—	—	—	—	—
	RNK 005	*Aspergillus*	—	—	—	—	—	—
	RNK 006	*Penicillium*	—	—	—	—	—	—
	RNK 007	*Penicillium*	—	—	—	—	—	—
	RNK 008	*Penicillium*	—	—	—	—	—	—
	RNK 009	*Penicillium*	—	—	—	—	—	—
	RNK 010	*Penicillium sp.*	—	—	—	—	—	—
	RNK 011	*Penicillium*	—	—	—	—	—	—
	RNK 012	*Alternaria*	—	—	—	—	—	—
	RNK 013	*Phoma sp.*	—	—	—	—	—	—
	RNK 014	*Aspergillus*	—	—	—	—	—	—
	RNK 015	*Cochliobolus sp.*	—	—	—	—	—	—
	RNK 016	*Alternaria*	—	—	+ (9)	—	—	—
	RNK 017	*Fusarium*	—	—	—	—	—	—
	RNK 018	*Alternaria sp.*	—	—	—	—	—	—
	RNK 019	*Alternaria*	—	—	—	—	—	—
	RNK 020	*Rhizopus*	—	—	—	—	—	—
	RNK 021	*Rhizopus*	—	—	—	—	—	—
	RNK 022	*Penicillium*	—	—	—	—	—	—
	RNK 023	*Plectosphaerella*	—	—	—	—	—	—
	PG 1	*Neurospora*	—	—	—	—	—	—
	PG 2	*Neurospora*	—	—	—	—	—	—
	PG 3	*Aspergillus*	—	—	—	—	—	—
	PG 4	*Ceratobasidium*	—	—	—	—	—	—

	PG 5	*Rhizoctonia*	—	—	—	—	—	—
	PG 6	*Colletotrichum*	—	—	—	—	—	—
	PG 7	*Neopestalotiopsis*	—	—	—	—	—	—
	PG 8	*Alternaria*	—	—	—	—	—	—
	PG 9	*Chaetomium*	—	+ (9)	+ (6)	—	—	—
	PG 10	*Humicola*	—	—	—	—	—	—
	PG 11	*Talaromyces*	—	—	—	—	—	—
	PG 12	*Alternaria*	—	—	—	—	—	—
	END 01,1	*Epicoccum*	—	—	—	—	—	—
	END 01,2	*Epicoccum*	—	—	—	—	—	—
	END 001	*Penicillium*	—	—	—	—	—	—
	END 002	*Aspergillus*	—	—	—	—	—	—
	END 03,1	*Cladosporium*	—	—	—	—	—	—
	END 03,2	*Cladosporium*	—	—	—	—	—	—
	END 04,1	*Cladosporium*	—	—	—	—	—	—
	END 04,2	*Cladosporium*	—	—	—	—	—	—
	END 005	*Neonectria*	—	—	—	—	—	—
	END 006	*Dactylonectria*	—	—	—	—	—	—
	END 007	*Neonectria*	—	—	—	—	—	—
	END 008	*Trichoderma*	—	—	—	—	—	—
	END 009	*Leptosphaerulina*	—	—	—	—	—	—
	END 010	*Penicillium*	—	—	—	—	—	—
	END 011	*Rhizoctonia*	—	—	—	—	—	—
	END 012	*Aspergillus*	—	—	—	—	—	—
	END 013	*Pezicula*	—	—	—	—	—	—
	END 014	*Penicillium*	—	—	—	—	—	—
	END 015	*Boeremia*	—	—	—	++ (11)	—	—
	END 016	*Alternaria*	—	—	—	—	—	—
	END 017,1	*Penicillium*	+ (9)	—	—	—	—	—
	END 017,2	*Alternaria*	—	—	—	—	—	—
	END 018,1	*Aspergillus*	—	—	—	—	—	—
	END 018,2	*Aspergillus*	—	—	—	—	—	—
	END 019	*Penicillium*	—	—	—	—	—	—
	END 020,1	*Penicillium*	—	—	—	—	—	—
	END 020,2	*Rhizopus*	—	—	—	—	—	—

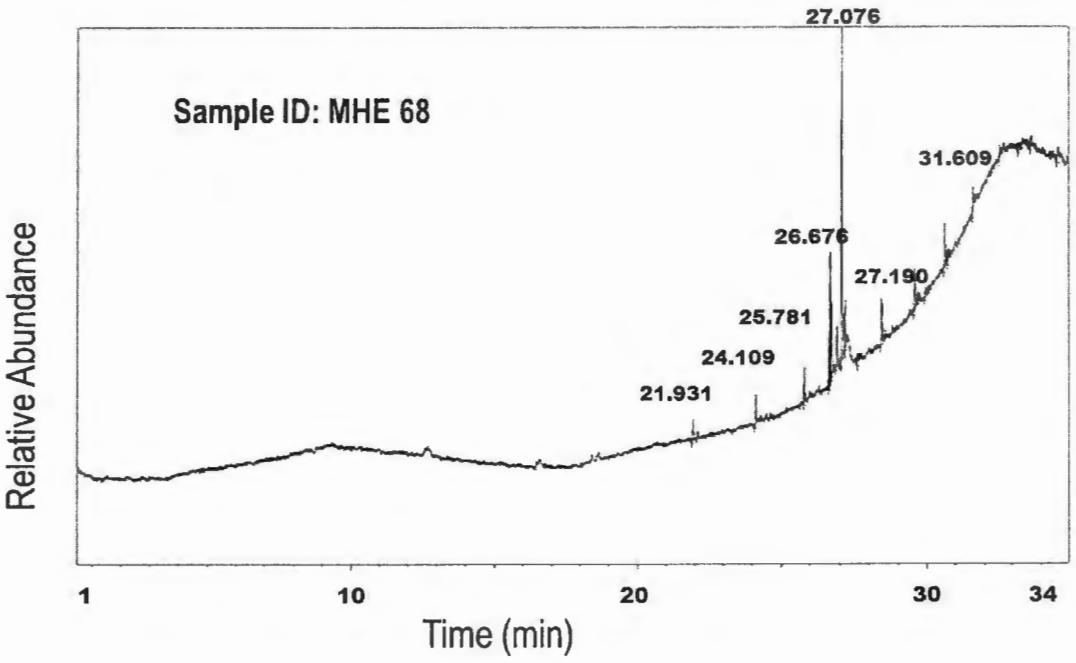
	END 021	*Penicillium*	+ (10)	—	—	—	—	—
	END 022	*Penicillium*	—	—	—	—	—	—
	END 023	*Purpureocillium*	—	—	—	—	—	—
	END 024	*Penicillium*	—	—	—	—	—	—
	END 031	*Neonectria*	—	—	—	—	—	—
	MHE 001	*Fusarium*	—	++ (11)	—	—	—	—
	MHE 002	*Fusarium*	—	—	—	—	—	—
	MHE 003	*Trichoderma*	—	—	—	—	—	—
	MHE 004	*Fusarium*						
	MHE 005	*Ceratobasidium*	—	—	—	—	—	—
	MHE 006	*Penicillium*	—	—	—	—	—	—
	MHE 007	*Penicillium*	—	—	—	—	—	—
	MHE 008	*Fusarium*	—	—	—	—	—	—
	MHE 009	*Fusarium*	—	—	—	—	—	—
	MHE 010	*Neurospora*	+ (9)	+ (8)	—	—	—	—
	MHE 011	*Penicillium*	+ (9)	—	—	—	—	—
	MHE 012	*Chaetomium*	—	—	—	—	—	—
	MHE 014	*Neurospora*	—	—	—	—	—	—
	MHE 015	*Fusarium*	—	—	—	—	—	—
	MHE 016	*Altemaria*	—	—	—	—	—	—
	MHE 017	*Neurospora*	—	—	—	—	—	—
	MHE 018	*Neurospora*	—	—	—	—	—	—
	MHE 019	*Neurospora*	—	—	—	—	—	—
	MHE 020	*Fusarium*	—	—	—	—	—	—
	MHE 021	*Aspergillus*	—	—	—	—	—	—
	MHE 022	*Penicillium*	—	—	—	—	—	—
	MHE 023	*Penicillium*	—	—	—	—	—	—
	MHE 024	*Aspergillus*	—	—	—	—	—	—
	MHE 025	*Penicillium*	—	—	—	—	—	—
	MHE 026	*Penicillium*	—	—	—	—	—	—
	MHE 027	*Talaromyces*	—	—	—	—	—	—
	MHE 028	*Albifimbria*	—	—	—	—	—	—
	MHE 029	*Penicillium*	—	—	—	—	—	—
	MHE 030	*Undifilum*	—	—	—	—	—	—
	MHE 031	*Penicillium*	—	—	—	—	—	—

	MHE 032	*Penicillium*	—	—	—	—	—	—
	MHE 033	*Aspergillus*	+ (2)	+ (9)	—	—	—	—
	MHE 034	*Penicillium*	—	—	—	—	—	—
	MHE 035	*Penicillium*	—	—	—	—	—	—
	MHE 036	*Penicillium*	—	—	—	—	—	—
	MHE 040	*Fusarium*	—	—	—	—	—	—
	MHE 041	*Neurospora*	—	—	—	—	—	—
	MHE 042	*Neurospora*	—	—	—	—	—	—
	MHE 043	*Geotrichum*	—	—	—	—	—	—
	MHE 044	*Fusarium*	—	—	—	—	—	—
	MHE 045	*Fusarium*	—	—	—	—	—	—
	MHE 046	*Fusarium*	—	—	—	—	—	—
	MHE 047	*Trichoderma*	—	—	—	—	—	—
	MHE 047,2	*Trichoderma*	—	—	—	—	—	—
	MHE 048	*Neocosmospora*	—	—	—	—	—	—
	MHE 049	*Fusarium*	—	—	—	—	—	—
	MHE 050	*Aspergillus*	—	—	—	—	—	—
	MHE 051	*Neurospora*	—	—	—	—	—	—
	MHE 052	*Alternaria*	—	—	—	—	—	—
	MHE 053	*Epicoccum*	—	—	—	—	—	—
	MHE 054	*Aspergillus*	—	—	—	—	—	—
	MHE 055	*Fusarium*	—	++ (12)	—	—	—	—
	MHE 056	*Fusarium*	—	—	+ (8)	—	++ (11)	—
	MHE 057	*Plectosphaerella*	—	—	—	—	—	—
	MHE 058	*Mucor*	—	—	—	—	—	—
	MHE 059	*Geotrichum*	+ (9)	—	—	—	—	—
	MHE 060	*Aspergillus*	—	—	—	—	—	—
	MHE 061	*Aspergillus*	—	—	—	—	—	—
	MHE 062	*Alternaria*						
	MHE 063	*Phomopsis*	—	—	—	—	—	—
	MHE 064	*Alternaria*	—	—	—	—	—	—
	MHE 065	*Mortierella*	—	—	—	—	—	—
	MHE 066	*Alternaria*	—	—	—	—	—	—
	MHE 067	*Alternaria*	—	—	—	—	—	—
	MHE 068	*Alternaria*	—	—	+ (8)	—	++ (11)	++ (12)

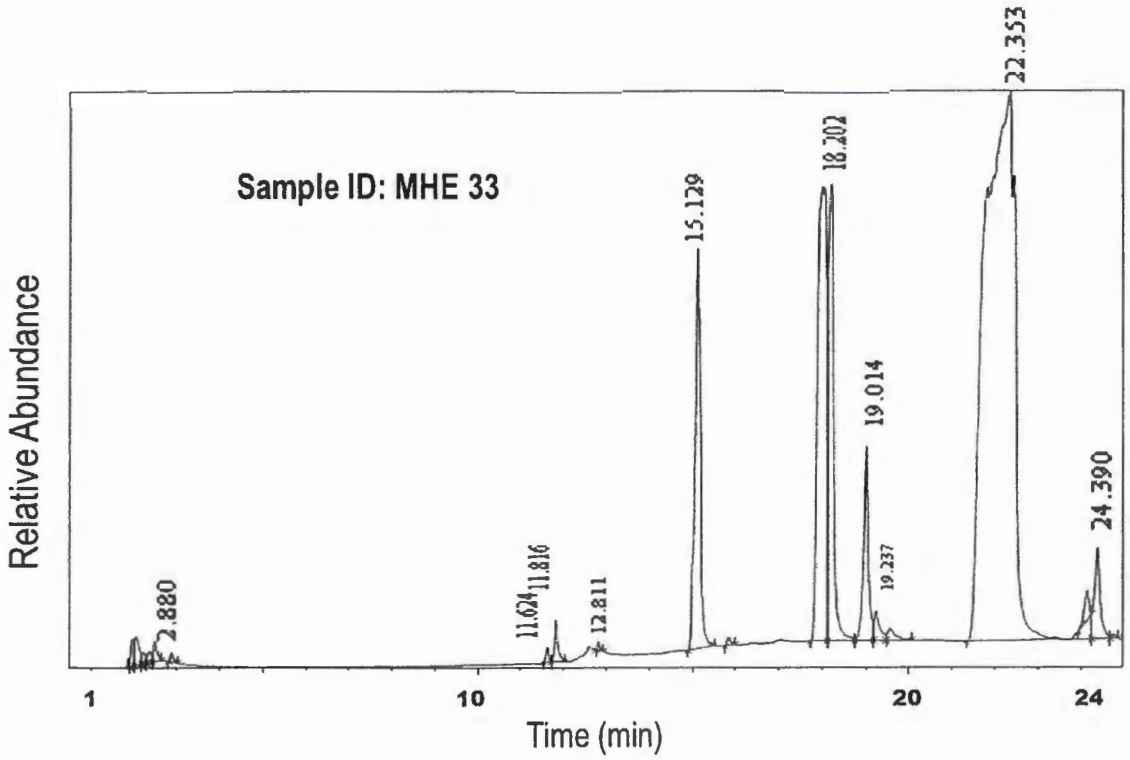
APPENDIX 5

CHROMATOGRAM OF SECONDARY METABOLITE OF INVESTATIVE SAMPLES

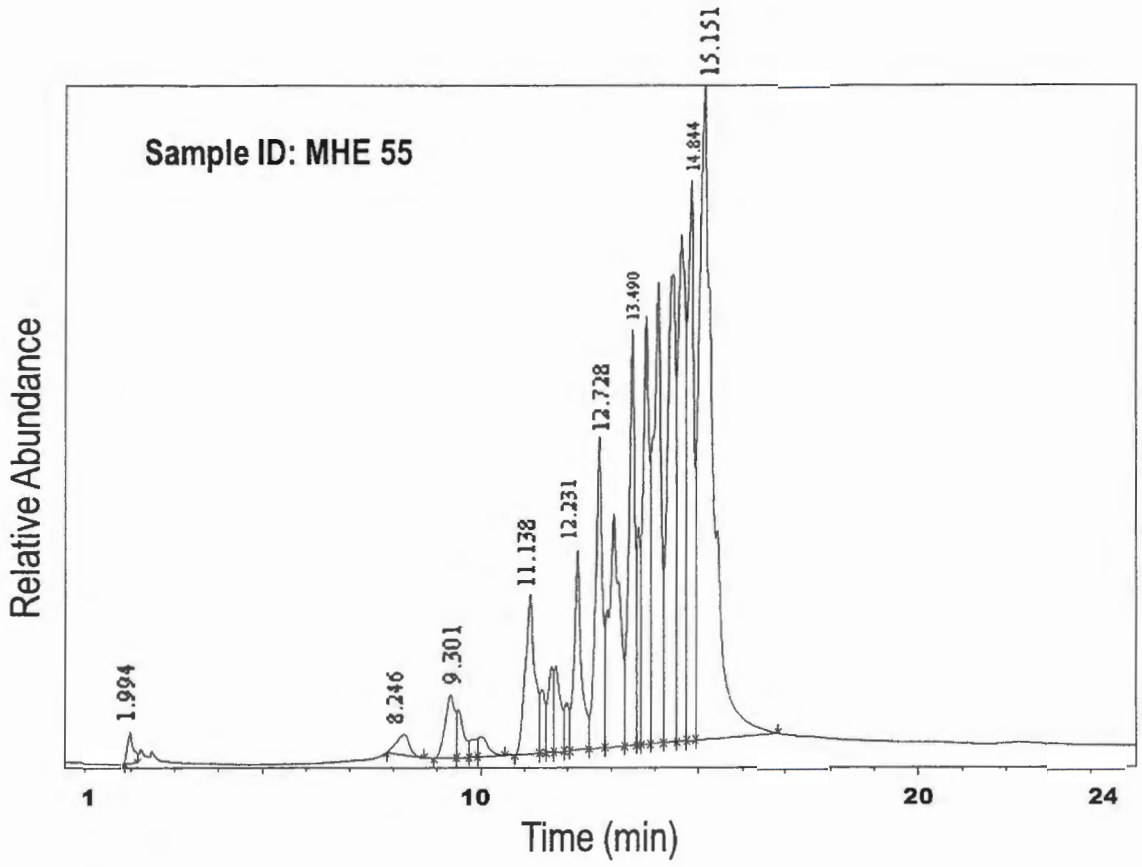
CHROMATOGRAM OF SECONDARY METABOLITE OF ACTIVE SAMPLES



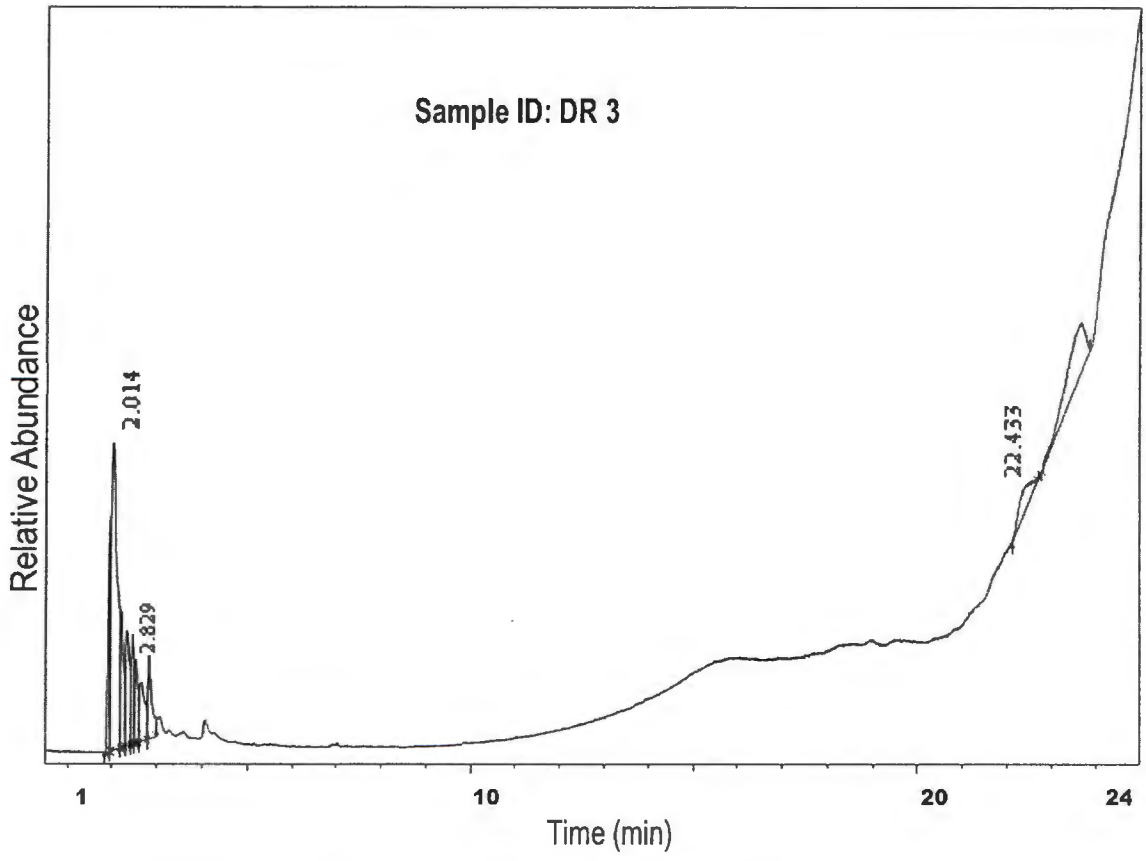
Peak	R. Time	Area	Height	Name
1	21.931	107935	49846	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexa
2	24.109	134156	70746	Hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-Dode
3	25.781	146379	84122	Cyclododecasiloxane,Tetracos
4	26.644	271510	178016	9,12-Octadecadienoicacid(Z,Z)-,M
5	26.676	593966	304562	9,12-Octadecadienoicacid(Z,Z)-,M
6	26.730	340337	172773	9,12-Octadecadienoicacid(Z,Z)-,M
7	26.917	287016	96415	9,12-Octadecadienoicacid(Z,Z)-,Methylest
8	27.076	1597988	779997	9,12-Octadecadienoicacid(Z,Z)-,M
9	27.190	250984	114397	Cyclodecasiloxane,Eicosamethyl-
10	27.601	68648	22586	1h-Purin-6-Amine,[(2-Fluoropheny
11	28.437	292869	108477	Cyclodecasiloxane,Eicosamethyl-
12	29.575	272854	99113	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18
13	29.937	78786	13195	1h-Purin-6-Amine,[(2-Fluoropheny
14	30.628	244851	102788	Cyclodecasiloxane,Eicosamethyl-
15	30.750	61412	23772	1h-Purin-6-Amine,[(2-Fluoropheny
16	31.609	107960	50978	Cyclodecasiloxane,Eicosamethyl-
17	32.568	64355	26327	Propanoicacid,3-[[Bis[(Trimethyl
18	33.220	84775	19795	2,3,4-Tri-O-Acetyl-6-Deoxy-6-Iodoh
19	33.687	94106	25879	1,2-Benzenediol,3,5-Bis(1,1-Dimeth
20	34.636	107673	25345	D-Mannitol,1-Decylsulfonyl-



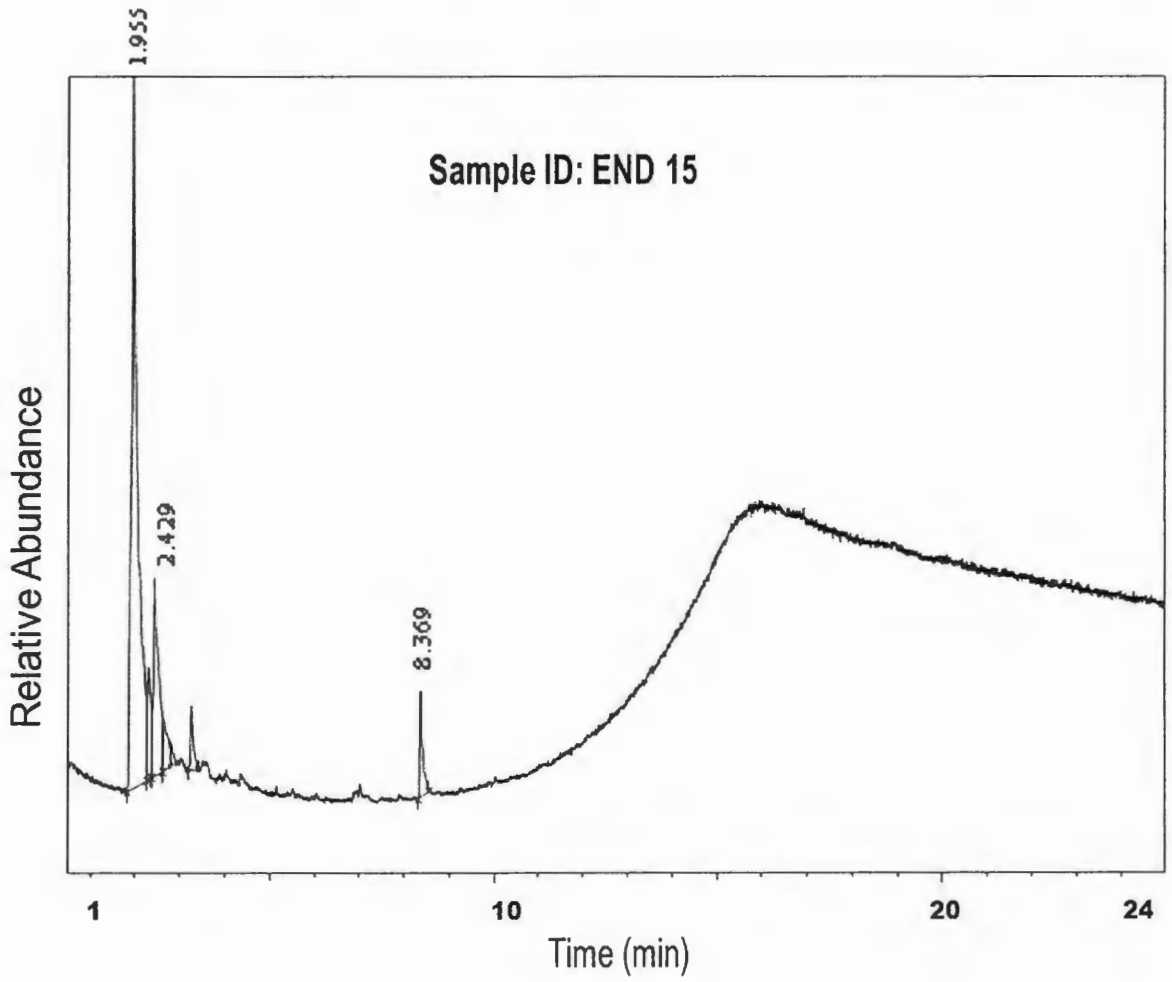
Peak	R. Time	Area	Height	Name
1	1.963	15818714	3014757	Hydrazine,Methyl-
2	2.053	23521710	3251848	2-Furanmethanol
3	2.233	8234907	1335174	Tricosane,2-Methyl-
4	2.372	8085687	1284442	2(3h)-Furanone,Dihydro-4-Hydroxy-
5	2.488	13327531	2349954	5-[Hydroxy[Methoxy(5-Oxotetr
6	2.880	5101353	1068453	Butanoicacid,3-Methyl-,Propylester
7	11.624	8551737	1737027	Glyceroltricaprylate
8	11.816	26701586	4716500	Dodecanoicacid,1,2,3-Propanetri
9	12.811	3857435	981562	Dodecanoicacid,1,2,3-Propanetriylester
10	15.129	398292018	45513729	Glyceroltricaprylate
11	15.853	4990782	920707	Dodecanoicacid,1,2,3-Propanetri
12	18.000	722549407	51426894	2-Lauro-1,3-Didecoin
13	18.202	461245982	51579808	Dodecanoicacid,1,2,3-Propanetri
14	19.014	167887267	21866925	Dodecanoicacid,1,2,3-Propanetriylester
15	19.237	29277032	3318112	Dodecanoicacid,1,2,3-Propanetri
16	19.563	16395144	1356706	Dodecanoicacid,1,2,3-Propanetri
17	22.353	3055212320	62250919	Dodecanoicacid,1,2,3-Propanetriylester
18	24.150	25943341	3352309	Dodecanoicacid,1,2,3-Propanetriylester
19	24.390	94086700	10236101	Dodecanoicacid,1,2,3-Propanetri
20	24.777	3049241	424577	Hexadecanoicacid,2-[(1-Oxodod



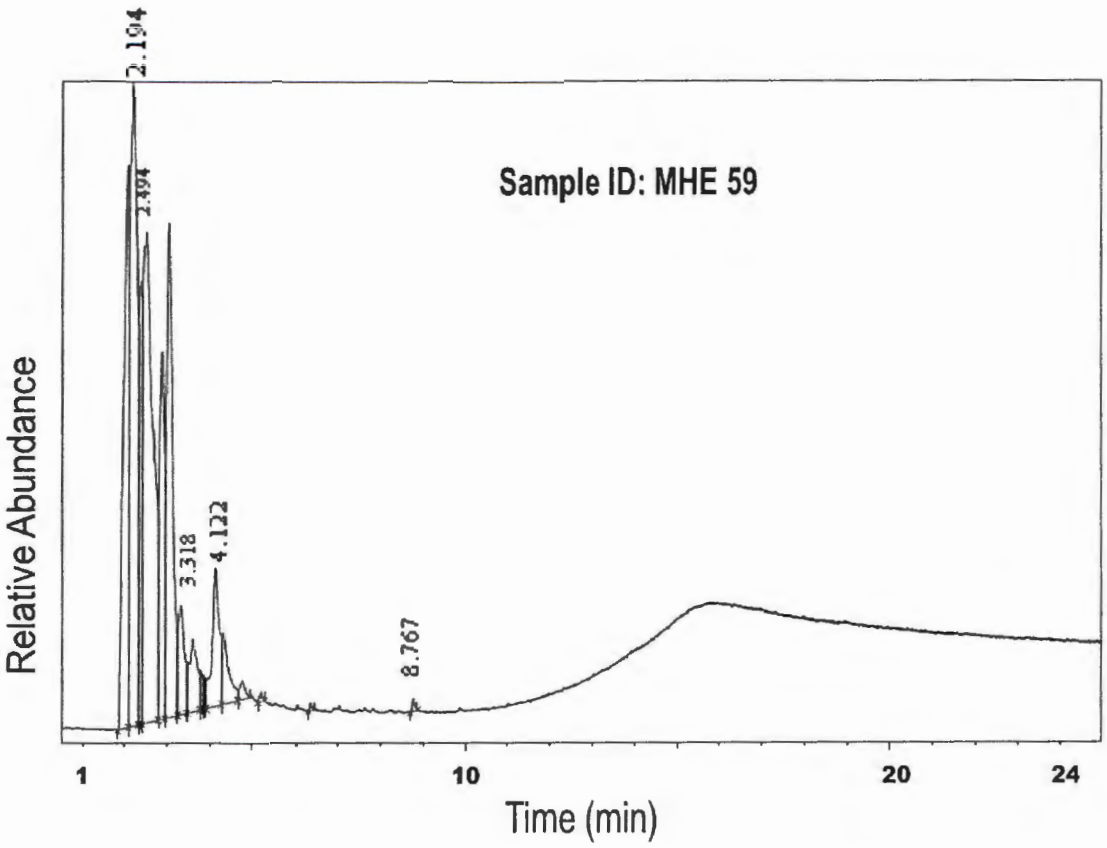
Peak	R. Time	Area	Height	Name
1	1.994	18725533	1994628	Cyclohexanol,2-(Methylaminomethyl)-,Tran
2	8.246	28115801	1352508	Dodecanoicacid,1,2,3-Propanetriylester
3	9.301	61541524	4061284	Dodecanoicacid,1,2,3-Propanetri
4	9.482	36172251	3053784	Dodecanoicacid,1,2,3-Propanetri
5	9.800	13447569	1135448	Dodecanoicacid,1,2,3-Propanetri
6	10.016	20641374	1265274	Dodecanoicacid,1,2,3-Propanetriylester
7	11.138	154920409	10218784	Dodecanoicacid,1,2,3-Propanetriylester
8	11.410	32586696	4037851	Dodecanoicacid,1,2,3-Propanetriylester
9	11.625	50442712	5461253	Dodecanoicacid,1,2,3-Propanetriylester
10	11.727	55681995	5515907	Dodecanoicacid,1,2,3-Propanetriylester
11	11.973	25027178	3101479	Dodecanoicacid,1,2,3-Propanetri
12	12.231	147496464	12706151	Dodecanoicacid,1,2,3-Propanetriylester
13	12.728	218009911	19877824	Dodecanoicacid,1,2,3-Propanetriylester
14	13.063	265744653	14880198	Dodecanoicacid,1,2,3-Propanetri
15	13.490	249083557	26448070	Dodecanoicacid,1,2,3-Propanetriylester
16	13.622	84333654	13909632	Dodecanoicacid,1,2,3-Propanetri
17	13.806	265907546	27275967	Dodecanoicacid,1,2,3-Propanetriylester
18	14.087	381846238	29440175	Dodecanoicacid,1,2,3-Propanetri
19	14.399	420865435	29797396	Dodecanoicacid,1,2,3-Propanetriylester
20	14.611	392434793	32375460	Dodecanoicacid,1,2,3-Propanetriylester
21	14.844	339045547	35748396	Dodecanoicacid,1,2,3-Propanetriylester
22	15.151	944622988	41799684	Dodecanoicacid,1,2,3-Propanetri



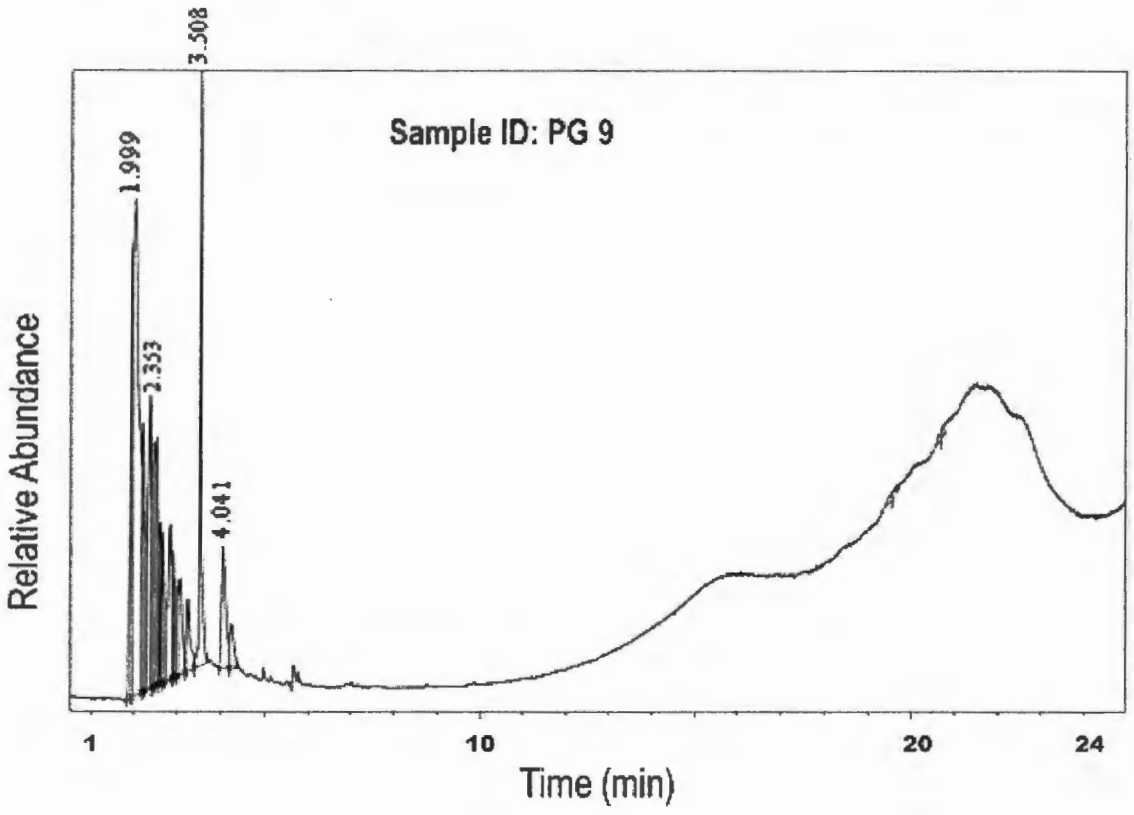
Peak	R. Time	Area	Height	Name
1	1.950	6119726	1693459	Peraceticacid
2	2.014	21279270	2224672	Dl-Glyceraldehydedimer
3	2.213	4584319	983873	1,3,5-Triazine-2,4,6-Triamine
4	2.332	5231658	832437	1,2,3-Propanetriol
5	2.456	3734315	790665	Valericanhydride
6	2.534	2837358	605530	1,2,3-Propanetriol,1-Acetate
7	2.654	3790843	426339	.Alpha.-D-Galactopyranoside,Methyl2-(Acet
8	2.829	3671268	599809	Butanedioicacid,2-Hydroxy-2-Methyl-,(S)-
9	22.433	3681193	165024	Oleicanhydride
10	23.671	11638536	343001	Hexadecanoicacid,2-[(1-Oxododecyl)Oxy]-1



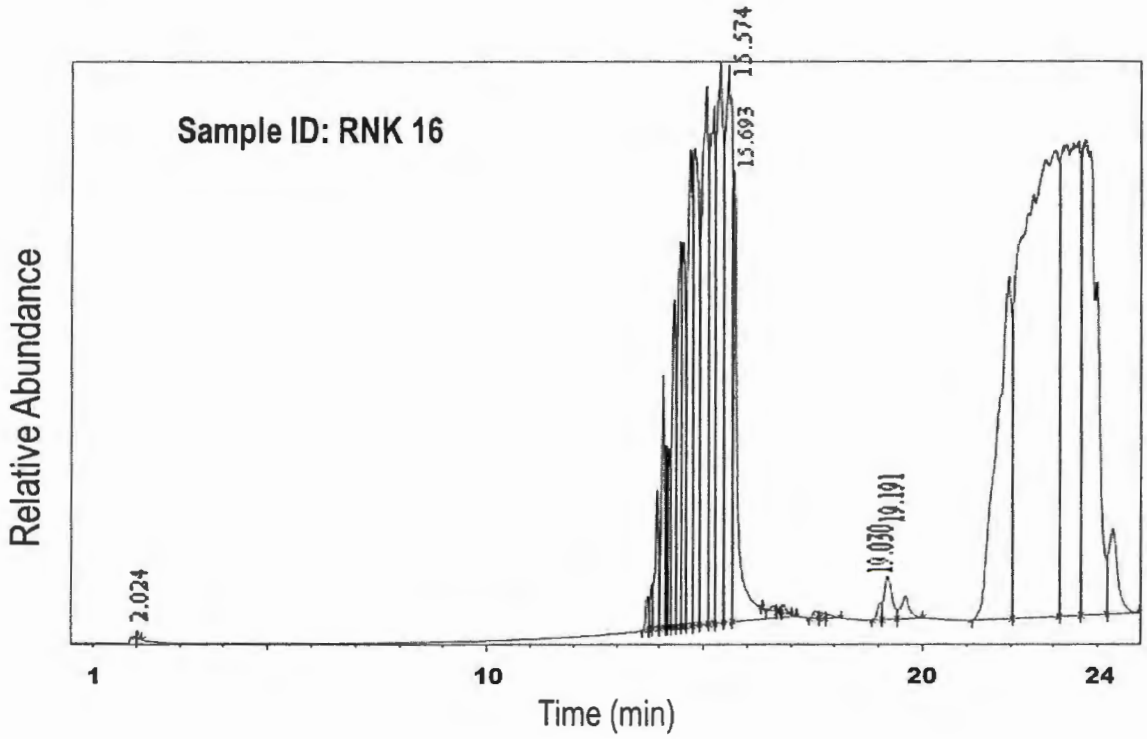
Peak	R. Time	Area	Height	Name
1	1.955	11619480	1286363	Lacticanhydride
2	2.304	1109601	202410	4-Hydroxydihydro-2(3h)-Furano
3	2.429	2953132	357100	2-Pentenoicacid,3-Methyl-,Met
4	2.647	670392	92290	Cyclohexane-1,3,5-Triol
5	3.259	492608	113971	Acetamide,N-Hexyl-
6	8.369	816311	191282	8,8,9-Trimethyl-3,5-Decadiene-2,7-



Peak	R. Time	Area	Height	Name
1	2.061	26468346	3017062	Peraceticacid
2	2.194	39275703	3455218	2h-Pyran-2,6(3h)-Dione
3	2.377	12913073	2374343	4h-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-
4	2.494	43093531	2631742	2(3h)-Furanone,Dihydro-5-(2-Octenyl)-,(Z)-
5	2.863	16035140	1968497	Butanoicacid,3-Methyl-,Propylester
6	3.027	27677952	2652459	Cyclohexane,1-Methyl-4-(1-Meth
7	3.318	5901430	588138	Guanosine
8	3.581	5116419	392243	.Beta.-D-Glucopyranose,1,6-Anhydro-
9	3.780	863033	213143	Benzenepropanol, 4-Hydroxy-.Al
10	3.840	735888	181647	Dicyclohexyl Pimelate
11	4.122	8485010	740945	5,5-D2-Trans-3,4-Dihydroxy-Cyclo
12	4.307	4167912	381004	1,3-Butanediol
13	4.741	927287	101544	Eicosenoicacid,Methylester
14	5.192	235862	50658	7-Oxabicyclo[4.1.0]Heptan-2-One,3
15	6.358	165309	43569	2-Furancarboxaldehyde,5-(Hyd
16	8.767	264798	70159	1,3-Bis(2-Pyrazinoxy)Propane

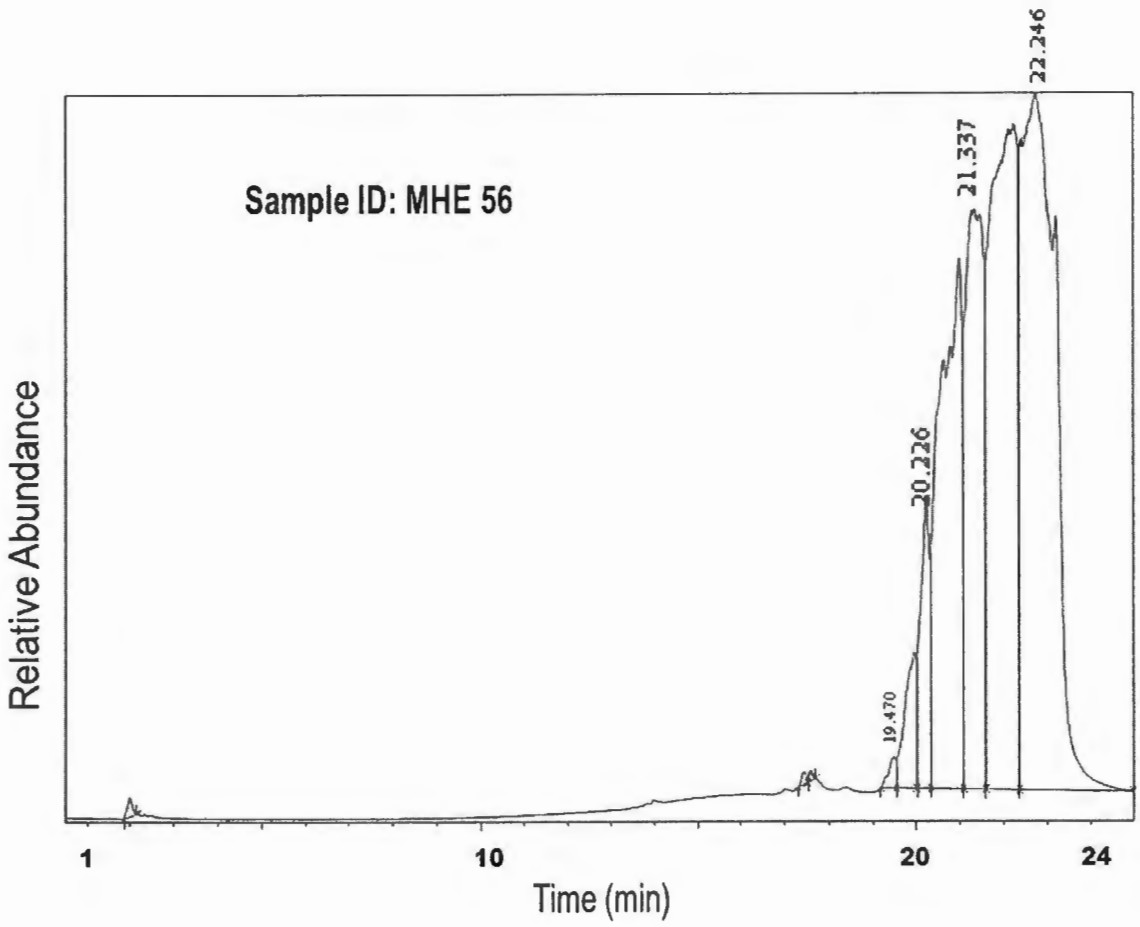


Peak	R.Time	Area	Height	Name
1	1.927	6275935	1967261	Aceticacid,[(Aminocarbonyl)A
2	1.999	21265804	2180660	Propyldecanoate
3	2.196	5548264	1180044	1,3,5-Triazine-2,4,6-Triamine
4	2.353	7680647	1284237	4h-Pyran-4-One,2,3-Dihydro-3,5-Di
5	2.440	5570442	1071011	5-Oxo-Tetrahydro-Furan-2-Carb
6	2.520	4399215	1091274	2,3-Dihydroxypropylacetate
7	2.606	2094612	710001	4h-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-
8	2.652	2831832	658004	Ethyl5-Hexenoate
9	2.830	4268101	678424	Butanoicacid,3-Methyl-,Propylester
10	2.898	1508086	556558	5-Methyl-1,3-Benzenediol
11	2.960	1778709	426683	1,2,3-Benzenetriol
12	3.054	2772997	425566	Lactoneg
13	3.234	1948604	322738	Guanosine
14	3.508	7115087	2604732	3-Furanaceticacid,4-Hexyl-2,5-Di
15	4.041	3407488	532529	5,5-D2-Trans-3,4-Dihydroxy-Cyclo
16	4.243	1223438	199280	2-Deoxypentopyranose
17	5.676	309230	79624	3,3-Dimethyl-5-Oxocyclohexane
18	19.570	189610	38387	5-(Acetyloxy)Cyclododecylace
19	20.687	186733	55593	4-Acetyloxyimino-6,6-Dimethyl-3

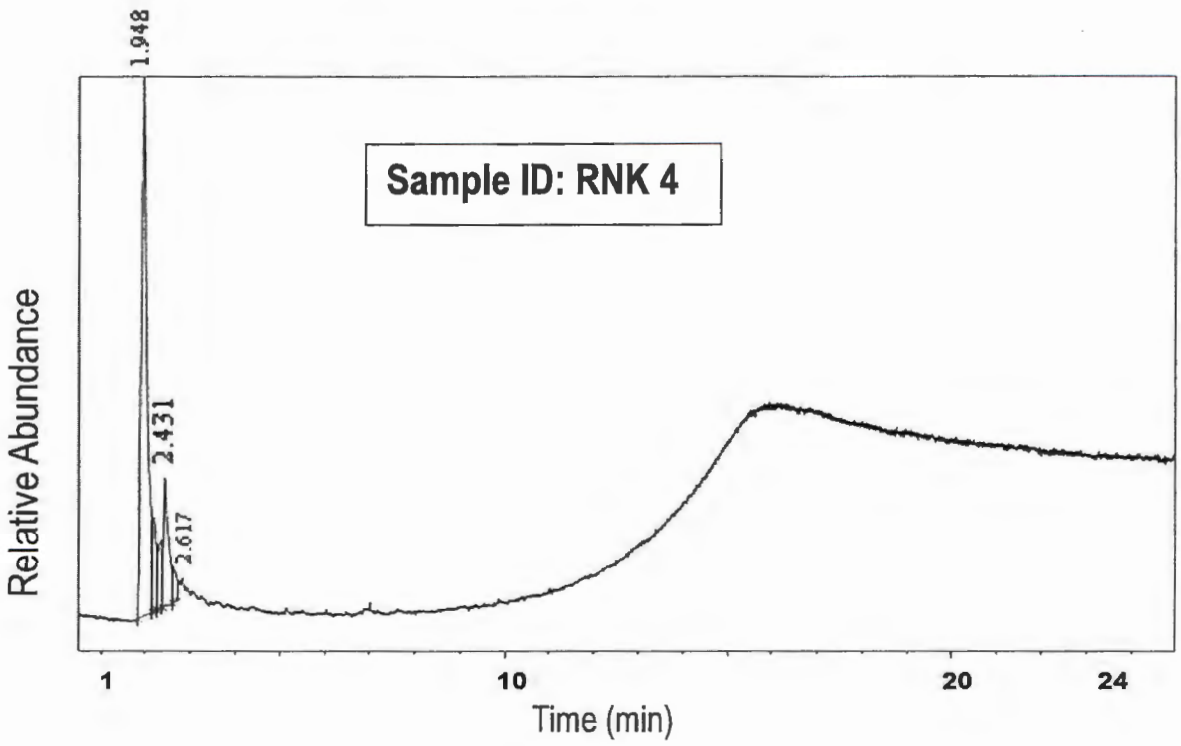


Peak	R.Time	Area	Height	Name
1	2.024	2215081	763794	L-Asparagine
2	13.719	18141354	3617020	Dodecanoicacid,1,2,3-Propanetriylester
3	13.835	23238125	4966185	Dodecanoicacid,1,2,3-Propanetriylester
4	13.947	82688076	15016529	Dodecanoicacid,1,2,3-Propanetri
5	14.076	134888635	27498453	2-Lauro-1,3-Didecoin
6	14.157	79144369	19801162	Dodecanoicacid,1,2,3-Propanetriylester
7	14.224	63031211	19411470	2-Lauro-1,3-Didecoin
8	14.331	229319991	35595668	2-Lauro-1,3-Didecoin
9	14.461	261174566	41959148	2-Lauro-1,3-Didecoin
10	14.534	239910163	41795429	2-Lauro-1,3-Didecoin
11	14.705	408417461	51776378	Bis[(3,5-Di-T-Butyl-1-Pyrazolyl)Et
12	14.793	483731156	51853088	Bis[(3,5-Di-T-Butyl-1-Pyrazolyl)Et
13	15.066	643865138	58455224	1,3,7,9,2,8-Parazabol,4,6,10,12-Tetr
14	15.227	447841479	56255701	Glycerol Tricaprylate
15	15.382	668780267	60947273	Dodecanoicacid,1,2,3-Propanetri
16	15.574	689339361	60450430	Lauricacid,2-(Hexadecyloxy)-3-(

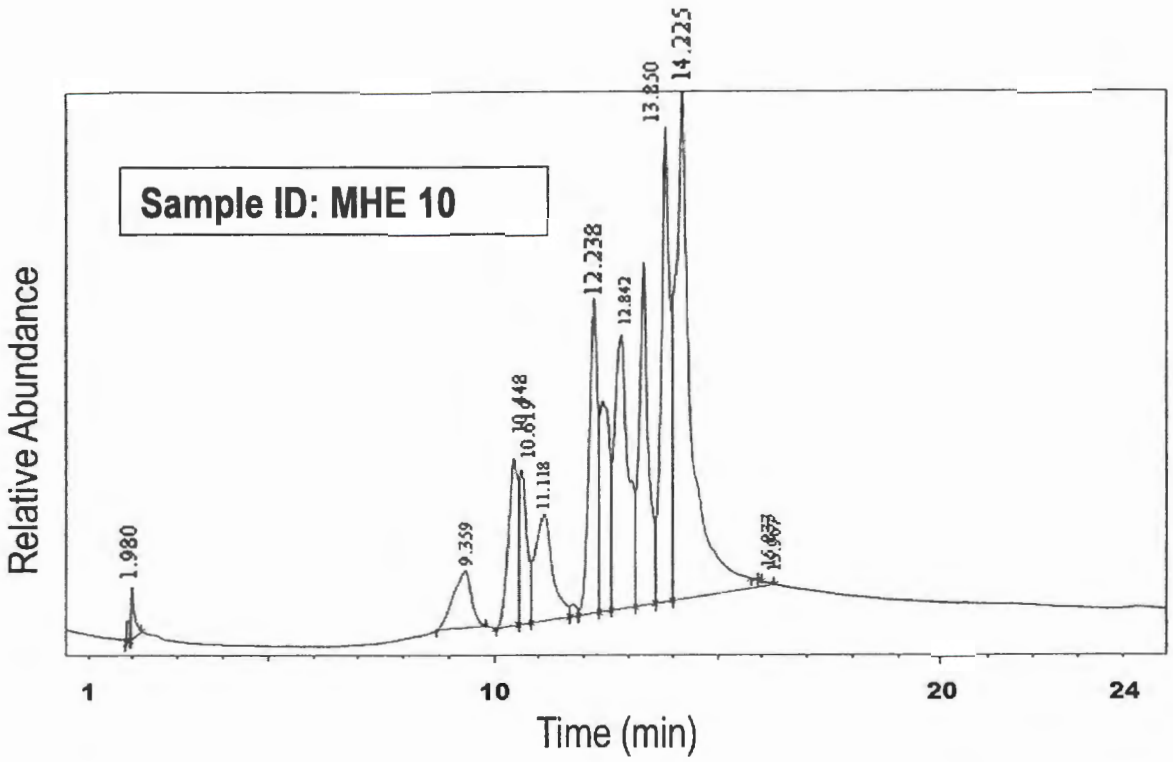
17	15.693	386190574	49020751	Eicosanoicacid,2-[(1-Oxohexade
18	16.317	10117	20978	Dodecanoicacid,1,2,3-Propanetri
19	16.595	4079557	570585	Dodecanoicacid,1,2,3-Propanetriylester
20	16.754	2852301	627857	Dodecanoicacid,1,2,3-Propanetri
21	16.835	5723563	971596	Dodecanoicacid,1,2,3-Propanetri
22	17.548	5857077	811111	Dodecanoicacid,1,2,3-Propanetri
23	17.703	5144374	604877	Dodecanoicacid,1,2,3-Propanetri
24	17.877	5150036	408862	Octanoicacid,1-Methyltridecylester
25	19.030	11678700	1794784	Dodecanoicacid,1,2,3-Propanetriylester
26	19.191	57762529	4679493	Dodecanoicacid,1,2,3-Propanetriylester
27	19.610	33745238	2528159	Dodecanoicacid,1,2,3-Propanetriylester
28	21.970	865278820	37135688	Dodecanoicacid,1,2,3-Propanetriylester
29	23.031	2932366871	50598968	Dodecanoicacid,1,2,3-Propanetri
30	23.542	1541386726	51263295	2-Lauro-1,3-Didecoin
31	23.718	1187851411	51595273	3h-2,3-Benzodiazepine,1-(3,4-Dimet
32	24.352	138160716	9102322	Dodecanoicacid,1,2,3-Propanetriylester



Peak	R. Time	Area	Height	Name
1	1.987	10894785	1367278	Butanoicacid,3-Hydroxy-
2	17.430	6714978	895234	Dodecanoicacid,1,2,3-Propanetri
3	17.591	4348071	727650	Dodecanoicacid,1,2,3-Propanetri
4	19.470	27587402	2175193	14-.Beta.-H-Pregna
5	19.950	171326298	9506833	Dodecanoicacid,1,2,3-Propanetriylester
6	20.226	307822552	20027509	Dodecanoicacid,1,2,3-Propanetriylester
7	20.992	1309466209	36978007	Dodecanoicacid,1,2,3-Propanetriylester
8	21.337	1165201752	40487547	Dodecanoicacid,1,2,3-Propanetri
9	22.246	2023096879	46547293	Dodecanoicacid,1,2,3-Propanetriylester
10	22.756	2674341707	48705729	Glyceryl Tridodecanoate

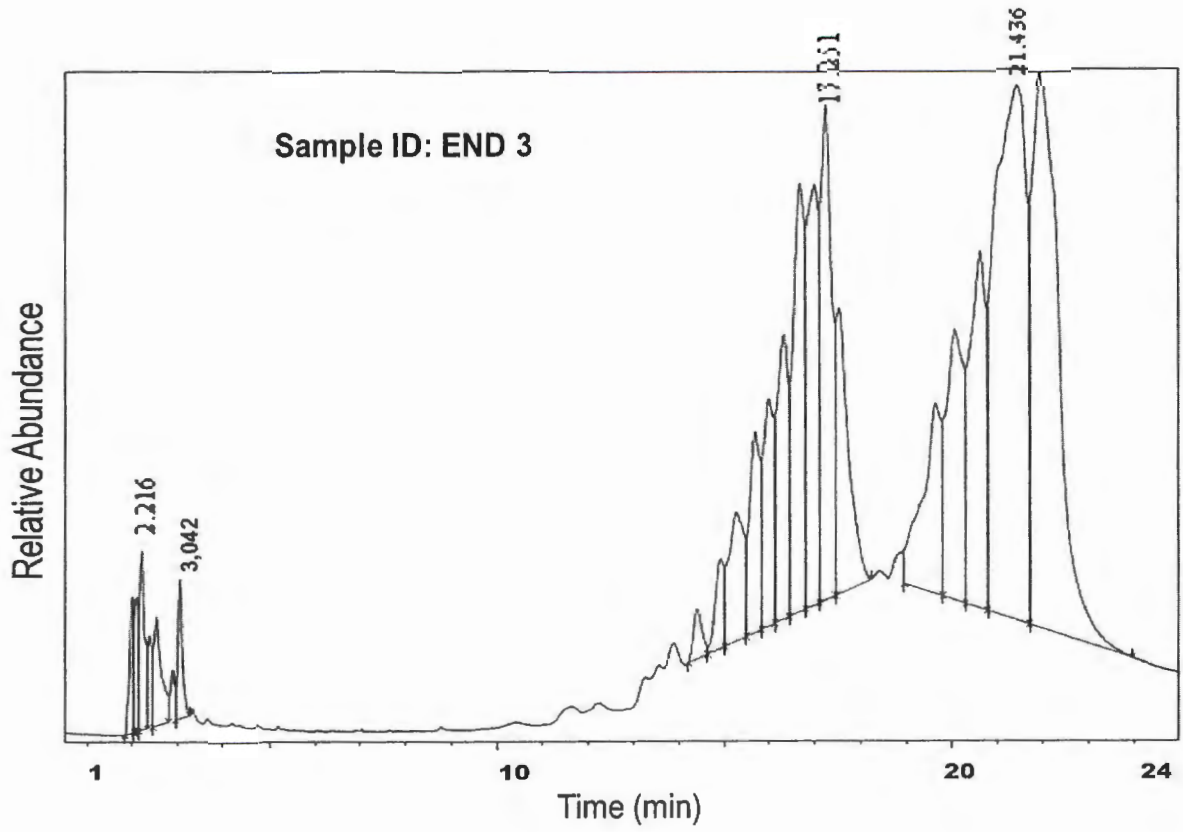


Peak	R. Time	Area	Height	Name
1	1.948	11472806	1284622	ETHANOL,2-NITRO-
2	2.197	1222848	223452	Octadecanoicacid,phenylmethylester
3	2.355	1146050	162938	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-
4	2.431	2249944	305544	5-Hydroxymethylfurfural
5	2.617	475548	88881	ACETAMIDE-N-D,N,N'-1,3-CYCLOHEX

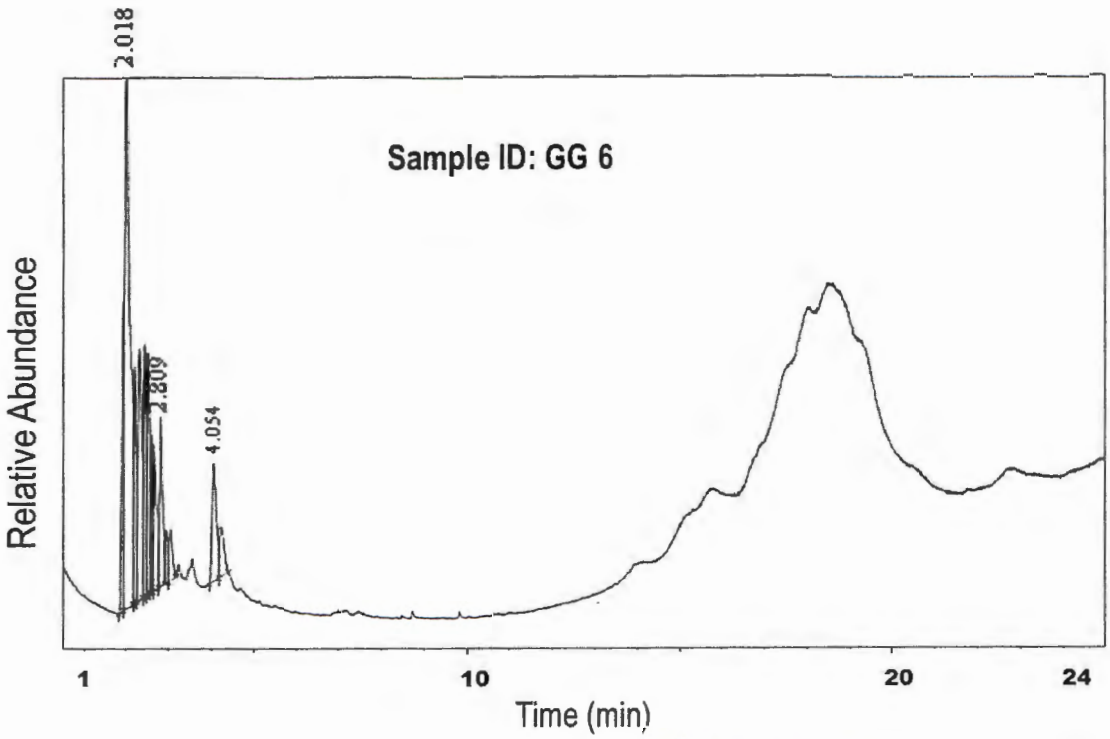


Peak	R. Time	Area	Height	Name
1	1.877	2600454	555537	Boric acid
2	1.980	7364571	1310552	9-Bromononanoicacid
3	9.359	40198770	1440164	Octadecanoicacid,3-[(1-Oxodod
4	10.448	62323244	4207847	Octadecanoicacid,3-[(1-Oxodod
5	10.619	47149711	3905278	Octadecanoicacid,3-[(1-Oxodod
6	11.118	72032235	2688539	Octadecanoicacid,3-[(1-Oxodod
7	11.762	2893618	313321	2,3-Bis(Tetradecanoyloxy)Propy
8	12.238	108247838	7944825	Dodecanoicacid,1,2,3-Propanetri
9	12.425	78085896	5322829	Octadecanoicacid,3-[(1-Oxodod
10	12.842	150733443	6924454	Dodecanoicacid,1,2,3-Propanetriylester
11	13.352	123146495	8620861	Dodecanoicacid,1,2,3-Propanetriylester
12	13.850	163122219	11921490	Dodecanoicacid,1,2,3-Propanetriylester
13	14.225	318209336	12807196	Dodecanoicacid,1,2,3-Propanetriylester
14	15.833	36078	16984	1,9-Dibutoxy-1,3,5,7,9-Pentaethylp
15	15.967	20049	16864	1h-Purin-6-Amine,[(2-Fluorophen

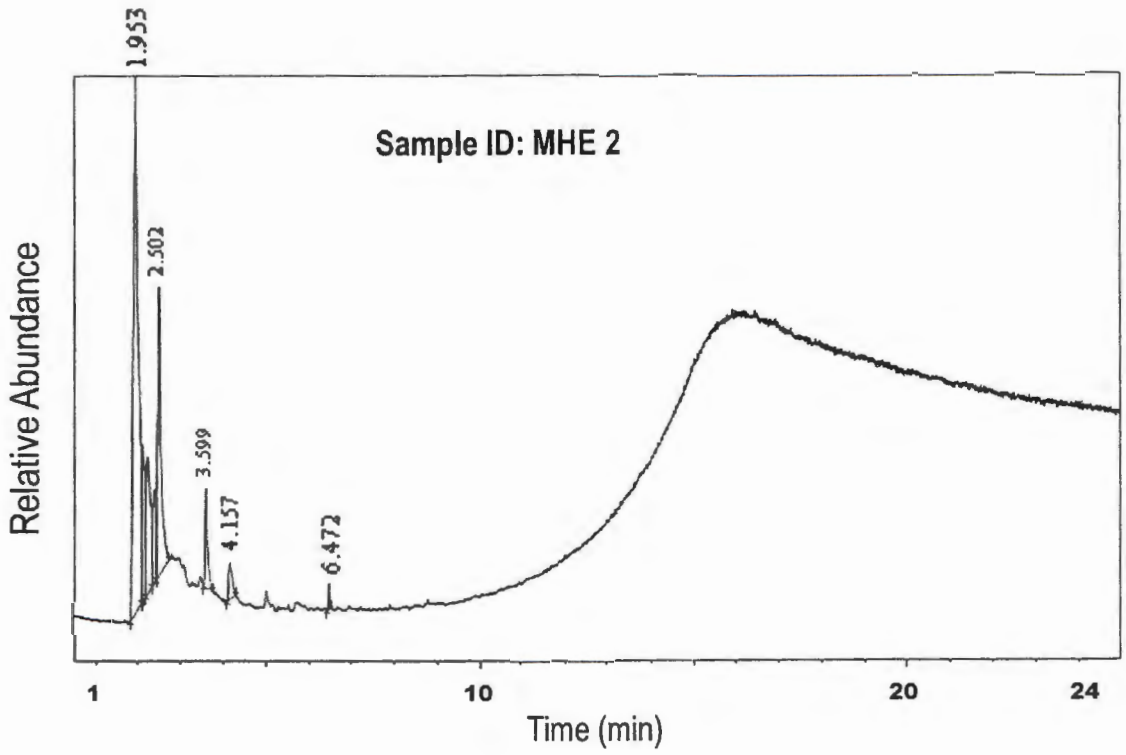
CHROMATOGRAM OF SECONDARY METABOLITE OF INACTIVE SAMPLES



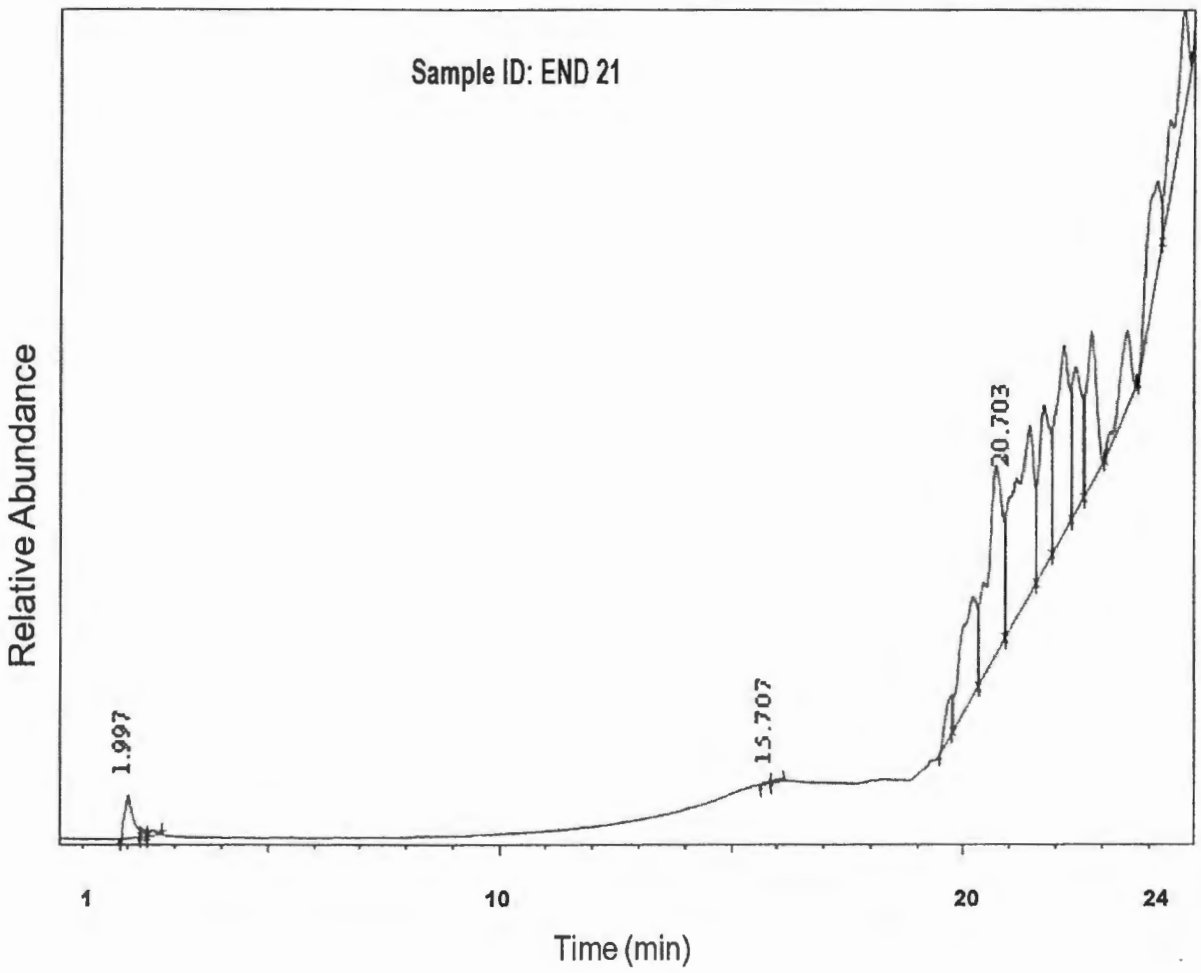
Peak	R. Time	Area	Height	Name
1	1.999	17499523	2690528	Methanamine,N-Methyl-
2	2.106	15071688	2651316	Propanal, 2,3-Dihydroxy-
3	2.216	29293236	3522035	2h-Pyran-2,6(3h)-Dione
4	2.539	24882092	2137517	2-(3-Butynyloxy)Tetrahydro-2h-
5	3.042	19450095	2739552	3-Methylenecyclopropane-Trans-1,2-Dicarbox
6	14.423	12939152	979001	Lauricacid,2-(Hexadecyloxy)-3-(Octadecylo
7	14.928	23481799	1761257	Lauricacid,2-(Hexadecyloxy)-3-(
8	15.272	60136800	2528001	Lauricacid,2-(Hexadecyloxy)-3-(
9	15.685	64301532	3946761	Lauricacid,2-(Hexadecyloxy)-3-(
10	15.989	71161790	4475716	Lauricacid,2-(Hexadecyloxy)-3-(Octadecylo
11	16.329	94559452	5600444	Lauricacid,2-(Hexadecyloxy)-3-(
12	16.686	149913591	8403590	Lauricacid,2-(Hexadecyloxy)-3-(
13	17.000	145912879	8247804	Lauricacid,2-(Hexadecyloxy)-3-(Octadecylo
14	17.251	162092290	9735793	Lauricacid,2-(Hexadecyloxy)-3-(
15	17.540	106430436	5620568	2-Hydroxy-3-(Palmitoyloxy)Prop
16	19.637	107847500	3739794	2,3-Bis(Tetradecanoyloxy)Propy
17	20.067	138763006	5301416	Octadecanoicacid,3-[(1-Oxohex
18	20.621	170143852	6986649	Octadecanoicacid,3-[(1-Oxohex
19	21.436	506749893	10525408	Octadecanoicacid,3-[(1-Oxohexadecyl)Oxy]
20	21.971	451367546	10971419	Octadecanoicacid,3-[(1-Oxododecyl)Oxy]-1,



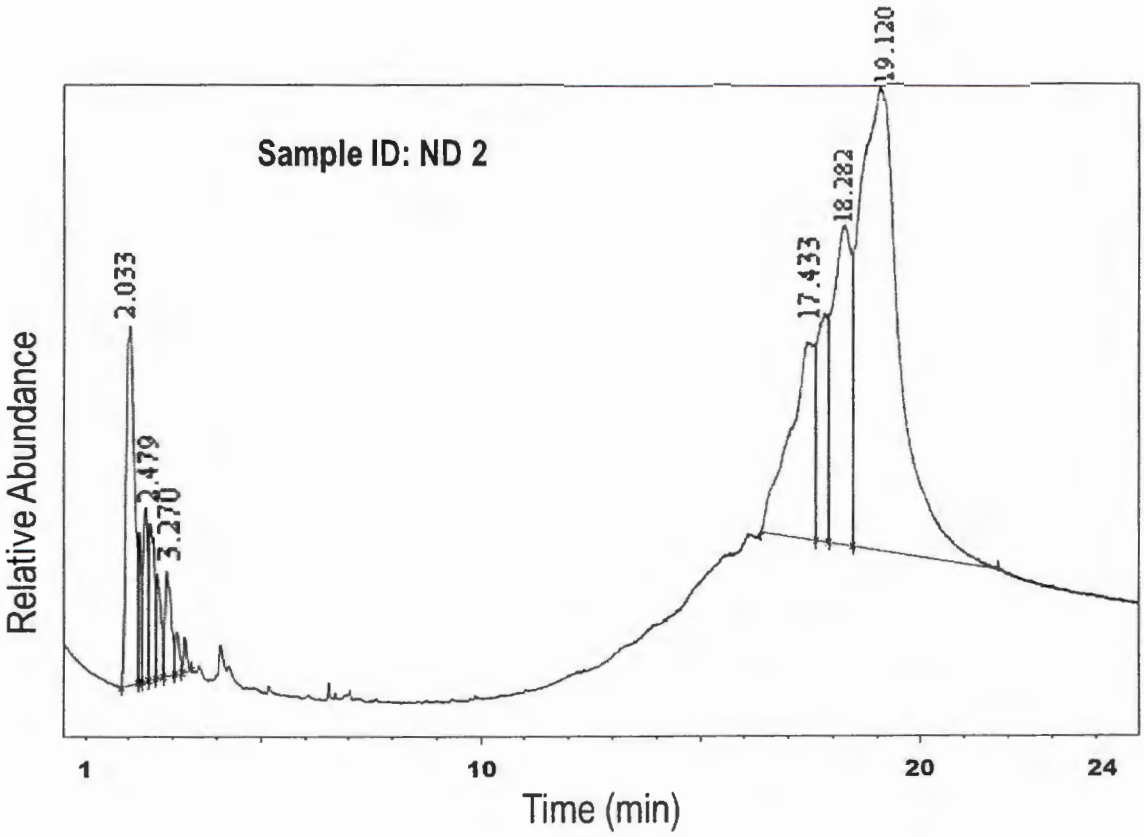
Peak	R. Time	Area	Height	Name
1	1.924	6784324	2015963	Propanedioicacid
2	2.018	30783892	3456283	Dl-Glyceraldehydedimer
3	2.196	7116773	1537196	3-Hepten-2-One,3-Methyl-
4	2.305	10314395	1634937	1,2,3-Propanetriol
5	2.435	7024788	1640640	5-(Hydroxy[Methoxy(5-Oxotetr
6	2.514	7310430	1573291	1,2,3-Propanetriol,1-Acetate
7	2.596	2920029	1022903	4h-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-
8	2.647	5273829	951229	1-Butoxy-1-Ethoxyethane
9	2.809	6073094	1104790	Butanedioicacid,2-Hydroxy-2-Methyl-,(S)-
10	3.050	1969316	334631	Lactoneg
11	4.054	5918839	767537	3-Deoxy-D-Mannoiclactone
12	4.240	2570625	325506	3-Deoxy-D-Mannonicacid



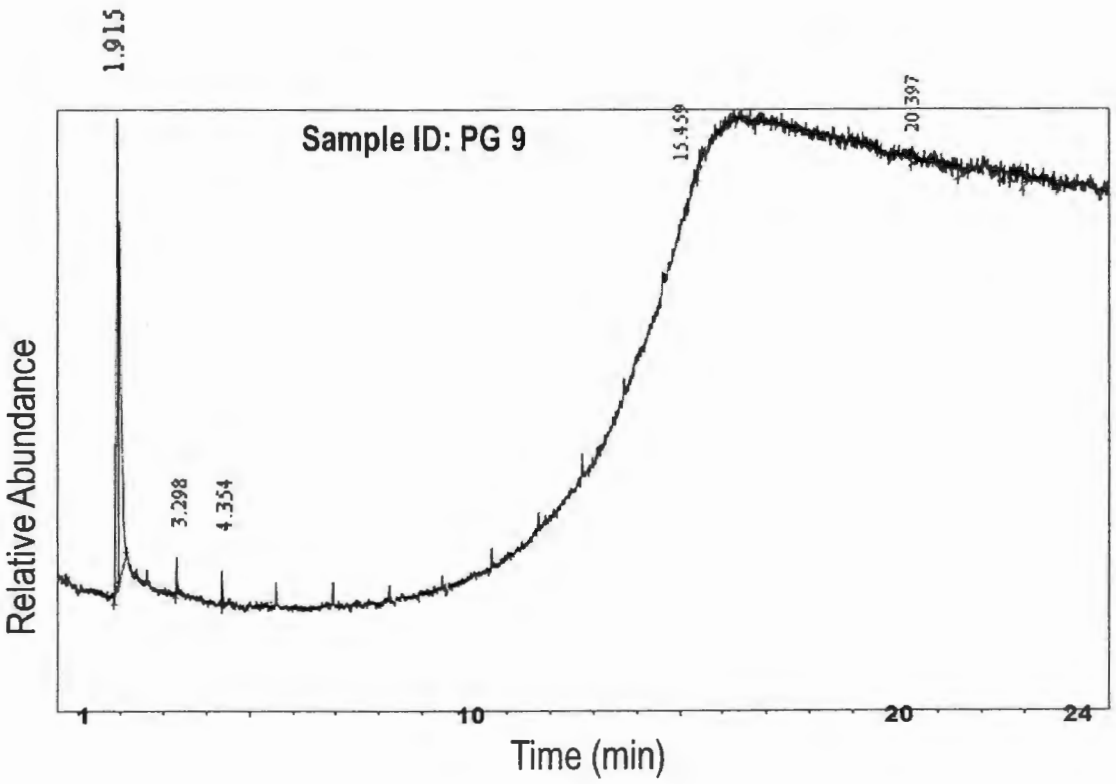
Peak	R. Time	Area	Height	Name
1	1.953	12407905	1478921	Hydrazine,Methyl-,Oxalate(1:1)
2	2.120	2158657	430296	2-Furancarboxaldehyde,5-Meth
3	2.233	2676977	380416	3-Hexen-2-One,3,4-Dimethyl-,(E)-
4	2.392	1405368	258881	4h-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-
5	2.502	4610880	796934	5-Hydroxymethylfurfural
6	3.599	1127859	276565	.Beta.-D-Glucopyranose,1,6-Anhydro-
7	4.157	566643	101701	D(-)Arabinose
8	6.472	143855	72639	N-Didehydrohexacarboxyl-2,4,5-



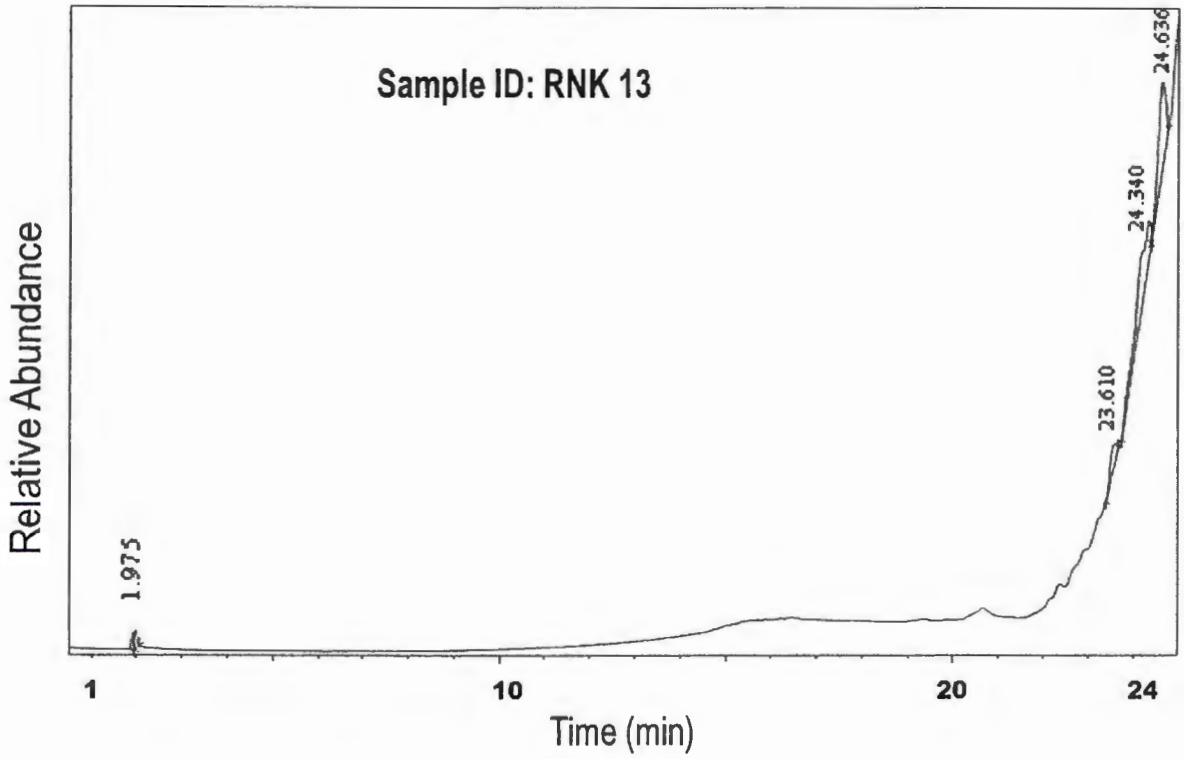
Peak	R. Time	Area	Height	Name
1	1.997	12532753	1035132	Benzeneethanamine,2,5-Difluoro-.Beta.,3,4-T
2	2.307	1262398	189242	Levoglucosenone
3	2.511	1556838	144488	5-Hydroxymethylfurfural
4	15.707	761899	66207	9-Octadecenoicacid(Z)-
5	15.967	716244	71778	1,7-Di(Dodec-9-Ynyl)-2,2,4,4,6,6-Hexamethyl
6	19.740	10747200	922761	Dodecanoicacid,1,2,3-Propanetriylester
7	20.207	59451893	2376290	Dodecanoicacid,1,2,3-Propanetriylester
8	20.703	106118408	4527655	Dodecanoicacid,1,2,3-Propanetriylester
9	21.427	130460549	4003216	Dodecanoicacid,1,2,3-Propanetri
10	21.742	66921757	3886667	Dodecanoicacid,1,2,3-Propanetriylester
11	22.177	93279899	4457246	Dodecanoicacid,1,2,3-Propanetriylester
12	22.431	48381430	3433620	Dodecanoicacid,1,2,3-Propanetriylester
13	22.779	53978474	3584201	Dodecanoicacid,1,2,3-Propanetri
14	23.546	33465753	1827340	2,3-Bis(Tetradecanoyloxy)Propy
15	24.177	52615068	2148068	Octadecanoicacid,3-[(1-Oxodod
16	24.762	53788842	2329031	Dodecanoicacid,1,2,3-Propanetri



Peak	R. Time	Area	Height	Name
1	2.033	30281992	2271811	Glyceraldehyde
2	2.234	4299858	948868	1,3,5-Triazine-2,4,6-Triamine
3	2.390	8164895	1096070	2,3-Dihydro-3,5-Dihydroxy-6-Meth
4	2.479	8695512	987269	2(3h)-Furanone,Dihydro-5-(2-Octenyl)-,(Z)-
5	2.645	4881299	662282	4h-Pyran-4-One,2,3-Dihydro-3,5-Dihydroxy-
6	2.852	6485202	666254	Butanoicacid,3-Methyl-,Propyl
7	3.088	1766265	267696	Lactoneg
8	3.270	1116229	221775	Guanosine
9	17.433	49765465	1243052	Hexadecanoicacid,2-[(1-Oxotetr
10	17.820	23156433	1445351	Octadecanoicacid,2-[(1-Oxohex
11	18.282	58563020	2025941	Octadecanoicacid,3-[(1-Oxohex
12	19.120	188207413	2948892	Octadecanoicacid,3-[(1-Oxodod



Peak	R. Time	Area	Height	Name
1	1.915	1654622	498298	Methane,Sulfinylbis-
2	1.968	1598441	379512	5.Beta.,7.Beta.H,10.Alpha.-Eudesm-11-En-1.Al
3	3.298	54888	39357	Cycloheptasiloxane,Tetradecamethyl-
4	4.354	58006	37020	Cyclooctasiloxane,Hexadecam
5	15.459	58329	23865	Siliconegrease,Siliconfett
6	20.397	56881	17952	Silikonfett
7	21.543	65650	18933	Dimethyl2-{4-[Ethyl(Dimethyl)Si
8	22.457	55020	19932	Siliconegrease,Siliconfett
9	22.726	72519	18173	2-(4-{[4-Oxo-3-Phenyl-2-(Phenylimi
10	22.960	72337	22220	1h-Purin-6-Amine,[(2-Fluorophen



Peak	R. Time	Area	Height	Name
1	1.975	2496624	716129	3-Phenylpropenoicacid,2',3'-Dimethoxy-2-Ac
2	23.610	10040713	756451	2,3-Bis(Tetradecanoyloxy)Propy
3	24.340	48495334	1570631	Dodecanoicacid,1,2,3-Propanetri
4	24.636	43118777	3529229	Lauricacid,2-(Hexadecyloxy)-3-(